Title: ANTI-CD277 ANTIBODIES AND USES THEREOF

Abstract: The present invention relates to anti-CD277 antibodies which: -activates or inhibit cytolytic function of Vγ9/ Vδ2 T cells, and/or -costimulates T cells together with CD3-TCR, and/or -costimulates T cells in addition to CD28-B7 costimulation, and/or -increases the activity and/or survival of monocytes and dendritic cells. The invention also relates to the use of said antibodies in therapy.
Anti-CD277 antibodies and uses thereof

FIELD OF THE INVENTION

The invention relates to anti-CD277 antibodies and uses thereof.

BACKGROUND OF THE INVENTION

White blood cells are cells of the immune system involved in defending the body against pathogens. Among these cells, lymphocytes, monocytes, and dendritic cells can be cited. Monocytes may migrate from the bloodstream to other tissues and differentiate into tissue resident macrophages or dendritic cells. Dendritic cells play a role as antigen presenting cells (APC) that activate lymphocytes. Among lymphocytes, T cells can be divided into γδ T cells and αβ T cells.

Vγ9/Vδ2 T cells are important effectors of the immune defence. They lyse directly pathogen infected or abnormal cells. In addition, they regulate immune responses by inducing dendritic cell (DC) maturation as well as the isotypic switching and immunoglobulin production. This important cell platform of the immune system is strictly regulated by surface receptors, chemokines and cytokines.

The priming of T cells is modulated by involvement of specialised cells and secretion of chemotactic cytokines. Nowadays, we know that T-cell activation is the result of two synergistic events. The first is the interaction between the receptor of T cell (TCR) and the major histocompatibility complex (MHC) conjugated with processed antigen on the surface of the antigen presenting cells (APC). The second event is a co-stimulatory antigen-independent signal involving B7 molecules. The lack of co-stimulatory signal induces anergy, i.e. the inhibition of T cells proliferation, cytokines secretion and cytotoxic activities. The study of these pathways may provide insight about the triggering of pathologic events, such as autoimmune or lymphoproliferative disorders.
The B7 family is an extended group of costimulatory molecules (Coyle et al., 2001). To the B7 family belong the ligands B7-1 (CD80) and B7-2 (CD86): their receptors are CD28, which leads to T cell activation (Linsley and Ledbetter, 1993, June, et al., 1994, Lenschow et al., 1996), and CTLA-4 (CD152), which competes with CD28 and transduces an inhibitory signal (Waterhouse et al., 1996). The critical role of CD152 as a negative regulator of T cell activation is demonstrated by the occurrence of lymphoproliferative disorders in CTLA-4 deficient mice (Waterhouse et al., 1995). Most data on the inhibitory function exerted by CD152 are gathered from studies of proliferation or cytokine production by naïve T lymphocytes during T cell priming (Linsley et al., 1992, Walunas et al., 1995, Walunas and Bluestone, 1998). In particular, CD152 is expressed following T-lymphocyte activation and inhibits the cytolytic functions of CTL clones obtained following PHA stimulation or Ag selection (Saverino et al., 1998).

B7-H1 (PD-L1, CD274) and B7-DC (PD-L2, CD273), whose receptor is PD-1 (CD279), proved to inhibit T-cell proliferation and cytokine secretion (Freeman et al., 2000, Latchman et al., 2001). Otherwise, different studies showed that PD-L1 and PD-L2 engagement increase T cell proliferation and IL-10 or IFN-γ production (Dong et al., 1999, Freeman et al., 2000, Latchman et al., 2001, Chapoval et al., 2001, Tseng et al., 2001). Other molecules related to the family B7 expressed on the surface of T cells are B7-H2 (ICOS-L), B7-H3, B7-H4 whose roles are not fully understood (Hutloff et al., 1999, Sun et al., 2002).

Henry et al. (1999) found that the region coding for butyrophilin (BT) is located at a telomeric position from the MHC class I region on human chromosome 6. In particular they described two genes Bt2 and Bt3, coding for a new group of co-stimulatory molecules (BT2.1, BT2.2, BT2.3, BT3.1, BT3.2 and BT3.3) belonging to the Ig superfamily (IgSF) (Williams and Barclay, 1988) and related to B7 family by sequence similarity analysis: in particular, it shows similarity with the Ig-V like extracellular domain of CD80 and CD86 (Linsley et al., 1992).

The BT3 family members appear in literature with different names: BT3.1 is also called BTF5 (Rhodes et al., 2001), or BTN3A1 (Ruddy et al., 1997), or more recently CD277 (Bensussan and Olive, 2005); BT3.2 is also called BTF4 (Rhodes et al., 2001), or BTN3A2 (Ruddy et al., 1997); and, finally, BT3.3 appears also as BTF3 (Rhodes et al.,
2001) or BTN3A3 (Ruddy et al., 1997). BT3 has two Ig-like extracellular domains that characterize the IgSF.

It has been proposed that b7 genes and MHC class I and II genes may have a common ancestral gene and could encode for proteins involved in similar function, such as T cell activation (Rhodes et al., 2001). BT3 molecules have been found on immune cells, such as T, B and NK cells, monocytes and dendritic cells as well as hematopoietic precursors and some neoplastic cell lines (Compte et al., 2004). As for other co-stimulatory molecules, their structure is characterized by three domains: an extracellular domain to bind the ligand, a transmembrane domain and an intracellular domain termed B30.2 which is presumably involved in the regulation of intracellular superoxide concentrations (Henry et al., 1998). So far, the ligand(s) of CD277 is still unknown.

To date, no satisfactory approach has been proven to induce potent immune responses against vaccines, especially in cancer patients. Methods have yet to be devised to overcome the immunosuppressive mechanisms observed in cancer patients, and during chronic infections. Treatment of autoimmune diseases and prevention of transplantation rejection in graft versus host diseases (GVHD) depends on immunosuppressive agents that have serious side effects, or are not always effective. New immunosuppressive agents are desired.

**SUMMARY OF THE INVENTION**

The present invention relates to an anti-CD277 antibody, which activates or inhibits the cytolytic function, cytokine production and proliferation of Vy9/V52 T cells. The present invention also relates to an anti-CD277 antibody, which costimulates T cells together with CD3-TCR, and/or which costimulates T cells in addition to CD28-B7 costimulation.

The present invention also relates to an anti-CD277 antibody, which increases the activity and/or survival of monocytes and dendritic cells.

Preferably, the antibody according to the invention is an anti-CD277 antibody, which:
- activates the cytolytic function of Vy9/V52 T cells, and
- costimulates T cells together with CD3-TCR, and
- costimulates T cells in addition to CD28-B7 costimulation, and
- increases the activity and/or survival of monocytes and dendritic cells.

Particularly, the present invention relates to an anti-CD277 antibody (chosen from mAbs 20.1, 7.2 and 103.2) which is obtainable from one of the hybridomas accessible under CNCM deposit number I-4401, I-4402 and I-4403.

The invention also relates to an anti-CD277 antibody which comprises the CDRs of mAb 20.1. The invention also relates to an anti-CD277 antibody which comprises the CDRs of mAb 7.2. The invention also relates to an anti-CD277 antibody which comprises the CDRs of mAb 103.2.

The invention also relates to one of the mAbs 20.1, 7.2 and 103.2, or a derivative thereof, for the use in therapy.

The invention relates to one of the mAbs 20.1 and 7.2, or a derivative thereof, for use for the treatment of a cancer or a chronic infection.

The invention relates to a vaccine for the treatment of a cancer or a chronic infection comprising one of the mAbs 20.1 and 7.2, or a derivative thereof. The invention relates to a kit for the treatment of a cancer or a chronic infection comprising:

a) mAb 20.1 or 7.2 or a derivative thereof; and
b) a vaccine for the treatment of a cancer or a chronic infection.

The invention relates to an anti-CD277 antibody as defined above for the use in therapy.

The invention finally relates to an anti-CD277 antibody which inhibits the functions of Vy9/Vδ2 T cells, particularly for use for the treatment of an autoimmune disease, transplantation rejection or a graft versus host disease.
DETAILLED DESCRIPTION OF THE INVENTION

Definitions

According to the present invention, "antibody" or "immunoglobulin" have the same meaning, and will be used equally in the present invention. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antigen binding site that immunospecifically binds an antigen. As such, the term antibody encompasses not only whole antibody molecules, but also antibody fragments or derivatives. Antibody fragments include but are not limited to Fv, Fab, F(ab')2, Fab', dsFv, scFv, Sc(Fv)2 and diabodies.

In natural antibodies, two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond. There are two types of light chain, lambda (λ) and kappa (κ). There are five main heavy chain classes (or isotypes) which determine the functional activity of an antibody molecule: IgM, IgD, IgG, IgA and IgE. Each chain contains distinct sequence domains.

The light chain includes two domains, a variable domain (VL) and a constant domain (CL). The heavy chain includes four domains, a variable domain (VH) and three constant domains (CH1, CH2 and CH3, collectively referred to as CH). The variable regions of both light (VL) and heavy (VH) chains determine binding recognition and specificity to the antigen. The constant region domains of the light (CL) and heavy (CH) chains confer important biological properties such as antibody chain association, secretion, trans-placental mobility, complement binding, and binding to Fc receptors (FcR). The Fv fragment is the N-terminal part of the Fab fragment of an immunoglobulin and consists of the variable portions of one light chain and one heavy chain. The specificity of the antibody resides in the structural complementarity between the antibody combining site and the antigenic determinant. Antibody combining sites are made up of residues that are primarily from the hypervariable or complementarity determining regions (CDRs). Occasionally, residues from nonhypervariable or framework regions (FR) influence the overall domain structure and hence the combining site. Complementarity Determining Regions or CDRs refer to amino acid...
sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. The light and heavy chains of an immunoglobulin each have three CDRs, designated L-CDR1, L-CDR2, L-CDR3 and H-CDR1, H-CDR2, H-CDR3, respectively. An antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. Framework Regions (FRs) refer to amino acid sequences interposed between CDRs.

The terms "chimeric antibody" refer to a genetically engineered fusion of parts of an animal antibody, typically a mouse antibody, with parts of a human antibody. Generally, chimeric antibodies contain approximately 33% mouse protein and 67% human protein. Developed to reduce the Human Anti-animal Antibodies response elicited by animal antibodies, they combine the specificity of the animal antibody with the efficient human immune system interaction of a human antibody.

According to the invention, the term "humanized antibody" refers to an antibody having variable region framework and constant regions from a human antibody but retains the CDRs of the animal antibody.

The term "Fab" denotes an antibody fragment having a molecular weight of about 50,000 and antigen binding activity, in which about a half of the N-terminal side of H chain and the entire L chain, among fragments obtained by treating IgG with a protease, papaine, are bound together through a disulfide bond.

The term "F(ab')2" refers to an antibody fragment having a molecular weight of about 100,000 and antigen binding activity, which is slightly larger than the Fab bound via a disulfide bond of the hinge region, among fragments obtained by treating IgG with a protease, pepsin.

The term "Fab' " refers to an antibody fragment having a molecular weight of about 50,000 and antigen binding activity, which is obtained by cutting a disulfide bond of the hinge region of the F(ab')2.

A single chain Fv ("scFv") polypeptide is a covalently linked VH::VL heterodimer which is usually expressed from a gene fusion including VH and VL encoding genes linked by a peptide-encoding linker.

"dsFv" is a VH:: VL heterodimer stabilised by a disulfide bond. Divalent and multivalent antibody fragments can form either spontaneously by association of monovalent scFvs,
or can be generated by coupling monovalent scFvs by a peptide linker, such as
divalent sc(Fv)2.
The term "diabodies" refers to small antibody fragments with two antigen-binding sites,
which fragments comprise a heavy-chain variable domain (VH) connected to a light-
chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker
that is too short to allow pairing between the two domains on the same chain, the
domains are forced to pair with the complementary domains of another chain and
create two antigen-binding sites.

By "purified" and "isolated" it is meant, when referring to a polypeptide (i.e. an antibody
according to the invention) or to a nucleotide sequence, that the indicated molecule is
present in the substantial absence of other biological macromolecules of the same
type.
The term "purified" as used herein preferably means at least 75% by weight, more
preferably at least 85% by weight, more preferably still at least 95% by weight, and
most preferably at least 98% by weight, of biological macromolecules of the same type
are present.
An "isolated" nucleic acid molecule which encodes a particular polypeptide refers to a
nucleic acid molecule which is substantially free of other nucleic acid molecules that do
not encode the polypeptide; however, the molecule may include some additional bases
or moieties which do not deleteriously affect the basic characteristics of the
composition.

In the context of the invention, the term "treating" or "treatment", as used herein,
means reversing, alleviating, inhibiting the progress of, or preventing the disorder or
condition to which such term applies, or one or more symptoms of such disorder or
condition.

