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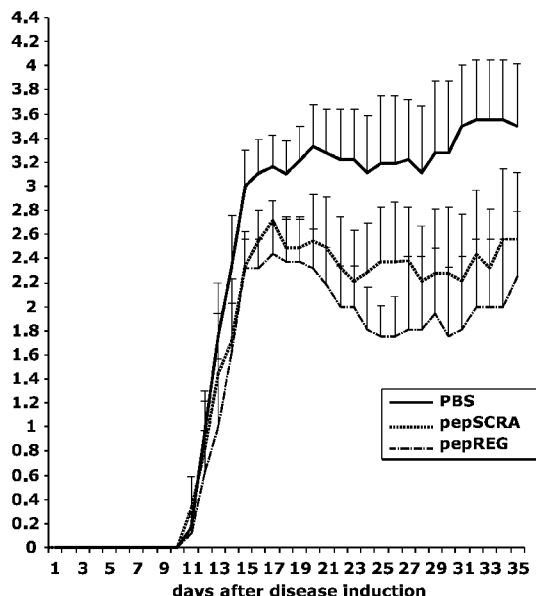


FIG. 7

(57) Abstract: The present invention stems from the finding that the extracellular domain of CD31 proteins present on blood leukocytes is shed and released in the circulation as a soluble form of CD31. The invention relates to peptides corresponding to fragments of CD31 that inhibit T-cell response, and to their use in the treatment of thrombotic disorders such as atherothrombosis and autoimmune disorders.

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USE OF CD31 PEPTIDES IN THE TREATMENT OF THROMBOTIC AND AUTOIMMUNE DISORDERS

FIELD OF THE INVENTION

5 The present invention stems from the finding that the extracellular domain of CD31 proteins present on blood leukocytes is shed and released in the circulation as a soluble form of CD31. The invention relates to peptides corresponding to fragments of CD31 that inhibit T-cell response, and to their use in the treatment of thrombotic and autoimmune disorders.

10

BACKGROUND

Thrombotic disorders

In a healthy person, a homeostatic balance exists between procoagulant (clotting) forces and anticoagulant and fibrinolytic forces. Numerous genetic, acquired, and environmental factors can tip the balance in favor of coagulation, leading to the pathologic formation of thrombi in veins (e.g. deep vein thrombosis), arteries (e.g. atherothrombosis, myocardial infarction, ischemic stroke), or cardiac chambers. Thrombi can obstruct blood flow at the site of formation or detach and embolize to block a distant blood vessel (e.g. pulmonary embolism, stroke).

20 Accumulating evidences show that atherothrombosis, a world-leading life-threatening disease, is linked to a defective immunoregulation driving a pathologic activation of blood leukocytes and a destructive inflammatory response within the vascular wall. Consequently, a restoration of immunoregulation at the blood-vessel interface would represent an innovative therapeutic option to fight
25 atherothrombosis.

Autoimmune disorders

In autoimmune disorders, the immune system produces antibodies to an endogenous antigen. Antibody-coated cells, like any similarly coated foreign
30 particle, activate the complement system, resulting in tissue injury. Autoimmune disorders include systemic lupus erythematoses (SLE), rheumatoid arthritis (RA),

multiple sclerosis (MS), inflammatory bowel disease (IBD), Graves' disease and diabetes mellitus.

Several mechanisms may account for the body's attack on itself. Autoantigens may become immunogenic because they are altered chemically, physically, or biologically. Certain chemicals couple with body proteins, making them immunogenic (as in contact dermatitis). Drugs can produce several autoimmune reactions by binding covalently to serum or tissue proteins (see below). Photosensitivity exemplifies physically induced autoallergy: Ultraviolet light alters skin protein, to which the patient becomes allergic. In animal models, persistent infection with an RNA virus that combines with host tissues alters autoantigens biologically, resulting in an autoallergic disorder resembling SLE.

Most human autoimmune diseases are specific antigen-driven T-cell diseases. T-cell clones responding to specific antigenic epitopes are responsible for the initiation and/or the propagation of these diseases. Similarly, specific antigen-driven T-cell responses are responsible for the rejection of organ allografts and the immune response to tumors. Activated T cells provide the "engine" for the chronic inflammation that is associated with autoimmune diseases, organ graft rejection and tumor immunity.

20 **CD31 (PECAM-1)**

Immune responses can be controlled by inhibitory immune receptors among which CD31 (PECAM-1), which is expressed exclusively and constitutively on cells at the blood-vessel interface.

CD31 consists of a single chain molecule comprising six Ig-like extracellular domains, a short transmembrane segment and a cytoplasmic tail containing two ImmunoTyrosine-based Inhibitory Motif (ITIM)s. The structure of CD31 is shown in the table below.

Domain	Position on SEQ ID No: 1
Signal peptide	1 to 27
Extracellular domain	28 to 601
First Ig-like extracellular domain	34 to 121
Second Ig-like extracellular domain	145 to 233
Third Ig-like extracellular domain	236 to 315
Fourth Ig-like extracellular domain	328 to 401
Fifth Ig-like extracellular domain	424 to 493
Sixth Ig-like extracellular domain	499 to 591
Juxta-membrane domain	592 to 601
Transmembrane domain	602 to 620
Cytoplasmic domain	621 to 738

The immunoregulatory properties of CD31 are supported by the fact that CD31 signalling drives mutual repulsion of blood leukocytes and modulates the balance between inhibitory and stimulatory signals of both innate and adaptive immune cells. Mechanical engagement of the distal Ig-like extracellular domains of CD31 induces outside-in inhibitory signalling triggered by the phosphorylation of its ITIMs, and the recruitment and activation of SH2-containing phosphatases.

Zehnder et al. (1995, *Blood*. 85(5):1282-8) identified a CD31 antibody that inhibited the mixed lymphocyte reaction (MLR) in a specific and dose-dependent manner. They further found that a CD31 peptide corresponding to the epitope of this antibody, i.e. to the 23 membrane-proximal amino acids of CD31, strongly inhibited the MLR. They hypothesized that the 23 membrane-proximal amino acids of CD31 constitutes a functionally important region, and that the CD31 peptide interferes with lymphocyte activation by competing for binding epitopes. However, Zehnder et al. failed to teach whether CD31-mediated signaling is activated or inhibited by the CD31 peptide.

Chen et al. (1997, *Blood*. 89(4):1452-9) showed that this peptide delayed onset of graft-versus-host disease (GVHD) and increased long-term survival in a murine model of the disease. They hypothesized that the CD31 peptide inhibits a

common pathway in T-cell activation. Again, Chen et al. failed to elucidate the role played by the CD31 peptide in T-cell activation. In particular, these previous works did not assess the putative effect of the peptide on the CD31 signaling cascade and more precisely on the phosphorylation state of the CD31 ITIMs.

5 By a yet unknown mechanism, CD31 is “lost” on certain circulating lymphocytes. Its loss is observed upon lymphocyte activation and it has been recently shown that the absence of lymphocyte CD31 signalling, in turn, heightens the pathologic immune responses involved in the development of atherothrombosis.

10 A soluble form of CD31, due to a variant transcript lacking the transmembrane segment, has also been reported and therefore it is currently thought that the individual amount of circulating CD31 is genetically determined. Consequently, a number of previous studies have attempted to find a correlation between plasma levels of soluble CD31 and the risk of atherothrombosis or other
15 autoimmune diseases. However, independently of the specific genetic polymorphisms analyzed, data showed a broad range of plasma CD31 values and the results of these different studies were contradicting.

There is therefore a need for better understanding the biological function of CD31. This would allow the provision of more efficient therapeutics for the
20 treatment of diseases linked with T-cell activation.

DESCRIPTION OF THE INVENTION

It has surprisingly been found that the assumed loss of CD31 on activated/memory T lymphocytes is actually incomplete and results from shedding
25 of CD31 between the 5th and the 6th extracellular Ig-like domains. The shed extracellular domain of CD31 (further referred to as “shed CD31”) is then released into the circulation, where it is present together with a soluble splice variant of CD31.

In addition, it has been shown that a high risk of atherothrombosis is linked
30 with the increase in shed CD31 and decrease in splice variant CD31 in the circulation, and not with the total level of circulating CD31.

The finding that CD31 is not lost on blood lymphocytes but only cleaved provides a unique opportunity to rescue its physiological immunoregulatory

function by targeting the residual portion of the molecule. Specifically, the present invention provides peptides corresponding to juxta-membrane amino acids of the ectodomain of CD31 that are able to rescue the physiological immunoregulatory function of CD31, even in patients having apparently lost CD31 from the surface of their circulating T lymphocytes.

It has been demonstrated that such peptides are capable of preventing disease progression and aneurysm formation in a mouse model for atherosclerosis. Shorter and more stable peptides restricted to the last 10 or 6 COOH-terminal amino acids of the known peptide of twenty-three amino acids display superior *in vitro* immunosuppressive properties (lower ED and lower intra and inter-assay variability) than the known peptide. These amino acids correspond to a short extracellular fragment comprised between the membrane and the 6th Ig-like domain of CD31.

The invention therefore provides peptides consisting of a fragment comprising the membrane juxta-proximal part of extracellular CD31 and part of the sixth Ig-like domain and the use of such peptides in the treatment of a thrombotic or an autoimmune as further described herein.

Such peptides have unique properties compared to soluble forms of CD31 comprising all or most Ig-like domains of CD31. Indeed, such peptides are highly homophilic since they have a Kd of 10^{-7} M, as assessed by BIAcore analysis. Hence they are able to engage CD31 signaling by bridging the membrane juxta-proximal part of extracellular CD31 that remains expressed after its cleavage, via a strong homo-oligomerization. In contrast to this, alternatively spliced soluble CD31 lacks the first 10 membrane juxta-proximal amino acids and shows weak homophilic binding with the 23-mer peptide (Kd of 17 μ M, as assessed by BIAcore analysis). Furthermore, *in vitro*, only the peptides according to the invention are capable of engaging the ITIM pathway downstream of the truncated isoform of CD31, and are thus capable of restoring CD31 signaling in T lymphocytes having apparently lost CD31.

CD31 peptides

It has been found that the six-amino-acid-long CD31 peptide of sequence SEQ ID NO: 2 and that a ten-amino-acid-long CD31 peptide of sequence SEQ ID

NO: 3 are capable of inhibiting T-cell activation. These two peptides correspond to fragments comprising the membrane juxta-proximal part of extracellular CD31, which is adjacent to the sixth extracellular Ig-like domain of CD31.

Therefore, the invention is directed to an isolated peptide comprising or
5 consisting of a fragment of CD31, wherein said fragment is selected from the group consisting of:

a) a fragment of at least 3, 6 or 10 amino acids of the sequence defined by amino acids 579 to 601 of SEQ ID NO: 1;

b) a fragment of at least 3, 6 or 10 amino acids of the sequence
10 corresponding to (a) in a non-human mammalian CD31; or

c) a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to (a);

with the proviso that said peptide of (c) does not consist of an amino acid sequence of SEQ ID NO: 5 or 6.

15 As used herein, the term "*peptide*" has the meaning usually given in the art. More specifically, the dividing line between proteins and peptides is usually set at a length of approximately 50 amino acids. Thus the peptides according to the invention preferably have a length of at most 50, 40, 35, 30, 25, 20, 15 or 10 amino acids.

20 As used herein, the term "*fragment*" of a reference sequence refers to a chain of contiguous nucleotides or amino acids that is shorter than the reference sequence. More specifically, a fragment of the sequence defined by amino acids 579 to 601 of SEQ ID NO: 1 is at most 22 amino acids long. Said fragment may have a length of e.g. 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21
25 or 22 amino acids. Preferably, said fragment has a length within the range of 3 to 18, 3 to 15, 3 to 10, 6 to 18, 6 to 15 or 6 to 10 amino acids.

The isolated peptide according to the invention may for example comprise or consist of a fragment having a sequence selected from the group consisting of
30 SMRTSPRSSTLAVRVFLAPWKK (amino acids 2 to 23 of SEQ ID NO: 5),
MRTSPRSSTLAVRVFLAPWKK (amino acids 3 to 23 of SEQ ID NO: 5),
RTSPRSSTLAVRVFLAPWKK (amino acids 4 to 23 of SEQ ID NO: 5),
TSPRSSTLAVRVFLAPWKK (amino acids 5 to 23 of SEQ ID NO: 5),
SPRSSTLAVRVFLAPWKK (amino acids 6 to 23 of SEQ ID NO: 5),

PRSSLAVRVFLAPWKK (amino acids 7 to 23 of SEQ ID NO: 5),
RSSLAVRVFLAPWKK (amino acids 8 to 23 of SEQ ID NO: 5),
SSLAVRVFLAPWKK (amino acids 9 to 23 of SEQ ID NO: 5),
STLAVRVFLAPWKK (amino acids 10 to 23 of SEQ ID NO: 5), TLAVRVFLAPWKK
5 (amino acids 11 to 23 of SEQ ID NO: 5), LAVRVFLAPWKK (amino acids 12 to 23
of SEQ ID NO: 5), AVRVFLAPWKK (amino acids 13 to 23 of SEQ ID NO: 5),
VRVFLAPWKK (amino acids 14 to 23 of SEQ ID NO: 5), RVFLAPWKK (amino
acids 15 to 23 of SEQ ID NO: 5), VFLAPWKK (amino acids 16 to 23 of SEQ ID
NO: 5), FLAPWKK (amino acids 17 to 23 of SEQ ID NO: 5), LAPWKK (amino
10 acids 18 to 23 of SEQ ID NO: 5), APWKK (amino acids 19 to 23 of SEQ ID NO: 5),
PWKK (amino acids 20 to 23 of SEQ ID NO: 5), WKK (amino acids 21 to 23 of
SEQ ID NO: 5), HASSVPRSKILTVRVILAPWKK (amino acids 2 to 23 of SEQ ID
NO: 6), ASSVPRSKILTVRVILAPWKK (amino acids 3 to 23 of SEQ ID NO: 6),
SSVPRSKILTVRVILAPWKK (amino acids 4 to 23 of SEQ ID NO: 6),
15 SVPRSKILTVRVILAPWKK (amino acids 5 to 23 of SEQ ID NO: 6),
VPRSKILTVRVILAPWKK (amino acids 6 to 23 of SEQ ID NO: 6),
PRSKILTVRVILAPWKK (amino acids 7 to 23 of SEQ ID NO: 6),
RSKILTVRVILAPWKK (amino acids 8 to 23 of SEQ ID NO: 6),
SKILTVRVILAPWKK (amino acids 9 to 23 of SEQ ID NO: 6), KILTVRVILAPWKK
20 (amino acids 10 to 23 of SEQ ID NO: 6), ILTVRVILAPWKK (amino acids 11 to 23
of SEQ ID NO: 6), LTVRVILAPWKK (amino acids 12 to 23 of SEQ ID NO: 6),
TVRVILAPWKK (amino acids 13 to 23 of SEQ ID NO: 6), VRVILAPWKK (amino
acids 14 to 23 of SEQ ID NO: 6), RVILAPWKK (amino acids 15 to 23 of SEQ ID
NO: 6), VILAPWKK (amino acids 16 to 23 of SEQ ID NO: 6), ILAPWKK (amino
25 acids 17 to 23 of SEQ ID NO: 6), SSMRTSPRSSTLAVRVFLAPWK (amino acids 1
to 22 of SEQ ID NO: 5), SSMRTSPRSSTLAVRVFLAPW (amino acids 1 to 21 of
SEQ ID NO: 5), SSMRTSPRSSTLAVRVFLAP (amino acids 1 to 20 of SEQ ID NO:
5), SSMRTSPRSSTLAVRVFLA (amino acids 1 to 19 of SEQ ID NO: 5),
SSMRTSPRSSTLAVRVFL (amino acids 1 to 18 of SEQ ID NO: 5),
30 SSMRTSPRSSTLAVRVF (amino acids 1 to 17 of SEQ ID NO: 5),
SSMRTSPRSSTLAVRV (amino acids 1 to 16 of SEQ ID NO: 5),
SSMRTSPRSSTLAVR (amino acids 1 to 15 of SEQ ID NO: 5),
SSMRTSPRSSTLAV (amino acids 1 to 14 of SEQ ID NO: 5), SSMRTSPRSSTLA

