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(54) Title: CD161 LIGAND, PILAR, FOR MODULATING ACTIVATION AND PROLIFERATION OF T CELLS

(57) Abstract: The invention relates to a CD161 ligand known as Proliferation- Induced Lymphocyte -Associated Receptor (PILAR), which is crucial for a robust expansion of human lymphocytes. PILAR is markedly up-regulated on both CD4 and CD8 T cells upon TCR engagement and increases the expression of anti-apoptotic genes and glucose transporters through CD161, which globally results in a dramatic enhancement of T cell proliferation. Agents which stimulate or block this activity are also provided as are methods for manipulating PILAR signaling in the treatment of disease.

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**CD161 LIGAND, PILAR, FOR MODULATING ACTIVATION AND
PROLIFERATION OF T CELLS**

5 Introduction

This application claims benefit of priority to U.S. Provisional Patent Application Serial Nos. 60/911,138, filed April 11, 2007, and 60/015,864, filed December 21, 2007, the contents of which are incorporated herein by
10 reference in their entireties.

This invention was made in the course of research sponsored by the National Center for Research Resources (Grant No. 2P20RR016437-06). The U.S. government may have certain rights in this invention.

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Background of the Invention

T cells are activated by engagement of their T cell receptor (TCR) by specific MHC-peptide complexes expressed on antigen-presenting cells, but other signals concurrently
20 delivered decide the appropriate immune response. CD28 represents the archetype of activating costimulatory receptor (Carreno & Collins (2002) *Annu. Rev. Immunol.* 20:29-53; June, et al. (1987) *Mol. Cell Biol.* 7:4472-4481; Thompson, et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:1333-
25 1337), but many other molecules contribute to support continued expansion and differentiation of T cells (Watts (2005) *Annu. Rev. Immunol.* 23:23-68; Markiewicz, et al. (2005) *J. Immunol.* 175:2825-2833; Groh, et al. (2001) *Nat. Immunol.* 2:255-260; Conejo-Garcia, et al. (2004) *Cancer*
30 *Res.* 64:2175-2182; Conejo-Garcia, et al. (2003) *Cancer Biol. Ther.* 2:446-451; Kahn & Koretzky (2006) *Nat. Immunol.* 7:1286-1288). In this context, the extent of naïve T cell proliferation is critically dependent on the cytokine

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milieu (Swain (1995) *J. Leukoc. Biol.* 57:795-798; Boyman, et al. (2006) *Science* 311:1924-1927).

Experimental results indicate that engagement of CD161, a C-type lectin receptor associated with NK cells (Lanier, et al. (1994) *J. Immunol.* 153:2417-2428), enhances IFN- γ and TNF- α production in the context of a TCR signal (Takahashi, et al. (2006) *J. Immunol.* 176:211-216; Aldemir, et al. (2005) *J. Immunol.* 175:7791-7795), while inducing the production of IL-12 by dendritic cells (Poggi, et al. (1997) *Eur. J. Immunol.* 27:2965-2970). Unlike murine lymphocytes, the expression of CD161 in human T cells identifies mostly memory lymphocytes, but only a small proportion of invariant NK T cells (Takahashi, et al. (2006) *supra*). The only previously identified ligand of CD161 is CLEC2D/LLT1 (Aldemir, et al. (2005) *supra*; Rosen, et al. (2005) *J. Immunol.* 175:7796-7799), another C-type lectin molecule mapping in the vicinity of NKG2D at the NK cluster. This narrow region of human chromosome 12 spans ~2 Mb and contains at least 18 different genes involved in immune responses that encode for leukocyte receptors exhibiting a C-type lectin-like motif in their extracellular part.

Summary of the Invention

The present invention is an isolated nucleic acid molecule, wherein said nucleic acid molecule (a) encodes a Proliferation-Induced Lymphocyte-Associated Receptor (PILAR) polypeptide comprising the amino acid sequence set forth in SEQ ID NO:9; or (b) hybridizes to a nucleic acid molecule encoding a PILAR polypeptide comprising the amino acid sequence set forth in SEQ ID NO:9. In some embodiments, a vector harbors the nucleic acid molecule. In another embodiment, the vector is in a host cell.

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The present invention is also an isolated PILAR polypeptide containing the amino acid sequence set forth in SEQ ID NO:9. In some embodiments, the PILAR polypeptide is in a host cell. In other embodiments, the PILAR polypeptide is in admixture with a pharmaceutically acceptable carrier.

The present invention is also an antibody which specifically binds to an isolated PILAR polypeptide.

A method for identifying an agent which modulates an activity of PILAR is also embraced by this invention. Such a screening assay involves contacting a PILAR polypeptide, or host cell expressing a PILAR polypeptide, with a test agent and determining whether the test agent modulates an activity of PILAR wherein a decrease in a PILAR activity is indicative of an agent which antagonizes PILAR and an increase in PILAR activity is indicative of an agent which agonizes PILAR.

Agents identified in accordance with this screening assay find application in methods for modulating T cell activation or proliferation, and methods for modulating immune responses in a subject. Subjects benefiting from such treatment include those with cancer, an autoimmune disease or inflammation.

Brief Description of the Drawings

Figure 1 shows the gene structure and amino acid sequence of PILAR. As depicted in Figure 1A, the *PILAR* gene spans five exons encoding a predicted transmembrane molecule that exhibits a truncated C-type Lectin domain in the extracellular region. Figure 1B shows the alignment of PILAR (SEQ ID NO:1) with two closely-related human molecules, namely the leukocyte markers CD69 (SEQ ID NO:2) and CLEC2D (SEQ ID NO:3). Sequence identity is indicated with "*". Figure 1C shows the amino acid sequence alignment

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of PILAR (SEQ ID NO:1), INPE5792 (SEQ ID NO:4) and CLEC2A (SEQ ID NO:5). Figure 1D shows the nucleic acid (SEQ ID NO:6) and deduced amino acid (SEQ ID NO:1) sequence for PILAR. Normal font represents the predicted intracellular domain; italic represents the predicted transmembrane domain; single underlining represents the predicted extracellular domain; and double underlining shows the location of a truncated C-type Lectin domain.

Figure 2 shows MILTENYI bead-purified, CFSE-labeled peripheral T cells stimulated with aAPCs coated with agonistic antibodies to CD3 (100 ng/ml, Figure 2A), or CD3/CD28 (100 ng/ml, Figure 2B; 0.5/100 ng/ml, Figure 2C), for 5 days, in the presence of 20 µg/ml of either blocking anti-PILAR antibodies (dotted line) or an irrelevant rabbit antibody (solid line). These results are representative of 4 independent experiments

Figure 3 shows that the expression of both PILAR and CD161 can be recovered on tumor-associated cytotoxic T cells ex vivo by activation with aAPCs. These results are representative of at least 2 independent experiments. Figure 3A shows IFN-γ ELISPOT analysis of PBMCs from an A2⁺ donor, stimulated for 7 days with A2⁺CD80⁺ aAPCs (10:1 ratio) pulsed with the CEF peptide pool (MABTECH, 2 µg/mL of each peptide), in the presence of 20 µg/ml of anti-PILAR (α-PILAR) or an irrelevant antibody (iAb). Figure 3B shows 2x10⁶ CFSE-labeled PBMCs/ml incubated for 7 days with the CEF peptide pool (1.75 µg/ml) in the presence of 20 µg/ml of anti-PILAR antibodies (dashed line) or an irrelevant antibody (solid line). Figure 3C shows PBMCs stimulated for 7 days with autologous monocyte-derived dendritic cells (10:1 ratio, 10⁶ total cells/ml), pulsed with pool of 138 Cytomegalovirus peptides (2 µg/mL; pp65 sequence, strain

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AD169; BD Biosciences), in the presence of 20 µg/ml of anti-PILAR (dotted line) or an irrelevant antibody (solid line).

Figure 4 shows that the expression of both PILAR (Figure 4A) and CD161 (Figure 4B) can be recovered on tumor-associated cytotoxic T cells *ex vivo* by activation with aAPCs. The gate was on CD3⁺CD8⁺ cells. Dotted line, tumor-derived unstimulated CD8⁺ T cells; solid line, the same cells after stimulation with CD3/CD28 for 5 days.

10 Detailed Description of the Invention

A novel human transmembrane ligand for CD161 has now been identified. This protein, designated Proliferation-Induced Lymphocyte-Associated Receptor (PILAR) is up-regulated upon early T cell activation and can both enhance TCR-dependent stimulation through CD161 and induce apoptotic T cell death through a second receptor. The results disclosed herein reveal a crucial role for PILAR in modulating the extent of cellular adaptive immune responses in humans. Accordingly, the present invention relates to a nucleic acid molecule encoding PILAR, PILAR polypeptide, anti-PILAR antibodies, PILAR agonists and antagonists, and methods for using such molecules to modulate immune responses in the treatment of cancer, autoimmune diseases or inflammation.