A "therapeutically effective amount" is intended for a minimal amount of active agent
which is necessary to impart therapeutic benefit to a subject. For example, a
"therapeutically effective amount" to a mammal is such an amount which induces,
ameliorates or otherwise causes an improvement in the pathological symptoms,
disease progression or physiological conditions associated with or resistance to
succumbing to a disorder.
As used herein, the term "prevention" refers to preventing the disease or condition from occurring in a subject which has not yet been diagnosed as having it. As used herein, the term "subject" denotes a mammal, such as a rodent, a feline, a canine, and a primate. Preferably a subject according to the invention is a human.

As used herein, the terms "cancer", "hyperproliferative" and "neoplastic" refer to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. Hyperproliferative and neoplastic disease states may be categorized as pathologic, i.e., characterizing or constituting a disease state, or may be categorized as non-pathologic, i.e., a deviation from normal but not associated with a disease state. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness.

The terms "cancer" or "neoplasms" include malignancies of the various organ systems, such as affecting lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract, as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus.

The inventors have deposited a murine anti-CD277 antibody (mAb 20.1) producing hybridoma at the Collection Nationale de Cultures de Microorganismes (CNCM, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France), in accordance with the terms of Budapest Treaty, on November 24, 2010.

The deposited hybridoma for mAb 20.1 has CNCM deposit number I-4402.

"mAb 20.1" refers to an isolated anti-CD277 antibody which is obtainable from the hybridoma accessible under CNCM deposit number I-4402. The expression "a derivative of mAb 20.1" refers to an anti-CD277 antibody which comprises the 6 CDRs of mAb 20.1.

The inventors have deposited a murine anti-CD277 antibody (mAb 7.2) producing hybridoma at the Collection Nationale de Cultures de Microorganismes (CNCM, Institut
Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France), in accordance with the terms of Budapest Treaty, on November 24, 2010.
The deposited hybridoma for mAb 7.2 has CNCM deposit number l-4401.

"mAb 7.2" refers to an isolated anti-CD277 antibody which is obtainable from the hybridoma accessible under CNCM deposit number l-4401. The expression "a derivative of mAb 7.2" refers to an anti-CD277 antibody which comprises the 6 CDRs of mAb 7.2.

The inventors have deposited a murine anti-CD277 antibody (mAb 103.2) producing hybridoma at the Collection Nationale de Cultures de Microorganismes (CNCM, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France), in accordance with the terms of Budapest Treaty, on November 24, 2010.
The deposited hybridoma for mAb 103.2 has CNCM deposit number l-4403.

"mAb 103.2" refers to an isolated anti-CD277 antibody which is obtainable from the hybridoma accessible under CNCM deposit number l-4403. The expression "a derivative of 103.2" refers to an anti-CD277 antibody which comprises the 6 CDRs of mAb 103.2.

**Antibodies of the invention and nucleic acids encoding them**

The present invention relates to an isolated anti-CD277 antibody, which activates the cytolytic function, cytokine production and proliferation of Vy9/Vδ2 T cells.
By activating the cytolytic function of Vy9/Vδ2 T cells, it is meant that a significant increase of the cytotoxicity of Vy9/Vδ2 T cells, i.e. a significant increase of the specific lysis of the target cells by Vy9/Vδ2 T cells, is observed.
By increasing the cytokine production by Vy9/Vδ2 T cells, it is meant that a significant increase of the cytokine production by Vy9/Vδ2 T cells is observed, as compared to control Vy9/Vδ2 T cells (i.e. non stimulated and non treated).
By increasing the proliferation of Vy9/Vδ2 T cells, it is meant that a significant increase of the proliferation of Vy9/Vδ2 T cells is observed, as compared to control Vy9/Vδ2 T cells (i.e. non stimulated and non treated).
Typically the activation of the cytolytic function of Vy9/V52 T cells may be measured according to the method described in example 3 (i.e. "Analysis of Vy9V52 T cell responses by direct cytotoxicity assay", and for example the results “CD277 potentiate the anti-tumor cytolysis mediated by Vy9V52 T cells”). Examples of isolated anti-CD277 antibodies, which activate the cytolytic function, cytokine production and proliferation of Vy9/V52 T cells, are mAbs 20.1 or 7.2, or derivatives thereof.

The present invention also relates to an anti-CD277 antibody, which costimulates T cells together with CD3-TCR. By costimulating T cells together with CD3-TCR, it is meant that the reaction cascade following the binding of CD3 with TCR is activated. Typically the costimulation of T cells together with CD3-TCR may be measured according to the method described in example 2 (particularly “CD277 costimulates CD3 signals”). Examples of isolated anti-CD277 antibodies, which costimulate T cells together with CD3-TCR, are mAbs 20.1 or 7.2, or derivatives thereof.

The present invention also relates to an anti-CD277 antibody, which costimulates T cells in addition to CD28-B7 costimulation. By costimulating T cells together with CD28-B7, it is meant that the reaction cascade following the binding of CD28 with B7 is activated. Typically the costimulation of T cells together with CD28-B7 may be measured according to the method described in example 2 (particularly “CD277 costimulates CD3 signals”). Examples of isolated anti-CD277 antibodies, which costimulate T cells together with CD28-B7, are mAbs 20.1 or 7.2, or derivatives thereof.

The present invention also relates to an anti-CD277 antibody, which increases the activity and/or survival of monocytes and dendritic cells. By increasing the activity of monocytes and dendritic cells, it is meant that said anti-CD277 antibody increases costimulatory molecules expression (like CD86, CD80 and HLA-DR) on the surface of monocytes and dendritic cells, and increases the proinflammatory responses induced by TLR ligands in these cells. Typically the increase of the activity and/or survival of monocytes and dendritic cells may be measured according to the method described in example 1 (particularly "Apoptosis detection" and "Cytokines production"). Examples of isolated anti-CD277 antibodies, which increase the activity and/or survival of monocytes and dendritic cells, are mAb 20.1, or derivatives thereof.
The present invention also relates to an isolated anti-CD277 antibody (mAb 20.1) which is obtainable from the hybridoma accessible under CNCM deposit number I-4402.

The present invention relates to the hybridoma accessible under CNCM deposit number I-4402.

The invention relates to an antibody which comprises the 6 CDRs of mAb 20.1.

In another embodiment, the invention relates to a derivative of mAb 20.1 which comprises the VL chains and the VH chains of mAb 20.1.

In another embodiment, the invention relates to a derivative of mAb 20.1 which is a chimeric antibody, which comprises the variable domains of mAb 20.1.

The present invention also relates to an isolated anti-CD277 antibody (mAb 7.2) which is obtainable from the hybridoma accessible under CNCM deposit number I-4401.

The present invention relates to the hybridoma accessible under CNCM deposit number I-4401.

The invention relates to an antibody which comprises the 6 CDRs of mAb 7.2.

In another embodiment, the invention relates to a derivative of mAb 7.2 which comprises the VL chains and the VH chains of mAb 7.2.

In another embodiment, the invention relates to a derivative of mAb 7.2 which is a chimeric antibody, which comprises the variable domains of mAb 7.2.

The present invention relates to an isolated anti-CD277 antibody, which inhibits the cytolytic function, cytokine production and proliferation of Vγ9/Vδ2 T cells.

By inhibiting the cytolytic function of Vγ9/Vδ2 T cells, it is meant that a significant decrease of the cytotoxicity of Vγ9/Vδ2 T cells, i.e. a significant decrease of the specific lysis of the target cells by Vγ9/Vδ2 T cells, is observed, as compared to control Vγ9/Vδ2 T cells (i.e. non stimulated and non treated).

By inhibiting the cytokine production by Vγ9/Vδ2 T cells, it is meant that a significant decrease of the cytokine production by Vγ9/Vδ2 T cells is observed, as compared to control Vγ9/Vδ2 T cells (i.e. non stimulated and non treated).

By inhibiting the proliferation of Vγ9/Vδ2 T cells, it is meant that a significant decrease of the proliferation of Vγ9/Vδ2 T cells is observed, as compared to control Vγ9/Vδ2 T cells (i.e. non stimulated and non treated).
Typically the inhibition of the cytolytic function of \( V_{y9/V52} \) T cells may be measured according to the method described in example 3 (i.e. "Analysis of \( V_{y9/V52} \) T cell responses by direct cytotoxicity assay"). An example of an isolated anti-CD277 antibody, which inhibits the cytolytic function, cytokine production and proliferation of \( V_{y9/V52} \) T cells, is mAbs 103.2, or derivatives thereof.

The present invention also relates to an isolated anti-CD277 antibody (mAb 103.2) which is obtainable from the hybridoma accessible under CNCM deposit number I-4403.

The present invention relates to the hybridoma accessible under CNCM deposit number I-4403.

The invention relates to an antibody which comprises the 6 CDRs of mAb 103.2.

In another embodiment, the invention relates to a derivative of mAb 103.2 which comprises the VL chains and the VH chains of mAb 103.2.

In another embodiment, the invention relates to a derivative of mAb 103.2 which is a chimeric antibody, which comprises the variable domains of mAb 103.2.

In an embodiment, an antibody of the invention is a monoclonal antibody.

In an embodiment, an antibody of the invention is a chimeric antibody.

In an embodiment, an antibody of the invention is a humanized antibody.

A further embodiment of the invention relates to a nucleic acid sequence encoding an antibody of the invention.

In a particular embodiment, the invention relates to a nucleic acid sequence encoding the VH domain or the VL domain of an antibody of the invention. Typically, said nucleic acid is a DNA or RNA molecule, which may be included in any suitable vector, such as a plasmid, cosmid, episome, artificial chromosome, phage or a viral vector.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (e.g. a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (e.g. transcription and translation) of the introduced sequence. So, a further object of the invention relates to a vector comprising a nucleic acid of the invention.
Such vectors may comprise regulatory elements, such as a promoter, enhancer, terminator and the like, to cause or direct expression of said antibody upon administration to a subject. Examples of promoters and enhancers used in the expression vector for animal cell include early promoter and enhancer of SV40, LTR promoter and enhancer of Moloney mouse leukemia virus, promoter and enhancer of immunoglobulin H chain and the like.

Any expression vector for animal cell can be used, so long as a gene encoding the human antibody C region can be inserted and expressed. Examples of suitable vectors include pAGE107, pAGE103, pHSG274, pKCR, pSGI beta d2-4- and the like.

Other examples of plasmids include replicating plasmids comprising an origin of replication, or integrative plasmids, such as for instance pUC, pcDNA, pBR, and the like. Other examples of viral vector include adenoviral, retroviral, herpes virus and AAV vectors. Such recombinant viruses may be produced by techniques known in the art, such as by transfecting packaging cells or by transient transfection with helper plasmids or viruses. Typical examples of virus packaging cells include PA317 cells, PsiCRIP cells, GPen+ cells, 293 cells, etc. Detailed protocols for producing such replication-defective recombinant viruses may be found for instance in WO 95/14785, WO 96/22378, US 5,882,877, US 6,013,516, US 4,861,719, US 5,278,056 and WO 94/19478.

A further object of the present invention relates to a cell which has been transfected, infected or transformed by a nucleic acid and/or a vector according to the invention. The term "transformation" means the introduction of a "foreign" (i.e. extrinsic or extracellular) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. A host cell that receives and expresses introduced DNA or RNA has been "transformed". The nucleic acids of the invention may be used to produce an antibody of the invention in a suitable expression system. The term "expression system" means a host cell and compatible vector under suitable conditions, e.g. for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell.

Common expression systems include E. coli host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors. Other examples
of host cells include, without limitation, prokaryotic cells (such as bacteria) and eukaryotic cells (such as yeast cells, mammalian cells, insect cells, plant cells, etc.). Specific examples include E. coli, Kluyveromyces or Saccharomyces yeasts, mammalian cell lines (e.g., Vero cells, CHO cells, 3T3 cells, COS cells, etc.) as well as primary or established mammalian cell cultures (e.g., produced from lymphoblasts, fibroblasts, embryonic cells, epithelial cells, nervous cells, adipocytes, etc.). Examples also include mouse SP2/0-Agl4 cell (ATCC CRL1581), mouse P3X63-Ag8.653 cell (ATCC CRL1580), CHO cell in which a dihydrofolate reductase gene (hereinafter referred to as "DHFR gene") is defective, rat YB2/3HL.P2.G11.16Ag.2O cell (ATCC CRL1662, hereinafter referred to as "YB2/0 cell"), and the like.