(amino acids 1 to 13 of SEQ ID NO: 5), SSMRTSPRSSTL (amino acids 1 to 12 of SEQ ID NO: 5), SSMRTSPRSST (amino acids 1 to 11 of SEQ ID NO: 5), SSMRTSPRSS (amino acids 1 to 10 of SEQ ID NO: 5), SSMRTSPRS (amino acids 1 to 9 of SEQ ID NO: 5), SSMRTSPR (amino acids 1 to 8 of SEQ ID NO: 5),
5 SSMRTSP (amino acids 1 to 7 of SEQ ID NO: 5), SSMRTS (amino acids 1 to 6 of SEQ ID NO: 5), SSMRT (amino acids 1 to 5 of SEQ ID NO: 5), SSMR (amino acids 1 to 4 of SEQ ID NO: 5), SSM (amino acids 1 to 3 of SEQ ID NO: 5), NHASSVPRSKILTVRVILAPWK (amino acids 1 to 22 of SEQ ID NO: 6), NHASSVPRSKILTVRVILAPW (amino acids 1 to 21 of SEQ ID NO: 6),
10 NHASSVPRSKILTVRVILAP (amino acids 1 to 20 of SEQ ID NO: 6), NHASSVPRSKILTVRVILA (amino acids 1 to 19 of SEQ ID NO: 6), NHASSVPRSKILTVRVIL (amino acids 1 to 18 of SEQ ID NO: 6), NHASSVPRSKILTVRVI (amino acids 1 to 17 of SEQ ID NO: 6), NHASSVPRSKILTVRV (amino acids 1 to 16 of SEQ ID NO: 6),
15 NHASSVPRSKILTVR (amino acids 1 to 15 of SEQ ID NO: 6), NHASSVPRSKILTV (amino acids 1 to 14 of SEQ ID NO: 6), NHASSVPRSKILT (amino acids 1 to 13 of SEQ ID NO: 6), NHASSVPRSKIL (amino acids 1 to 12 of SEQ ID NO: 6), NHASSVPRSKI (amino acids 1 to 11 of SEQ ID NO: 6), NHASSVPRSK (amino acids 1 to 10 of SEQ ID NO: 6), NHASSVPRS (amino acids 1 to 9 of SEQ ID NO:
20 6), NHASSVPR (amino acids 1 to 8 of SEQ ID NO: 6), NHASSVP (amino acids 1 to 7 of SEQ ID NO: 6), NHASSV (amino acids 1 to 6 of SEQ ID NO: 6), NHASS (amino acids 1 to 5 of SEQ ID NO: 6), NHAS (amino acids 1 to 4 of SEQ ID NO: 6), NHA (amino acids 1 to 3 of SEQ ID NO: 6), SMRTSPRSSTLAVRVFLAPWK (amino acids 2 to 22 of SEQ ID NO: 5), MRTSPRSSTLAVRVFLAPW (amino acids
25 3 to 21 of SEQ ID NO: 5), RTSPRSSTLAVRVFLAP (amino acids 4 to 20 of SEQ ID NO: 5), TSPRSSTLAVRVFLA (amino acids 5 to 19 of SEQ ID NO: 5), SPRSSTLAVRVFL (amino acids 6 to 18 of SEQ ID NO: 5), PRSSTLAVRVF (amino acids 7 to 17 of SEQ ID NO: 5), RSSTLAVRV (amino acids 8 to 16 of SEQ ID NO: 5), SSTLAVR (amino acids 9 to 15 of SEQ ID NO: 5), STLAV (amino acids
30 10 to 14 of SEQ ID NO: 5), TLA (amino acids 11 to 13 of SEQ ID NO: 5), HASSVPRSKILTVRVILAPWK (amino acids 2 to 22 of SEQ ID NO: 6), ASSVPRSKILTVRVILAPW (amino acids 3 to 21 of SEQ ID NO: 6), SSVPRSKILTVRVILAP (amino acids 4 to 20 of SEQ ID NO: 6),

SVPRSKILTVRVILA (amino acids 5 to 19 of SEQ ID NO: 6), VPRSKILTVRVIL (amino acids 6 to 18 of SEQ ID NO: 6), PRSKILTVRVI (amino acids 7 to 17 of SEQ ID NO: 6), RSKILTVRV (amino acids 8 to 16 of SEQ ID NO: 6), SKILTVR (amino acids 9 to 15 of SEQ ID NO: 6), KILTV (amino acids 10 to 14 of SEQ ID NO: 6) and ILT (amino acids 11 to 13 of SEQ ID NO: 6).

In a preferred embodiment, said fragment corresponds to a juxta-membrane fragment, i.e. to a fragment immediately adjacent to the transmembrane domain of CD31. In other words, said fragment preferably corresponds to a fragment including the C-terminal extremity of the sequence consisting of amino acids 579 to 601 of SEQ ID NO: 1 or of the corresponding sequence in a non-human mammalian CD31. For example, a peptide consisting of a juxta-membrane fragment of 15 amino acids of the sequence defined by amino acids 579 to 601 of SEQ ID NO: 1 consists of amino acids 587 to 601 of SEQ ID NO: 1.

In a specific embodiment, peptides comprising the transmembrane domain of CD31 or part thereof are excluded from the scope of the present invention.

The sequence of CD31 peptides according to the invention is preferably derived from the sequence of human or murine CD31. However, the sequence of CD31 may be derived from any non-human mammalian CD31 sequence. Figure 1 shows an alignment between the human, murine, bovine and pig CD31 sequences. The sequence corresponding to the sequence defined by amino acids 579 to 601 of SEQ ID NO: 1 is highlighted by a box. The skilled in the art can easily identify the corresponding sequence in another non-human mammalian CD31 protein by performing a sequence alignment with the sequences shown in Figure 1.

In addition to the CD31 fragment, the peptide may optionally comprise sequences heterologous to CD31. These heterologous sequences may e.g. correspond to a carrier molecule such as the Keyhole Limpet Hemocyanin (KLH), bovine serum albumine (BSA), ovalbumin (OVA), thyroglobulin (THY) or the multiple antigenic peptide (MAP).

In a preferred embodiment, the peptide comprises or consists of a peptide selected from the group consisting of:

- the six-amino-acid-long peptide shown as SEQ ID NO: 2, which is present both in human and murine CD31 sequences;

- the ten-amino-acid-long peptide shown as SEQ ID NO: 3, the sequence of which is derived from the mouse CD31 sequence; and
- the ten-amino-acid-long peptide shown as SEQ ID NO: 4, the sequence of which is derived from the human CD31 sequence.

5 In another preferred embodiment, the peptide comprises or consists of an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to any one of SEQ ID NOs: 2, 3 or 4.

CD31 peptides according to the invention have the biological activity of exerting a dose-dependent inhibition of T-cell proliferation *in vitro* and/or of
10 inhibiting the mixed-lymphocyte reaction (MLR). Their biological activity may for example be measured as described in Example 3, Example 6 or in Zehnder et al. (1995, Blood. 85(5):1282-8).

The T-cell proliferation assay may comprise comparing the radioactivity incorporated into T-cells cultured either in the presence or in the absence of the
15 compound to be tested. This assay may for example be performed as follows:

- providing a multi-well plate comprising complete medium supplemented with anti-CD3 antibodies;
- supplementing the wells with increasing concentrations of the compound to be tested;
- 20 – plating peripheral blood mononuclear cells (e.g. of spleen cells);
- culturing the cells for about 72 hours;
- adding (^3H) thymidine and culturing the cells for about 16 hours;
- measuring the radioactivity; and
- comparing the radioactivity measured in the presence of the compound to be
25 tested with the radioactivity measured in the absence of said compound, and/or in the presence of a reference compound, and/or in the presence of a negative control.

Alternatively, the T-cell proliferation assay may comprise comparing expression levels of the early activation marker CD69 in T-cells cultured either in
30 the presence or in the absence of the compound to be tested. This assay may for example be performed as follows:

- providing purified CD4+ cells (e.g. purified from C57Bl6 mice);

- stimulating said CD4+ cells by addition of anti-CD3 purified antibodies and bone marrow derived dendritic cells;
- culturing the cells for about 18 hours; and
- analyzing said cells for the expression of the early activation marker CD69, e.g. by flow cytometry; and
- comparing CD69 expression in the presence of the compound to be tested.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query amino acid sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% (5 of 100) of the amino acid residues in the subject sequence may be inserted, deleted, or substituted with another amino acid.

Methods for comparing the identity and homology of two or more sequences are well known in the art. Thus for instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1, for example the programs BESTFIT and GAP, may be used to determine the % identity between two polynucleotides and the % identity and the % homology between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman and finds the best single region of similarity between two sequences. Other programs for determining identity and/or similarity between sequences are also known in the art, for instance the BLAST family of programs, accessible through the home page of the NCBI at world wide web site ncbi.nlm.nih.gov) and FASTA.

Peptides consisting of an amino acid sequence "at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical" to a reference sequence may comprise mutations such as deletions, insertions and/or substitutions compared to the reference sequence. In case of substitutions, the substitution preferably corresponds to a conservative substitution as indicated in the table below. In a preferred embodiment, the peptide consisting of an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a reference sequence only differs from the reference sequence by conservative substitutions. In another

preferred embodiment, the peptide consisting of an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a reference sequence corresponds to a naturally-occurring allelic variant of the reference sequence. In still another preferred embodiment, the peptide consisting of an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a reference sequence corresponds to a homologous sequence derived from another non-human mammalian species than the reference sequence.

Conservative substitutions	Type of Amino Acid
Ala, Val, Leu, Ile, Met, Pro, Phe, Trp	Amino acids with aliphatic hydrophobic side chains
Ser, Tyr, Asn, Gln, Cys	Amino acids with uncharged but polar side chains
Asp, Glu	Amino acids with acidic side chains
Lys, Arg, His	Amino acids with basic side chains
Gly	Neutral side chain

In a preferred embodiment, the peptide according to the invention consists of any one of the sequences shown in the table below:

species	Swissprot accession number	position	sequence	SEQ ID NO :
Human	P16284	592-601	VRVILAPWKK	4
Mouse	Q08481	581-590	VRVFLAPWKK	3
Rat	Q3SWT0	580-589	VRVFLAPWKK	10
Pig	Q95242	593-602	VRVYLAPWKK	11
Bovin	P51866	591-600	VRVYL-PLEK	12

CD31 peptides according to the invention may be prepared by any well-known procedure in the art, such as solid phase synthesis, liquid phase synthesis or genetic engineering. As a solid phase synthesis, for example, the amino acid corresponding to the C-terminus of the peptide to be synthesized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from the C-terminus to the N-terminus, and one where the amino acids bound to the resin or the protective group of the amino groups of the peptides are released, the peptide chain is thus extended in this manner. After

synthesis of the desired peptide, it is subjected to the deprotection reaction and cut out from the solid support.

The CD31 peptides of the invention may optionally comprise chemical modifications improving their stability and/or their biodisponibility. Such chemical
5 modifications aim at obtaining peptides with increased protection of the peptides against enzymatic degradation *in vivo*, and/or increased capacity to cross membrane barriers, thus increasing its half-life and maintaining or improving its biological activity. Any chemical modification known in the art can be employed according to the present invention. Such chemical modifications include but are
10 not limited to:

- modifications to the N-terminal and/or C-terminal ends of the peptides such as e.g. N-terminal acylation (preferably acetylation) or desamination, or modification of the C-terminal carboxyl group into an amide or an alcohol group;
- modifications at the amide bond between two amino acids: acylation (preferably
15 acetylation) or alkylation (preferably methylation) at the nitrogen atom or the alpha carbon of the amide bond linking two amino acids;
- modifications at the alpha carbon of the amide bond linking two amino acids such as e.g. acylation (preferably acetylation) or alkylation (preferably methylation) at the alpha carbon of the amide bond linking two amino acids.
- 20 – chirality changes such as e.g. replacement of one or more naturally occurring amino acids (L enantiomer) with the corresponding D-enantiomers;
- retro-inversions in which one or more naturally-occurring amino acids (L-enantiomer) are replaced with the corresponding D-enantiomers, together with an inversion of the amino acid chain (from the C-terminal end to the N-terminal
25 end);
- azapeptides, in which one or more alpha carbons are replaced with nitrogen atoms; and/or
- betapeptides, in which the amino group of one or more amino acid is bonded to the β carbon rather than the α carbon.

30 By an “isolated” peptide, it is intended that the peptide is not present within a living organism, e.g. within human body. However, the isolated peptide may be part of a composition or a kit. The isolated peptide is preferably purified.

The compounds of the invention may be produced by any well-known

procedure in the art, including chemical synthesis technologies and recombinant technologies.

Examples of chemical synthesis technologies are solid phase synthesis and liquid phase synthesis. As a solid phase synthesis, for example, the amino acid
5 corresponding to the C-terminus of the peptide to be synthesized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from the C-terminus to the N- terminus, and one where the amino
10 acids bound to the resin or the protective group of the amino groups of the peptides are released, the peptide chain is thus extended in this manner. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoe (t-butoxycarbonyl), Cl-Z (2-chlorobenzoyloxycarbonyl), Br-Z (2-bromobenzoyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4, 4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2, 3, 6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Clz-Bzl (2, 6-dichlorobenzyl) for the amino groups; NO₂ (nitro) and Pmc (2,2, 5,7, 8-pentamethylchromane-6-sulphonyl) for the guanidino groups); and tBu (t-butyl) for the hydroxyl groups).
15 After synthesis of the desired peptide, it is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried with hydrogen fluoride or tri-fluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method.
20

Alternatively, the peptide may be synthesized using recombinant techniques.
25 In this case, a nucleic acid encoding a peptide according to the invention (further referred to as "a nucleic acid according to the invention") is cloned into an expression vector. The nucleic acid of the invention is preferably placed under the control of expression signals (e.g. a promoter, a terminator and/or an enhancer) allowing its expression. The expression vector is then transfected into a host cell
30 (e.g. a human, CHO, mouse, monkey, fungal or bacterial host cell), and the transfected host cell is cultivated under conditions suitable for the expression of the peptide.

The method of producing the peptide may optionally comprise the steps of

purifying said peptide, chemically modifying said peptide, and/or formulating said peptide into a pharmaceutical composition.

The invention also encompasses a method for identifying a peptidomimetic of a peptide according to the invention, said peptidomimetic being a candidate
5 compound for the treatment and/or prevention of a thrombotic or an autoimmune disorder, comprising the steps of:

- a) providing a peptidomimetic; and
- b) determining whether said peptidomimetic exerts:
 - 10 i. a dose-dependent inhibition of T-cell proliferation *in vitro* and/or of the mixed-lymphocyte reaction (MLR);
 - ii. an immunosuppressant activity; and/or
 - iii. an antiplatelet activity.

wherein, if said peptidomimetic exerts a dose-dependent inhibition of T-cell proliferation *in vitro* and/or of the mixed-lymphocyte reaction (MLR), an
15 immunosuppressant activity and/or an antiplatelet activity, said peptidomimetic is a candidate compound for the treatment and/or prevention of a thrombotic or an autoimmune disorder.

Step (a) of the above method may comprise designing and synthesizing said peptidomimetic.

20 Step (b) of the above method for identifying a peptidomimetic of a peptide according to the invention may comprise or consist of (i); (ii); (iii); (i) and (ii); (i) and (iii); (ii) and (iii); or (i) and (ii) and (iii).