25 In accordance with the present invention, an isolated nucleic acid molecule encoding a PILAR polypeptide is intended to include a genomic DNA, RNA or cDNA encoding the PILAR polypeptide or fragment thereof. The nucleic acid molecule is isolated in the sense that the molecule is separated or substantially free from at least some of the other components of the naturally occurring organism, such as for example, the cell structural components or other

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polypeptides or nucleic acids commonly found associated with the molecule.

In one embodiment, the nucleic acid molecule encodes a PILAR polypeptide having the amino acid sequence as set forth in SEQ ID NO:1. In another embodiment, the nucleic acid molecule encodes a PILAR polypeptide having the amino acid sequence as set forth in SEQ ID NO:9. In a particular embodiment the nucleic acid molecule has a nucleotide sequence as set forth in SEQ ID NO:6. In still other embodiments, the nucleic acid molecule of the present invention hybridizes to a nucleic acid molecule encoding a PILAR polypeptide of SEQ ID NO:1 or SEQ ID NO:9, or nucleic acid molecule as set forth in SEQ ID NO:6 under stringent conditions. In accordance with this embodiment, stringent hybridization is carried by conducting the hybridization reaction at 42°C in a solution containing 50% formamide, 5X SSC, and 1% SDS and washing at 65°C in a solution containing 0.2X SSC and 0.1% SDS See, e.g., Sambrook et al., *Molecular Cloning, A Laboratory Manual* (2d Ed. 1989) (Cold Spring Harbor Laboratory).

Alternatively stated, the isolated nucleic acid molecules of the invention encompass nucleic acid molecules encoding PILAR polypeptides that have at least about 85%, 90%, 95%, 97%, 98% or higher amino acid sequence identity across the entire length of the polypeptide sequences specifically disclosed herein and, in some embodiments, further encode a functional PILAR polypeptide as defined herein.

As is known in the art, a number of different programs can be used to identify whether a nucleic acid molecule or polypeptide has sequence identity or similarity to a known sequence. Sequence identity and/or similarity can be determined using standard techniques known in the art,

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including, but not limited to, the local sequence identity algorithm of Smith & Waterman (1981) *Adv. Appl. Math.* 2:482, by the sequence identity alignment algorithm of Needleman & Wunsch (1970) *J. Mol. Biol.* 48:443, by the
5 search for similarity method of Pearson & Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI),
10 the Best Fit sequence program described by Devereux, et al. (1984) *Nucl. Acid Res.* 12:387-395, either using the default settings, or by inspection.

An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of
15 related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle (1987) *J. Mol. Evol.* 35:351-360; the method is
20 similar to that described by Higgins & Sharp (1989) *CABIOS* 5:151-153.

Another example of a useful algorithm is the BLAST algorithm, described in Altschul, et al. (1990) *J. Mol. Biol.* 215:403-410 and Karlin, et al. (1993) *Proc. Natl.*
25 *Acad. Sci. USA* 90:5873-5787. A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul, et al. ((1996) *Methods in Enzymology*, 266:460-480). WU-BLAST-2 uses several search parameters, which can be set to the default values. The parameters are dynamic
30 values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values

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can be adjusted to increase sensitivity. An additional useful algorithm is gapped BLAST as reported by Altschul, et al. ((1997) *Nucleic Acids Res.* 25:3389-3402).

5 A percentage amino acid sequence identity value can be determined by the number of matching identical residues divided by the total number of residues of the longer sequence in the aligned region. The longer sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment
10 score are ignored).

The alignment can include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the polypeptides specifically disclosed herein, it is
15 understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical amino acids in relation to the total number of amino acids. Thus, for example, sequence identity of sequences shorter than a sequence specifically disclosed
20 herein, will be determined using the number of amino acids in the shorter sequence, in one embodiment. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as, insertions, deletions, substitutions, etc.

25 For the purposes of the present invention, a functional PILAR polypeptide is one that retains at least one biological activity normally associated with this polypeptide, e.g., binding to CD161 and/or stimulating T cell activation or proliferation. Alternatively, a
30 functional PILAR polypeptide retains all of the activities possessed by the unmodified peptide. By retains biological activity, it is meant that the polypeptide retains at least about 50%, 60%, 75%, 85%, 90%, 95%, 97%, 98%, 99%, or more,

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of the biological activity of the native polypeptide (and can even have a higher level of activity than the native polypeptide). A non-functional polypeptide is one that exhibits essentially no detectable biological activity normally associated with the polypeptide (e.g., at most, only an insignificant amount, e.g., less than about 10% or even 5%).

A PILAR polypeptide or PILAR protein as used herein, is intended to be construed broadly and encompasses a protein capable of binding CD161 and enhancing CD3/CD28-dependent T cell proliferation and cytotoxic T cell activation. In one embodiment, the PILAR polypeptide contains a transmembrane domain near the N-terminus of the polypeptide, and has an overall length of about 120 to about 190 amino acid residues. In particular embodiments, the length of the polypeptide is about 174 amino acid residues. In some embodiments, the polypeptide has a C-terminal amino acid sequence as set forth in SEQ ID NO:9. In other embodiments, the PILAR polypeptide can be defined as having or lacking the intracellular and transmembrane domains. In accordance with certain aspects of this embodiment, the PILAR polypeptide encompasses the C-terminal 124 amino acid residues of SEQ ID NO:1.

The term PILAR polypeptide also includes a modified (e.g., mutated) PILAR that retains biological function (i.e., have at least one biological activity of the native PILAR polypeptide, e.g., binding to CD161), and functional PILAR fusion polypeptides (e.g., a PILAR-GST protein fusion or a PILAR-His tagged protein).

To modify the PILAR amino acid sequence specifically disclosed herein, amino acid substitutions can be based on any characteristic known in the art, including the relative similarity or differences of the amino acid side-chain

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substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. In particular embodiments, conservative substitutions (*i.e.*, substitution with an amino acid residue having similar properties) are made in the amino acid sequence encoding PILAR.

In making amino acid substitutions, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (see, Kyte and Doolittle (1982) *J. Mol. Biol.* 157:105). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, and the like.

Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle (1982) *supra*), and these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is also understood in the art that the substitution of amino acids can be made on the basis of hydrophilicity. U.S. Patent No. 4,554,101 states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. As detailed in U.S. Patent No. 4,554,101, the following hydrophilicity values

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have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 $\pm$ 1); glutamate (+3.0 $\pm$ 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 $\pm$ 1); alanine (-0.5);
 5 histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

As will be appreciated by those skilled in the art, there can be variability in the nucleic acid molecules that
 10 encode any one of the PILAR polypeptides disclosed herein due the addition of regulatory sequences (e.g., non-translated sequences, such as intronic sequences and 5'- and 3'-untranslated sequences, promoters, enhancers, and the like) and the degeneracy of the genetic code. The
 15 degeneracy of the genetic code, which allows different nucleic acid sequences to code for the same polypeptide, is well-known in the literature (see Table 1).

TABLE 1

Amino Acid	3-Letter Code	1-Letter Code	Codons
Alanine	Ala	A	GCA GCC GCG GCT
Cysteine	Cys	C	TGC TGT
Aspartic acid	Asp	D	GAC GAT
Glutamic acid	Glu	E	GAA GAG
Phenylalanine	Phe	F	TTC TTT
Glycine	Gly	G	GGA GGC GGG GGT
Histidine	His	H	CAC CAT
Isoleucine	Ile	I	ATA ATC ATT
Lysine	Lys	K	AAA AAG
Leucine	Leu	L	TTA TTG CTA CTC CTG CTT
Methionine	Met	M	ATG
Asparagine	Asn	N	AAC AAT
Proline	Pro	P	CCA CCC CCG CCT
Glutamine	Gln	Q	CAA CAG
Arginine	Arg	R	AGA AGG CGA CGC CGG CGT
Serine	Ser	S	AGC ACT TCA TCC TCG TCT
Threonine	Thr	T	ACA ACC ACG ACT
Valine	Val	V	GTA GTC GTG GTT
Tryptophan	Trp	W	TGG
Tyrosine	Tyr	Y	TAC TAT

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As indicated, the nucleic acid molecule encoding PILAR can also be associated with appropriate expression control sequences, e.g., transcription/translation control signals and polyadenylation signals to regulate transcription and translation of the PILAR polypeptide. It will be appreciated that a variety of promoter/enhancer elements can be used depending on the level and tissue-specific expression desired. The promoter can be constitutive or inducible (e.g., the metallothionein promoter or a hormone inducible promoter), depending on the pattern of expression desired. The promoter can be native or foreign and can be a natural or a synthetic sequence. By foreign, it is intended that the transcriptional initiation region is not found in the wild-type host into which the transcriptional initiation region is introduced. The promoter is chosen so that it will function in the target host cell(s) of interest. In particular embodiments, the promoter functions in immune cells (e.g., T cells including CD4⁺ and CD8⁺ T cells and APCs such as dendritic cells, macrophages, or B cells) or in cells that can be used to express nucleic acid molecules encoding PILAR for the purposes of large-scale protein production. Likewise, the promoter can be specific for certain cells and tissues in that PILAR is only expressed in these cells or tissues. In some embodiments, the PILAR coding sequence is operatively associated with the well-known cytomegalovirus (CMV) major immediate-early promoter, albumin promoter, Elongation Factor 1- α (EF1- α) promoter, PyK promoter, MFG promoter, Rous sarcoma virus promoter, or glyceraldehyde-3-phosphate promoter.