The present invention also relates to a method of producing a recombinant host cell expressing an antibody according to the invention, said method comprising the steps of:

(i) introducng in vitro or ex vivo a recombinant nucleic acid or a vector as described above into a competent host cell,
(ii) culturing in vitro or ex vivo the recombinant host cell obtained, and
(iii) optionally, selecting the cells which express and/or secrete said antibody. Such recombinant host cells can be used for the production of antibodies of the invention.

Methods of producing antibodies of the invention

Antibodies of the invention may be produced by any technique known in the art, such as, without limitation, any chemical, biological, genetic or enzymatic technique, either alone or in combination.

Knowing the amino acid sequence of the desired sequence, one skilled in the art can readily produce said antibodies, by standard techniques for production of polypeptides. For instance, they can be synthesized using well-known solid phase method, preferably using a commercially available peptide synthesis apparatus (such as that made by Applied Biosystems, Foster City, California) and following the manufacturer's instructions. Alternatively, antibodies of the invention can be synthesized by recombinant DNA techniques well-known in the art. For example, antibodies can be
obtained as DNA expression products after incorporation of DNA sequences encoding the antibodies into expression vectors and introduction of such vectors into suitable eukaryotic or prokaryotic hosts that will express the desired antibodies, from which they can be later isolated using well-known techniques.

In particular, the invention further relates to a method of producing an antibody of the invention, which method comprises the steps consisting of:
(i) culturing a transformed host cell according to the invention under conditions suitable to allow expression of said antibody; and
(ii) recovering the expressed antibody.

In another particular embodiment, the method comprises the steps of:
(i) culturing the hybridoma deposited as CNCM I-4401, CNCM I-4402 or CNCM I-4403 under conditions suitable to allow expression of the antibody; and
(ii) recovering the expressed antibody.

Antibodies of the invention are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

In a particular embodiment, the human chimeric antibody of the present invention can be produced by obtaining nucleic sequences encoding VL and VH domains as previously described, constructing a human chimeric antibody expression vector by inserting them into an expression vector for animal cell having genes encoding human antibody CH and human antibody CL, and expressing the coding sequence by introducing the expression vector into an animal cell. As the CH domain of a human chimeric antibody, it may be any region which belongs to human immunoglobulin, but those of IgG class are suitable and any one of subclasses belonging to IgG class, such as IgG1, IgG2, IgG3 and IgG4, can also be used. Also, as the CL of a human chimeric antibody, it may be any region which belongs to Ig, and those of kappa class or lambda class can be used. Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques are well known in the art (See patent documents US5,202,238; and US5,204,244).
The humanized antibody of the present invention may be produced by obtaining nucleic acid sequences encoding CDR domains, as previously described, constructing a humanized antibody expression vector by inserting them into an expression vector for animal cell having genes encoding (i) a heavy chain constant region identical to that of a human antibody and (ii) a light chain constant region identical to that of a human antibody, and expressing the genes by introducing the expression vector into an animal cell.

The humanized antibody expression vector may be either of a type in which a gene encoding an antibody heavy chain and a gene encoding an antibody light chain exists on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a humanized antibody expression vector, easiness of introduction into animal cells, and balance between the expression levels of antibody H and L chains in animal cells, humanized antibody expression vector of the tandem type is preferred. Examples of tandem type humanized antibody expression vector include pKANTEX93 (WO 97/10354), pEE18 and the like.

Methods for producing humanized antibodies based on conventional recombinant DNA and gene transfection techniques are well known in the art. Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596), and chain shuffling (U.S. Pat. No.5,565,332). The general recombinant DNA technology for preparation of such antibodies is also known (see European Patent Application EP 125023 and International Patent Application WO 96/02576).

The Fab of the present invention can be obtained by treating an antibody which specifically reacts with CD277 with a protease, papaione. Also, the Fab can be produced by inserting DNA encoding Fab of the antibody into a vector for prokaryotic expression system, or for eukaryotic expression system, and introducing the vector into a procaryote or eucaryote (as appropriate) to express the Fab.

The F(ab')2 of the present invention can be obtained treating an antibody which specifically reacts with CD277 with a protease, pepsin.
Also, the F(ab')2 can be produced by binding Fab' described below via a thioether bond or a disulfide bond.

The Fab' of the present invention can be obtained by treating F(ab')2 which specifically reacts with human CD277 with a reducing agent, dithiothreitol. Also, the Fab' can be produced by inserting DNA encoding Fab' fragment of the antibody into an expression vector for prokaryote, or an expression vector for eukaryote, and introducing the vector into a prokaryote or eukaryote (as appropriate) to perform its expression.

The scFv of the present invention can be produced by obtaining cDNA encoding the VH and VL domains as previously described, constructing DNA encoding scFv, inserting the DNA into an expression vector for prokaryote, or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote (as appropriate) to express the scFv. To generate a humanized scFv fragment, a well known technology called CDR grafting may be used, which involves selecting the complementary determining regions (CDRs) from a donor scFv fragment, and grafting them onto a human scFv fragment framework of known three dimensional structure (see, e.g., WO98/45322; WO 87/02671; US5,859,205; US5,585,089; US4,816,567; EP0173494).

Amino acid sequence modification(s) of the antibodies described herein are contemplated. For example, it may be desirable to improve the binding affinity and/or other biological properties of the antibody. It is known that when a humanized antibody is produced by simply grafting only CDRs in VH and VL of an antibody derived from a non-human animal in FRs of the VH and VL of a human antibody, the antigen binding activity is reduced in comparison with that of the original antibody derived from a non-human animal. It is considered that several amino acid residues of the VH and VL of the non-human antibody, not only in CDRs but also in FRs, are directly or indirectly associated with the antigen binding activity. Hence, substitution of these amino acid residues with different amino acid residues derived from FRs of the VH and VL of the human antibody would reduce of the binding activity.

In order to resolve the problem, in antibodies grafted with human CDR, attempts have to be made to identify, among amino acid sequences of the FR of the VH and VL of
human antibodies, an amino acid residue which is directly associated with binding to the antibody, or which interacts with an amino acid residue of CDR, or which maintains the three-dimensional structure of the antibody and which is directly associated with binding to the antigen. The reduced antigen binding activity could be increased by replacing the identified amino acids with amino acid residues of the original antibody derived from a non-human animal.

Modifications and changes may be made in the structure of the antibodies of the present invention, and in the DNA sequences encoding them, and still obtain a functional molecule that encodes an antibody with desirable characteristics. In making the changes in the amino sequences, the hydrophobic index of amino acids may be considered. The importance of the hydrophobic amino acid index in conferring interactive biologic function on a protein is generally understood in the art. It is accepted that the relative hydrophobic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydrophobic index on the basis of their hydrophobicity and charge characteristics these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophane (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

A further embodiment of the present invention also encompasses function-conservative variants of the antibodies of the present invention. "Function-conservative variants" are those in which a given amino acid residue in a protein or enzyme has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, polarity, hydrogen bonding potential, acidic, basic, hydrophobic, aromatic, and the like).

Amino acids other than those indicated as conserved may differ in a protein so that the percent protein or amino acid sequence similarity between any two proteins of similar
function may vary and may be, for example, from 70 % to 99 % as determined according to an alignment scheme such as by the Cluster Method, wherein similarity is based on the MEGALIGN algorithm.

A "function-conservative variant" also includes a polypeptide which has at least 60 % amino acid identity as determined by BLAST or FASTA algorithms, preferably at least 75 %, more preferably at least 85%, still preferably at least 90 %, and even more preferably at least 95%, and which has the same or substantially similar properties or functions as the native or parent protein to which it is compared. Two amino acid sequences are "substantially homologous" or "substantially similar" when greater than 80 %, preferably greater than 85 %, preferably greater than 90 % of the amino acids are identical, or greater than about 90 %, preferably greater than 95 %, are similar (functionally identical) over the whole length of the shorter sequence. Preferably, the similar or homologous sequences are identified by alignment using, for example, the GCG (Genetics Computer Group, Program Manual for the GCG Package, Version 7, Madison, Wisconsin) pileup program, or any of sequence comparison algorithms such as BLAST, FASTA, etc.

For example, certain amino acids may be substituted by other amino acids in a protein structure without appreciable loss of activity. Since the interactive capacity and nature of a protein define the protein's biological functional activity, certain amino acid substitutions can be made in a protein sequence, and, of course, in its DNA encoding sequence, while nevertheless obtaining a protein with like properties. It is thus contemplated that various changes may be made in the antibodies sequences of the invention, or corresponding DNA sequences which encode said antibodies, without appreciable loss of their biological activity.

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydrophobic index or score and still result in a protein with similar biological activity, i.e. still obtain a biological functionally equivalent protein. As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like.
Exemplary substitutions which take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine. Another type of amino acid modification of the antibody of the invention may be useful for altering the original glycosylation pattern of the antibody.

By "altering" is meant deleting one or more carbohydrate moieties found in the antibody, and/or adding one or more glycosylation sites that are not present in the antibody.

Glycosylation of antibodies is typically N-linked. "N-linked" refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. Addition of glycosylation sites to the antibody is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above- described tripeptide sequences (for N-linked glycosylation sites). Another type of covalent modification involves chemically or enzymatically coupling glycodies to the antibody. These procedures are advantageous in that they do not require production of the antibody in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. For example, such methods are described in WO87/05330.

Removal of any carbohydrate moieties present on the antibody may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the antibody to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the antibody intact.
Enzymatic cleavage of carbohydrate moieties on antibodies can be achieved by the use of a variety of endo-and exo-glycosidases.

Another type of covalent modification of the antibody comprises linking the antibody to one of a variety of non proteinaceous polymers, eg., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in US Patent Nos. 4,640, 835; 4,496, 689; 4,301, 144; 4,670, 417; 4,791, 192 or 4,179,337. It may be also desirable to modify the antibody of the invention with respect to effector function, e.g. so as to enhance antigen-dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) of the antibody. This may be achieved by introducing one or more amino acid substitutions in an Fc region of the antibody. Alternatively or additionally, cysteine residue(s) may be introduced in the Fc region, thereby allowing inter-chain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and/or antibody-dependent cellular cytotoxicity (ADCC) (Caron PC. et al. J Exp Med. 1992 Oct 1;176(4):1191-5 and Shopes B. J Immunol. 1992 May 1;148(9):2918-22).

20 Therapeutic uses of the antibodies of the invention

The inventors have demonstrated that some antibodies, like mAbs 20.1 and 7.2, activate the cytolytic function, cytokine production and proliferation of Vγ9/Vδ2 T cells, and thereby may be used to overcome the immunosuppressive mechanisms observed in cancer patients and during chronic infections. Some antibodies costimulate T cells together with CD3-TCR, or costimulate T cells in addition to CD28-B7 costimulation; said antibodies may be used in the same therapeutic applications. Some antibodies increase the survival of monocytes and dendritic cells; said antibodies may be used in the same therapeutic applications.

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The invention relates to mAbs 20.1 or 7.2 or a derivative thereof for the use in therapy. The invention relates to mAbs 20.1 or 7.2 or a derivative thereof for use for treating a cancer or a chronic infection.
The invention also relates to a method for treating a cancer or a chronic infection, wherein said method comprises the step of administering to a subject in need thereof a therapeutically effective amount of mAbs 20.1 or 7.2 or of a derivative thereof.

Examples of cancers include, but are not limited to, hematological malignancies such as B-cell lymphoid neoplasm, T-cell lymphoid neoplasm, non-Hodgkin lymphoma (NHL), B-NHL, T-NHL, chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), mantle cell lymphoma (MCL), NK-cell lymphoid neoplasm and myeloid cell lineage neoplasm.

Examples of non-hematological cancers include, but are not limited to, colon cancer, breast cancer, lung cancer, brain cancer, prostate cancer, head and neck cancer, pancreatic cancer, bladder cancer, colorectal cancer, bone cancer, cervical cancer, liver cancer, oral cancer, esophageal cancer, thyroid cancer, kidney cancer, stomach cancer, testicular cancer and skin cancer.

Examples of chronic infections include, but are not limited to, viral, bacterial, parasitic or fungal infections such as chronic hepatitis, lung infections, lower respiratory tract infections, bronchitis, influenza, pneumonias and sexually transmitted diseases. Examples of viral infections include include, but are not limited to, hepatitis (HAV, HBV, HCV), herpes simplex (HSV), herpes zoster, HPV, influenza (Flu), AIDS and AIDS related complex, chickenpox (varicella), common cold, cytomegalovirus (CMV) infection, smallpox (variola), Colorado tick fever, dengue fever, ebola hemorrhagic fever, foot and mouth disease, lassa fever, measles, marburg hemorrhagic fever, infectious mononucleosis, mumps, norovirus, poliomyelitis, progressive multifocal leukencephalopathy (PML), rabies, rubella, SARS, viral encephalitis, viral gastroenteritis, viral meningitis, viral pneumonia, West Nile disease and yellow fever.