Methods for determining whether said peptidomimetic exerts a dose-dependent inhibition of T-cell proliferation *in vitro* and/or of the mixed-lymphocyte
25 reaction (MLR), an immunosuppressant activity and/or an antiplatelet activity are well known to the skilled in the art.

The determination whether said peptidomimetic exerts a dose-dependent inhibition of T-cell proliferation *in vitro* and/or of the mixed-lymphocyte reaction (MLR) may for example be performed as described in Example 3.

30 Immunosuppressant and antiplatelet activity can for example be the evaluated by analyzing the expression of activation surface markers (e.g. CD62P for platelets and/or CD69 for leukocytes) or of soluble activation markers (e.g. TXA2 for platelets and/or IL-2 for Lymphocytes).

As used herein, the term « *peptidomimetic* » refers to a compound containing non-peptidic structural elements that mimics the biological action of a CD31 peptide according to the invention. Methods for designing and synthesizing peptidomimetics of a given peptide are well-known in the art and include e.g. those described in Ripka and Rich (Curr Opin Chem Biol. 1998; 2(4):441-52) and in Patch and Barron (Curr Opin Chem Biol. 2002; 6(6):872-7).

Use of CD31 peptides for the treatment of thrombotic and autoimmune disorders

It has been found that CD31 peptides corresponding to fragments comprising part of the sixth extracellular Ig-like domain of CD31 are capable of activating CD31-mediated signaling, even in CD31⁻ (i.e. CD31^{shed}) T lymphocytes. In addition, such peptides are capable of preventing disease progression and aneurysm formation in a mouse model for atherosclerosis.

Therefore, the invention is directed to an isolated peptide comprising or consisting of

- a) amino acids 579 to 601 of SEQ ID NO: 1;
- b) the amino acids corresponding to (a) in a non-human mammalian CD31;
- c) a fragment of at least 6 amino acids of (a);
- d) a fragment of at least 6 amino acids of (b); or
- e) a sequence at least 80% identical to (a) or (c);

for use in activating CD31-mediated signaling. These peptides preferably exert a dose-dependent inhibition of T-cell proliferation *in vitro*. The activation of CD31-mediated signaling may be an *in vitro* or an *in vivo* activation.

As used throughout the present specification, the term “CD31-mediated signaling” refers to a signaling pathway in which CD31 is involved. Such pathways are well known in the art and include those described e.g. in Newman and Newman (2003 Arterioscler Thromb Vasc Biol 23:953-964) and in Newton-Nash and Newman (1999. J Immunol 163:682-688).

The invention is further directed to an isolated peptide comprising or consisting of:

- a) amino acids 579 to 601 of SEQ ID NO: 1;
- b) the amino acids corresponding to (a) in a non-human mammalian CD31;

- c) a fragment of at least 6 amino acids of (a);
- d) a fragment of at least 6 amino acids of (b); or
- e) a sequence at least 80% identical to (a) or (c);

for use in the treatment of a thrombotic or an autoimmune disorder. These peptides preferably exert a dose-dependent inhibition of T-cell proliferation *in vitro*.

As used throughout the present specification, the term "thrombotic disorder" includes but is not limited to atherothrombosis, atherosclerosis, acute coronary syndrome, ischemic stroke, peripheral arterial disease and abdominal aortic aneurysm.

As used throughout the present specification, the term "autoimmune disorder" includes but is not limited to rheumatoid arthritis (RA), multiple sclerosis (MS), inflammatory bowel disease (IBD), systemic lupus erythematoses (SLE), Graves' disease and diabetes mellitus.

In a preferred embodiment of the invention, said thrombotic or autoimmune disorder is associated with a loss of CD31⁺ T lymphocytes phenotype. Indeed, it has been surprisingly found that CD31 peptides restore CD31 signaling even in individuals with a CD31⁻ T lymphocytes phenotype. Therefore, in the context of the present invention, CD31 peptides are preferably used to treat a subgroup of individuals and/or patients having a CD31⁻ T lymphocytes phenotype.

As used herein, the term "CD31⁻ T lymphocytes phenotype" is used interchangeably with the term "CD31^{shed} T lymphocytes phenotype". These terms refer to the phenotype of an individual having apparently lost CD31 on its circulating T cells when conventional prior art methods for detecting CD31, e.g. such as those described in Stockinger et al. (Immunology, 1992, 75(1):53-8), Demeure et al. (Immunology, 1996, 88(1):110-5), Caligiuri et al. (Arterioscler Thromb Vasc Biol, 2005, 25(8):1659-64) or Caligiuri et al. (Arterioscler Thromb Vasc Biol, 2006, 26(3):618-23) are used. In such methods, the antibody used for detecting CD31 binds to an epitope located on any one of the 1st to the 5th extracellular Ig-like domains.

Preferably, individuals having a CD31⁻ T lymphocytes phenotype have lost at least 50%, 60%, 65%, 70%, 75%, 80%, 90% or 95% of their circulating CD4⁺/CD31⁺ T lymphocytes. In other words, at least 50%, 60%, 65%, 70%, 75%, 80%, 90% or 95% of their circulating T lymphocytes are CD31^{shed} lymphocytes.

Either the plasmatic rate or the cellular rate of CD31⁻ T lymphocytes, compared to CD31⁺ T lymphocytes, may be measured.

The CD31 peptide used for activating CD31-mediated signaling and/or treating a thrombotic or an autoimmune disorder may be any one of the peptides described in the above paragraph entitled "CD31 peptides".

Alternatively, the CD31 peptide used for activating CD31-mediated signaling and/or treating a thrombotic or an autoimmune disorder comprises or consists of:

- a) amino acids 579 to 601 of SEQ ID NO: 1;
- b) the amino acids corresponding to (a) in a non-human mammalian CD31;
- c) a sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to (a) or (b);

The peptides used herein may have a length comprised between e.g. 10-100, 15-80, 20-60, 25-50 and 20-40 amino acids. These peptides preferably comprise or consist of fragments immediately adjacent to the transmembrane domain of CD31. The peptide may for example correspond to a peptide consisting of an amino acid sequence at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 5 or 6. The peptide preferably consists of SEQ ID NO: 5 or 6. These peptides may optionally comprise chemical modifications improving their stability and/or their biodisponibility.

The invention is also directed to a method of treating or preventing a thrombotic or an autoimmune disorder comprising the step of administering an effective amount of a peptide as described herein, or a nucleic coding therefore, to an individual in need thereof. Said individual in need thereof preferably suffers from or is at risk of suffering from a thrombotic or an autoimmune disorder. Most preferably, said individual has a CD31⁻ T lymphocytes phenotype.

By "effective amount", is meant an amount sufficient to achieve a concentration of peptide which is capable of preventing, treating or slowing down the disease to be treated. Such concentrations can be routinely determined by those of skilled in the art. The amount of the compound actually administered will typically be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight, and response of the individual patient,

the severity of the patient's symptoms, and the like. It will also be appreciated by those of skilled in the art that the dosage may be dependent on the stability of the administered peptide.

The individuals to be treated in the frame of the invention are preferably human individuals. However, the veterinary use of CD31 peptides for treating other mammals is also contemplated by the present invention.

Pharmaceutical compositions

The CD31 peptides described herein may be formulated into a pharmaceutical composition. Thus the invention contemplates a pharmaceutical composition comprising any one of the above CD31 peptides and a physiologically acceptable carrier. Physiologically acceptable carriers can be prepared by any method known by those skilled in the art.

Pharmaceutical compositions comprising at least one peptide of the invention include all compositions wherein the peptide(s) are contained in an amount effective to achieve the intended purpose. In addition, the pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Suitable pharmaceutically acceptable vehicles are well known in the art and are described for example in Remington's Pharmaceutical Sciences (Mack Publishing Company, Easton, USA, 1985), which is a standard reference text in this field. Pharmaceutically acceptable vehicles can be routinely selected in accordance with the mode of administration, solubility and stability of the peptides. For example, formulations for intravenous administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. The use of biomaterials and other polymers for drug delivery, as well the different techniques and models to validate a specific mode of administration, are disclosed in literature.

The peptides of the present invention may be administered by any means that achieve the intended purpose. For example, administration may be achieved by a number of different routes including, but not limited to subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intracerebral, intrathecal,

intranasal, oral, rectal, transdermal, buccal, topical, local, inhalant or subcutaneous use.

Dosages to be administered depend on individual needs, on the desired effect and the chosen route of administration. It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The total dose required for each treatment may be administered by multiple doses or in a single dose.

Depending on the intended route of delivery, the compounds may be formulated as liquid (e.g., solutions, suspensions), solid (e.g., pills, tablets, suppositories) or semisolid (e.g., creams, gels) forms.

In a preferred embodiment, the compositions are presented in unit dosage forms to facilitate accurate dosing. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a pre-determined quantity of active material calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient. Typical unit dosage forms include pre-filled, pre-measured ampoules or syringes of the liquid compositions or pills, tablets, capsules or the like in the case of solid compositions. In such compositions, the compound of the invention is usually a minor component (from about 0.1 to about 50% by weight or preferably from about 1 to about 40% by weight) with the remainder being various vehicles or carriers and processing aids helpful for forming the desired dosing form.

The compounds of this invention can also be administered in sustained release forms or from sustained release drug delivery systems.

The expression "physiologically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which is administered. For example, for parenteral administration, the above active ingredients may be formulated in unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

Besides the pharmaceutically acceptable carrier, the compositions of the invention can also comprise minor amounts of additives, such as stabilizers,

excipients, buffers and preservatives.

The invention also contemplates a pharmaceutical composition comprising a nucleic acid encoding the peptide of the invention in the frame of e.g. a treatment by gene therapy. In this case, the nucleic acid is preferably present on a vector, on
5 which the sequence coding for the peptide is placed under the control of expression signals (e.g. a promoter, a terminator and/or an enhancer) allowing its expression. The vector may for example correspond to a viral vector such as an adenoviral or a lentiviral vector.

The invention further provides kits comprising a pharmaceutical composition
10 comprising a CD31 peptide of the invention and instructions regarding the mode of administration. These instructions may e.g. indicate the medical indication, and/or the route of administration, and/or the dosage, and/or the group of patients to be treated.

15 All references cited herein, including journal articles or abstracts, published or unpublished patent application, issued patents or any other references, are entirely incorporated by reference herein, including all data, tables, figures and text presented in the cited references.

Although having distinct meanings, the terms “comprising”, “having”,
20 “containing” and “consisting of” have been used interchangeably throughout this specification and may be replaced with one another.

The invention will be further evaluated in view of the following examples and figures.

25 **BRIEF DESCRIPTION OF THE FIGURES**

Figure 1 shows an alignment between the sequences of human, mouse, pig and bovine CD31. The sequence defined by amino acids 579 to 601 of SEQ ID NO: 4 (human CD31) and the corresponding sequence in mouse, pig and bovine CD31 is highlighted by a box.

30 Figure 2 shows a representative example of 10-color flow-cytometry analysis of human peripheral blood cells from a healthy donor. Isotype controls of antibodies anti-CD31 dom1 and anti-CD31 dom6 are shown in the insets. Lymphocytes, Monocytes and Granulocytes were gated within the FSC/SSC

scatter. B (CD20 A700+) and T (CD3 PE-TR+) lymphocytes were identified and gated within the "Lymphocytes" and CD8+ (PerCP) and CD4+ (APC) subpopulations were gated within T lymphocytes. CD8+ and CD4+ T cells were further analyzed for the expression of HLA-DR and CD45RA and accordingly subdivided in activated (1), memory (2) and naïve (3) cells. All leukocytes were positive for CD31 dom6. Lack of dom1 increased from naïve (3) to memory (2) to activated (1) T cells.

Figure 3 shows that the apparent loss of CD31 on lymphocytes is due to its extracellular shedding. a. Solubilized cell membrane-bound CD31 molecules were extracted from cultured Jurkat CD4+ T cells and coupled to fluorescent beads. The percentage of dom1- bead-bound molecules is <6% in resting conditions and >99% 5' after TCR engagement. b. Most soluble CD31 in culture supernatant (□) of TCR-activated T cells and in human plasma (■) consists of a single truncated fragment comprising dom1-dom5 and lacking dom6. Negligible levels of truncated CD31 lacking both dom5 and dom6 could be detected only in plasma.

Figure 4 shows that a peptide homotypic of the residual extracellular fragment on CD31^{shed}T induces CD31-ITIM phosphorylation. a. Proliferative response to TCR engagement of human peripheral blood mononuclear cells in the presence of increasing doses of CD31 peptide 551-574. *p<0.05 vs dose "0". b. Flow cytometry assessment of 686ITIM phosphorylation on solubilized membrane-bound CD31 from cultured Jurkat CD4+ T cells. Solubilized proteins were captured by E9-PECAM-1.2 (dom6) functional CBA beads and detection was carried out by anti-pY686 rabbit sera followed by AlexaFluor®488-anti-rabbit secondary antibody. The histogram shows the Median Fluorescent Intensity (MFI) ± the % of the variability coefficient (CV%) of Alexafluor®488 (pY686) over 2000 E9-PECAM-1.2 acquired beads. Pervan= positive control (pervanadate); CD3/CD28= anti-CD3 and anti-CD28 antibodies (1µg/ml each); peptide= CD31 peptide 551-574 (100µM).

Figure 5 shows that CD31 homotypic peptide 551-574 inhibits T-cell responses. a. BIAcore® analysis of mouse CD31 peptide 551-574 homophilic binding at two-fold stepwise dilutions of the analyte (12.5, 25, 50 and 100 µM). Data are normalised against the control channels and expressed as ΔRU (Resonance Units). b. TCR-induced intracellular calcium mobilisation determined

by flow cytometry in Fluo-3AM-loaded spleen cells. Data are expressed as MFI (530/30 nm). Grey arrow = addition of either anti-CD3/CD28 antibodies and crosslinker alone (■=control) or with peptide 551-554 at 100µM (●) or with anti-mouse CD31 antibodies (○). c. Proliferation in response to TCR-stimulation in CD31+/+ (black columns) and CD31-/- (white columns) spleen cells. A dose-dependent inhibition is observed in CD31+/+ cells while only the highest dose of the peptide affect proliferation of CD31-/- splenocytes. No effect was observed with 100µM dose of the scramble peptide on CD31+/+ cells (crisscross column). *p<0.05 vs previous peptide dose. d. Immunosuppressive effect of the peptide in the DTH model. *p<0.01 vs scramble (crisscross column) and 10 µM dose of peptide 551-574. Data are expressed as mean ± SEM.

Figure 6 shows that CD31 peptide biotherapy prevents acceleration of atherosclerosis and aneurysm formation. a. Mean ± SEM of Atherosclerotic lesion surface area in serial cross-sections of the aortic root at 200, 400, 600, 800 µm from the appearance of the first cusp in control (■) and peptide 551-574 (□) treated mice. *p<0.05 vs control, ANOVA, repeated measure. b. The presence of an abdominal aortic aneurysm (AAA) was macroscopically, blindly evaluated by A.G. and A. N. after careful dissection of the adventitial tissue. An aneurysm was present in 8/10 (Exp #1) or 6/8 (Exp #2) control mice as opposed to only 1/8 peptide-treated mice in both experiments (p<0.001 by Chi squared test). The images below show an example of aneurysm (AAA; arrows) as compared to the absence of aneurysm (no AAA) of the abdominal aorta. c. Flow cytometry analysis of the effect of the peptide (50µM) on CD8+ T cell (% cytotoxicity) and macrophage (MMP-2/9 activity) functions. Data represent mean ± SEM of cultures from 3-4 mous/group. *p<0.05 vs control.

Figure 7 shows the clinical score of EAE activity (paralysis level). “pepREG” refers to the peptide of SEQ ID NO: 3. “pepSCRA” refers to the peptide of SEQ ID NO: 14.