PILAR can be expressed not only directly, but also as a fusion protein with a heterologous polypeptide, i.e. a

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signal sequence for secretion and/or other polypeptide which will aid in the purification of PILAR. In one embodiment, the heterologous polypeptide has a specific cleavage site to remove the heterologous polypeptide from
5 PILAR.

In general, a signal sequence can be a component of the vector and should be one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For production in a prokaryote, a prokaryotic signal
10 sequence from, for example, alkaline phosphatase, penicillinase, or heat-stable enterotoxin II leaders can be used. For yeast secretion, one can use, *e.g.*, the yeast invertase, alpha factor, or acid phosphatase leaders, the *Candida albicans* glucoamylase leader (EP 362,179), or the
15 like (see, for example WO 90/13646). In mammalian cell expression, signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal can be used.

20 Other useful heterologous polypeptides which can be fused to PILAR include those which increase expression or solubility of the fusion protein or aid in the purification of the fusion protein by acting as a ligand in affinity purification. Typical fusion expression vectors include
25 those exemplified herein as well as pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse maltose E binding protein or protein A, respectively, to the target recombinant protein.

The nucleic acid molecule encoding PILAR can be
30 incorporated into a vector, *e.g.*, for the purposes of cloning or other laboratory manipulations, recombinant protein production, or gene delivery. In particular embodiments, the vector is an expression vector. By the

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term express, expresses or expression of a nucleic acid coding sequence, in particular a PILAR coding sequence, it is meant that the sequence is transcribed, and optionally, translated. Typically, according to the present invention, transcription and translation of the coding sequence will result in production of a PILAR polypeptide.

Generally, vectors of the present invention include bacterial artificial chromosomes, cosmids, yeast artificial chromosomes, phage, plasmids, lipid vectors and viral vectors (e.g., retrovirus, alphavirus, vaccinia virus, adenovirus, adeno-associated virus, or herpes simplex virus), wherein the choice of vector is made based on a number of factors known in the art, including age and species of the target host cell, *in vitro* vs. *in vivo* delivery, level and persistence of expression desired (e.g., transient or stable), intended purpose (e.g., for therapy or drug screening), the target cell or organ, route of delivery, size of the isolated nucleic acid molecule, safety concerns, and the like.

For example, for expression of PILAR in the yeast *S. cerevisiae*, vectors such as pYepSec1 (Baldari, et al. (1987) *EMBO J.* 6:229-234), pMFa (Kurjan and Herskowitz (1982) *Cell* 30:933-943), pJRY88 (Schultz, et al. (1987) *Gene* 54:113-123), and pYES2 (INVITROGEN Corporation, San Diego, CA) can be employed. Baculovirus vectors available for expression of nucleic acids to produce proteins in cultured insect cells (e.g., Sf9 cells) include the pAc series (Smith, et al. (1983) *Mol. Cell. Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39). Examples of mammalian expression vectors include pCDM8 (Seed (1987) *Nature* 329:840) and pMT2PC (Kaufman, et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions

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are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40. When expressing in a mammalian subject is desired, viral vectors
5 are generally employed.

As used herein, the term viral vector or viral delivery vector can refer to a virus particle that functions as a nucleic acid delivery vehicle, and which contains the vector genome packaged within a virion.
10 Alternatively, these terms can be used to refer to the vector genome when used as a nucleic acid delivery vehicle in the absence of the virion. Protocols for producing recombinant viral vectors and for using viral vectors for nucleic acid delivery can be found in *Current Protocols in*
15 *Molecular Biology*, Ausubel, F. M. et al. (eds.) Greene Publishing Associates, (1989) and other standard laboratory manuals (e.g., *Vectors for Gene Therapy*. In: *Current Protocols in Human Genetics*. John Wiley and Sons, Inc.: 1997). See also, Muzyczka et al. (1992) *Curr. Topics Micro.*
20 *Immunol.* 158:97-129; U.S. Patent No. 6,146,874; and Miller (1990) *Blood* 76:271.

The present invention also provide for delivering, and optionally expressing, a nucleic acid molecule encoding PILAR in a broad range of host cells, including both
25 dividing and non-dividing cells *in vitro* (e.g., for large-scale recombinant protein production or for use in screening assays) or *in vivo* (e.g., for recombinant large-scale protein production, for creating an animal model for disease, or for therapeutic purposes). Vectors can be
30 introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms transformation and transfection refer to a variety of art-recognized techniques for

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introducing foreign nucleic acids (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, microinjection, DNA-loaded liposomes, LIPOFECTAMINE-DNA complexes, cell sonication, gene bombardment using high velocity microprojectiles, and viral-mediated transfection. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory manuals.

Often only a small fraction of cells (in particular, mammalian cells) integrate the foreign DNA into their genome. In order to identify and select these integrants, a nucleic acid molecule that encodes a selectable marker (e.g., resistance to antibiotics) can be introduced into the host cells along with the nucleic acid of interest. In particular embodiments, selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acids encoding a selectable marker can be introduced into a host cell on the same vector as that comprising the nucleic acid of interest or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid molecule can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die).

Host cells for use in screening assays or recombinant PILAR protein production can be a bacterial, protozoan, plant, yeast, fungus, or animal cell. In one embodiment, the cell is an animal cell (e.g., insect, avian or mammalian), and in another embodiment a mammalian cell (e.g., an immune cell such as an APC). In particular

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embodiments, an isolated nucleic acid molecule encoding PILAR is introduced into a cultured cell, e.g., a cell of a primary or immortalized cell line for recombinant protein production. The recombinant cell is used to produce the
5 PILAR polypeptide, which is collected from the cells or cell culture medium. Likewise, recombinant protein can be produced in, and optionally purified from an organism (e.g., a microorganism, animal or plant) being used essentially as a bioreactor.

10 Thus, the present invention also provides a transgenic non-human animal containing an isolated nucleic acid molecule encoding PILAR, which can be produced according to methods well-known in the art. The transgenic non-human animal can be any species, including avians and non-human
15 mammals. In accordance with the invention, suitable non-human mammals include mice, rats, rabbits, guinea pigs, goats, sheep, pigs and cattle. Mammalian models for cancer and autoimmune diseases can also be used.

A nucleic acid encoding PILAR is stably incorporated
20 into cells within the transgenic animal (typically, by stable integration into the genome or by stably maintained episomal constructs). It is not necessary that every cell contain the transgene, and the animal can be a chimera of modified and unmodified cells, as long as a sufficient
25 number of cells contain and express the PILAR transgene so that the animal is a useful screening tool (e.g., so that administration of test agents give rise to detectable response).

Methods of making transgenic animals are routinely
30 practiced in the art. DNA constructs are introduced into the germ line of an avian or mammal to make a transgenic animal. For example, one or several copies of the construct can be incorporated into the genome of an embryo by

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standard transgenic techniques. In an exemplary embodiment, a transgenic non-human animal is produced by introducing a transgene into the germ line of the non-human animal. Transgenes can be introduced into embryonal target cells at various developmental stages. Different methods are used depending on the stage of development of the embryonal target cell.

Introduction of the transgene into the embryo can be accomplished by any of a variety of means known in the art such as microinjection, electroporation, lipofection or a viral vector. For example, the transgene can be introduced into a mammal by microinjection of the construct into the pronuclei of the fertilized mammalian egg(s) to cause one or more copies of the construct to be retained in the cells of the developing mammal(s). Following introduction of the transgenic construct into the fertilized egg, the egg can be incubated *in vitro* for varying amounts of time, or reimplanted into the surrogate host, or both. One common method is to incubate the embryos *in vitro* for about 1-7 days, depending on the species, and then reimplant them into the surrogate host. The progeny of the transgenically manipulated embryos can be tested for the presence of the construct (e.g., by Southern blot analysis) of a segment of tissue. An embryo having one or more copies of the exogenous cloned construct stably integrated into the genome can be used to establish a permanent transgenic animal line carrying the transgenically added construct. Methods of producing transgenic avians are also known in the art, see, e.g., U.S. Patent No. 5,162,215.