Examples of bacterial infections include include, but are not limited to, pneumonia, bacterial meningitis, cholera, diphtheria, tuberculosis, anthrax, botulism, brucellosis, campylobacteriosis, typhus, gonorrhea, listeriosis, lyme disease, rheumatic fever, pertussis (Whooping Cough), plague, salmonellosis, scarlet fever, shigellosis, syphilis, tetanus, trachoma, tularemia, typhoid fever, and urinary tract infections.

Examples of parasitic infections include include, but are not limited to, malaria, leishmaniasis, trypanosomiasis, chagas disease, cryptosporidiosis, fascioliasis, filariasis, amebic infections, giardiasis, pinworm infection, schistosomiasis, taeniasis,
toxoplasmosis, trichinellosis, and trypanosomiasis. Examples of fungal infections include, but are not limited to, candidiasis, aspergillosis, coccidioidomycosis, cryptococcosis, histoplasmosis and tinea pedis.

mAbs 20.1 or 7.2 or a derivative thereof may be used as a vaccine adjuvant for the treatment of a cancer or a chronic infection.

The invention relates to a vaccine for the treatment of a cancer or a chronic infection comprising mAbs 20.1 or 7.2 or a derivative thereof.

The invention relates to a kit for the treatment of a cancer or a chronic infection comprising:

a) mAbs 20.1 or 7.2 or a derivative thereof; and

b) a vaccine for the treatment of a cancer or a chronic infection.

The two elements of the kit may be administered concomitantly or sequentially over time.

Examples of vaccine for the treatment of a cancer or a chronic infection include, but are not limited to vaccines against viral, bacterial, parasitic or fungal infections such as HIV and HBV and vaccines against viral associated cancers (for instance HPV or HBV) or anti cancer vaccines for instance used to treat patients with melanoma, leukemia, breast cancers, lung cancers.

Furthermore, the inventors have generated anti-CD277 antibodies, which inhibit the cytolytic function, the cytokine production and the proliferation of Vγ9/Vδ2 T cells. These anti-CD277 antibodies may be used as immunosuppressive agents.

In a further embodiment, the invention relates to an anti-CD277 antibody, which inhibits the cytolytic function, the cytokine production and the proliferation of Vγ9/Vδ2 T cells, for the use in therapy. In particular, the invention relates to an anti-CD277 antibody, the cytolytic function, the cytokine production and the proliferation of Vγ9/Vδ2 T cells, for the treatment of an autoimmune disease, transplantation rejection or a graft versus host disease.
The invention also relates to a method for treating an autoimmune disease, transplantation rejection or a graft versus host disease, wherein said method comprises the step of administering to a subject in need thereof a therapeutically effective amount of anti-CD277 antibody, which inhibits the cytolytic function, the cytokine production and the proliferation of Vy9/Vδ2 T cells.

Typically the anti-CD277 antibodies, which inhibit the cytolytic function, the cytokine production and the proliferation of Vy9/Vδ2 T cells, may be mAb 103.2 or a derivative thereof.

Examples of autoimmune diseases which may be treated include but are not limited to rheumatoid arthritis (RA), insulin dependent diabetes mellitus (Type 1 diabetes), multiple sclerosis (MS), Crohn's disease, systemic lupus erythematosus (SLE), scleroderma, Sjogren's syndrome, pemphigus vulgaris, pemphigoid, addison's disease, ankylosing spondylitis, aplastic anemia, autoimmune hemolytic anemia, autoimmune hepatitis, coeliac disease, dermatomyositis, Goodpasture's syndrome, Graves' disease, Guillain-Barre syndrome, Hashimoto's disease, idiopathic leucopenia, idiopathic thrombocytopenic purpura, male infertility, mixed connective tissue disease, myasthenia gravis, pernicious anemia, phacogenic uveitis, primary biliary cirrhosis, primary myxoedema, Reiter's syndrome, stiff man syndrome, thyrotoxicosis, ulcerative colitis, and Wegener's granulomatosis.

Typically an anti-CD277 antibody, which inhibits the cytolytic function, the cytokine production and the proliferation of Vy9/Vδ2 T cells, may be used in combination with other immunosuppressive and chemotherapeutic agents such as, but not limited to, prednisone, azathioprine, cyclosporin, methotrexate, and cyclophosphamide.

The invention also relates to pharmaceutical composition comprising an antibody of the invention.

Therefore, an antibody of the invention may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.
"Pharmacologically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous or intraocular administration and the like.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment. To prepare pharmaceutical compositions, an effective amount of the antibody may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.
Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

An antibody of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.
Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The preparation of more, or highly concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose.

These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject
being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The antibodies of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered. In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration; time release capsules; and any other form currently used.

In certain embodiments, the use of liposomes and/or nanoparticles is contemplated for the introduction of antibodies into host cells. The formation and use of liposomes and/or nanoparticles are known to those of skill in the art.

Nanocapsules can generally entrap compounds in a stable and reproducible way. To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1 µm) are generally designed using polymers able to be degraded in vivo. Biodegradable polyalkyl-cyanoacrylate nanoparticles that meet these requirements are contemplated for use in the present invention, and such particles may be easily made.

Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)). MLVs generally have diameters of from 25 nm to 4 µm.

Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core. The physical characteristics of liposomes depend on pH, ionic strength and the presence of divalent cations.

The invention will further be illustrated in view of the following figures and example.
FIGURES

Figure 1:
A: *BT3 receptors are constitutively expressed on the surface of monocytes and iDCs.*
The expression on freshly isolated monocytes and iDC using both clone 20.1 anti-BT3 mAb are shown. The experiment shown is representative of five donors studied (the intensity of fluorescence variation among the five experiments was <2%).
B: *BT3 ligation provides a survival signal for monocytes and iDC.*
Freshly isolated monocytes (*upper row*) and iDC (*lower row*) were stimulated with plastic-coated mAb (anti-CD19, and anti-BT3) or 20 ng/ml GM-CSF as indicated. After stimulation for 72 hours, cells were harvested and analyzed for Annexin V binding, as a marker of apoptotic cells. Numbers in the corners correspond to the percentage of positive cells for Annexin V. Data shown are representative of five independent experiments.

C: *BT3 engagement increases costimulatory molecules expression on the surface of monocytes.*

D: *BT3 engagement increases costimulatory molecules expression on the surface of iDC.*

Figure 2: *Regulation of AKT phosphorylation and ERK phosphorylation on CD277 activated CD4+ T cells.*
Purified CD4+ T cells from thawed PBMCs from 4 healthy donors stimulated or not with antibody-coated Epoxy dynabeads (1μg/ml of anti-CD3 plus various concentrations of anti-CD277.20.1clone or IgG1 (isotype control). Every 2, 5, 10 and 30 minutes, the intracellular phosphorylation of AKT and B, ERK on CD4+ T cells are measured by flow cytometry. Data are representative of four independent studies.
Results are represented as MFI (mean fluorescence intensity expression) on CD4+ T cells at different time points after treatments for 2-30 minutes.

Figure 3: *CD277 is a costimulatory molecule of CD4+ T cells*
CD4+ T cells were purified from PBMCs from 4 healthy donors. Purified CD4+ T cells were stimulated with antibody-coated Epoxy dynabeads (1μg/ml of anti-CD3 and 2μg/ml of anti-CD28 plus various concentrations of anti-CD277 (20.1) or IgG1 (isotype
control) Supernatants were collected on day 2 of culture for cytokine assay by ELISA. INFγ, IL-2 and IL-10 production assay.

**Figure 4:** Expression of CD277 in lymph nodes

Expression profiles of CD277 and PD1 in lymphoid organs. Living T cells from lymphoid tissue are distinguished by staining anti-CD14 (CD14 negative cells) and vivid. T follicular Helper cells were further gated using staining with mAbs CD4, ICOS and CXCR5. Results are presented by MFI (mean fluorescence intensity). Data are representative of 7 independent studies. The p values were calculated using the Mann-Witney paired test to compare differences between CD277 or PD1 expressions on T cells subsets.

* p < 0.05; ** 0.001 < p < 0.01; *** p < 0.001. p > 0.05 is not shown.

**Figure 5:** Anti-CD277 mAb 20.1 stimulates the expansion of Vγ9Vδ2 T cells

A: ex vivo expansion (15 days+IL-2): PBMC were stimulated by 20.1 mAb abd IL-2 and Vγ9Vδ2 T cells were monitored by staining with Vγ9Vδ2 T cells specific mAb.

B: CFSE assay (4 days+IL-2): PBMC were stimulated by 20.1 mAb abd IL-2 and Vγ9Vδ2 T cells proliferation was monitored CFSE analysis.

**Figure 6:** Activating (20.1) and inhibitory (103.2) CD277 mAbs regulating Vγ9Vδ2 T cells were monitored by staining with Vγ9Vδ2 cells specific mAb.

A: Antagonist CD77 mAb 103.2 inhibits the phosoantigen mediated activation of Vγ9Vδ2

B: Antagonist CD77 mAb 103.2 does not inhibit the CD3 mediated activation of Vγ9Vδ2

C: 103.2 BTN3 mAb inhibits phosoantigen-induced activation of Jurkat TCR Vγ9/Vδ2 T cells as determined by CD69 expression.

**Figure 7:** CD277 potentiate the anti-tumor cytolysis mediated by Vγ9Vδ2 T cells

Tumor cell lines were incubated with Vγ9Vδ2 with or without 20.1 agonist mAb. The activation of Vγ9Vδ2 was evaluated by CD107a/b degranulation.
EXAMPLE 1

Materials and methods

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Cell cultures

Blood samples were obtained from 5 healthy volunteers enrolled among the staff members, after informed consent. Monocytes were separated from peripheral blood mononuclear cells, and isolated using the MACS CD14 isolation kit (Miltenyi Biotech, Auburn, CA USA). Monocytes were cultured for 5 days with 200 ng/ml of recombinant human GM-CSF and 10 ng/ml recombinant human IL-4 (Schering-Plough Research Institute). These cells were termed immature dendritic cells (IDCs). To achieve the stimulation, monocytes and IDCs were cultured with 10 ng/ml of LPS, ligand for TLR4, 30 µg/ml of R848 (Sigma-Aldrich, Milano, Italy), ligand for TLR7/8 and with 2.5 mg/ml of poly (I:C) (Sigma-Aldrich), ligand for TLR3, and were harvested after 48h. The cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 5mM L-glutamine and 50 IU/ml penicillin-streptomycin (from here on referred to as complete medium).

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Flow cytometry and Antibodies

Monocytes and IDCs before and after stimulation were analysed by immunofluorescence flow cytometry (FACScalibur Becton Dickinson, Milano, Italy) to verify their activation state. To this end, mAb specific for CD80, CD86, HLA-DR, CD1a, CD14 (BD Becton Dickinson), BT3 (clone 20.1, IgG1) and BT3 Fab2 were previously selected, purified and characterised (Bensussan and Olive, 2005, Compte et al., 2004) or irrelevant molecules (anti-CD19, Becton Dickinson, and anti-CD31, clone Moon-1, provided by F. Malavasi, both IgG1) were used. All the available anti-BT3 mAbs are unable to discriminate among the three members of this family (BT3.1, BT3.2, or BT3.3), probably due to their high sequence homology (Compte et al., 2004, Bensussan and Olive, 2005). As further control, to block the possible effect of trace amounts of endotoxin, polymyxin B (10 µg/ml, Sigma-Aldrich) was added to the anti-BT3 mAb 20.1 before adding the cells.

As loading control for Western blot analyses, anti-tubulin mAb (clone: 6-11B-1, IgG1, Invitrogen, Milano, Italy) was used.
**Apoptosis detection**

Monocytes and iDC were cultured on plastic coated with anti-BT3 mAb (clone 20.1) or with isotype-matched irrelevant mAb. As control, the cells were stimulated with 20 ng/ml recombinant human GM-CSF (Schering-Plough Research Institute). After three days, cells were harvested, stained with Annexin V-FITC (Bender MedSystems, Wien, Austria) and analysed by flow cytometry.

**Quantitative RT-PCR**

qRT-PCR analysis was performed with the Applied Biosystems 7900HT Fast Real-Time PCR system using Taqman detection. Briefly, total RNA was isolated from THP-1 cells using the standard TRIzol reagent protocol (Invitrogen Life Technologies). 2 µg of the obtained RNA was reverse transcribed using oligo(dT). Each PCR assay was performed in a 25 µl reaction containing 2x TaqMan universal Mix (Applied Biosystems) reagent, 20X primers and 2 µl cDNA (equivalent to 40 ng of total RNA). Thermal cycle conditions were 95 °C for 15 s and 60 °C for 60 s (40 cycles). Capture of fluorescence was recorded on the ABI Prism 7900HT scanner and the Ct (threshold cycle) was calculated for each assay (Sequence Detection System Software 2.3, Applied Biosystems). Data are normalized using GAPDH as endogenous control ($\Delta$Ct = Ct\text{target} - Ct\text{GAPDH}). A higher $\Delta$Ct correspond to a lower expression of the analysed gene. BT3.1, BT3.2, BT3.3 and GAPDH TaqMan Gene Expression assays were purchased from Applied Biosystems.