30 **BRIEF DESCRIPTION OF THE SEQUENCES**

SEQ ID NO: 1 corresponds to the sequence of human CD31.

SEQ ID NOs: 2, 3, 4, 10, 11 and 12 correspond to CD31 peptides according to the invention.

SEQ ID NOs: 5 and 6 correspond to CD31 peptides for use in the methods according to the invention.

SEQ ID NO: 7 corresponds to the sequence of murine CD31.

SEQ ID NO: 8 corresponds to the sequence of bovine CD31.

5 SEQ ID NO: 9 corresponds to the sequence of pig CD31.

SEQ ID NOs: 13 and 14 correspond to scramble peptides used as controls.

EXAMPLES

Example 1: material and methods

10 Assessment of CD31⁺ and CD31^{shed} blood leukocytes. Ten-color flow cytometry was performed on peripheral blood leukocytes from 5 healthy individuals either in basal conditions or after overnight stimulation with soluble 1 µg/ml of purified anti-CD3 antibody (R&D Systems). Ten-color flow cytometry was performed after erythrocyte hypotonic lysis (10 minutes at 37°C 1:10 v:v in
15 Tris 10 mM, NH₄Cl 155 mM, KHCO₃ 10 mM, pH 7.4) on heparinized peripheral blood leukocytes from 5 healthy individuals, fixed in PBS / Formaldehyde 1% / FCS 1% for 4 minutes at 37°C prior to processing. All experiments on human blood were approved by the International Ethical committee (see world wide web page clinicaltrials.gov; Identifier: NCT00430820). Pelleted cells were incubated for
20 30 minutes at room temperature and protected from light with a cocktail of fluorescent monoclonal antibodies directed to CD3 (PE-Texas Red), CD4 (PE-Cy7), CD8 (PerCP), HLA-DR (APC-Cy7), CD45RA (Pacific Blue), and CD31 (WM59, PE) from BD Biosciences and anti-CD20 (AlexaFluor[®]700) and anti-CD31 (PECAM 1.2, FITC) from Invitrogen (1µl of each). At least 50,000 events were
25 acquired in the lymphocyte gate using a BD LSRII[®] equipped with 3 lasers (405, 488 and 633 nm) and analysed with BD DIVA[®] 6.0 software.

Subtractive measurement of soluble CD31. To detect the splice variant and truncated CD31 in plasma and the culture supernatant, a cytokine bead array (CBA[®], BD) has been customized. Three differently functional CBA beads (A9, D5
30 and E9) were coupled with either one of the following purified monoclonal anti-CD31 antibodies JC70A (domain 1, DAKO), MEM-05 (domain 5, Zymed) and PECAM 1.2 (domain 6, Invitrogen). The coupled beads were then incubated with the plasma of the same 5 healthy controls (Figure 2) or the culture supernatant

and positive binding of circulating CD31 was detected by a fourth anti-CD31 monoclonal antibody, WM-59 (domains 1-2) coupled to PE (BD). The concentration of plasma CD31 including at least domain 1 (JC70A), or domains 1 to 5 (MEM-05) or all the extracellular domains 1 to 6 of CD31 (PECAM 1.2) was determined by analysing the median fluorescent intensity of the detecting antibody on ≥ 1000 gated beads on samples and serial dilutions of the same standard (recombinant, full length extracellular CD31, R&D Systems). The standard curve was obtained for each of the beads using the same known concentrations of the recombinant CD31 in order to overcome any bias due to differences in binding affinity of the diverse antibodies. The concentration in ng/ml of CD31 determined with PECAM 1.2 coupled beads (dom 1-6) was subtracted from the one obtained using MEM-05 coupled beads to obtain the amount of circulating CD31 lacking dom6 (dom 1-5). The latter was subtracted from the concentration of CD31 obtained using the JC70A-coupled beads to calculate the value of soluble CD31 lacking both dom 5 and 6 but containing at least domains 1 and 2 (dom 1-2).

Assessment of CD31-ITIM phosphorylation. Log-phase Jurkat cells (10^7 cells/condition) were either left unstimulated (negative control) or incubated with pervanadate (positive control) or stimulated with anti-CD3 and anti-CD28 antibodies (R&D Systems, 1 $\mu\text{g/ml}$ each) in the presence or absence of peptide 551-574 (100 μM), or incubated with the peptide alone during 20 minutes. Cells were then lysed with 1 ml of RIPA buffer on ice for 30 minutes, ultracentrifuged and 16 μl of the supernatant was incubated with PECAM 1.2-coated Functional E9 CBA[®] beads (BD) for 2 hours at room temperature. Beads were subsequently washed with CBA washing buffer and incubated with 2 μl of undiluted rabbit anti-CD31 phospho-tyrosine 686 (pY686) sera followed by two washings and incubation with AlexaFluor[®]488-conjugated (Fab')₂ fragments (1:100 in CBA washing buffer) of goat-anti-rabbit IgG (Invitrogen). The beads (2000/condition) were finally analysed by flow cytometry in the FITC channel (530/30 nm) and data are expressed as Median fluorescence intensity (MFI) \pm the percentage of the coefficient of variability (% CV) calculated with the DIVA 6.0[®] software (BD). Duplicate lysate aliquots and serial dilutions of recombinant CD31 were incubated with the PECAM 1.2-coated beads and the amount of dom1+ cell-bound CD31

was revealed using anti-CD31 WM59-R-PE (dom1) and PECAM 1.2-FITC (dom6) antibodies (data shown in Figure 3a).

Fluorescent peptide binding. For visualisation of peptide binding to CD31⁺ and CD31^{shed} CD4⁺ T cells, freshly purified peripheral blood leukocytes prepared as above were washed with a buffered solution containing 2mM EDTA (to avoid endocytosis of the peptide) and incubated overnight at room temperature in a dark humidified chamber with 50µM FITC-labelled CD31 peptide 551-574 and 1:10 dilution of fluorescent monoclonal anti-CD31 (PE) and anti-CD4 (APC) antibodies (BD Biosciences) in a poly-D-Lysine coated ibidi® 8-well culture chamber (Biovalley). Cells were then washed twice, nuclei counterstained with DAPI and digital images of a 0.3µm intracellular section were acquired on a Zeiss Axiovert M200 microscope (x63 immersion objective) equipped with the ApoTome® and a cooled monochromatic digital camera (Zeiss).

Calcium mobilisation assay. Spleen cells from C57Bl/6 mice were prepared as described in Caligiuri et al. (2005 Arterioscler Thromb Vasc Biol 25:1659-1664). Cells were incubated with Fluo-3AM (Invitrogen, # F1242) as per the instructions of the manufacturer. Fluorescence of calcium-bound tracer was measured in the FITC channel on an LSRII® cytometer (BD Biosciences) prior to and during 60 seconds following the addition of hamster anti-mouse CD3/CD28 monoclonal antibodies (40µg/ml each) and rat/hamster compBead® (1:50) either alone or in the presence of rat anti-mouse CD31 antibody (clone 390, 10 µg/ml) or in the presence of CD31 peptide 551-574 (100µM). Negative controls included rat IgG isotype control and scramble peptide. Antibodies and compBeads® were from BD Biosciences.

Plasmon Surface Resonance. Homophilic binding association and dissociation constants were calculated by surface plasmon resonance (BIAcore® 2000, GE). In brief, peptide 551-574 was coated at 3400 resonance units (RU) on CM5 chips according to the manufacturer's instructions. Soluble peptide 551-574 (12.5, 25, 50 and 100 µM in 200 µl of 10 mM HEPES pH 7.4, 150 mM NaCl, 0.005% Tween 20) was injected at 20 µl/min at 25°C, on the peptide-coated channel and on an uncoated channel. Dissociation was monitored for 300 seconds. Association (kon) and dissociation (koff) constants were calculated

using the BIAevaluation[®] 3.0 Software (GE). Injection of peptide 551-574 on a channel coated with the scramble peptide yielded negligible signal.

Evaluation of immunoregulation *in vitro*. CD8⁺ T cell-mediated cytolytic activity against allogeneic mouse aortic smooth muscle cells and measurement of macrophage gelatinase (MMP-2/9) activity were performed as previously described for human cells in Caligiuri et al. (2006 *Arterioscler Thromb Vasc Biol* 26:618-623) using kits and reagents from Invitrogen. Briefly, primary cultures of FVB/N mouse aorta smooth muscle cells were labelled with the lipophylic tracer DIO (green) and co-cultured for 3 hours with CD8⁺ T cell-enriched spleen cells from C57Bl/6 mice (n=3 scramble peptide and n=3 peptide 551-574, 50µM). Cytolysis was evaluated by intracellular accumulation of propidium iodide (PI). Cells were analysed by flow cytometry and the % of cytolysis was calculated by expressing the number of dead (PI⁺) cells among the target (DIO⁺) cells. Intracellular MMP-2/9 (gelatinase) activity was measured by flow cytometry in 7-day bone-marrow derived macrophages from C57Bl/6 mice (n=3 scramble peptide and n=3 peptide 551-574, 50µM) three hours after the incorporation of OregonGreen[®] gelatine (MFI). T-cell proliferation was performed using either human peripheral blood mononuclear cells or spleen cells from C57Bl/6 (CD31^{+/+}) and CD31^{-/-} mice (Charles River France) as previously described (Caligiuri et al. 2005 *Arterioscler Thromb Vasc Biol* 25:1659-1664). Briefly, cells were plated in triplicates at 0.2x10⁶ cells/well in a U bottom 96-well plate in complete medium (RPMI 1640, 1% pyruvate, 1% glutamine, 1% penicillin-streptomycyne-fungizone, 10% decompemented fetal calf serum, all from Invitrogen) containing 1µg/ml anti-mouse CD3/CD28 or 5µg/ml anti-human CD3 antibodies (BD) as appropriate. CD31 (551-574) and scramble peptide at 25, 50 and 100 µM final concentration were deposited in the wells just before cell plating. Plated cells were cultured for 72 hours in 5% CO₂ at 37°C. (³H) thymidine (0.5µCi/well) was added for the last 16 hours and proliferation evaluated using a Tomtec harvester and analysis on a Wallac micro beta counter. Data are expressed as mean±SEM of cpm in triplicates.

Evaluation of immunoregulation *in vivo*. Delayed type hypersensitivity (DTH) suppression was evaluated as described in the "Current Protocols in Immunology (2001) 4.0.1-4.0.2 Unit 4.2". Briefly, TNCB (2-chloro-1,3,5-trinitrobenzene, Fluka

#79874) was dissolved in acetone/olive oil (1:1 v/v) at a concentration of 10 mg/ml. BALB/c mice (n=6/group) were primed by painting the shaved regions of the abdomen a with a total 0.2 ml of the preparation (n=6/group). The experiment included 3 groups for peptide 551-574 (10, 50, 100 μ M) and 1 group treated with
 5 scramble peptide at 100 μ M). Five days after priming, 10 μ l of the TNCB-solvent mixture was painted on the right pinna, 30 minutes after subcutaneous (interscapular) administration of the peptide 551-574 or the scramble peptide. Ear thickness increases were calculated by subtracting the thickness of the right and the left pinna of each mouse (right-left/left x 100), measured at 24 h with a dial
 10 caliper ("Pocotest", Kroeplin Längenmesstechnik). The measure was performed 5 times on each ear and averaged for further analysis. The immunosuppressive effect of the peptide was calculated as % suppression = $(1 - \Delta TE / \Delta TS) \times 100$, where $\Delta T =$ (ear thickness 24 hr after elicitation) – (baseline ear thickness), E=sensitised animals, S=treated animals. Data are expressed as mean \pm SEM.

15 Detection of atherosclerotic lesion size and aneurysm formation. Male 28-week old apolipoprotein E^{-/-} mice (n=8-10 mice/group) from our breeding facility were maintained on a regular chow diet and kept under standard conditions. Acceleration of atherosclerosis and aneurysm formation was induced by subcutaneous angiotensin II (Sigma, #A9525) infusion (1mg/kg/d) for 28 days
 20 using osmotic minipumps (Alzet, #2004) as previously described (Daugherty et al. J Clin Invest 105:1605-1612). All experiments were approved by our institutional Ethical committee. Atherosclerotic lesion size was measured as previously described (Caligiuri et al. 2005 Arterioscler Thromb Vasc Biol 25:1659-1664). These experiments were repeated twice with similar results.

25 Peptides. All experiments on human material were carried out using the human peptide sequence while the mouse equivalent was used in all mouse experiments. The sequences of the peptides are shown in the table below.

Human	NH2 - NHASSVPRSKILTVRVILAPWKK - COOH	SEQ ID NO: 6
Mouse	NH2 - SSMRTSPRSSTLAVRVFLAPWKK - COOH	SEQ ID NO: 5
Scramble	NH2 - SMPAVRSRFSATSLVTLKSRWPK - COOH	SEQ ID NO: 13

Example 2: The apparent loss of CD31 at the surface of blood lymphocytes is due to its shedding between the 5th and 6th extracellular Ig-like domains

In order to establish whether the loss CD31 was restricted to part or
5 extended to the totality of its 6 extracellular Ig-like domains, a multicolor flow
cytometry analysis of whole blood leukocytes from 5 healthy donors using two
different antibodies specifically recognizing the membrane-distal and membrane-
proximal Ig-like domains of the molecule was performed. To be able to
discriminate between the different leukocyte populations and assess their state of
10 maturation and activation, a panel of lineage markers as well as the expression of
CD45RA and HLA-DR were simultaneously used. While the expression of CD31,
as detected by a monoclonal antibody specific for the first domains of CD31 (clone
WM-59, dom1-2) was recognized on naïve but not on activated/memory blood T
cells, all cells expressed the membrane-proximal extracellular fragment of the
15 molecule detected by another monoclonal antibody specific for the 6th Ig-like
domain of CD31 (clone PECAM 1.2, dom6), irrespective of their state of
maturation/activation (Figure 2).

Flow cell cytometry showed that T-cell receptor (TCR) engagement induces a
shift of >80% of blood resting T cells from a CD31 dom1⁺/dom6⁺ to a dom1⁻/dom6⁺
20 (CD31^{shed}) phenotype. Molecular analysis of the membrane proteins from cultured
T-cell lysates demonstrated that >99% of the T cell-bound CD31 molecules drop
the distal portion containing dom1 already 5' minutes after TCR stimulation *in vitro*
(Figure 3a). Analysis of the supernatant showed that, simultaneously, a single
truncated soluble protein limited to the first 5 Ig-like domains of CD31,
25 accumulates in the culture supernatant (Figure 3b). Furthermore, the analysis of
the plasma of the same healthy donors showed that major part of soluble CD31 in
plasma was constituted of a truncated molecule comprising Ig-like domains 1 to 5
and specifically lacking the membrane-proximal 6th domain (Figure 3b) that always
remains anchored to the apparently CD31-negative (CD31 dom1⁻) lymphocytes
30 both *in vitro* and *in vivo*. Only a minimal fraction of soluble CD31 contained all 6
extracellular domains predicted in the previously reported (Goldberger et al. J Biol
Chem 269:17183-17191) variant spliced form both in culture supernatant and in
plasma (Figure 3b). No significant other cleavage of the molecule occurs upstream

of the 5th domain since the latter was virtually always present concomitantly with the first domain in the truncated soluble CD31 proteins (Figure 3b).