When it is desirable to produce large amounts of PILAR protein, generally the PILAR protein is recombinantly produced and purified. When PILAR is expressed in a recombinant cell other than one of human origin, the PILAR

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is completely free of proteins or polypeptides of human origin. However, it may be necessary to purify PILAR from recombinant host cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to
5 PILAR. As a first step, the culture medium or cell lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions are then separated. PILAR can then be purified from the soluble protein fraction. PILAR thereafter can then be purified from
10 contaminant soluble proteins and polypeptides with, for example, the following suitable purification procedures: by fractionation on immunoaffinity or ion-exchange columns; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE;
15 chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, SEPHADEX G-75; ligand affinity chromatography, and protein A SEPHAROSE columns to remove contaminants such as IgG.

Whether purified or expressed by a recombinant host
20 cell, the PILAR polypeptide of the present invention can be used in screening assays for identifying antagonists and agonists of PILAR. Cell-free screening assays generally involve contacting purified PILAR (e.g., in the presence or absence of CD161) with a test agent and determining whether
25 the test agent binds and/or modulates an activity of PILAR as compared to a PILAR polypeptide which has not been contacted with the test agent. Test agents in the cell-free assay can be synthesized or otherwise affixed to a solid substrate, such as plastic pins, glass slides, plastic
30 wells, and the like. Further, isolated PILAR can be free in solution, affixed to a solid support, or expressed on a cell surface for conducting cell-free screening assays. Alternatively, a PILAR fusion protein can be provided to

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facilitate binding of PILAR to a matrix. For example, glutathione-S-transferase fusion proteins can be adsorbed onto glutathione SEPHAROSE beads (Sigma Chemical, St. Louis, MO) or glutathione-derivatized microtitre plates, which are then combined with the test agent, and the mixture incubated under conditions conducive to complex formation (e.g., at physiological conditions for salt and pH). Such cell-free screening assays can be used to identify PILAR agonists, which mimic or stimulate binding of PILAR to CD161 or other alternative receptor, as well as PILAR antagonists, which block binding of PILAR to CD161 or other alternative receptor. In this regard, it is contemplated that PILAR agonists and PILAR antagonists can bind to either CD161 or PILAR.

In an alternative embodiment, a PILAR agonist or antagonist is identified in a cell-based assay. The steps involved in this screening assay of the invention include, contacting a test cell which expresses a recombinant PILAR polypeptide with a test agent, and determining the whether the test agent modulates an activity of PILAR. Activities of PILAR which can be assayed in accordance with this screening method include binding of PILAR to CD161 or other alternative receptor, proliferation or activation of T cells (e.g., naïve or cytotoxic T cells), expression of IL-10, or surface expression of CD28, as compared to a control cell (e.g., a cell not contacted with the test agent). Such cell-based screening assays can be used to identify PILAR agonists, which mimic or stimulate binding of PILAR to CD161 or other alternative receptor, or increase expression of PILAR. Likewise, such cell-based screening assays can be used to identify PILAR antagonists, which block binding of PILAR to CD161 or other alternative receptor or inhibit the expression of PILAR.

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While the cell-based assay can be carried out using any suitable cell including bacteria, yeast, insect cells (e.g., with a baculovirus expression system), avian cells, mammalian cells, or plant cells, in particular embodiments, the test cell is a mammalian cell (e.g., an APC). Screening assays can also be carried out *in vivo* in animals. Cells modified to express a recombinant PILAR can be transiently or stably transformed with the nucleic acid molecule encoding PILAR. Stably transformed cells can be generated by stable integration into the genome of the organism or by expression from a stably maintained episome (e.g., Epstein Barr Virus derived episomes). The test cells of the screening method of the invention can be cultured under standard conditions of temperature, incubation time, optical density, plating density and media composition corresponding to the nutritional and physiological requirements of the cells. However, conditions for maintenance and growth of the test cell can be different from those for assaying candidate agents in the screening methods of the invention. Any techniques known in the art can be applied to establish the optimal conditions.

Screening assays of the invention can be performed in any format that allows rapid preparation and processing of multiple reactions such as in, for example, multi-well plates of the 96-well variety. Stock solutions of the agents as well as assay components are prepared manually and all subsequent pipetting, diluting, mixing, washing, incubating, sample readout and data collecting is done using commercially available robotic pipetting equipment, automated work stations, and analytical instruments for detecting the output of the assay.

In addition to the reagents provided above, a variety of other reagents can be included in the screening assays

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of the invention. These include reagents like salts, neutral proteins, e.g., albumin, detergents, etc. Also, reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, 5 anti-microbial agents, and the like can be used.

Test agents which can be screened in accordance with the methods of the present invention are generally derived from libraries of agents or compounds. Such libraries can contain either collections of pure agents or collections of 10 agent mixtures. Examples of pure agents include, but are not limited to, proteins, antibodies, aptamers, polypeptides, peptides, nucleic acids, oligonucleotides, siRNA, carbohydrates, lipids, synthetic or semi-synthetic chemicals, and purified natural products. Examples of agent 15 mixtures include, but are not limited to, extracts of prokaryotic or eukaryotic cells and tissues, as well as fermentation broths and cell or tissue culture supernates. In the case of agent mixtures, the methods of this invention are not only used to identify those crude 20 mixtures that possess the desired activity, but also provide the means to monitor purification of the active agent from the mixture for characterization and development as a therapeutic drug. In particular, the mixture so identified can be sequentially fractionated by methods 25 commonly known to those skilled in the art which can include, but are not limited to, precipitation, centrifugation, filtration, ultrafiltration, selective digestion, extraction, chromatography, electrophoresis or complex formation. Each resulting subfraction can be 30 assayed for the desired activity using the original assay until a pure, biologically active agent is obtained.

Additional screens, such as well-established computational screens, are also contemplated for use in

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conjunction with the screening method disclosed herein. Such screens could employ using the agents disclosed herein as lead compounds for the generation of libraries of compounds which modulate the activity of PILAR.

5 Exemplary agents of the instant invention include antagonistic and agonist antibodies which specifically bind to PILAR and modulate T cell proliferation. Such antibodies can be either polyclonal or monoclonal. Moreover, such antibodies can be natural or partially or wholly
10 synthetically produced. All fragments or derivatives thereof (e.g., Fab, Fab', F(ab')₂, scFv, Fv, or Fd fragments) which maintain the ability to specifically bind to and recognize PILAR are also included. The antibodies can be a member of any immunoglobulin class, including any
15 of the human classes: IgG, IgM, IgA, IgD, and IgE.

The PILAR-specific antibodies can be generated using classical cloning and cell fusion techniques. See, for example, Kohler and Milstein (1975) *Nature* 256:495-497; Harlow and Lane (1988) *Antibodies: A Laboratory Manual*,
20 Cold Spring Harbor Laboratory, New York. Alternatively, antibodies which specifically bind PILAR are derived by a phage display method. Methods of producing phage display antibodies are well-known in the art (e.g., Huse, et al. (1989) *Science* 246(4935):1275-81).

25 Selection of PILAR-specific antibodies is based on binding affinity and can be determined by various well-known immunoassays including, enzyme-linked immunosorbent, immunodiffusion chemiluminescent, immunofluorescent, immunohistochemical, radioimmunoassay, agglutination,
30 complement fixation, immunoelectrophoresis, and immunoprecipitation assays and the like which can be performed *in vitro*, *in vivo* or *in situ*. Such standard techniques are well-known to those of skill in the art

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(see, e.g., "Methods in Immunodiagnosis", 2nd Edition, Rose and Bigazzi, eds. John Wiley & Sons, 1980; Campbell et al., "Methods and Immunology", W.A. Benjamin, Inc., 1964; and Oellerich, M. (1984) *J. Clin. Chem. Clin. Biochem.* 22:895-
5 904).

Once fully characterized for specificity, the antibodies of the invention can be assayed for their ability to agonize or antagonize binding of PILAR to CD161 or other alternative receptor via techniques such as ELISA,
10 co-immunoprecipitation experiments and the like.

PILAR agonists and PILAR antagonists (collectively referred to hereafter as agents) identified in accordance with the assay methods of the present invention, as well as vectors, nucleic acids, and PILAR polypeptides disclosed
15 herein (collectively referred to hereafter as molecules) will be useful in various applications including modulating T cell proliferation and activation, surface levels of CD28 and Th1 cytokine secretion. Such method generally involve contacting an immune cell, such as an antigen presenting
20 cell, with an agent or molecule that increases or decreases the expression or activity (including binding to CD161) of PILAR so that T cell proliferation or activation, surface levels of CD28 or Th1 cytokine secretion is modulated. Methods for analyzing these responses are well-known in the
25 art and disclosed herein. In particular embodiments, an agent or molecule that blocks PILAR expression or activity or the PILAR-CD161 interaction is used to decrease T cell proliferation or activation, lower surface levels of CD28 and decrease Th1 cytokine secretion; whereas an agent or
30 molecule that increases PILAR expression or activity or the PILAR-CD161 interaction is used to stimulate T cell proliferation or activation, increase surface levels of CD28 and increase Th1 cytokine secretion.