**Statistical analyses**

Statistical analysis was performed by GraphPad Prism4 software 4.0 (GraphPad Software Inc., CA, USA) using the Student’s t test. For the evaluation of cytokine production, data were analyzed with one-way analysis of variance (ANOVA). For the evaluation of apoptosis induction, data were analyzed with a non-parametric test. The significance level was set at $P<0.05$. 


Results

BT3 receptors are constitutively expressed on the surface of monocytes and iDCs.

In order to investigate the expression of BT3, we chose a panel of healthy donors to obtain monocytes and iDC. BT3 expression was evaluated on freshly isolated monocytes and iDC using clone 20.1 anti-BT3 mAb (Figure 1A). Figure 1A shows BT3 expression on freshly isolated monocytes and iDC using clone 20.1 anti-BT3 mAb. The differentiation of monocytes in DC was verified by expression of cell surface markers: CD14 was down-regulated and remained low in both immature and mature DC, whereas CD1a and HLA-DR was up-regulated (data not shown). The experiment shown is representative of five on different donors. The level of expression of BT3 was slightly higher on monocytes. In addition, we analysed the expression of BT3 following stimulation of these cells with LPS, poly (I:C) and R848, ligands for TLR4, TLR3 and TLR7/8, respectively. The activation via TLR (Toll Like Receptors) did not significantly alter the cell surface expression of BT3 (data not shown). We can derive that BT3 receptors are constitutively expressed on the surface of monocytes and iDC, independently on their activation state.

Finally, the qRT-PCR analysis confirmed the immunophenotypic results showing that all the three receptors of the BT3 family are expressed by THP-1 cells. In detail, we found BT3.2 as the most represented one (ten times more BT3.2 than BT3.1 and BT3.3) (data not shown).

BT3 ligation provides a survival signal for monocytes and iDC in culture.

As BT3 is a stably expressed receptor family, we investigated the ability of BT3 engagement to modify the life span of ex vivo monocytes and iDC cultured in the absence of survival factors (i.e. completed medium with serum) for 72 hours. Cell stimulation by plastic-coated mAb was used to engage BT3 on the surface of monocytes and iDC. Approximately 77.98% of monocytes treated with the mAb specific
for BT3 survived in the absence of endogenous survival factors for 3 days (range 51.13-77.98%, \( p=0.0040 \)) (Figure 1B, upper row, shows a representative experiment of five). This effect was slightly lower than that obtained by GM-CSF treatment (81.22%, range 60.41-83%, \( p=0.0278 \)). In control cultures, i.e. in the presence of plastic-coated irrelevant mAb (anti-CD19 mAb), the survival effect was minimal and a very high proportion of apoptotic monocytes (Annexin-V-positive cells) was observed (about 95.48% of apoptotic cells was measured in medium alone and about 88.79% in plastic-coated irrelevant mAb condition, survival range 0.8-11.74% and 1.08-11.21% respectively, \( p \) N.S.). Similar results were obtained by analysing the effect on iDC survival (Figure 1B, lower row, shows a representative experiment of five). Activation of iDC via BT3 was able to partially inhibit iDC apoptosis during a 72-h culture period (56.75% of healthy-vital cells versus 85.97% in the survival GM-CSF treatment, range 53.13-60.71%, \( p=0.0044 \), and 71.01-85.97%, \( p=0.0040 \) respectively). Irrelevant isotype-matched mAb did not alter the naturally occurring apoptosis of DC (if we compare medium alone and anti-CD19 mAb condition of a representative experiment of five, range 0.8-24.12% and 0.9-26.97% of surviving cells respectively, \( p=0.3452 \)). Altogether, these results suggest that BT3 receptors are able to promote monocytes and iDC survival and to extend the duration of cells responses by attenuating apoptosis. For both cell types, the survival differences following anti-BT3 versus control isotype-matched mAb was statistically significant (as for GM-CSF treatment). In addition, a dose response effect of anti-BT3 mAb on survival of both monocytes and iDC is apparent (data not shown). The lowest anti-BT3 mAb dilution able to promote a good survival capability was 10\( \mu \)g/ml.

BT3 engagement increases costimulatory molecules expression on the surface of monocytes and iDC.

To investigate the role of BT3 in primary inflammatory responses, freshly isolated blood monocytes and monocyte-derived dendritic cells were stimulated by mAb, coated on tissue culture plates, or by TLR ligands known to stimulate monocytes and dendritic cells (LPS). After 24h stimulation, coated anti-BT3 mAb was able to trigger the activation of monocytes, as shown by up-regulation of the cell surface costimulatory molecule CD86 (Figure 1C). The isotype-matched control mAb (whose antigen is not
expressed on these cells), had no significant effect. Thus, the activation observed using anti-BT3.1 mAb was specific and not due to the engagement of FcR. Cross-linking was required for cellular activation, because no effect was observed with non-coated (soluble) anti-BT3 mAb (data not shown). In addition, the activating effect of anti-BT3 mAb was maintained in the presence of polymyxin B, thus excluding the role of trace levels of endotoxins.

Interestingly, CD86 up-regulation upon BT3 stimulation was similar to that observed with TLR ligand LPS. BT3 stimulation also triggered up-regulation at the monocyte surface of CD80 and HLA-DR molecules (Figure 1C). Consistently, we found increased expression of CD80, CD86 and HLA-DR on iDC (Figure 1D). In addition, similar results were obtained using anti-BT3 Fab mAb (as can be depicted from figures 1C and 1D on monocytes and DC respectively). These results allow us to rule out that a signal through FcRs cannot be responsible of cells activation. The irrelevant mAb (anti-CD19) was a negative control, whereas stimulation via TLR4 ligand for LPS was a positive control.

EXAMPLE 2

Materials and methods

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteer donors provided by the “Etablissement Français du Sang” (EFS-Marseille-France) and isolated by fractionation over a density gradient of Lymphoprep® (Abcys). Human CD4⁺ T cells were negatively selected from isolated PBMCs by depletion of non-CD4⁺ T cells with magnetic beads using the T cell isolation kit II from Miltenyi Biotec®. Isolated CD4 cells were used for further experiments when purity was superior to 90%.

Generation of monoclonal antibodies (mAbs)
The mouse anti-human programmed death-1 (PD-1) mAb and three different clones of mouse anti-human CD277 were purified from ascites in our laboratory: Anti-PD-1
(clone PD1 3.1 with an IgG1 isotype) (ghiotto et al. Int Immunol., 2010), Anti-CD277 (clone 103.2 with an IgG2a isotype, clones 20.1 and 7.2 both with an IgG1 isotype) (compte et al.). The anti-CD277 (clone 20.1) mAb was labelled with Alexa Fluor 647 using a commercial kit (Invitrogen, Eugene, OR).

Expression profile of CD277 on T cells subpopulations

Thawed human PBMCs from four healthy donors were stained with 5µl of the following mouse anti-human monoclonal antibodies per million of cells: ECD-conjugated anti-CD3, PC5-conjugated anti-CD14, PC5-conjugated anti-CD19 (to select CD3⁺CD14⁺CD19⁻ cells) (all from Beckman Coulter, Marseilles, France), Pacific Blue-conjugated anti-CD4, Alexa700-conjugated anti-CD8 (all from BD Pharmingen (San Diego, USA)), APC-Alexa750-conjugated anti-CD27 (from Caltag, Invitrogen, USA), PC7-conjugated anti-CD45RA (from BD Biosciences), Alexa647-conjugated anti-CD277 (clone 20.1, homemade). APC-conjugated IgG1 (Beckman Coulter) was used as control and LIVE/DEAD Fixable Dead Cell Stain Kit was used for viability. Cells were incubated 20 minutes at 4°C then washed twice in phosphate-buffered saline (PBS, Lonza) fixed with 2% paraformaldehyde, and analyzed on a FACSARia flow cytometer (BD Biosciences). Data were analyzed using FlowJo Software (TreeStar, Ashland, OR).

Kinetic of CD277’s expression profile on naive CD4⁺ T cells

Purified CD4⁺ T cells (200 X 10^3 cells/well) from thawed human PBMCs were cultured during 96h in RPMI 1640 10% FBS in flat bottom 96-well plates (Microtest™ 96, Becton Dickinson) with previously plate-immobilized mouse anti-human CD3/CD28 or not (unstimulated). Anti-CD3 (clone OKT3) and anti-CD28 (clone CD28.2, home-made) were used at 0.3µg/ml and 10µg/ml respectively. Cells were placed into an atmosphere of 5% CO₂ at 37°C in a humidified incubator. Every 24h cells were transferred in a conic bottom 96-well plate (Nunc™, Denmark) and stained with the following mAbs 30 minutes at 4°C: 3µl of purified anti-PD-1 (clone PD-1 3.1, home-made) (giotto et al, int immunol. 2010), washed 3 times in PBS- FBS 0.2%- azide 0.02%, then stained with PE-conjugated goat anti-mouse (1/80, Beckman Coulter), washed and stained with 3µl of each following mAbs 30 minutes at 4°C: PC7-conjugated anti-CD4, FITC-conjugated anti-CD3 (all from BD Biosciences) Alexa647-conjugated anti-CD277 and 6µl of 7-AAD (BD Biosciences) for viability. Purified IgG1 or APC-conjugated IgG1 were used as
controls. Immunostained cell samples fixed with 2% paraformaldehyde were analyzed on a BD FACS Canto (BD Bioscience). Data were analyzed using FlowJo Software (TreeStar, Ashland, OR).

5 **Expression of CD277 in lymph nodes**

Cells teased from lymph nodes were collected from 7 patients who had given informed consent. Mononuclear cells were obtained by crushing fresh tissue samples in RPMI 1640 10% FBS. Detection of Folliculat T Helper cells (TFH) cells was performed by incubation for 20 minutes at 4°C with PE-conjugated anti-ICOS, biotinylated anti-CXCR5 (all from BD Biosciences), PC5-conjugated anti-CD14, Pacific blue-conjugated anti-CD4 (all from Beckman Coulter) and LIVE/DEAD Fixable Dead Cell Stain Kit®. Cells were then washed in PBS and incubated with anti–biotin–allophycocyanin–Alexa Fluor 750 (Invitrogen, Carlsbad, CA) for 20 minutes at 4°C. After staining, each cell preparation was washed twice in PBS, fixed with 2% paraformaldehyde, and analyzed on a FACSAria flow cytometer (BD Biosciences). Data were analyzed using FlowJo Software (TreeStar, Ashland, OR).

**Expression profile of CD277 on NK cells**

Thawed alive NK cells (Live dead® negative cells) were selected based on the expression of CD56+CD3- from healthy human PBMCs after 20 minutes incubation at 4°C with the anti-CD277-Alexa647 (clone 20.1). Cells were further washed twice in PBS (PBS, Lonza), then fixed with 2% formaldehyde and analyzed on a FACSAria® flow cytometer (BD Biosciences). Data were analyzed using FlowJo Software (TreeStar, Ashland).

25 **Functional assays on CD4+ T cells using plate-immobilized mAbs**

Purified CD4+ T cells (200 X 10^3 cells/well) from thawed human PBMCs were cultured in RPMI 1640 10% FBS in flat bottom 96-well plates (Microtest™ 96, BD) with previously plate-immobilized mouse anti-human CD3 (clone OKT3)/CD28 (clone CD28.2) or anti-CD3/anti-CD277 (clone 20.1) or anti-CD3/isotypic control (IgG1). Purified anti-CD3 was used at 0, 3μg/ml. Anti-CD28, anti-CD277 and isotypic control were used at 10μg/ml. Cells were placed into an atmosphere of 5% CO2 at 37°C in a humidified incubator. After 2 days of culture, cytokines production (Interleukine-2, IL-2 and Interferon gamma, IFN-γ) was measured by ELISA assay according to the
manufacturer's protocol (OptEIA, human IFN-γ or IL-2 Set, BD Pharmingen). After 5
days, cells were stained with 3µl of PE-conjugated anti-CD25 (BD Biosciences), and
5µl of 7-AAD for 30 minutes at 4°C then washed twice in PBS, fixed with 2%
paraformaldehyde and analyzed on a BD FACS Canto (BD Bioscience). Data were
analyzed using FlowJo Software (TreeStar, Ashland, OR).