Example 3: A peptide contained in the residual extracellular CD31 fragment on CD31^{shed} T cells enhances phosphorylation of CD31-ITIM

A CD31 dom6-derived synthetic peptide corresponding to the juxta-membrane 23 aminoacids (551-574) of the ectodomain of the human molecule binds both to CD31 dom1⁺ and to CD31 dom1⁻ (CD31^{shed}) CD4⁺ T lymphocytes *ex vivo*. Importantly, the binding of this peptide on T cells has functional consequences on immune cell control since it exerted dose-dependent inhibition of human peripheral blood T-cell proliferation *in vitro* (Figure 4a). To assess whether the inhibitory effect of the peptide could be mediated by homophilic binding and engagement of the CD31 signaling, the level of phosphorylation of the CD31 ITIM tyrosine at position 686 (₆₈₆ITIM) in cultured T cells was evaluated. Stimulation of the TCR by anti-CD3 and anti-CD28 antibodies alone or the sole presence of the peptide induced a minor increase of CD31 pY686 (Figure 4b) but concomitant TCR-stimulation in the presence of the peptide boosted the phosphorylation the CD31 ₆₈₆ITIM by a factor of >23 (Figure 4b).

Example 4: CD31 homotypic peptide 551-574 inhibits a large array of cell-mediated immune responses *in vitro* and *in vivo*

To further test our hypothesis, *in vivo* experiments were performed, and the murine equivalent of CD31 (551-574) peptide was therefore synthesized. Its properties were evaluated *in vitro* and *in vivo*. Its homophilic interaction by surface plasmon resonance analysis was first established (Figure 5a). The association and dissociation constants were of $134 \pm 120 \text{ M}^{-1}\text{s}^{-1}$ and $1.58\text{E-}03 \pm 1.07\text{E-}03 \text{ s}^{-1}$, respectively and the affinity at equilibrium was $17.8 \pm 9.7 \mu\text{M}$, in agreement with previous measurement done on recombinant human CD31 homo-dimerization using other systems. It was next assessed whether the mouse CD31 (551-574) peptide was able to inhibit calcium mobilization in response to the co-engagement of the CD3 and of the co-stimulatory molecule CD28 in spleen lymphocytes. The data showed that homophilic targeting of the juxta-membrane portion of CD31 with this small peptide is as an efficient immunoregulatory strategy as it is the co-

ligation of CD3 and CD28 with the distal portion of CD31 by cross-linking the molecules using specific monoclonal antibodies (Figure 5b). The engagement of the CD31 molecular pathway by this peptide could attain effective suppression of antigen-driven lymphocyte responses *in vitro* and *in vivo*. It was found that peptide 551-574 inhibits in a dose-dependent manner the proliferation of spleen cells in response to stimulation of the T cell-receptor *in vitro* (Figure 5c). At low dose, the effect of the peptide was exclusively due to its homophilic binding with CD31 molecules since no effect was observed on spleen cells from CD31^{-/-} mice (Figure 3c). However, at high concentrations the peptide can also bind to other (low affinity) heterophilic ligands as suggested by its effect at 100µM dose also on cells lacking CD31 (Figure 5c). The scramble peptide, used at the highest dose, was ineffective (Figure 5c). Finally, the therapeutic immunosuppressive potential of this peptide *in vivo* in the context of matched, histocompatible antigen-driven immune responses was evaluated using a model of delayed type of hypersensitivity. Distal recall of an hapten-elicited specific immune response was suppressed in a dose-dependent manner by peptide 551-574 in this model (Figure 5d). Effective immunosuppression was achieved by a single subcutaneous administration of 50 µM of the peptide 551-574 while the scramble peptide was ineffective (Figure 5d).

20

Example 5: CD31^{shed} cell-targeting peptide biotherapy prevents disease progression and aneurysm formation in atherosclerotic mice

The model of angiotensin II infusion into aged apolipoprotein E^{-/-} mice (25) was used, this model closely mimicking atherothrombosis in humans. Daily subcutaneous administration of 50 µM of the peptide prevented both the acceleration of plaque growth in the aortic root (Figure 6a) and the formation of atherothrombotic abdominal aortic aneurysms (AAA) in this model (Figure 6b). *in vitro*, the CD31-derived peptide 551-574 inhibited both the activity of matrix degrading enzymes in bone-marrow derived macrophages and CD8⁺ T cell-dependent cytotoxicity of murine aortic smooth muscle cells (Figure 6c).

30

Example 6: *In silico* and *in vitro* validation of the 10 amino acid-long peptide candidate peptide "PepReg"

A shorter nested peptide corresponding to the ten COOH terminal amino acids was tested. This peptide is shown as SEQ ID NO: 3 and referred to as "PepReg". As shown in the table below, this peptide was expected to be more stable than the CD31 551-574 peptide of 23 amino acids.

Table: Characteristics of PepReg vs the 23aa CD31 peptide *in silico*

	PepReg CD31 564-574 (10aa)	CD31 551-574 (23aa)
sequence	VRVFLAPWKK (SEQ ID NO: 3)	SSMRTSPRSSTLAVRV FLAPWKK (SEQ ID NO: 5)
Number of amino acids	10	23
Molecular weight	1243.5	2606.0
Theoretical pI	11.17	12.31
Formula	C62H98N16O11	C116H193N35O31S1
- (Asp, Glu) /+ (Arg, Lys) charges	0/3	0/5
Instability index	25.38	65.09
Estimated half-life (mammalian reticulocytes, <i>in vitro</i>)	100 hours	1.9 hours
Aliphatic index	107.00	68.83

The immunosuppressive properties of PepReg (10aa) vs the 23 aa parent peptide were evaluated *in vitro*. Negatively purified CD4+ cells from C57Bl6 mice were stimulated by soluble anti-CD3 purified antibodies and bone marrow derived dendritic cells. Cells from triplicate wells were analyzed for the expression of the early activation marker CD69 after 18 hours culture in complete RPMI medium supplemented with 10% fetal calf serum. Flow cytometry showed that PepReg was at least as efficient as the 23aa peptide in suppressing T cell activation as determined by the percentage of CD4 cells expressing CD69. Interestingly, the effect was more reproducible (smaller standard deviation) and was observed with lower doses (50µg/ml vs 100µg/ml) of PepReg (10aa) as compared to the parent (23aa) peptide. The suppression of T cell proliferation by PepReg was analyzed by [H3] thymidin incorporation and persisted up to 7 days of culture at 37°C in 10%

serum demonstrating that the data regarding the stability of the peptide obtained *in silico* were validated *in vitro*.

Example 7: In vivo validation of the 10 amino acid-long peptide candidate peptide “PepReg” in the EAE model

Experimental Autoimmune Encephalomyelitis (EAE), also called Experimental Allergic Encephalomyelitis, is an animal model of Multiple Sclerosis.

Twelve-week old female C57BL/6J mice were immunized with 300µg of MOG35-55 peptide emulsified in Complete Freund’s Adjuvant 1:1 by volume containing 800µg of nonviable desiccated Mycobacterium tuberculosis H37RA. A final volume of 200µl of the emulsion was injected subcutaneously at 4 sites (50µl/site) over the flanks. In addition, 300ng of Pertussis toxin was injected intravenously (retro-orbital plexus) on the same day and 2 days later. Clinical signs of EAE were assessed daily by the following scoring system: 0, no signs; 1, hindlimb weakness; 2, hindlimb weakness and tail paralysis; 3, hindlimb and tail paralysis; 4, hindlimb and tail paralysis and forelimb weakness; 5, moribund state; and 6, death. The peak (waxing phase) occurred around day 21. In this C57BL/6J mouse model, there was no waning phase as assessed in our laboratory up to day 41.

The experiment was carried out with ten mice per group. The mice of each group were treated with either of:

- PBS;
- PepReg (SEQ ID NO: 3); or
- PepScra (SEQ ID NO: 14).

The dosing was of 50µg of peptide per mice and per day (i.e. about 2 mg/Kg per day). The peptide was administered by a subcutaneous injection.

Disease protection was associated with reduced infiltration of IL17+ and IFNγ+ T helper CD4+ cells and increased proportion of regulatory CD25+/foxP3 CD4+ cells in the central nervous system of the mice.

As shown on Figure 7, PepReg is capable of arresting disease development in the waxing phase (Day 15) and reducing disease extension in the plateau phase (Days 21 through to 35).

The scrambled peptide was also beneficial, although less than PepReg. This result suggests that the amino acid composition rather than the sequence *per se* is important for the beneficial effect. This result has important implications for the development of peptidomimetics.

5

Example 8: Detection of shed CD31 in plasma from patients suffering from atherothrombosis and in unaffected individuals

The total amount of CD31, the amount of shed CD31 and the amount of spliced CD31 has been measured both in eleven individuals suffering from atherothrombosis and in twenty-three unaffected individuals.

The group "Atherothrombosis" comprised eleven individuals suffering from chest pain even at rest and presenting an abnormal coronarography.

The group "No Atherothrombosis" comprised twenty-three individuals. A sub-analysis was carried out on the group "No Atherothrombosis", which was found to comprise:

- eight individuals presenting a normal coronarography and a normal carotid echodoppler in spite of chest pain;
- four individuals presenting a normal coronarography in spite of chest pain, but in whom atherosclerosis was detected by carotid echodoppler; and
- eleven individuals suffering from chest pain only on effort and presenting an abnormal coronarography (i.e. suffering from coronary atherosclerosis without thrombosis).

The total amount of CD31, the amount of shed CD31 and the amount of spliced CD31 was determined as follows.

1. The total amount (1µl/test) of beads (E9, coupled with clone JC70A, DAKO) was transferred to a conical tube and centrifuged at 200g for 5 minutes. The supernatant was carefully discarded and replaced with same amount of serum enhancement buffer (BD #51-9002150), and incubated at room temperature for 15 minutes.

2. The fluorescently-labeled antibody antibody mix (PE-WM59; FITC-HC1/6; PB-PECAM1.2) was prepared, each at 1µg/ml, 1 µl each/condition.

3. 1 tube precondition was prepared, each containing 3 µl of a standard dilution or a plasma sample. The reconstituted beads were centrifuged at 200g for

5 minutes, the supernatant was discarded and the serum enhancement buffer was replaced with the fluorescently-labeled antibody mix. 3µl of this solution was distributed in each of the tubes containing the standard dilution and samples, and the solution incubated for 1 hour at 4°C in the dark.

- 5 4. 150µl of Washing buffer (BD # 51-9003797) were added to each tube, and the signal was acquired.

As shown in the table below, the percentage of shed CD31 was higher in individuals suffering from atherothrombosis than in unaffected individuals, in spite of the fact that all individuals were suffering from chest pain.

CD31 Plasma Level (ng/ml)	total	splice	shed
Atherothrombosis (n=11)	11.55±0.7	-7.02±2.69	18.57±2.67
No Atherothrombosis (N=23)	11.58±0.49	5.26±1.850	6.31±1.85
T-test Prob>F	0.9756	0.0007	0.0006

Total CD31 amounts were similar in the four groups, while the amount of shed CD31 and the amount of spliced CD31 were significantly different in each paired group comparison. Shed CD31 was increased in individuals with abnormal coronarography, with highest values in those suffering from atherothrombosis. Splice CD31 was still present in patients suffering from atherosclerosis without atherothrombosis, while it was almost undetectable in patients suffering from atherothrombosis.

These results demonstrate that high levels of CD31 soluble splice variants associated with low levels of shed CD31 indicates that the patient suffers from non specific chest pain, eventually associated with carotid plaques. A slight increase of shed CD31 levels associated with normal or reduced levels of CD31 soluble splice variants indicates that the patient suffers from atherosclerosis. An important increase of shed CD31 levels associated with undetectable amounts of CD31 soluble splice variants indicates that the patient suffers from atherothrombosis.

25

Example 9: Discussion of the results

Dysimmune diseases are linked to lack of appropriate control of immune responses. Atherosclerosis and its complications are not only due to metabolic disturbances but are increasingly recognized as a dysimmune disease and an

important current issue is the identification of interventional tools able to restore immunoregulation. It has been previously shown that atherothrombotic manifestations such as plaque rupture and thrombosis (Caligiuri et al. 2005 Arterioscler Thromb Vasc Biol 25:1659-1664) or aneurysm complication (Caligiuri et al. 2006 Arterioscler Thromb Vasc Biol 26:618-623) are associated with a significant reduction of CD31+ T lymphocytes in the peripheral blood. In these previous works, we documented that lack of CD31 signaling on lymphocytes elicited pro-atherothrombotic immune responses whilst the presence of CD31 on T cells was able to inhibit them.

Here it is demonstrated that the assumed loss of the molecule on activated/memory T lymphocytes is actually incomplete and results from shedding of CD31 between the 5th and 6th extracellular Ig-like domains. CD31 shedding occurred immediately after cell activation on T lymphocytes and was accompanied by the accumulation of the truncated molecule in the supernatant together with trace levels of the spliced variant produced by the cells. This finding was unsuspected because all commercially available tests to detect plasma CD31 use antibodies directed to CD31 domains 1 to 5, and therefore cannot discriminate between the spliced variant (containing all the 6 extracellular domains) and the truncated (domains 1 to 5) forms of CD31. The subtractive immunosorbent assay described herein is able to discriminate between the two forms of soluble CD31 and precisely quantify the proportion of each of them in the plasma. This assay showed that the major part of plasma CD31 comprises domains 1 to 5 but lacks the membrane-proximal 6th domain, which remains anchored to blood CD31 dom1- lymphocytes. Therefore, it is proposed to refer to these lymphocytes as CD31shed rather than CD31 "negative" cells. Previous work *in vitro* had indicated that CD31 shedding at an unidentified position N-terminal from the transmembrane segment of the molecule can occur in endothelial cells undergoing apoptosis (Ilan et al. 2001. Faseb J 15:362-372). For the first time, it is shown herein that shedding is responsible for the CD31 (incomplete) loss on blood lymphocytes and that the circulating CD31 consists mainly of a truncated form derived from its cleavage between the Ig-like domains 5 and 6, rather than of the secreted spliced variant form. Genetic polymorphisms for CD31 have been described, but the predictive value of soluble CD31 levels was conflicting either in atherothrombosis

or other dysimmune diseases. In fact, while the amount of the spliced form can be predicted by different genetic variants, the proportion of the form resulting from protein shedding is not determined by CD31 gene polymorphism. It is proposed that the disparity between the different studies was due to fact that circulating
5 CD31 is a mixture of the genetic variant and of the truncated form and discrimination between the two forms of CD31 was not possible. The subtractive method described herein will allow the differentiation of the prognostic value determined by genetic variants of CD31 independently of that linked to CD31 shedding.

10 The fact that CD31 is not completely lost on blood lymphocytes offers a unique opportunity to rescue its physiological immunoregulatory function by targeting the residual extracellular portion of the molecule. Indeed, it has been documented herein that this can be achieved by a homotypic peptide-based therapy, both *in vitro* and *in vivo*. Homophilic binding of this peptide dramatically
15 enhanced the phosphorylation the CD31 686ITIM and inhibited their TCR-induced proliferation. Induction of CD31 ITIM phosphorylation by antibody-mediated cross-linking of CD31 and CD3 surface molecules was previously known to inhibit calcium mobilization induced by anti-CD3 antibodies in human T-cell lines. Remarkably, it has been found that targeting the juxta-membrane portion of CD31
20 whith the small homotypic peptide is as an efficient immunoregulatory strategy as it is the co-ligation of CD3 and CD28 with the distal portion of CD31 by cross-linking the molecules with antibodies. A selective small synthetic peptide strategy is obviously simpler and might also be safer than using large proteins, such as monoclonal antibodies and cross-linkers, for the biotherapy of immunological
25 disorders (Isaacs 2007. Curr Opin Pharmacol 7:418-425).

With this idea in mind, it was assessed whether the engagement of the CD31 molecular pathway by this peptide could attain effective suppression of antigen-driven lymphocyte responses *in vitro* and *in vivo* in the context of matched, histocompatible antigen-driven immune responses. Distal recall of an hapten-
30 elicited specific immune response was suppressed in a dose-dependent manner by a single subcutaneous administration of the peptide. A similar protective effect of a single peptide shot was also observed in experimental autoimmune

encephalomyelitis (a mouse model of multiple sclerosis) and lasted for up to 5 days.