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In so far as the molecules and agents of the invention ultimately modulate CD161 activity, the present invention also relates to methods stimulating and decreasing immune responses in a subject. Such methods involve administering
5 to a subject in need of treatment an effective amount of an agent or molecule disclosed herein thereby stimulating or decreasing immune responses in the subject. In most cases the subject being treated will be a human being, but treatment of agricultural animals, e.g., livestock and
10 poultry, and companion animals, e.g., dogs, cats and horses, is expressly covered herein. The dosage or effective amount of an agent or molecule is an amount which achieves the desired outcome of reducing at least one sign or symptom of a disease or disorder involving CD161
15 activity. By way of illustration, PILAR antagonists can be used to ameliorate symptoms in autoimmune disease (e.g., diabetes mellitus type 1, systemic lupus erythematosus, Sjögren's syndrome, Hashimoto's thyroiditis, Graves' disease, and rheumatoid arthritis) or inflammation by
20 preventing the activation of autoreactive T cells via PILAR. Conversely, PILAR agonists can be used to mimic PILAR signaling to improve the anti-tumor immune response resulting from tumor-specific T cell activation.

To evaluate the efficacy of any one of these molecules
25 or agents of this invention, one of skill will appreciate that a model system of any particular disease or disorder involving CD161 activity can be utilized to evaluate the adsorption, distribution, metabolism and excretion of a compound as well as its potential toxicity in acute, sub-
30 chronic and chronic studies.

For therapeutic use, it is generally desirable that the agents and molecules of the present invention be provided to a subject in a pharmaceutically acceptable

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carrier and at an appropriate dose. Such pharmaceutical compositions can be prepared by methods and contain carriers which are well-known in the art. A generally recognized compendium of such methods and ingredients is
5 Remington: The Science and Practice of Pharmacy, Alfonso R. Gennaro, editor, 20th ed. Lippincott Williams & Wilkins: Philadelphia, PA, 2000. A pharmaceutically acceptable carrier, composition or vehicle, such as a liquid or solid filler, diluent, excipient, or solvent encapsulating
10 material, is involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be acceptable in the sense of being compatible with the other ingredients of the formulation and not injurious to the
15 subject being treated.

Examples of materials which can serve as pharmaceutically acceptable carriers include sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives,
20 such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil;
25 glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic
30 saline; Ringer's solution; ethyl alcohol; pH buffered solutions; polyesters, polycarbonates and/or polyanhydrides; and other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents,

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emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present
5 in the compositions.

Pharmaceutical compositions appropriately formulated for parenteral (for example, by intravenous, intraperitoneal, subcutaneous or intramuscular injection), topical (including buccal and sublingual), oral,
10 intranasal, intravaginal, or rectal administration can be prepared according to standard methods.

The selected dosage level will depend upon a variety of factors including the activity of the particular agent or molecule employed, the route of administration, the time
15 of administration, the rate of excretion or metabolism of the particular agent being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular agent employed, the age, sex, weight, condition, general health and prior medical
20 history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For
25 example, the physician or veterinarian could start doses of an agent at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. Moreover, given the efficacy antibodies and of an VEGFR1
30 siRNA developed by Sirna Therapeutics (San Francisco, CA) for the treatment of AMD, one of skill in the art can appreciate dosing of such molecules useful for achieving

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the desired therapeutic result with no systemic or local adverse events.

The invention is described in greater detail by the following non-limiting examples.

5

Example 1: Materials and Methods

Tissues and Cells. Tissues were obtained and single-cell suspensions were prepared by gently forcing minced fresh specimens through a 70 μ m-pore mesh and subjected to
10 FICOLL gradient centrifugation. Peripheral blood lymphocytes were obtained by leukapheresis/elutriation and MILTENYI bead-purified (Conejo-Garcia, et al. (2004) *Cancer Res* 64:2175-2182). DCs were generated by incubating magnetically purified CD14⁺ cells for 7 days with
15 Granulocyte-macrophage colony stimulating factor (20 ng/ml; PEPROTECH, Rocky Hill, NJ) and IL-4 (50 ng/ml; R&D Systems, Minneapolis, MN).

Immunohistochemistry was performed using anti-CD161 monoclonal antibodies (DX12 and B199.2) and the ABC kit
20 (CHEMICON, Temecula, CA). Horse serum (1/10 dilution) was used as a negative control.

K562 cells were transduced with CD32 according to established methods (Maus, et al. (2002) *Nat. Biotechnol.* 20:143-148). Expression of CD161 and PILAR was achieved
25 with the pLENTI6/V5-D-TOPO lentiviral system (INVITROGEN, Carlsbad, CA). 293 cells were stably transfected with a pCEFL-kz plasmid encoding Hemagglutinin (HA)-PILAR. Artificial APCs (aAPCs) were gamma-irradiated (100 Gy), washed and loaded with anti-CD3 (OKT3; eBioscience, San
30 Diego, CA) or anti-CD3 plus anti-CD28 (15E8; CHEMICON, Temecula, CA) antibodies at room temperature. T cells were added at a 10:1 ratio. A2⁺ aAPCs were generated by

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transducing K562 cells with lentiviral human CD80 and HLA-A*0201. Positive cells were selected by FACS sorting.

Antibodies and cytokines. Flow cytometry was performed using anti-CD8 (OKT8), anti-CD28 (CD28.2), anti-CD45RA (HI100), anti-CD80 (2D10.4) and anti-CD62L (DREG56) antibodies from eBioscience; anti-CCR7 (3D12) and anti-CD3 (SP34-2) antibodies from BD Biosciences; anti-CD161 (B199.2) and anti-HLA-A2 (BB7.2) from Serotec (Raleigh, NC); and custom rabbit polyclonal anti-PILAR antibody plus anti-rabbit Ig-FITC (Biomedex, Foster City, CA). Cytokines in supernatants from stimulated T cells were quantified in a BIO-PLEX assay (BIO-RAD, Hercules, CA) using the Human-27-Plex panel. Plates were read in a BIO-PLEX Array Reader (BIO-RAD).

Immunoprecipitation and Immunoblotting. K562 cells transduced with CD161 and 293T cells stably transfected with HA-PILAR were lysed on ice for 45 minutes at 4°C in 500 µl of lysis buffer (50 mM Tris-Cl, pH 7.5, 15 mM EDTA, 100 mM NaCl, 0.1% (w/v) TRITON X-100, 1 mM dithiothreitol and 1 mM PEFABLOC SC (Roche, Mannheim, Germany). The lysates were incubated on ice for 90 minutes with 20 µl anti-CD-161 (DX12) or 3 µl anti-HA (HA.11; Covance, Berkeley, CA) monoclonal antibodies, or an irrelevant rabbit IgG (NeoMarkers, Fremont, CA). Following centrifugation at 13,000 x g for 10 minutes at 4°C, the supernatants were incubated with 25 µl Protein G/protein A-agarose beads (CALBIOCHEM, San Diego, CA) for 45 minutes at 4°C with continuous rotation and washed (30 seconds, 1000g). Pellets were resuspended in 30 µl Laemmli buffer, boiled, loaded onto a 15% SDS-PAGE, transferred to a nitrocellulose membrane, blocked and incubated with the indicated primary antibody. Immunoreactive bands were developed using horseradish peroxidase-conjugated secondary antibodies

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(BIO-RAD, Hercules, CA) and chemiluminescent substrate (Pierce, Rockford, IL).

Characterization of Genomic, cDNA and Amino Acid Sequences of PILAR. The predicted amino acid sequence of the following genes encoding a C-type Lectin domain (Smart #00034 of the EMBL database) were aligned with ClustalW software to create a pattern with the residues conserved in at least 6 of 9 sequences and coded by a single exon: AF097358, AF461811, AY486483, Z22576, AF175206, AY358499, DQ049594, HSA133532 and AF133299.

Genomic sequences at chromosome 12p12-13 were translated into the 6 possible ORFs by using ORF Finder software and were scanned for the presence of the pattern with PattenProt software.