Functional assay on CD4+ T cells with aAPC and carboxyfluorescein diacetate
succinimidyl diester (CFSE) labeling
Human CD4+ T cells were purified by negative selection from PBMCs using magnetic
beads (Miltenyi Biotec) according to the manufacturer's protocol. CD4+ T cells were
routinely more than 97% pure.
CD4+ T cells were labelled with 0.5µM CFSE (Invitrogen) for 10 min at 37°C, washed
and stimulated (1.5x10^5 cells/well) with aAPC at a ratio of 1:1 (cells to beads)
comprised of magnetic beads in triplicate in 96-well round-bottom plates (Falcon; BD
Biosciences). Cultures were incubated at 37°C, 5% CO2 for 5 days and then
proliferation of CFSE labelled CD4+ T cells were measured by flow cytometry (FACS
Canto, Beckman Coulter).

Functional assay on NK cells, cytolytic activity.
Fresh NK cells were sorted with Easy Sep® negative selection kit and incubated over
night in medium completed with sub-optimal concentrations of IL-2 (100 U/ml) and IL-
15 (10ng/ml). NK cell receptors functions were tested in re-directed cytolytic
experiments against the FcγR positive P815 mastocytoma murine cell line. Briefly,
effector cells were incubated with P815 cells pre-coated for 30 minutes with the mAb of
interest (irrelevant mouse IgG1: 11 µg/ml, anti-NKp46: 1µg/ml, anti-DNAM: 5 µg/ml,
anti-CD277 20.1: 10 µg/ml) according to a 1:1 Effector:Target (E/T) ratio. Cytotoxic
tests were performed in 4-hours assays in the presence of GolgiStop® and soluble
CD107 (a&b)-FITC (both from BD Biosciences), afterward cells were stained for
surface markers (CD56-PeCy7 (Beckman Coulter, Immunotech), fixed and
permeabilized (Cytofix/Cytoperm®) then stained with intracellular mAb (IFN-γ
(Beckman Coulter, Immunotech)). Cells were finally re-suspended in PBS 2% para-
formaldehyde and extemporaneously analyzed on a BD FACS Canto® (BD
Biosciences, San Jose, CA). The degree of activation of NK cells was measured based
on the percentage of cells positive for CD107a and CD107b (degranulation) and/or the production of inflammatory cytokine (IFN-γ).

*Artificial APC (aAPC)*

Magnetic beads (Dynabeads M-450 Epoxy, Dynal Biotech) were coated with the following mAbs as described in Serriari et al. (serriari, ji 2010): anti-CD3 (OKT3), anti-human CD28 (CD28.2), and/or various concentrations of anti-human CD277 (CD277, 20.1) or IgG1 or anti-MHC class I (MHC I) (YJ4) or IgG1. These aAPCs were coated with suboptimal anti-CD3 Ab (5%), suboptimal levels of anti-CD28 Ab (10%), and either IgG1 Ab (CD3/CD28/IgG1), anti-CD277 Ab (CD3/CD28/CD277 + IgG1) or anti-MHC class I (CD3/28/ anti-MHC class I + IgG1), constituting the remaining 85% of protein added to the bead. The amount of protein was kept constant at 20 mg/ml by the addition of control IgG1.

*ELISA for cytokine analysis*

To determine the production of cytokines, cell-free supernatants were collected at 48 h and assayed for IL-2, IL-10 and IFN-γ by ELISA using OptEIA kits (BD Pharmingen) according to the manufacturer’s instructions.

*Immunohistochemistry*

CD277 immunostainings were performed on total frozen sections of reactive lymph nodes as previously described (25). The final dilution for CD277.20.1 mAbs was 1/800. Negative control samples were prepared by omitting the primary mAb.

*Screening of the different btn3a isoforms transcripts in PBMC from healthy subjects*

Public and personal Affymetrix U133+2 data sets of purified CD8, CD4, GD et NK cells were collected. CD8 and CD4 data were retrieved from the public GEO datasets (Sharp et al.) (http://www.ncbi.nlm.nih.gov/gds), while NK and gamma delta sets were personal. We used Robust Multichip Average (RMA) with the non-parametric quantile algorithm as normalization parameter. RMA was applied to the raw data collected from the various series. Quantile normalization and Loess’ correction were done in R using Bioconductor and associated packages. The probe set corresponding to the three isoforms of BTN3A were retrieved from the normalized data sets and the corresponding log values were linearized for graphical representation. We used the
respective Affymetrix probesets corresponding to BTN3A1, BTN3A2 and BTN3A3 isoforms: 201623_s_at, 213282_at, 204171_at

Stimulation, phosphoflow and FACS staining

Human CD4⁺ T cells were purified by negative selection from PBMCs using magnetic beads (Miltenyi Biotec) according to the manufacturer’s protocol. CD4 T cells were routinely more than 97% pure. Cells were incubated 24 hours in RPMI 1640 10% FBS at 37°C.

CD4⁺ T cells were washed and stimulated at different times (2, 5, 10 and 30 minutes) with aAPC at a ratio of 1:3 (cells to beads) comprised of magnetic beads (Dynabeads M-450 Epoxy, Dynal Biotech) were coated with the following mAbs: anti-CD3 (OKT3), anti-human CD28 (CD28.2), and/or various concentrations of anti-human CD277 (CD277.20.1 clone) or isotype control IgG1. These aAPCs were coated with suboptimal anti-CD3 Ab (5%), suboptimal levels of anti-CD28 Ab (10%), and either IgG1 Ab (CD3/CD28/IgG1), anti-CD277.20.1 Ab (CD3/CD28/CD277.20.1 + IgG1), constituting the remaining 85% of protein added to the bead. The amount of protein was kept constant at 20 mg/ml by the addition of control IgG1.

We examined the effect of mAbs CD277 clones engagement on AKT and ERK phosphorylation in CD4⁺ T cells by phosphoflow proteomic method [27], which uses state-specific antibodies to detect target phosphoproteins by fluorescence-activated cell sorting (FACS). We assessed the activity of the signalling pathways after exposure of the cells to beads stimulations. We apply a sandwich-labeling method, which involves the application of a biotin-conjugated secondary antibody followed by detection with fluorescently conjugated streptavidin. We demonstrated the activity of the PI3K-pathway by measuring the phosphorylation of AKT at SER 473 and ERK at p44/42 MAPK T (202/y204) after stimulation with beads with different doses of the CD277 mAb and at four different times (2, 5, 10 and 30 minutes). Cells were permeabilized, fixed and analyzed after intracellular staining of the target phosphoproteins (AKT and ERK) by the use of cytofix/cytoperm KIT and perm/wash buffer (BD Biosciences).

FACS data were acquired on a FACSCanto flow cytometer (BD Biosciences) using Diva software. FACS data were analyzed using Flowjo software (TreeStar, Ashland, OR).
Statistical analysis

All data were analyzed using GraphPad Prism version 5.00 for (GraphPad, San Diego, CA) and microsoft excel (microsoft office). The Mann-Whitney test matched nonparametric test was used to examine: the variations of CD277 and PD-1 expression from lymphoid tissue on living T lymphocyte subsets (in Figure 3), the variation of AKT and ERK phosphorylation on CD277 stimulated T lymphocyte cells (in Figure 4-5), and the difference of secretion of cytokines on T cells (in Figure 6). The Comparisons were made between different conditions of stimulation. The Wilcoxon paired test was used to compare between different conditions of stimulation on NK cells (in figure 7). Differences were considered as statistically significant when p < 0.05.

Results

Expression profile of CD277 on T cells subpopulations and NK cells

We had previously reported that CD277 was expressed on T and NK cells (compte et al). We decided to investigate its expression on known sub-populations of peripheral lymphocytes from healthy donors (n = 4). Using multi-parametric flow cytometry, we thus analysed the CD3⁺CD4⁺ and the CD3⁺CD8⁺ subpopulations (data not shown). The staining with the monoclonal anti-CD277 (data not shown) revealed a strong expression of CD277 on both CD4⁺ helper T cells and cytotoxic CD8⁺ T cells (data not shown). We also looked at the expression of CD277 on memory and naïve populations of T lymphocytes based on the differential expression of CD27 and CD45RA expression (data not shown). Here again, we did not detect any significant difference between the subsets (data not shown).

In parallel, we monitored CD277 expression on another population of lymphocytes belonging to the innate immune system, the NK (data not shown). We found that 100% of NK cells also expressed high level of CD277, independently of their CD56Bright (helper) or CD56Dim (cytotoxic) phenotype, showing that molecules related to the B7/CD28 family are similarly found on the two major subsets of NK cells.
Modulation of CD277 on activated T cells and NK cells

As some T cells co-signaling molecules expression could be regulated after T Cell Receptor (TCR) activation, like the induction of PD1, a co-regulatory molecule, we were wondering whether CD277 could also be modulated under activation. To test this hypothesis, we compared the expression profile of CD277 and PD1 under CD3/CD28 activation of CD4⁺ T cells.

Purified CD4⁺ T cells from four healthy donors were thus incubated from 24 to 96 hours with monoclonal antibodies directed against CD3 and CD28 or with the respective control isotypes. As expected, the CD3/CD28 treatment results in a seven-fold increase of PD1 expression after 72 hours of culture, whereas CD277 expression was not modified at any time point (data not shown). Similar results were obtained with CD8 T cells (data not shown).

In parallel we were wondering whether CD277 expression could be modulated on NK cells. We thus stimulated NK cells with usual NK cells stimulating cytokines (IL-2 and IL-15). We compared the expression profile of CD277 and HVEM. HVEM is highly expressed on NK cells and decreased upon NK cells stimulation. Our result showed that CD277 expression was not modulated after NK cells activation, contrary to our positive control HVEM (data not shown).

Altogether, these results demonstrated that T cells and NK cells constitutively express CD277, but its expression is not modulated in vitro after T or NK cells co-stimulation.

Expression profile of CD277 on CD4 T⁺H cells in lymph nodes

Multiple immune T cell populations are found in lymphoid organs were they play specialized functions. Among them, Follicular T helper cells (T⁺H) are present in the germinal centers were they play an important role in B cell differentiation. These cells express the chemokine receptor CXCR5 and high levels of the cosignaling molecules ICSO but also PD-1 and BTLA. CD277 expression could be differentially regulated in lymph nodes. (Figure 4)

Total frozen sections of reactive lymph nodes were also immuno stained for CD277. The results of immunohistochemical analysis showed a strong positivity on both inter-follicular T-cells area and mantle zone B-cells indicating that they were positive on T
cells as well as B cells. Surprisingly, the pattern of staining was totally different in the germinal center. Most of the GC were negative indicating that B cells lost the expression of CD277 during the differentiation process. However, few scattered cells were staining resembling the Tfh staining. We confirmed this after performing a flow cytometry analysis. The Tfh cells (CXCR5+ICOS+ PD-1+) were positive for CD277. In addition CD277 was equally present on the CXCR5- conventional T cells, whereas there is no significant staining in Germinal Center (GC) B-cells or cells (data not shown).

The conclusion of this first part of the study is that CD277 is expressed on all subtypes of T lymphocytes in the peripheral blood as well as in lymph nodes and NK cells, but its expression is not modulated under stimulation as it is often the case for the molecules of the B7/CD28 family, or any other molecules involved in lymphocytes regulation.

AKT and ERK phosphorylation is augmented after CD277 engagement on CD4+ T cells

To investigate the co-stimulatory role of CD277 on lymphocytes function, we searched whether CD277 triggering was able to induce the phosphorylation of the two most important kinases of the lymphocyte signaling pathway: ERK from the mitogen-activated protein kinase and the serine threonine kinase AKT. It is known that Akt and ERK signaling plays a central role in T cell functions including proliferation, protein synthesis and regulation of apoptosis.

First, we showed that the triggering of CD277 20.1 induced the phosphorylation of AKT and ERK (Figure 2), demonstrating that CD277 stimulation is involved in the regulation of T cell activation (purified CD4+ T cells were stimulated with antibody-coated Epoxy dynabeads with mAb anti-CD3 plus mAb anti-CD277.20.1)

Second, in order to clarify the implication of CD277 as a co-modulator of the TCR signaling pathway, we stimulated purified CD4+ T cells with various concentrations of mAb to CD277.20.1 clone or isotype control IgG1, together with anti-CD3 plus anti-CD28 at different times (2, 5, 10 and 30 minutes). We observed that the cross-linking of CD277 with mAb CD277.20.1 clone strongly up regulated the phosphorylation of AKT
and ERK induced by CD3 +CD28 stimulation. This effect was dose and time dependent (data not shown).

In this part of the paper, we thus demonstrated that CD277 triggering potentializes the TCR signal. We next decided to investigate the functional consequences of the activation of this signaling pathway.