Consequently, it was evaluated whether rescuing of CD31-mediated immunoregulation by the synthetic peptide 551-574 could be employed in a
5 biotherapy to fight atherothrombosis since CD31-mediated immunoregulation is typically lost in this disease. It was chosen to use the angiotensin-induced model of atherothrombosis because the abrupt acceleration of atherosclerotic plaque growth and the development of abdominal aortic aneurysms complicated by a thrombus in this model are produced simply by excess bioavailability of a
10 physiological peptide - angiotensin II - and does not require the use of surgery, gene transfer or high fat diet, each of which could considerably bias the interpretation of the results. The CD31-peptide prevented both the acceleration of plaque growth in the aortic root and the formation of atherothrombotic abdominal aortic aneurysms in this model. Such a dramatic protective effect was superior to
15 any therapeutic molecule ever tested and equivalent to that achieved by genetic manipulation.

Macrophages and lymphocytes represent the most important immune cells involved in the development of atherothrombosis. The local function of these cells injure the cellular and extracellular components of the arterial layers resulting in
20 either plaque rupture and luminal thrombosis, when occurring in the fibrous cap of atherosclerotic plaques, or aneurysm formation and eventually rupture, when happening in the outer layers of the artery. Degradation of the extracellular matrix is essentially due to the activity of macrophage-derived matrix metalloproteases while death of arterial smooth muscle cells is putatively caused by T cell-mediated
25 cytolysis. Remarkably, CD31+ T lymphocytes exert an important immunosuppressive function on both these phenomena which are conversely aggravated by CD31shed T cells. An aberrant reduction of CD31+ cells in patients with atherothrombosis underlies the defective immunoregulation observed in the disease. Here it is shown that the CD31-derived peptide 551-574 inhibits both the
30 activity of matrix degrading enzymes and T cell-dependent cytolysis of the arterial wall cells. The immunoregulation conveyed by this peptide is as efficient as that exerted by immunoregulatory CD31+ T cells and hence may counterweight the

loss of the physiologic CD31-dependent immunoregulation in human atherothrombosis.

This is the first time that a peptide-based biotherapy is envisaged to correct the defective immunoregulation characteristic of atherothrombosis and prevent development of the disease in patients. In addition, such biotherapy may broaden over to other debilitating dysimmune diseases. In particular, experimental studies have suggested that CD31-signalling might play a protective role in rheumatoid arthritis (Wong et al. 2005. J Clin Immunol 25:19-28), multiple sclerosis (Graesser et al. 2002. J Clin Invest 109:383-392) and non-alcoholic fatty liver disease (Goel et al. 2007. Am J Physiol Gastrointest Liver Physiol 293:G1205-1214).

Example 10: Evaluation of ninety six peptides according to the invention

The ninety six peptides consisting of a fragment having a sequence selected from the group consisting of:

- amino acids 2 to 23, 3 to 23, 4 to 23, 5 to 23, 6 to 23, 7 to 23, 8 to 23, 9 to 23, 10 to 23, 11 to 23, 12 to 23, 13 to 23, 14 to 23, 15 to 23, 16 to 23, 17 to 23, 18 to 23, 19 to 23, 20 to 23 and 21 to 23 of SEQ ID NO: 5; and
- amino acids 2 to 23, 3 to 23, 4 to 23, 5 to 23, 6 to 23, 7 to 23, 8 to 23, 9 to 23, 10 to 23, 11 to 23, 12 to 23, 13 to 23, 14 to 23, 15 to 23, 16 to 23 and 17 to 23 of SEQ ID NO: 6; and
- amino acids 1 to 22, 1 to 21, 1 to 20, 1 to 19, 1 to 18, 1 to 17, 1 to 16, 1 to 15, 1 to 14, 1 to 13, 1 to 12, 1 to 11, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4 and 1 to 3 of SEQ ID NO: 5; and
- amino acids 1 to 22, 1 to 21, 1 to 20, 1 to 19, 1 to 18, 1 to 17, 1 to 16, 1 to 15, 1 to 14, 1 to 13, 1 to 12, 1 to 11, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4 and 1 to 3 of SEQ ID NO: 6; and
- amino acids 2 to 22, 3 to 21, 4 to 20, 5 to 19, 6 to 18, 7 to 17, 8 to 16, 9 to 15, 10 to 14 and 11 to 13 of SEQ ID NO: 5; and
- amino acids 2 to 22, 3 to 21, 4 to 20, 5 to 19, 6 to 18, 7 to 17, 8 to 16, 9 to 15, 10 to 14 and 11 to 13 of SEQ ID NO: 6;

are tested for confirming that these peptides induce CD31-ITIM phosphorylation. CD31-ITIM phosphorylation is assessed by studying the effect of increasing doses of the peptides according to the invention on CD31-ITIM phosphorylation of cultured Jurkat cells stimulated with anti-CD3 and/or anti-CD28 antibodies, as described in Example 3.

It is confirmed that the above peptides inhibit T-cell proliferation using the protocol described by Caligiuri et al. (2005 Arterioscler Thromb Vasc Biol 25:1659-1664).

It is confirmed that the peptides can achieve effective immunosuppression using the protocol described in the Current Protocols in Immunology (2001, 4.0.1-4.0.2 Unit 4.2).

The efficiency of the peptides for treating a thrombotic disorder is confirmed in the model of angiotensin II infusion into aged apolipoprotein E-/- mice, which closely mimicks atherothrombosis in humans.

The efficiency of the peptides for treating an autoimmune disorder is confirmed in the Experimental Autoimmune Encephalomyelitis (EAE) model, which is an animal model of Multiple Sclerosis, and in a model for rheumatoid arthritis.

Example 11: Evaluation of the peptides according to the invention in an animal model of the rheumatoid arthritis (RA)

It is confirmed that the peptides according to the invention (e.g. PepReg and/or the peptides described in Example 10) are capable of arresting disease development and/or reducing disease extension in an animal model of Rheumatoid arthritis (RA).

Rheumatoid arthritis (RA) is a chronic and systemic inflammatory autoimmune disorder that causes the immune system to attack the joints. The disease is characterized by aggressive synovial hyperplasia (pannus formation) and inflammation (synovitis), which, if left untreated, lead to progressive destruction of joint cartilage and bone. The destructive lesions result from both immune responses and non-antigen-specific innate inflammatory processes. Several studies have shown that CD31 plays a critical role in this disease since the disease onset and progression is accelerated in its absence.

DBA/1 mice are used in this experiment. Induction of RA is initiated on 12 week-old mice. On day 0, mice are immunized intradermally at the base of the tail with 150 µg of bovine type II collagen (CII) emulsified with an equal volume of Freund's complete adjuvant containing 200 µg of H37RA Mycobacterium tuberculosis. On day 21, mice are given a booster (intradermal injection of 150 µg of bovine CII in Freund's incomplete adjuvant). Simultaneously, mice receive an intravenous injection of 50µg of LPS. Mice are followed up for two months. Following immunization, the animals develop an autoimmune polyarthritis that is characterized by severe cartilage and bone erosions. Mouse collagen-induced RA shares several clinical, histopathological and immunological features with human RA.

The peptides according to the invention are administered following one of the below treatment schemes.

- Scheme 1: Preventive administration. Two doses (50 and 100 mg/kg) of the peptide is administered to the mice one day before the CII immunization and then either daily, or twice a week, or weekly, for the study period (2 months). A peptide with a scrambled sequence (i.e. a peptide comprising the same amino acids as the peptide according to the invention, but not the same sequence) is administered to a control group of mice. Treatment is pursued for one month.
- Scheme 2: Curative administration. The peptide (50 and 100 mg/kg) and the scramble peptide are administered to mice after the beginning of the symptoms, either daily, or twice a week or weekly until the end of the study. Equivalent groups of mice are kept in conventional housing facilities and are bled weekly from a tail vein to monitor bleeding time and specific pathogen antibody raise in sera (CDTA, Orleans).

Arthritis development is monitored by physical examination 3 times per week. Inflammation in each of the 4 paws is graded on a scale of 0 to 3, and the scores for the 4 paws will be cumulated (yielding a maximum score of 12 per mouse). The arthritis index is calculated by dividing the total score in the experimental mice by the number of arthritic mice.

Lesions in the joints are also followed. Ankle joints of mice are excised 6 weeks after immunization and fixed in 10% buffered formalin, decalcified in 10% EDTA, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The intensity of synovial hyperplasia, cellular inflammation, and pannus formation is evaluated, and arthritis is graded in a blinded manner on a scale of 0 to 4.

Immunohistochemistry is further used to track and to phenotype inflammatory cells infiltrated in the joints.

The immunoregulation status is evaluated by measuring levels of serum IgG1 and IgG2a to CII. The measurement is performed by enzyme-linked immunosorbent assay (ELISA). The proliferation of T cells isolated from draining lymph nodes and the spleen is tested by the incorporation of ³H thymidine in response to CII-loaded dendritic cells.

Cell populations in the lymphoid organs and in the synovia is analyzed by flow cytometry.

This experiment allows confirming that continuous administration of the peptides according to the invention prevents onset of RA. The curative phase allows evaluating the therapeutic potential of the peptides for treating RA in patients that do not respond to the current biologicals (i.e. 40% of the patients). Dose and frequency of the administrations able to drive a regression of the inflammatory cells in the joints, and regression of the clinical score, are also determined.

CLAIMS

1. An isolated peptide consisting of:
 - 5 a) a fragment of at least 6 amino acids of the sequence defined by amino acids 579 to 601 of SEQ ID NO: 1;
 - b) a fragment of at least 6 amino acids of the sequence corresponding to (a) in a non-human mammalian CD31; or
 - 10 c) a fragment consisting of a sequence at least 80% identical to (a), wherein said peptide exerts a dose-dependent inhibition of T-cell proliferation *in vitro* and does not consist of an amino acid sequence of SEQ ID NO: 5 or 6.

2. The peptide according to claim 1, wherein said peptide consists of:
 - 15 a) a fragment of 6 to 15 amino acids of the sequence defined by amino acids 579 to 601 of SEQ ID NO: 1;
 - b) a fragment of 6 to 15 amino acids of the sequence corresponding to (a) in a non-human mammalian CD31; or
 - c) a fragment consisting of a sequence at least 80% identical to (a), wherein said peptide exerts a dose-dependent inhibition of T-cell proliferation *in vitro*.

- 20 3. The peptide according to claim 1 or 2, wherein said fragment is a juxta-membrane fragment.

4. The peptide according to any one of claims 1 to 3, wherein said peptide comprises an amino acid sequence at least 80% identical to SEQ ID NO: 2, 3 or 4.

- 25 5. The peptide according to claim 4, wherein said peptide comprises any one of SEQ ID NOs: 2, 3 or 4.

6. The peptide according to any one of claims 1 to 5, wherein said peptide comprises at least one chemical modification improving its stability and/or its biodisponibility.

- 30 7. An isolated nucleic acid encoding the peptide according to any one of claims

1 to 6.

8. A pharmaceutical composition comprising the peptide according to any one of claims 1 to 6 or the nucleic acid of claim 7 and a physiologically acceptable carrier.
- 5 9. A peptide comprising or consisting of:
- a) amino acids 579 to 601 of SEQ ID NO: 1;
 - b) the amino acids corresponding to (a) in a non-human mammalian CD31;
 - c) a fragment of at least 6 amino acids of (a);
 - 10 d) a fragment of at least 6 amino acids of (b); or
 - e) a sequence at least 80% identical to (a) or (c), wherein said peptide exerts a dose-dependent inhibition of T-cell proliferation *in vitro*;
- for use in activating CD31-mediated signaling *in vivo*.
10. A peptide comprising or consisting of:
- 15 a) amino acids 579 to 601 of SEQ ID NO: 1;
 - b) the amino acids corresponding to (a) in a non-human mammalian CD31;
 - c) a fragment of at least 6 amino acids of (a);
 - d) a fragment of at least 6 amino acids of (b); or
 - 20 e) a sequence at least 80% identical to (a) or (c), wherein said peptide exerts a dose-dependent inhibition of T-cell proliferation *in vitro*;
- for use in the treatment of a thrombotic or an autoimmune disorder.
11. The peptide according to claim 9 or 10, wherein said peptide has a length of at most 30 amino acids.
- 25 12. The peptide according to any one of claims 9 to 11, for use in the treatment of individuals having a CD31⁻ T lymphocytes phenotype.
13. The peptide according to any one of claims 9 to 12, wherein said peptide comprises at least one chemical modification improving its stability and/or its biodisponibility.

14. The peptide according to any one of claims 9 to 13, wherein said peptide consists of:
- a) the peptide according to any one of claims 1 to 6;
 - b) a peptide comprising an amino acid sequence at least 80% identical to SEQ ID NO: 5 or 6; or
 - c) a peptide comprising an amino acid sequence of SEQ ID NO: 5 or 6.
15. The peptide according to any one of claims 9 to 14, wherein said disorder is a thrombotic disorder selected from the group consisting of atherothrombosis, atherosclerosis, acute coronary syndrome, ischemic stroke, peripheral arterial disease and abdominal aortic aneurysm.
16. The peptide according to any one of claims 9 to 14, wherein said disorder is an autoimmune disorder selected from the group consisting of rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, systemic lupus erythematoses, Graves' disease and diabetes mellitus.
17. A method for identifying a peptidomimetic of a peptide according to any one of claims 1 to 6 being a candidate compound for the treatment and/or prevention of a thrombotic or an autoimmune disorder, comprising the steps of:
- a) providing a peptidomimetic; and
 - b) determining whether said peptidomimetic exerts a dose-dependent inhibition of T-cell proliferation *in vitro* and/or of the mixed-lymphocyte reaction (MLR);
- wherein, if said peptidomimetic exerts a dose-dependent inhibition, said peptidomimetic is a candidate compound for the treatment and/or prevention of a thrombotic or an autoimmune disorder.
18. The method according to claim 17, wherein step (b) further comprises determining whether said peptidomimetic exerts an immunosuppressant and/or an antiplatelet activity.