RACE-PCR was performed with the human spleen marathon-ready cDNA kit (CLONTECH, Carlsbad, CA), following manufacturer's instructions. The following internal primers were used:

Forward.Outer: 5'-CAG GGG ACT GGC TTG GAG TGA GAG AT-3' (SEQ ID NO:10);

Forward.Inner: 5'-TCT GAT GAT ACC AGA AAT TGG ACA GCC AGT-3' (SEQ ID NO:11);

Reverse.Outer: 5'-ATG GGC CCT CAC CAG AGG TTC CGT AT-3' (SEQ ID NO:12);

Reverse.Inner: 5'-TGC TGC AAA TCC ACT TGA TAT CAA TAA A-3' (SEQ ID NO:13).

Generation of an Anti-PILAR Antibody. The structure of PILAR was predicted with RASMOL software. Based on a putative structural similarity, a good matching of cysteines and the absence of high content of gaps, a CD69-like fold was assigned to the new sequence. A rabbit polyclonal antibody was generated against a fragment of PILAR located on a region of the extracellular domain not

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conserved on structurally related molecules. Moreover, this region putatively forming a β -strand at the C-terminus (Ser-Phe-Ala-Phe-Leu-Ser-Ala-Asp-Gly-Val-His-Ser-Ser-Arg-Gly-Phe-Ile-Asp-Ile-Lys; SEQ ID NO:14).

5 *Real-Time Quantitative PCR.* PILAR expression was analyzed by TAQMAN PCR analysis according to established methods (Boyman, et al. (2006) *Science* 311:1924-1927; Poggi, et al. (1998) *Eur. J. Immunol.* 28:1611-1616; Currier, et al. (2002) *J. Immunol. Methods* 260:157-172).

10 PILAR expression was analyzed with the following primers: PILAR.F, 5'-GTT AGC GCC TTG CCA TGA TTA-3' (SEQ ID NO:15); PILAR.R, 5'-AGG AAG CAC ATA AGG CCA ATC TT-3' (SEQ ID NO:16); and the probe PILAR.P, 5'-(FAM)CTT CAT ACA TCG GAT AGT TCC CAA GTT GAT ACA(TAMRA)-3' (SEQ ID NO:17). The cDNA

15 load was normalized to human *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH) with primers GAPDH F: 5'-CCT GCA CCA CCA ACT GCT TA-3' (SEQ ID NO:18) and GAPDH R: 5'-CAT GAG TCC TTC CAC GAT ACC A-3' (SEQ ID NO:19) and the probe GAPDH.P: 5'-(FAM)CCT GGC CAA GGT CAT CCA TGA CAA C(TAMRA)-

20 3' (SEQ ID NO:20).

Generation of Soluble Fusion Proteins. The cDNAs that encode the extracellular domains of PILAR (124 amino acid residues) and CD161 (160 amino acid residues) were PCR amplified from mRNA isolated from human spleen using the

25 primer sets PILAR.F: 5'-aag ctt AAG CAT GCT AAA CCT GTG GCA TGT TC-3' (SEQ ID NO:21); PILAR.R: 5'-gaa ttc AAA ATA TTT AGG TTT GCT GCA AAT C-3' (SEQ ID NO:22); and CD161.F: 5'-aag ctt ATA CAG AAA TCA TCA ATA GAA AAA TGC-3' (SEQ ID NO:23); CD161.R: 5'-gga tcc GAG TCA GGA TAC ACT TTA TTT CTC

30 ACA-3' (SEQ ID NO:24), respectively, which introduced restriction sequences for *HindIII* and *EcoRI* (PILAR) and for *HindIII* and *BamHI* (CD161). The fragments were subcloned into pSecTag (INVITROGEN) in-frame with a human Ig leader

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sequence (upstream) and a Myc epitope (downstream), followed by a 6x His-tag, to generate the constructs Ig-PILAR-Myc and Ig-CD161-Myc. The sequence amplified with primers BTN.F: 5'-aag ctT CAG TTT TCT GTG CTT GGA C-3' (SEQ ID NO:25) and BTN.R: 5'-gaa ttc TCT GGG CGC TCC TGA AGA AG-3' (SEQ ID NO:26), corresponding to the extracellular domain of BTN3A1 (GENBANK Accession No. NM_007048) was cloned, expressed and purified in the same manner and used as a negative control.

293 cells were independently transfected with the plasmids pIg-PILAR-Myc, pIg-CD161-Myc and pIg-BTN-Myc using LIPOFECTAMINE (INVITROGEN), following manufacturer's instructions. Supernatants from the 2 transfections were harvested at day 3 and 7 and the secreted fusion proteins were purified from culture medium by using a standard Ni column (GE Healthcare), following manufacturer's instructions. After dialysis against PBS, protein concentration was determined by the Bradford assay.

ELISPOT Analysis. For ELISPOT analysis, 2×10^6 PBMCs/ml from an A2⁺ donor were incubated for 7 days (10:1) ratio with CEF peptide pool-pulsed (2 µg/ml) A2⁺ aAPCs or autologous monocyte-derived dendritic cells (generated by incubating magnetically purified CD14⁺ cells for 7 days with Granulocyte-macrophage colony stimulating factor (20 ng/ml, PEPROTECH) and IL-4 (50 ng/ml, R&D Systems)). Interferon-γ ELISPOT was performed on day 8 against irradiated A2⁺ aAPCs or autologous monocyte-derived dendritic cells (10:1 ratio), pulsed with 2 µg/mL antigens.

Flat-bottomed, 96-well nitrocellulose-lined plates (Millipore MultiScreen, Millipore, Bedford, MA) were coated with IFN-γ monoclonal antibody (MD-1; eBioscience, San Diego, CA) and incubated overnight at 4°C. After washing with Coating Buffer (eBioscience, San Diego, CA), plates

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were blocked with 10% FBS serum for 2 hours at 37°C. Effector and target cells were incubated together for 20 hours in RPMI medium 1640 supplemented with 10% FBS. After incubation, the plates were thoroughly washed with 0.05% 5 TWEEN 20 in PBS to remove cells, and biotinylated secondary IFN- γ monoclonal antibody (4S.B3; eBioscience) was added to each well. After incubation for 2 hours at 37°C, the plates were washed and developed with Avidin-horseradish peroxidase (eBioscience) for 1 hour at room temperature. 10 After washing, fresh substrate (3-amino-9-ethyl carbazole; Sigma, St. Louis, MO) was added and the plates incubated for approximately 20 minutes.

Example 2: Identification PILAR

15 Amino acid sequence alignment of known or predicted molecules mapping at chromosome 12p12-p13 and exhibiting a C-type Lectin domain was conducted. The alignment revealed the identification of the conserved pattern Cys-Pro-(Xaa)₂-Trp-(Xaa)₂-[Tyr-Phe]-(Xaa)₃-Cys-Tyr-(Xaa)₂-Ser-(Xaa)₅-Trp- 20 (Xaa)₂-Ser-(Xaa)₃-Cys (SEQ ID NO:7), wherein Xaa is any amino acid residue. This motif was used to screen the genomic sequences (~2 Mb) mapping at the human chromosome 12p12-p13, translated in their six possible open reading frames. A continuous genomic sequence was identified and 25 was found to encode an amino acid sequence with 74% amino acid sequence identity with this aforementioned pattern (Cys-Ser-Gly-Asp-Trp-Leu-Gly-Val-Arg-Asp-Lys-Cys-Phe-Tyr-Phe-Ser-Asp-Asp-Thr-Arg-Asn-Trp-Thr-Ala-Ser-Lys-Ile-Phe-Cys (SEQ ID NO:8). This 29 amino acid sequence was found on a 30 provisional GENBANK mRNA entry identified as *C-type lectin domain family 2, member A* (CLEC2A; Accession Number: NM_207375). However, because the entire open reading frame of the identified genomic clone could not be PCR-amplified

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from cDNA derived from five different leukocyte subsets, including human B cells, T cells, NK cells, dendritic cells or monocytes, it was concluded that the identified clone corresponds to a splice variant which differed from CLEC2A.

5 To identify a corresponding mRNA for the genomic clone, a leukocyte-specific cDNA library was created by RACE-PCR with cDNA from human spleen. The full-length cDNA sequence of the newly identified gene had five exons encoding a transmembrane molecule containing a truncated extracellular

10 C-type lectin-like domain (Figure 1A). As depicted in Figure 1B, sequence similarity searches identified the immunoreceptors CD69 and CLEC2D/LLT1 as two closely-related human molecules. Moreover, as shown in Figure 1C, the protein shares 100% amino acid sequence identity with the
15 N-terminal 137 amino acid residues of CLEC2A and INPE5792 (GENBANK Accession No. AAQ89483), but is divergent from these protein in the remaining 37 amino acid residues, *i.e.*, Phe-Glu-Ile-Ile-Gly-Asn-Gly-Ser-Phe-Ala-Phe-Leu-Ser-Ala-Asp-Gly-Val-His-Ser-Ser-Arg-Gly-Phe-Ile-Asp-Ile-Lys-

20 Trp-Ile-Cys-Ser-Lys-Pro-Lys-Tyr-Phe-Leu (SEQ ID NO:9). The predicted amino acid sequence of this novel protein identifies the presence of a short cytoplasmic domain and a truncated C-type Lectin domain (Figure 1D).