**CD277 costimulates CD3 signals**

We next investigated the effect of CD277 engagement on cytokine production and activation markers regulation mediated by CD3 mediated signals. Purified CD4+ T cells from at least 4 healthy donors were cultured during 24 to 72 hours with anti-CD3/anti-CD28 or anti-CD3/anti-CD277 (clone 20.1) or anti-CD3/IgG1 (control condition). After 24 hours of culture, IL-2 and IFN-γ production by CD4+ T cells were measured by ELISA. As expected, these two cytokines were produced in large amount after CD3/CD28 stimulation by comparison with the control condition (p= 0.0079, p=0.0317, data not shown). Although IL-2 level produced by CD3/CD277 co-activated CD4+ T cells was lower than the one obtained with CD3/CD28 co-stimulation, the amount of IL-2 induced by CD3/CD277 co-activation was nonetheless significantly highest than the one with the IgG control (p= 0.0159, data not shown). Moreover, IFN-γ secretion was strongly enhanced by CD3/CD277 co-activation compared to the control situation, and surprisingly, the production was even greater than the one obtained after CD3/CD28 co-activation (data not shown). Furthermore, after 3 days of culture, a similar effect was obtained regarding the expression profile of the activation marker CD25 after CD3/CD277 co-stimulation. This CD4+ T cells co-stimulation induced a significant increase to 45% of CD25 activated positive cells, whereas CD3/CD28 co-activation only induced an increase to 25% of activated CD25 positive cells compared with the controlled condition (data not shown).

Altogether, these results strongly suggest that CD277 is a co-stimulatory molecule of T lymphocytes activation signal.
CD277 further enhances CD3-CD28 costimulation and can act as a third signal to enhance T cell proliferation and cytokine production.

Then, we investigated the consequences of CD277 cosignals on T cell proliferation and cytokine production induced by CD3+CD28 signals. We stimulated purified CD4⁺ T cells with various concentrations of mAb to CD277, together with anti-CD3 plus anti-CD28. We kept the amount of antibody on the beads constant by adding isotype control IgG1 and anti-MHC class I (MHC I). We demonstrated that the cross-linking of CD277 on CD4⁺ T cells strongly activated CD4⁺ T cell proliferation mediated by anti-CD3 plus anti-CD28 in a dose-dependent way. Indeed, we measured the proliferation of CD4 cells by measuring the dilution of cytosolic dye CFSE (data not shown). We found that 60% of cells stimulated with anti-CD3 plus anti-CD28 and IgG1 entered division by day 5. The cross-linking of CD277 (at 17μg/ml) strongly enhanced CD4⁺ T cells division already induced by anti-CD3 plus anti-CD28 in a dose dependent way, such as 90% of cells entered division (data not shown).

In parallel, our results also showed that the engagement of CD277 increased the proliferation (data not shown) and the secretion of cytokines induced by anti-CD3 and anti-CD28 stimulation in a dose-dependent way (Figure 3).

Alltogether, these data support a role of costimulatory molecule for CD277 even after optimal costimulation provided by CD28.

Is CD277 also a co-stimulatory molecule of NK cells?

In parallel, we investigated whether a similar co-stimulatory effect was obtained in NK cells. We thus stimulated two of the most important receptors of NK cells (anti-NKp46 or anti-CD16), in presence of isotypic control or CD277 monoclonal antibody (20.1) or anti-DNAM (positive control of co-stimulation of the activation receptors) or anti-NKG2A (positive control of co-inhibition of the activation receptors). First, CD277 alone did not have any effect on NK cell stimulation. Second, the monoclonal mAb 20.1 directed against the CD277 molecules failed to potentialize any effects on NK cells activation as DNAM or NKG2A did. Both cytotoxicity (data not shown) and IFN-γ secretion (data not shown) were not affected, whether the primo-stimulation was performed with NKp46 or
CD16. This result was quite surprising, but obviously CD277 is not involved in the regulation of NK cells activation, contrary to what was observed with T cells.

5 *Btna Isoforms expressed by lymphocytes*

Considering that CD277 has three isoforms *btn3a1, btn3a2 and btn3a3*, with (*btn3a1 and btn3a3*), or without (*btn3a2*) the B30.2 domain, we decided to look at the mRNA expression of each isoforms on T lymphocytes to determine whether each isoform has an equimolar expression pattern. Using available data from GEO omnibus that we further confirmed by Q-PCR, we found that *btn3a1* is the main form expressed by T lymphocytes whereas the decoy form (*btn3a2*) is mostly expressed on NK cells (data not shown). This result was validated by quantitative PCR on 4 healthy donors. We thus emitted the hypothesis that the absence of co-stimulation in response to CD277 stimulation of NK cells might be attributed to this form of BTN3A.

**EXAMPLE 3**

20 **Materials and methods**

*Antibodies and Fab fragmentation*

Anti-CD277: anti-BT3-20.1 and 103.2 mAb were generated and validated as previously described [10]. Fab fragments of anti-BT3-20.1 were generated and purified with the Immunopure Fab Preparation Kit following the manufacturer’s recommendation (Pierce). Protein purity was assessed by nonreducing SDS-PAGE.

*Construction of phylogenetic trees*

Phylogenetic analyses were performed using the automated genomic annotation platform FIGENIX (FIGENIX Annotation Platform: [http://figenix2.up.univmrs.fr/Figenix/index.jsp]) to retrieve sequences and alignments and perform phylogenetic reconstruction. The pipeline used applied three different methods of phylogenetic tree reconstruction, i.e. Maximum Parsimony [38], Maximum
likelihood [39] and Neighbour Joining [40], and a midpoint rooted consensus tree was built. Bootstrapping was carried out with 1000 replications. Bootstrap values are reported for each method (for a detailed description of the pipelines and models used, see [41]).

5

Cell lines and expansion of γδ T cells

P815 (mouse mastocytoma cell line), K562 (chronic myeloid leukaemia cell line), Raji and Daudi (Burkitt lymphoma cell lines) were cultured in RPMI 1640 medium (Invitrogen) and 10% foetal calf serum (FCS) (Eurobio). The PBMC from healthy donors were distributed at 106/ml in 24-well culture plates at 37°C in 5% CO2 in RPMI 1640 medium and 10% FCS. Polyclonal Vγ9Vδ2 T cells were specifically expanded with 3μmol/l of Phosphostim (BrHPP molecule, Innate Pharma, Marseille, France) and 100 U/ml IL-2 (Chiron, Basel, Switzerland) for 12 days. Phosphostim was added once at the onset of the culture. Every 2 days, one-half of the culture medium volume was replaced with fresh medium containing 100 U/ml IL-2.

The last day, the percentage of γδ T cells was evaluated using anti Vδ2 FITC and anti-CD3-Cy7. Only cells cultures that reached more than 90% of γδ T Cells, were selected to be used in functional tests.

20

Flow Cytometry

We used for γδ T cells labelling in purity test after expansion, anti-CD3 PE-Cy7 and anti-Vδ2 FITC mAb (BD Pharmingen). For CD277 characterisation in different subsets PBMC were incubated with the following antibodies and molecules: anti-CD3 PE-Cy5 (BD Pharmingen), anti-CD4 PB (BD Pharmingen), anti-CD8 PB (BD Pharmingen), anti-Vδ2 FITC (BD Pharmingen), anti-CD27 APC-Alexa Fluor 750 (CALTAG Laboratory), anti-CCR7 PECy7 (BD Pharmingen), anti-CD28 PE (Beckman Coulter), anti-CD45RA ECD (Beckman Coulter), LIVE/DEAD® Fixable Dead Cell Stain Kit (L34957, Invitrogen) and anti-CD277 labelled with Alexa Fluor 647 (Protein labelling Kit Alexa Fluor 647, Molecular Probes, Invitrogen). For analysis of CD107a and CD107b expression, γδ T cells and target cells were co-incubated at 37°C with anti-CD107a FITC and anti-CD107b FITC in presence of monensin (10μM, GolgiStop, BD Bioscience). Cells were collected and washed with PBS, 4 hours after incubation. γδ T cells were labelled with anti-pan γδ TCR PE and anti-CD3 PECy7 mAbs (BD Pharmingen). All samples were
measured on FACSCanto or FACSARia flow cytometers (BD Biosciences) using FACSDiva software. Analyses were performed with FlowJo software (Tree Star).

**Supplementary material:**

Intracellular staining was performed according to the recommended by BD Phamrigen Fix and Perm Kit (BD Biosciences). 100 µl of γδ T cells at 2.106 cells/ml were plated on 96-well plates. They were incubated in presence or not of BrHPP at 3 µM and with anti-CD277 or control isotypes at 10 µg/ml, for 30 min at 4°C. Cells were stimulated at different times at 37°C. Stimulation were stopped adding 100µl Cytofix/Cytopher solution at 37°C for 10min. Intracellular phosphorylated proteins were stained with purified monoclonal rabbit antibodies: anti-pZap 70, anti-pAKT and anti-pErk from Cell Signaling Technology (Danvers, USA); labelled with Biotin-SP-conjugated F(ab')2 Donkey anti-Rabbit (Jackson); and revealed with streptavidin-PE (Beckman Coulter).

**Stimulation and expansion assay**

PBMC were plated and stimulated as described in the paragraph: cell lines and expansion of γδ T cells, except cells were stimulated with anti-CD277 mAbs (10 µg/ml); or control isotypes (10 µg/ml); and with or without BrHPP at different concentrations. Cultures were stopped 9 days after. Percentages of Vγ9Vδ2 were measured the first day and the last day of culture as described above. Expanded γδ T cells effectors were stimulated with or without different doses of BrHPP added in anti-CD277 mAbs at 10 µg/ml; or with OKT3 (4 ng/ml) associated with anti-BT3 19.5 mAb in different concentrations. Activation of degranulation was measured by CD107 labelling assay as described above.

**Redirected activation assay**

2.10⁵ P815 mastocytoma mouse cells were incubated 30 min, with mouse controls isotypes or anti-CD277 mAbs (10 µg/ml) or/and with anti-CD3 (OKT3, 4 ng/ml). After washing, P815 were incubated 4h at 37°C, with expanded Vγ9Vδ2 T effectors cells at the same concentration. A Flow-based CD107a degranulation assay was performed as described above.

**ELISA**

Supernatants from redirected activation essay were collected after 4h co-culture. IFNγ, TNFα and IL-17 release was tested by ELISA Kits (OptEIA kits from BD Phamrigen
for IFNγ and TNFα. Cytokines were detected with plate reader Multiskan RC (Labsystems). Cytokines concentrations were determined from standard curves established with recombinant standards.

5 Analysis of Vγ9Vδ2 T cell responses by direct cytotoxicity assay:
Target cells were labelled with 20μCi of 51Cr (PerkinElmer) for 1h at 37°C. After washing target cells were incubated with effectors Vγ9Vδ2 T cells for 4h at 37°C in different ratio. Incubations were performed in presence of specific or isotype control mAbs or Fab fragments. The radioactivity released by target cells was measured, 4h later, on beta plate counter. The percentage of specific 51Cr lysis was calculated using the following equation:
percent specific lysis = 100 x [(test release) – (spontaneous release)]/[(maximal release) - (spontaneous release)].

15 Statistical analysis:
StatXact software (version 8 PC) produced by the Cytel, was used for all statistical analyses. Significance values for comparisons between groups were determined by the nonparametric Mann and Whitney analysis. Assuming an unequal variance with 95% confidence levels, and p values <0.05 were considered significant.

Skint-1 and CD277 belong to the same super family as B7

Using Skint-1 IgV protein domain sequence in NCBI pBlast, we searched similarity between Skint-1 and other human proteins in databases. The sequence alignments obtained show that Skint-1 is relatively similar to CD277 with 38% of identity (data not shown). To clarify the relationships between this Skint and Btn families, we performed phylogenetic analysis based on their IgV sequences, using the Figenix automated genomic annotation platform.

The phylogeny shows that Skint-1 and CD277 form a monophylogenetic group with genes or genes family implied in immune response regulation: BTN 1 to 3, BTN-L2 ERMAP; B-G and MOG (data not shown). This group also includes CD80 and CD86 from to B7 family, equally known for its involvement in immune response regulation. Every group forms a subfamily.
When we focused on the Skint subfamily (data not shown), phylogenetic analysis based on Skint-1 full sequence evidences at least eight paralog genes in rodents including five in mice in sharp contrast with primates where there is only one copy. This result suggests an important role of the skint family in rodents. We performed the same analysis using CD277 sequence (data not shown). We show that CD277 and his second isoform: BTN3-A3, have a common ancestor in embranchment between horse and primates. Interestingly, these two isoforms are present in primates but are absent in rodents.