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HUMAN	MQPRWAQGATMWLGVLLTLLLCSSLEGQENSFTINSVDMKSLPDWTVQNGKNLTLOCFAD	60
MOUSE	-----MLLADGLTLVLYASLQAEENSFTINSIHMESLPSWEVMNGQOLTLECLVD	50
BOVIN	MQLRWTQRGMMWLGALLTLLLCSSLKGQENSFTINSIHMQLPHSTVQNGENLTLOCLVD	60
PIG	MRLRWTQGGNMWLGVLLTLLLCSSLEGQENSFTINSIHMEMLPGQEVHNGENLTLOCLVD	60
HUMAN	VSTTSHVKPQHQLFVKDDVLFYNTISSMKSTESYFIPFVRIYDSGTYKCTVIWNKKEKTT	120
MOUSE	ISTTSKRSRQHRVLFYKDDAMVYNVTSREHTESYVIPQARVPHSGGKYKCTVMLNNKKEKTT	110
BOVIN	VSTTSRVKPLHQVLFYKDDVLLHNVSRRNTESYLIPIHVRVCDSGRYKCNVILNNKKEKTT	120
PIG	VSTTSVVKPQHQLFVKDDVLFHNVSSTRKNTESYFISEARVYNSGRYKCTVILNNKKEKTT	120
HUMAN	AEYQLLVEGVSPFRVTLDDKKEALQGGIVRVNCSVPEKAPVHFTIEKLELNEKMKVKKRE	180
MOUSE	IEYEVKVGVSFKPVTLLDKKEVTEGGVVTVNCSLQBEKPIFFKIEKLEVGTKFKVRRID	170
BOVIN	FEYEVWVKGVSDPRVTLDDKKEVIEGGVVVNCVPEKAPVHFTIEKLELNIIRGAKKKRE	180
PIG	AEYKVVVEGVSNPRVTLDDKKEVIEGGVVKVTCVPEKAPVHFTIEKLELNVDRVKQRRR	180
HUMAN	KNSRDQNFVILEFPVEEQDRVLSERCQARIISGIHMQTSESTKSELVTVTESFSTPKFHI	240
MOUSE	KTS-NENFVLMFPIEAQDHLVLRFCQAGILSGFKLQSESEPIRSEYVTVQESFSTPKFBI	229
BOVIN	KTSQNQNFVTLFETVVEEQDRVIRFQCQAKLFGSGSNVSSRPIQSDLVTVRESFSNPKFHI	240
PIG	KTANNQNSVTLFETVVEEQDRVILFSCQANVIFGTRVEISDSVRSDLVTVRESFSNPKFHI	240
HUMAN	SPTGMIMEGAQLHIKCTIQVTHLAQEPPEIIQKDKAIVAHNRHGKNAVYSVMAMVEHSG	300
MOUSE	KPPGMIIEGDQLHIRCIQVQVTHLVQEFTEIIQKDKAIVATSKQSSSEAVYSVMAMVEYSG	289
BOVIN	IPEGKVMEGDDLQVQKCTVQVTHQAQSFPEIIQKDKAIVAHNSLSSSEAVYSVMATTEHNG	300
PIG	SPKGVIEGDQLLIKCTIQVTHQAQSFPEIIQKDKAIVAHNSRNGSEAVYSVMATVEHNS	300
HUMAN	NYTCKVESSRISKVSSIVVNI TELFSKPELSSSFTHLDQGERLNLSCSIPGAPP-ANFTI	359
MOUSE	HYTCKVESNRISKASSIMVNI TELFPKPKLEFSSSRLDQGELELDLSCSVSGTPV-ANFTI	348
BOVIN	NYTCKVEASRISKVSSVVVNVTELFSPKPKLESSATHLDQGEDLNLSCSIPGAPP-ANFTI	359
PIG	NYTCKVEASRISKVSSIMVNI TELESRPKPKSSATRLDQGESLRLWCSIPGAPPANFTI	360
HUMAN	QKEDTIVSQTDFTKIASKSDSGTYICTAGIDKVVKKSNTVQIVVCEMLSQPRISYDAQF	419
MOUSE	QKEETVLSQYQNFESKIAEESDSGEYSCTAGIGKVVKRSGLVPIQVCEMLSKPSIFHDAKS	408
BOVIN	QKGGMTVSQTQNFTRVSEWDSGLYTCVAGVGRVFKRSNTVQITVCEMLSKPSIFHDSRS	419
PIG	QKGGMMMLQDQNLTKVASERDSGTYTCVAGIGKVVKRSNEVQIIVCEMLSKPSIFHDSGS	420
HUMAN	EVIKGQTEVRCESISGTLPLISYQLLKTSKVLENSTKNSNDPFAVKDNPTEDEVYQCQAD	479
MOUSE	EIIKGHALGISCSQSENGTAPIYHLMKAKSDFQTLVTSNDPATFTDKPTRDMEYQCRAD	468
BOVIN	EVIKGQTEVSCQSVNGTAPIFYQLSNTSKPVANQSVGSKNKPALFRVKEPKDVEYCCSAD	479
PIG	EVIKGQTEVSCQSINGTSPISYQLLKGSDLLASQNVSNPEAVFKDNPTEDEVYQCQIAD	480
HUMAN	NCHSHAKMLSEVLRVKVIAPVDEVQTSILSSKVVESGEDIVLQCAVNEGSGPITYKIFYRE	539
MOUSE	NCHSHPAVFSEILRVRVIAPVDEVWISILSSENEVQSGSEMVLRCVKEGTSPIITFQFYKE	528
BOVIN	NCHSHSKMFSEVLRVKVIAPVDEAQL-VVLKGEVEPEGEPIVFYCSVNEGSPITYKIFYKE	538
PIG	NCHSHAGMPSKVLRVKVIAPVDEVKLSILSSEVESGQAVLQCSVKEGSGPITYKIFYKE	540
HUMAN	KEGKPFYQMTSNATQAFWTKQKASKEQEYCTAFNRANHASSVPRSKILTVRVVILAPW	599
MOUSE	KEDRPFHQAVVNDTQAFWHNKQASKKQEGQYYCTASNRASSMRTSPRSSTLAVRVVFLAPW	588
BOVIN	KESKPFYQDTINATQIMWHKTTASKEYEGQYYCTASNRANLSKHVIQSNLTVRVVYL-PL	597
PIG	KENKPFHQVTLNDTQAIWHKPKASKKQEGQYYCLASNRATPSKNFLQSNILAVRVVILAPW	600
HUMAN	KKGLIAVVVIIGVITALLIIAAKCYFLRKAKAKQMPVEMSRPAVPLLNSNNEK-MSDPNME	658
MOUSE	KKGLIAVVVIGVVIATLIVAACKCYFLRKAKAKQKPVEMSRPAAPLLNSNSEK-ISEPSVE	647
BOVIN	EKGLIAVVVIIGVIVTLVLGAKCYFLKAKAKQMPVEMSRPAVPLLNSNNEKTLSDAGTE	657
PIG	KKGLIAVVVIAVITAVLLIGAREFYFLKSKAKAKQMPVEMCRPAAPLLNSNNEKTLSDPNTF	660

FIG.1a

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HUMAN	ANSHYGHNDVVRNHAMKPIINDNKEPLNSDVQYTEVQVSSAESHKDLGKKD--TETVYSEV 716
MOUSE	ANSHYGYDDVSGNDAVKPINQNKDFQNM DVEYTEVEVSSLEPHQALGTRA--TETVYSEI 705
BOVIN	ADRHYGYNEDVGNHAMKPLNENKEPLTLDVEYTEVEVTSPEPHQGLGKGTETETVYSEI 717
PIG	ANRHYGYNEDVGNHAMKPLNENKEPLTLDVEYTEVEVTSPEPHRGLGKTKG--TETVYSEI 718
HUMAN	RKAVPDAVESRYSRTEGSLDGT 738
MOUSE	RKVDPNIMENRYSRTEGSLNGT 727
BOVIN	RKADPDFVENRYSRTEGSLDGS 739
PIG	RKADPDLVENRYSRTEGSLDGT 740

FIG.1b

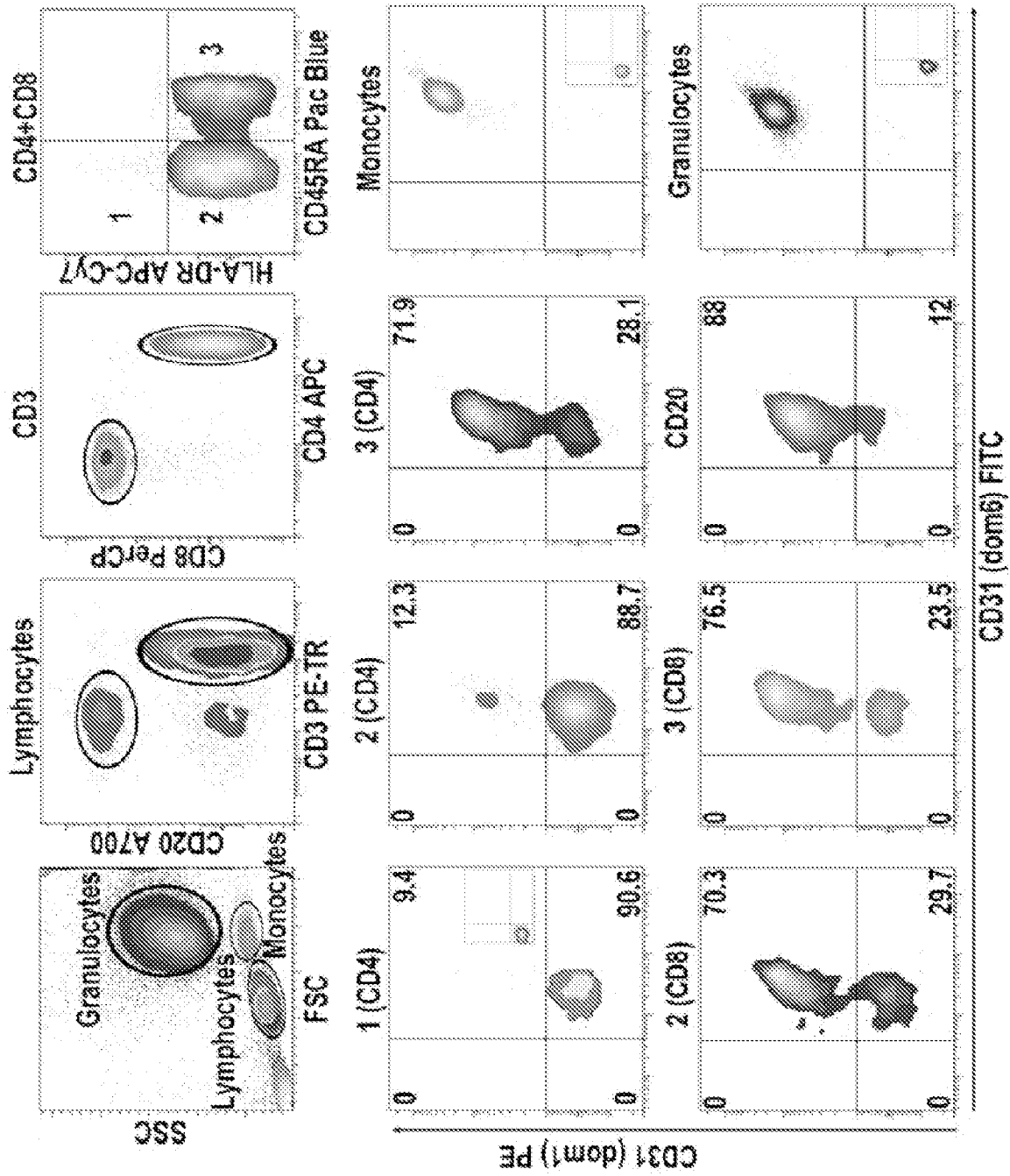


FIG.2

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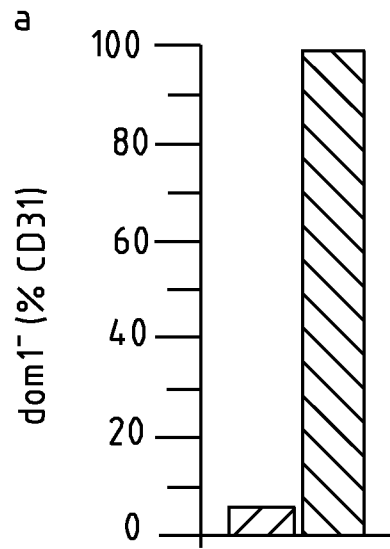
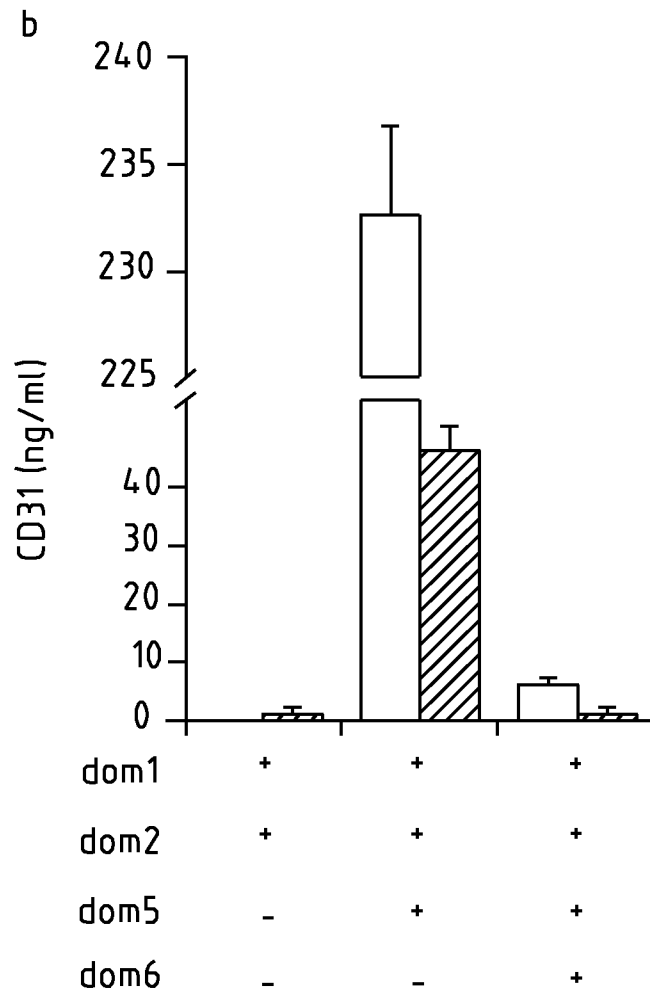


FIG.3



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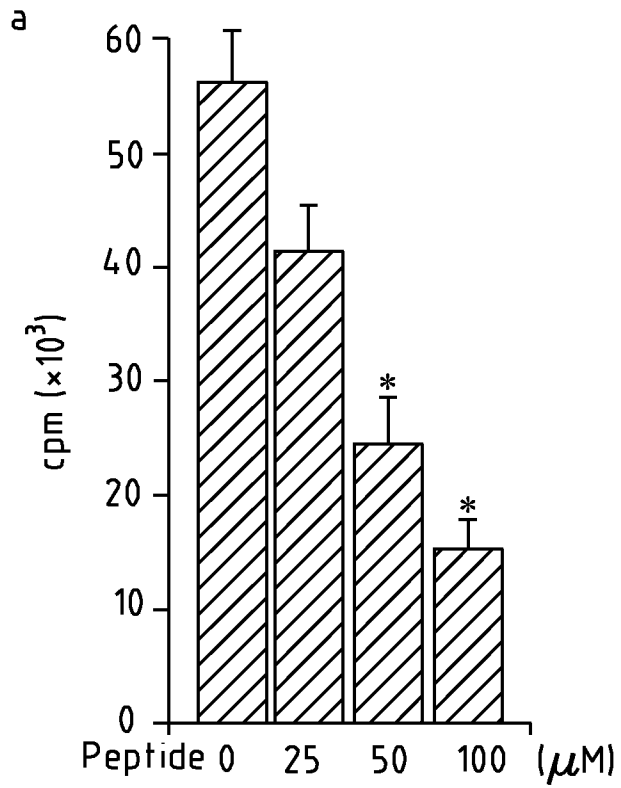
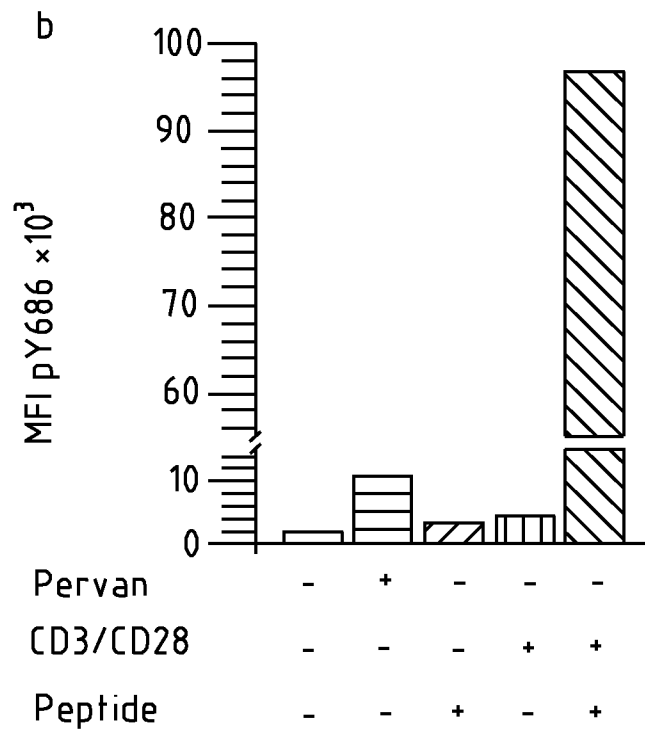