25 **Example 3: PILAR is a Ligand for the CD161 Immunoreceptor**

Real-Time Q-PCR analysis showed that, in addition to spleen, the novel sequence was constitutively expressed on human thymus and small intestine, indicating that this protein may be expressed in lymphocytes. Consequently, PCR
30 analysis showed the mRNA signal in T and B cells, but not on epithelial tumor cell lines. In addition, lower mRNA levels were detected in the genital tract (*i.e.*, testis and ovary), where lymphocyte numbers are low. No detectable

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expression was found in any other tissue tested (e.g., breast, brain, liver, muscle, or colon). The highest expression of the new protein was identified on CD8⁺ T cells, followed by B lymphocytes and CD4⁺ T cells. No detectable mRNA levels were found on myeloid leukocytes or natural killer (NK) cells.

Because of the homology with CLEC2D/LLT1, the only known ligand for CD161, it was contemplated that the new molecule could also bind to CD161. To demonstrate this interaction, 293 cells were stably transfected with a construct encoding the novel sequence fused in-frame with a hemagglutinin epitope at the 5'-end (i.e., the novel sequence was inserted into the pCEFL-kz plasmid (Crespo, et al. (1997) *Nature* 385:169-172)). Likewise, CD161 was transduced into K562 cells using a pLXSN retrovirus encoding CD161 and selected with neomycin. Lysates from both lines were then incubated for 2 hours and immunoprecipitation was performed separately with an anti-hemagglutinin antibody (HA11, Covance, Berkeley, CA) or an anti-CD161 antibody in the presence of protein G/protein A agarose beads. Western blot analysis revealed that lysates immunoprecipitated with anti-hemagglutinin antibodies contained a ~40 kD band reacting with the anti-CD161 antibody, while immunoprecipitation with anti-CD161 allowed the detection of a ~20 kD band that reacted with the anti-hemagglutinin antibody. No band was detectable when CD161-expressing K562 cells were incubated with the parental 293 cells, or when 293 cells expressing the novel sequence were incubated with plain K562 cells. In different experiments, it was confirmed that the anti-CD161 antibody reacted with the expected ~40 kD band only when the lysates were immunoprecipitated with the anti-hemagglutinin antibody, but not when the immunoprecipitation was performed with an

-36-

irrelevant antibody. Therefore, the novel transmembrane molecule binds specifically to CD161.

Confirming the PILAR/CD161 interaction, a soluble chimeric CD161-Myc epitope protein, but not an irrelevant-Myc protein, specifically bound to K562 cells ectopically expressing PILAR and not the PILAR-K562 cell line. Moreover, a chimeric PILAR protein specifically bound to CD161-transduced K562 cells. Together, these data demonstrate that the novel transmembrane molecule interacts with CD161.

Example 4: Transient Up-Regulation of PILAR on Activated Lymphocytes

Based on the similarities with CD69, it was surmised that expression of the novel protein disclosed herein could be transiently up-regulated upon lymphocyte activation (Sancho, et al. (2005) *Trends Immunol.* 26:136-140). To demonstrate this, peripheral CD8⁺ T cells (10⁶) were isolated and incubated with phytohemagglutinin (PHA; 10 µg/mL) for different periods of time. The new CD161 ligand was maximally up-regulated at 9 hours after activation of peripheral T cells, with the mRNA message decreasing thereafter. Comparable results were found with CD4⁺ T cells.

To verify this pattern of up-regulation after CD3/CD28-dependent stimulation, a previously-described artificial antigen-presenting cell (aAPC) system was employed (Conejo-Garcia, et al. (2004) *supra*; Conejo-Garcia, et al. (2003) *supra*; Maus, et al. (2002) *Nat. Biotechnol.* 20:143-148). Briefly, the method involves the use of (MHC-1 deficient) K562 cells transduced with CD32, which can subsequently be coated with agonistic CD3 and CD28 antibodies (100 ng/mL). Consistent with the up-regulation upon stimulation with PHA, CD3/CD28 activation

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also induced a maximum over-expression of the new CD161 ligand at 9 hours, with a dramatic decrease compared to constitutive mRNA levels found on naïve lymphocytes after T cell activation for more than 20 hours. Based on this expression pattern and the presence of a transmembrane domain, the new CD161 ligand was designated Proliferation-Induced Lymphocyte-Associated Receptor (PILAR).

FACS analysis performed with an endotoxin-free PILAR-specific antibody confirmed that PILAR surface expression was detectable in less than 2% of naïve T cells. However, after CD3/CD28-stimulation for three days, PILAR became detectable on CD4 T cells and reached a maximum level on both proliferating CD4 and CD8 T cells (but not in cells that did not proliferate) at day 7. Similar uncoupled mRNA and protein expression are frequently observed on primed T cells, as they initially undergo translational attenuation (Cham, et al. (2003) *J. Biol. Chem.* 278:17044-17052; Scheu, et al. (2006) *Nat. Immunol.* 7:644-651; Mao, et al. (1992) *J. Biol. Chem.* 267:20444-20450). Correspondingly, a rapid PILAR mRNA degradation was found shortly after T cell activation; PILAR mRNA was nearly undetectable at 45 minutes after actinomycin D treatment. In agreement with previous reports, CD161 was not found on naïve T cells, but it was clearly detectable on both CD4 and CD8 T cells upon CD3/CD28 stimulation.

Example 5: PILAR Enhances TCR-Mediated Proliferation of Human T Cells

Since PILAR was quickly up-regulated on early activated T cells, the effect of increased PILAR availability on T cell proliferation was determined by ectopically expressing PILAR on the PILAR⁻ aAPCs. To define activating conditions of different stringency, a

-38-

proliferation assay was performed using decreasing concentrations of anti-CD3 mAb (500 ng - 0.5 pg/ml), and a constant concentration of 100 ng/ml of anti-CD28 monoclonal antibody. Based on 5-day proliferation analysis of naive T cells, concentrations of anti-CD3 of 100ng/ml and 0.5ng/ml were defined as "optimal" and "limiting", respectively. Hence, under optimal TCR-stimulation conditions but in the absence of CD28 signaling, ectopic PILAR dramatically enhanced the proliferation of naïve T cells (53% increase in total proliferating cells). Because co-stimulatory molecules prevent the induction of apoptosis by increasing Bcl-xL (Sperling, et al. (1996) *J. Immunol.* 157:3909-3917), expression was next compared on T cells stimulated with agonistic CD3 antibodies in the presence or the absence of PILAR. Results of this analysis indicated that increasing PILAR availability augmented Bcl-xL levels and, to a much lesser extent, c-FLIP, after 24 hours of stimulation. Together, these data indicate that PILAR enhances the expansion of TCR-stimulated T cells by increasing their survival through enhanced expression of anti-apoptotic proteins. Of note, PILAR appears not to be expressed by myeloid cells indicated that, *in vivo*, T cells present PILAR to each other for engagement to CD161 in a reciprocal manner. Similar T-T co-stimulatory interactions occur between CD27 and CD70 (Huang, et al. (2006) *J. Immunol.* 176:7726-7735), although they appear to be more relevant for secondary responses (Watts (2005) *Annu. Rev. Immunol.* 23:23-68; Borst, et al. (2005) *Curr. Opin. Immunol.* 17:275-281).

Significantly, PILAR overexpression profoundly increased the production of IFN- γ and the IFN-responsive chemokine CXCL10 by stimulated T cells (Fig.3C). Similarly, the inflammatory chemokines CCL3, CCL4 and CCL5, as well as

-39-

GM-CSF, which increases the production of myeloid cells, were dramatically upregulated by ectopic PILAR signaling. In contrast, PILAR had no effect on CXCL8 or G-CSF secretion. These results indicate a fine balance between activation and inhibition that regulates T cell homeostasis. IL-12 strongly upregulates CD161 expression on NK cells (Poggi, et al. (1998) *Eur. J. Immunol.* 28:1611-1616), and CD161 signaling, in turn, induces the secretion of IFN- γ .