Human Skint-1 gene is a likely pseudogene since two stop codons are present within its sequence. The first stop codon is located immediately downstream to the signal peptide, at the beginning of the IgV sequence and the second downstream of the first transmembrane domain [7]. It is unlikely to be due to sequencing errors because we find the very same mutations in Pongo abelii DNA sequence. These data indicated that Skint genes were not necessary for primate species and suggested that their function could be performed by other genes.

In a reciprocal way, CD277 gene was absent in Ratus norvegicus and in Mus musculus DNA sequence using NCBI ntBlast. Indeed, within the BTN families only BTN2 sequence was present in these two species. These results indicated that CD277 really disappeared in rodents and consequently were not necessary for survival of these species. This suggests that CD277 function has been assumed by other gene(s).

Both functional redundancies in rodents and primates common ancestors could be on one hand at the origin of disappearance of CD277 in rodents and on the other hand of Skint-1 in primates.

To summarize, both molecules have similarities in their extracellular region within both IgV and IgC domains, but differ by the number of transmembrane domains and above all by their intracytoplasmic region (data not shown). Indeed, CD277 has only one transmembrane domain instead of three for Skint-1. Furthermore, in their intracytoplasmic region, CD277 has a B30.2 domain. This domain is absent in Skint-1.

CD277 is expressed in Vy9Vδ2 T cells and not modulated by their differentiation.
We had previously shown that CD277 was expressed on conventional T lymphocytes suggesting a role of CD277 in the regulation of T cell response. We tested CD277 expression on γδ T cells as well as their described subpopulations corresponding to different stages of differentiation starting from the naïve compartment. Hence using 8 colour flow cytometry, we determined 4 major subpopulations: Naives cells (CD45RA+/CD27+); Central Memory cells (CD45RA-/CD27+); Effectors Memory (CD45RA-/CD27-/CCR7-) and Effectors Memory RA+ (CD45RA+/CD27-/CCR7-).

We also evaluated CD28 that discriminated two subsets among N, CM, EM as well as EMRA+ gd T cells. Its function is not clear although in αβ T cells it is associated to CTL functions.

95% of Vy9Vδ2 T cells express CD277 with higher mean fluorescence intensity than αβ T cells suggesting an important role in homeostasis of γδ T cells. Interestingly in all of Vy9Vδ2 subpopulation, every cells express CD277. Its level of expression is approximately the same in the analysed γδ T cell subpopulations. The only slight increase was found in the naive CD45RA+/CD27+/CD28+/CCR7+, but this variation was not significant in the series if healthy volunteers tested.

Theses results indicate that CD277 is not modulated in Vy9Vδ2 T cells differentiation and suggest a putative role at the different stages of Vy9Vδ2 T cells activation.

Anti-CD277 mAb 20.1 induces proliferation of Vy9Vδ2 T cells whereas 103.2 inhibits the stimulation mediated by optimal TCR stimulation by phoshoantigens.

To investigate the function of CD277 in γδ T cells we used mAbs directed against CD277. In a first setting we tested whether CD277 triggering might affect the stimulation of γδ T cells by optimal doses of the phoshoantigen BrHPP. We cultured PBMC with IL-2 (100U/ml) and BrHPP (3000nM).

After 15 days of culture Vy9Vδ2 T cells were expanded (Figure 5A) upon anti-CD277 mAb 20.1 addition. This effect was associated with the proliferation of the Vy9Vδ2 cells as demonstrated by the analysis of CFSE labeled cells (Figure 5B). This result suggests a stimulatory function of CD277 20.1 on Vy9Vδ2 T cells activation.
We then tested the function of CD277 mAb 103.2 on the phoshoantigen mediated activation of Vγ9Vδ2 cells. 103. mAb inhibited completely the Vγ9Vδ2 T cell activation mediated by phoshoantigens but CD3mAbs (Figure 6A and 6B). Similar effects were demonstrated in Jurkat cells expressing Vγ9Vδ2TcR

CD277 modulate the degranulation in Vγ9Vδ2 T cells mediated by the TcR complex

These data prompted us to test whether the function of CD277 could be detected in another functions of γδ T cells such as cytokine production, cytotoxicity and degranulation.

We stimulated γδ T cells by phoshoantigens and tested the expanded cells for their effector functions. We first used the modulation of CD107 expression as an indicator of degranulation. This test corresponds in part to the ability of cells to elicit their cytolytic function. In this experiment, we measured the effect of the CD277 engagement with either suboptimal (0.06 nM) or optimal (4000 nM) doses of the phoshoantigen BrHPP. Increasing doses of BrHPP induces increasing CD107 expression up to 40%. Using a low dose of BrHPP unable to induce CD107 expression, addition of anti-CD277 increased CD107 expression up to (20%). However, with high dose of BrHPP, anti-CD277 inhibited CD107 expression induced by BrHPP which decreased from 40% to 20%. We tested another system of γδ T cell stimulation using anti-CD3 and anti-CD277 immobilised mAbs. CD277 stimulation alone induced CD107 expression (30%). CD3 mAb induced a dose dependent CD107 expression that reached plateau at 50 ng/ml in this setting.

We next tested CD107 expression using a steady dose of CD277 mAb with increasing concentration of CD3 mAb. CD107 expression was increased when combining CD277 with increasing doses of anti-CD3 up to 10ng/ml. However, at higher doses of CD3 mAb CD107 expression decreased in a dose dependent manner.

Altogether these data demonstrate that CD277 modulates the ability of γδ T cells to express CD107 and hence to degranulate following phoshoantigen or anti-CD3 stimulation. CD277 effect is biphasic: enhancing at low stimulation levels and decreasing at higher levels of TCR crosslinking the γδ T cell degranulation activity.
Crosslinking of CD277 triggers degranulation together with INFγ and TNFα release.

We next performed redirected stimulation of expanded Vy9V52 T cells using CD277 mAb. The anti-CD277 alone induces CD107 expression after 4 hours of stimulation in a dose dependent manner (10%< 30%). The optimal activation is obtained with 10μg/ml dose of anti-CD277 whereas the ID50 of the mAb for its target is 3μg/ml (data not shown).

Moreover, CD227 engagement induced the robust release of IFNγ and TNFα that was detected early as soon as 4 hours stimulation. So the degranulation induced by CD277 comes along with Th1 cytokines. This degranulation and cytokines release induction results of CD277 stimulation by anti-CD277 crosslinking without the need for TCR engagement.

CD277 potentiate the anti-tumor cytolysis mediated by Vy9V52 T cells.

Finally, we tested whether CD277 could be involved in the anti-tumor function of Vy9V52 T cells activation. To verify this hypothesis, we used anti-CD277 mAb and anti-CD277 Fab fragments in cytotoxicity assay against various tumor cell lines including Daudi, K562 or Raji cells lines.

20.1 Mab potentiated or revealed the function Vy9V52 T cells against the targets as determined by the CD107a/b degranulation assay (Figure 7). In contrast 103.2 mAb prevented the activation of Vy9V52 T cells in the same experimental setting (data not shown).

These results show that CD277 mAbs potentiate or inhibit the anti-tumor cytolysis mediated by responding Vy9V52 T cells.
REFERENCES

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.


References cited in Example 3 are the following:


Immuno1 175, 2144-51(2005).


8. Stammers, M. et coll. BTL-II: a polymorphic locus with homology to the butyrophilin gene family, located at the border of the major histocompatibility complex class II and class III regions in human and mouse. *Immunogenetics* 51, 373-82(2000).


CLAIMS

1. An anti-CD277 antibody, which:
   - activates the cytolytic function, cytokine production and proliferation of Vγ9/Vδ2 T cells, and/or
   - costimulates T cells together with CD3-TCR, and/or
   - costimulates T cells in addition to CD28-B7 costimulation, and/or
   - increases the activity and/or survival of monocytes and dendritic cells.

2. An anti-CD277 antibody, which:
   - activates the cytolytic function, cytokine production and proliferation of Vγ9/Vδ2 T cells, and
   - costimulates T cells together with CD3-TCR, and
   - costimulates T cells in addition to CD28-B7 costimulation, and
   - increases the activity and/or survival of monocytes and dendritic cells.

3. An anti-CD277 antibody obtainable from the hybridoma accessible under CNCM deposit number I-4402 or I-4401.

4. An anti-CD277 antibody comprising the CDRs of the anti-CD277 antibody of claim 3.

5. An anti-CD277 antibody according to any one of claims 1 to 4 for the use in therapy.

6. An anti-CD277 antibody according to claim 5 for use for the treatment of a cancer or a chronic infection.

7. A vaccine for the treatment of a cancer or a chronic infection comprising an anti-CD277 antibody according to any one of claims 1 to 4.

8. A kit for the treatment of a cancer or a chronic infection comprising:
   a) an anti-CD277 antibody according to any one of claims 1 to 4; and
   b) a vaccine for the treatment of a cancer or a chronic infection.

9. A hybridoma cell line selected from the group consisting of CNCM I-4401, CNCM I-4402 and CNCM I-4403.

10. An anti-CD277 antibody, which inhibits the cytolytic function, cytokine production and proliferation of Vγ9/Vδ2 T cells.
11. An anti-CD277 antibody obtainable from the hybridoma accessible under CNCM deposit number I-4403.

12. An anti-CD277 antibody comprising the CDRs of the anti-CD277 antibody of claim 11.

13. An anti-CD277 antibody according to any one of claims 10 to 12 for the use in therapy.

14. An anti-CD277 antibody according to claim 13 for use for the treatment of an autoimmune disease, transplantation rejection or a graft versus host disease.
Figure 1A

Monocytes

iDC

anti-BT3 mAb (clone 20.1)
Figure 1B

anti-BT3
(clone 20.1)

26.7 ± 10.8

61.0 ± 11.2

GM-CSF

18.8 ± 6.4

14.0 ± 7.7

anti-CD19

88.8 ± 10.5

75.3 ± 8.8

Medium

95.5 ± 4.5

78.4 ± 6.7

Monocytes

iDC
Figure 1D

None  BT3 mAb (clone 20.1)  BT3 Fab  LPS

iDC
Figure 3

% divided cells

0 10 20 30 40 50 60 70 80 90 100

NS 17 5 10 17 μg/ml

IgG1 CD277 (20.1)

CD3+CD28+IgG1
Figure 6

A

TNF-\(\alpha\) (% positive cells)

agonist
\(\alpha\)-BT3

isotypic control

antagonist
\(\alpha\)-BT3

BrHPP (\(\mu\)M)

B

\(\alpha\)-CD3 (\(\mu\)g/mL)

C

CD69 (gMFI)

[Graphs showing concentration-response curves for TNF-\(\alpha\) and CD69 expression under different conditions.]
## International Search Report

**International application No**
PCT/IB2010/003417

### A. Classification of Subject Matter

**INV.** A61K39/395 C07K16/28

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)**

A61K C07K

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

Electronic database consulted during the international search (name of database and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

### C. Documents Considered to Be Relevant

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>COMPTE ELSA ET AL: &quot;Characterization of BT3 molecules belonging to the B7 family expressed on immune cells&quot;, EUROPEAN JOURNAL OF IMMUNOLOGY, WILEY - V C H VERLAG GMBH &amp; CO. KGAA, DE, vol. 34, no. 8, 1 August 2004 (2004-08-01), pages 2089-2099, XP009147171, ISSN: 0014-2980 the whole document</td>
<td>1-9</td>
</tr>
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- **"** Special categories of cited documents:
  - "A" document defining the general state of the art which is not considered to be of particular relevance
  - "E" earlier document but published on or after the international filing date
  - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  - "O" document referring to an oral disclosure, use, exhibition or other means
  - "P" document published prior to the international filing date but later than the priority date claimed

- **"T"** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

- **"X"** document of particular relevance; the claimed invention cannot be considered without the document, or the claimed invention cannot be considered to involve an inventive step when the document is taken alone

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**Date of the actual completion of the international search**

18 April 2011

**Date of mailing of the international search report**

08/07/2011

**Name and mailing address of the ISA/European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV RIJSWIJK**

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**Authorized officer**

Fellows, Edward

Form PCT/ISA210 (second sheet) (April 2005)
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☑ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-9(partially)

Remark on Protest
☐ The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.
This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-9(partially)
   An anti-CD277 antibody obtainable from the hybridoma accessible under CNCM deposit number I-4402 and related subject-matter. ---

2. claims: 1-9(partially)
   An anti-CD277 antibody obtainable from the hybridoma accessible under CNCM deposit number I-4401 and related subject-matter. ---

3. claims: 10-14(completely); 9(partially)
   An anti-CD277 antibody obtainable from the hybridoma accessible under CNCM deposit number I-4403 and related subject-matter. ---