FIG.4



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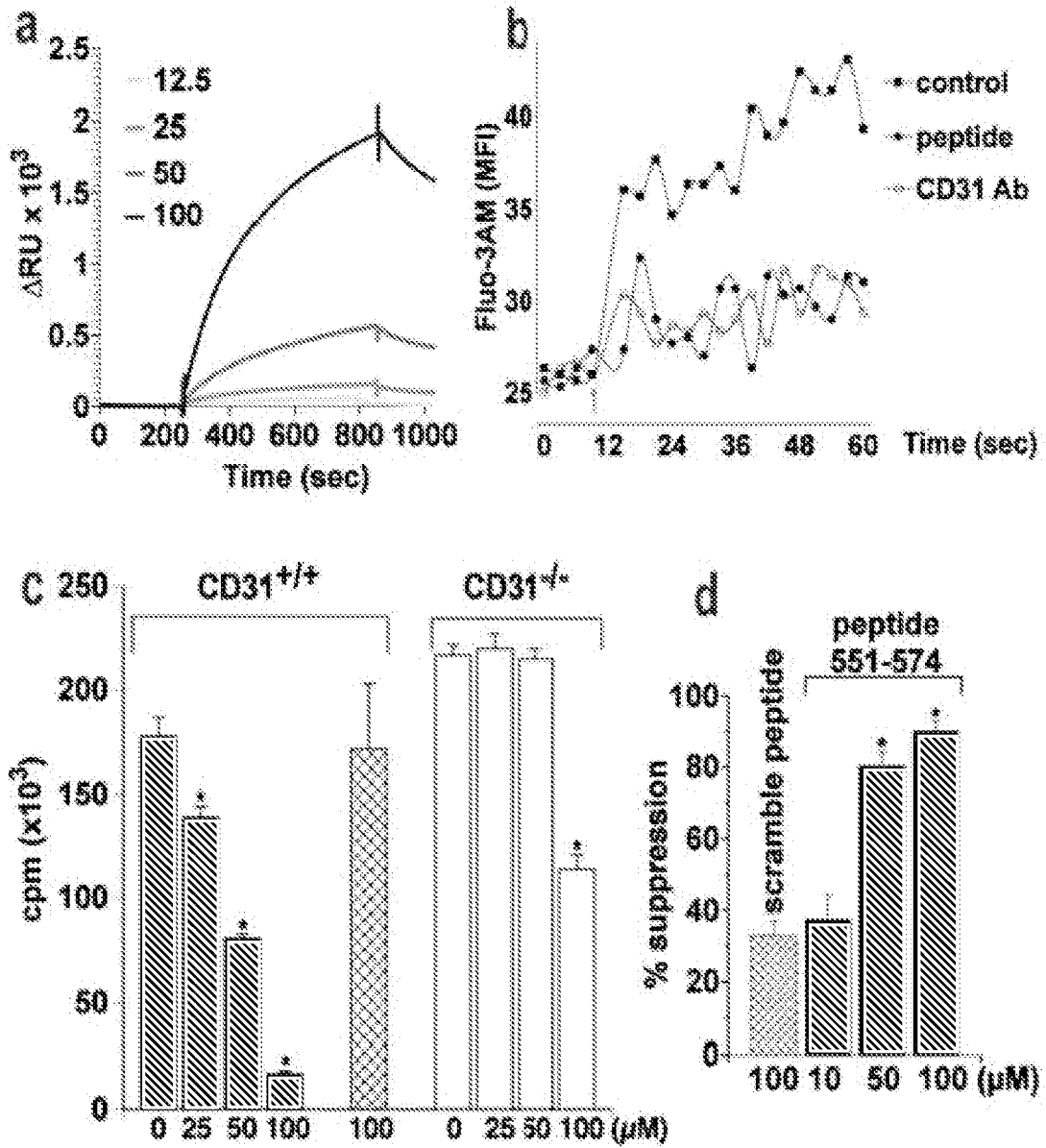


FIG.5

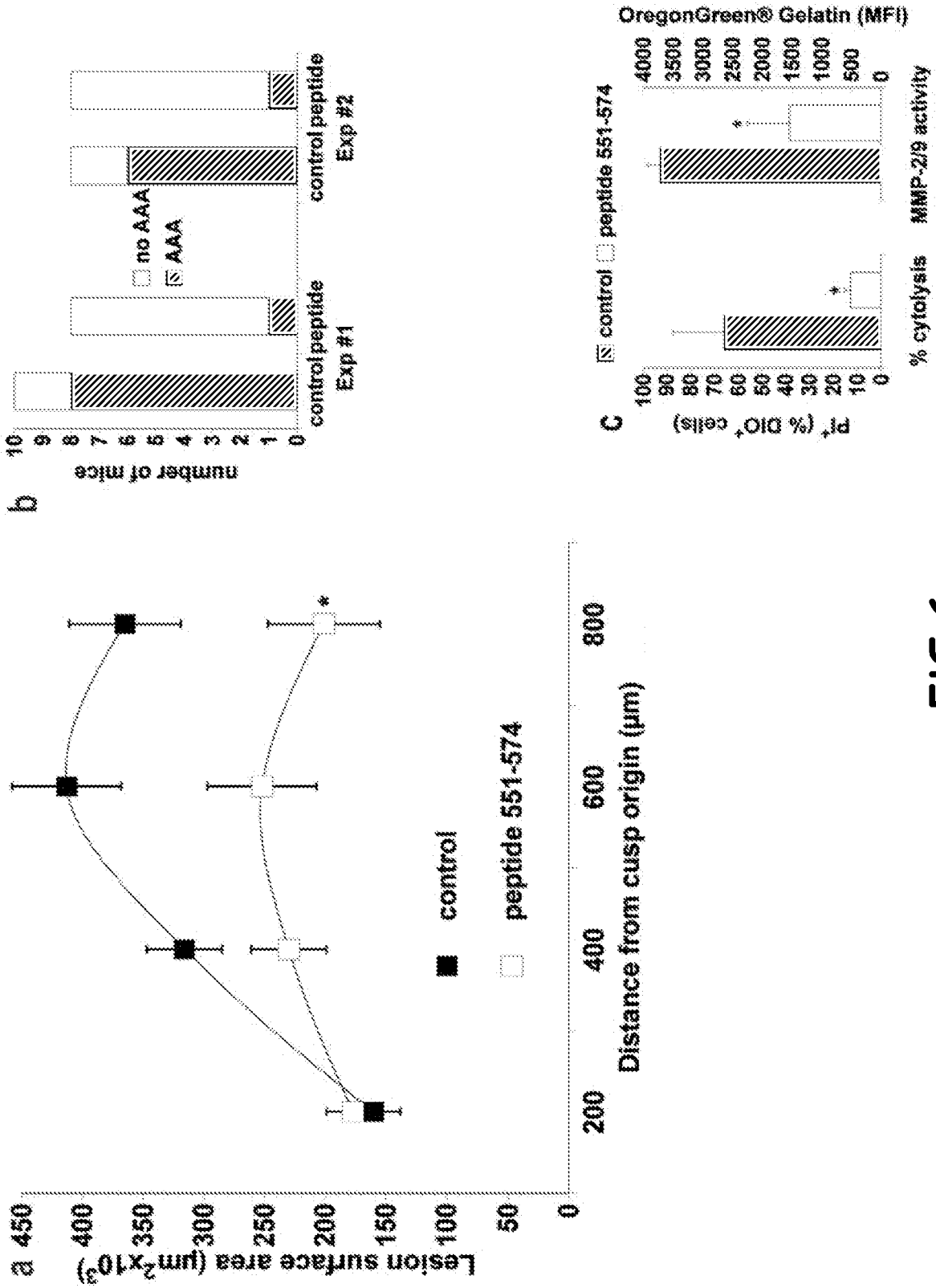


FIG.6

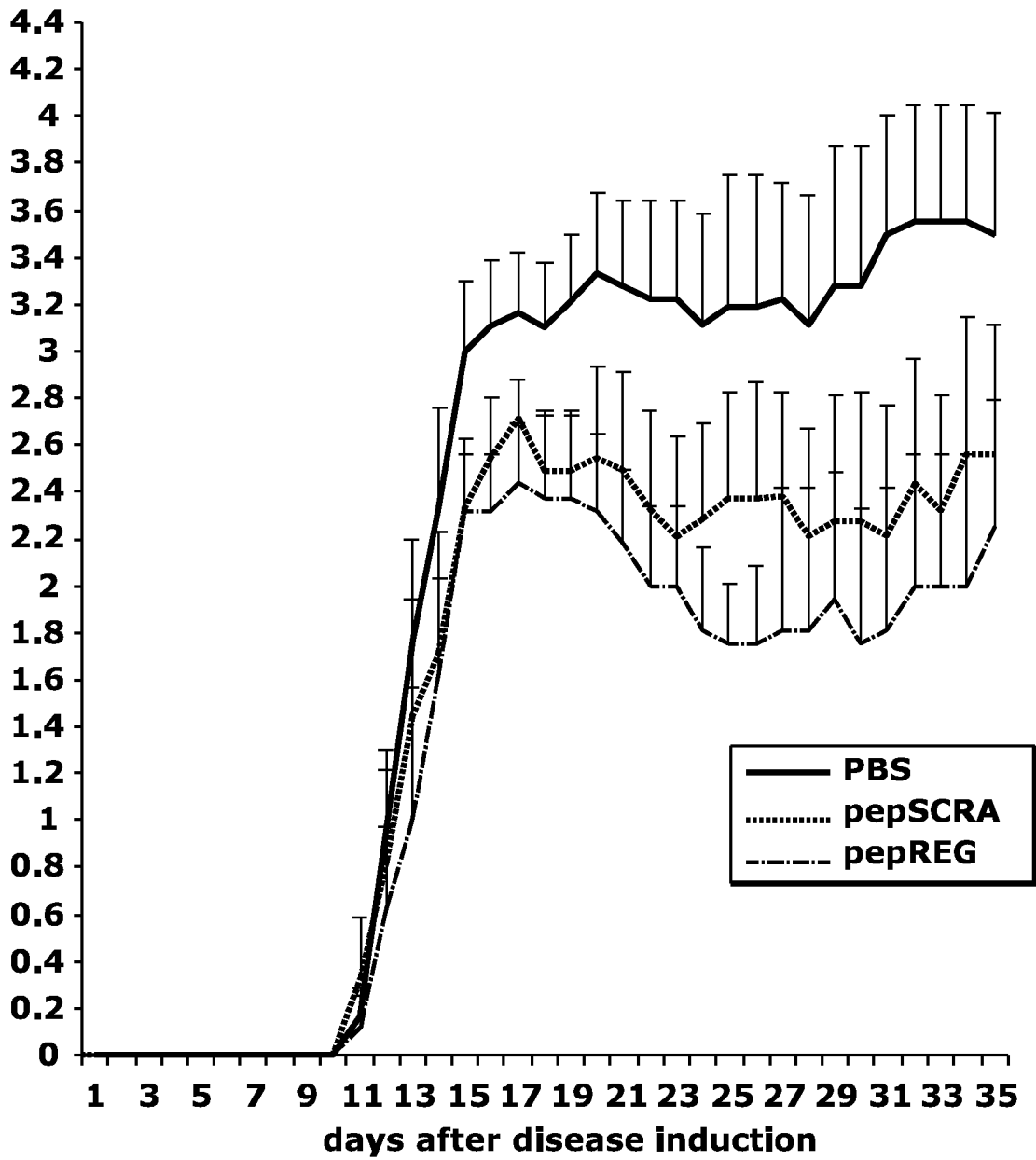


FIG.7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/058188

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K14/705 A61K38/17		
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) C07K A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, WPI Data, BIOSIS, EMBASE, Sequence Search, EMBL		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	REINKE ET AL: "Short-term sPECAM-Fc treatment ameliorates EAE while chronic use hastens onset of symptoms" JOURNAL OF NEUROIMMUNOLOGY, ELSEVIER SCIENCE PUBLISHERS BV, XX, vol. 186, no. 1-2, 1 May 2007 (2007-05-01), pages 86-93, XP022181758 ISSN: 0165-5728 pages 87-92	9, 10, 14, 16
Y	* same passages * ---	1-18
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input checked="" type="checkbox"/> See patent family annex.		
* Special categories of cited documents :		
A document defining the general state of the art which is not considered to be of particular relevance *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 novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family	
Date of the actual completion of the international search	Date of mailing of the international search report	
8 September 2009	21/09/2009	
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Steffen, Pierre	

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2009/058188

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	-& LIAO F ET AL: "Transgenic mice expressing different levels of soluble platelet/endothelial cell adhesion molecule-IgG display distinct inflammatory phenotypes." JOURNAL OF IMMUNOLOGY (BALTIMORE, MD. : 1950) 15 NOV 1999, vol. 163, no. 10, 15 November 1999 (1999-11-15), pages 5640-5648, XP002515386 ISSN: 0022-1767 page 5641; figure 1	9
X	-& LIAO F ET AL: "Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo." THE JOURNAL OF EXPERIMENTAL MEDICINE 7 APR 1997, vol. 185, no. 7, 7 April 1997 (1997-04-07), pages 1349-1357, XP002515387 ISSN: 0022-1007 page 1350 - page 1351; figure 2	9
X	US 6 087 331 A (NEWMAN PETER J [US] ET AL) 11 July 2000 (2000-07-11) columns 3-11; sequences 3-6	9,10, 13-15
Y	* same passages *	1-18
X	SATO S ET AL: "Clinical significance of soluble CD31 in patients with systemic sclerosis (SSc): association with limited cutaneous SSc." THE JOURNAL OF RHEUMATOLOGY NOV 2001, vol. 28, no. 11, November 2001 (2001-11), pages 2460-2465, XP002515388 ISSN: 0315-162X page 2460 - page 2464	9,10,14
Y	* same passages *	1-18
X	CHEN Y ET AL: "Administration of a CD31-derived peptide delays the onset and significantly increases survival from lethal graft-versus-host disease." BLOOD 15 FEB 1997, vol. 89, no. 4, 15 February 1997 (1997-02-15), pages 1452-1459, XP002515389 ISSN: 0006-4971 page 1542 - page 1547	9
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2009/058188

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZEHNDER J L ET AL: "Involvement of CD31 in lymphocyte-mediated immune responses: importance of the membrane-proximal immunoglobulin domain and identification of an inhibiting CD31 peptide." BLOOD 1 MAR 1995, vol. 85, no. 5, 1 March 1995 (1995-03-01), pages 1282-1288, XP002515390 ISSN: 0006-4971 cited in the application page 1282 - page 1288	9
Y	* same passages *	1-18
Y	KALINOWSKA A ET AL: "PECAM-1, a key player in neuroinflammation." EUROPEAN JOURNAL OF NEUROLOGY : THE OFFICIAL JOURNAL OF THE EUROPEAN FEDERATION OF NEUROLOGICAL SOCIETIES DEC 2006, vol. 13, no. 12, December 2006 (2006-12), pages 1284-1290, XP002515391 ISSN: 1468-1331 the whole document	1-18
Y	WOODFIN ABIGAIL ET AL: "PECAM-1: a multi-functional molecule in inflammation and vascular biology." ARTERIOSCLEROSIS, THROMBOSIS, AND VASCULAR BIOLOGY DEC 2007, vol. 27, no. 12, December 2007 (2007-12), pages 2514-2523, XP002515392 ISSN: 1524-4636 the whole document	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2009/058188

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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