Example 6: PILAR/CD161 Interactions are Critical for CD3-Mediated Proliferation

Paradoxically, increased PILAR availability in the presence of CD3 plus CD28 signaling decreased T cell proliferation. It was contemplated that PILAR enhances T cell proliferation through CD161 under suboptimal activating conditions and triggers an alternative regulatory pathway through a different receptor in vigorously activated lymphocytes. To demonstrate this, human naïve T cells were activated under optimal CD3 stimulation conditions in the absence or the presence of ectopic PILAR and anti-CD161 antibodies. Strikingly, in the presence of ectopic PILAR, >37% lymphocytes underwent apoptosis when an anti-CD161 antibody, but not a control Ig, was added to the media, as indicated by annexin V staining. Apoptosis was initiated within 24 hours of T cell activation and also occurred in naïve T cells exposed to PILAR expressed on the aAPCs. The presence of the anti-CD161 antibody did not result in detectable apoptosis or cell death in the absence of ectopic PILAR, ruling out a direct cytotoxic effect. These data indicate that PILAR induces T cell apoptosis through a second receptor present on naïve T cells that is upregulated shortly after TCR-

-40-

stimulation, and that this apoptotic cell death is prevented by the engagement of CD161. Since PILAR decreases the proliferation of CD3/CD28 co-stimulated T cells, it is believed that this alternative receptor is also upregulated upon CD28 signaling.

Collectively, these results point to a model whereby PILAR, depending on the receptor preferentially expressed at different stages of T cell activation, either increases T cell survival through CD161, or induces apoptotic death through a different mediator.

Example 7: PILAR Blockade Abrogates the Expansion of Both CD4 and CD8 T Cells

To further demonstrate the critical role of PILAR on T cell proliferation, it was determined whether PILAR blockade affected TCR-mediated signaling. In the presence of anti-PILAR antibodies, the CFSE signal of magnetically purified T cells expanded under optimal conditions of CD3 stimulation was significantly lower than the signal from cells that were incubated with an irrelevant Ig. Furthermore, this signal was comparable to that of unstimulated lymphocytes (Figure 2). A high level of co-stimulation (anti-CD28 at 100 ng/ml) was required to rescue the inhibition of proliferation mediated by PILAR blockade, although a decrease in the strong CD3/CD28-mediated expansion was still apparent. Comparable results were obtained with higher concentrations of plate-bound antibody. Under limiting conditions of TCR engagement, a dramatic impairment of proliferation was observed after the addition of an anti-PILAR antibody, even in the presence of co-stimulation (Figure 2C). Additionally, PILAR blockade abrogated the generation of IFN- γ -secreting, antigen-specific HLA-A2 CD8 T cells responding to a cocktail of 23

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immunogenic, class I restricted 8-11mers (Currier, et al. (2002) *J. Immunol. Methods* 260:157-172) presented by A2⁺ aAPCs or autologous dendritic cells (Figure 3A), as well as the proliferation of peripheral blood mononuclear cells (PBMCs) induced by these specific antigens (Figure 3B). Addition of anti-PILAR antibodies also impaired the proliferation of CD4 and CD8 T cells induced by autologous DCs pulsed with 138 different Cytomegalovirus 15-mers with 11 amino acid overlaps (Figure 3C). These data confirm that endogenous PILAR, depending on the relative strength of the TCR signal and the degree of co-stimulation, is critical for the proliferation of naïve T cells.

Notably, a reduction in the surface expression of CD28 was observed when naïve T cells were stimulated with CD3/CD28 for 2 days in the presence of anti-PILAR antibody, compared to cells cultured with an irrelevant antibody. A major decrease in the expression of surface CD62L, which mediates lymphocyte homing during inflammation, was also observed upon PILAR blockade. A comparable decrease was observed at day 7 and when lymphocytes were activated through PHA in the presence or the absence of anti-PILAR antibody. Taken together, these results indicate that, *in vivo*, PILAR modulates the capacity of T lymphocytes to home to lymph nodes through CD62L, where they can undergo a robust expansion in the presence of increased CD28 signaling.

In addition, incubation of purified naïve T cells with anti-PILAR antibodies in the absence of TCR stimulation resulted in the up-regulation of IL-10 at both the mRNA and protein levels, compared to lymphocytes incubated with identical concentrations of an irrelevant antibody. No significant effect on the expression of TGF- β was observed, whereas a 50% decrease in the levels of interferon- γ was

-42-

detected. These data indicate that 2% of naïve T cells expressing PILAR can also regulate the production of cytokines such as IL-10 under resting conditions through autocrine and paracrine interactions.

5

Example 8: CD3/CD28 Signaling Recovers PILAR and CD161 Expression on Human Tumor-Infiltrating T Cells

A majority of the T cells isolated from human tumors have an effector memory phenotype (Broderick, et al. (2006) *Clin. Immunol.* 118:159-169). Since the expression of CD161 in healthy donors identifies mostly T cells with effector memory differentiation (Takahashi, et al. (2006) *supra*), rather than natural killer (NK) T cells, it was contemplated that the tumor microenvironment could prevent the anti-tumor immune response by down-regulating the expression of the PILAR/CD161 pair on tumor-infiltrating T cells.

To demonstrate this effect, it was first confirmed that >92% CD8 T cells present in single-cell suspensions prepared from seven different human ovarian carcinoma specimens exhibited a CCR7⁻CD69⁻CD45RA^{+/-} (effector memory) phenotype, although some specimens contained a small proportion of naïve T cells, ranging from 3 to 7%. The great majority of these cells were negative for the early activation marker CD69, which is expressed transiently by fully activated effector T cells. They did not express the invariant (V α 24-J α Q) TCR chain, indicating that, unlike other cancer types (Terabe, et al. (2006) *Cancer Res.* 66:3869-3875), NK T cells do not play a significant role in the physiopathology of established ovarian carcinomas. Although a significant infiltration of CD161⁺ cells were observed in both the stroma and the tumor islets of most specimens analyzed, CD161 was found on less than 1% of CD8

-43-

T cells by flow cytometry. In contrast, variable levels of CD161 were found on autologous tumor-derived CD4 T cells. As CD161 expression in healthy donors is mostly found on effector memory cells (Takahashi, et al. (2006) *J. Immunol.* 176:211-216), these data indicate that CD161-mediated stimulation is specifically abrogated by the tumor microenvironment on effector T cells. Furthermore, most tumors contained CD4 and CD8 tumor-infiltrating T cells that expressed PILAR, indicating that CD4⁺ lymphocytes, which include CD25⁺Foxp3⁺ regulatory T cells (Sato, et al. (2005) *Proc. Natl. Acad. Sci. USA* 102:18538-18543), may be more susceptible to PILAR-mediated expansion. In contrast, CD8⁺ lymphocytes, the only cell type known to exert immune pressure against ovarian cancer growth (Sato, et al. (2005) *supra*), may be prone to PILAR-mediated apoptotic death.

Importantly, CD3/CD28-mediated T cell stimulation resulted in a significant upregulation of surface CD161 on CD8 T cells (Figure 4A). These data indicate that anti-tumor T cells can be recovered from tumor-induced immunosuppression *ex vivo* and converted into activated cytotoxic cells with a strong proliferative capacity. The observation that the expression of PILAR on CD8 T cells increased dramatically upon activation supports this conclusion (Figure 4B). In summary, PILAR represents a crucial modulator of the extent of cellular immune responses in humans. The results provide a mechanistic rationale for the manipulation of PILAR signaling, which may unveil new interventions against autoimmunity, inflammation and cancer.

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What is claimed is:

1. An isolated nucleic acid molecule, wherein said nucleic acid molecule

5 (a) encodes a Proliferation-Induced Lymphocyte-Associated Receptor (PILAR) polypeptide comprising the amino acid sequence set forth in SEQ ID NO:9; or

(b) hybridizes to a nucleic acid molecule encoding a PILAR polypeptide comprising the amino acid sequence set
10 forth in SEQ ID NO:9.

2. A vector comprising the isolated nucleic acid molecule of claim 1.

15 3. A host cell comprising the vector of claim 2.

4. An isolated PILAR polypeptide comprising the amino acid sequence set forth in SEQ ID NO:9.

20 5. A host cell comprising the isolated PILAR polypeptide of claim 4.

6. A composition comprising the isolated PILAR polypeptide of claim 4 in admixture with a pharmaceutically
25 acceptable carrier.

7. An antibody which specifically binds to the isolated PILAR polypeptide of claim 4.

30 8. A method for identifying an agent which modulates an activity of PILAR comprising contacting a PILAR polypeptide, or host cell expressing a PILAR polypeptide, with a test agent and determining whether the test agent

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modulates an activity of PILAR wherein a decrease in a PILAR activity is indicative of an agent which antagonizes PILAR and an increase in PILAR activity is indicative of an agent which agonizes PILAR.

5

9. An agent identified by the method of claim 8.

10. A method for stimulating T cell activation or proliferation comprising contacting an immune cell with
10 PILAR or a PILAR agonist thereby stimulating T cell activation or proliferation.

11. A method for decreasing T cell activation or proliferation comprising contacting an immune cell with a
15 PILAR antagonist thereby decreasing T cell activation or proliferation.

12. A method for stimulating immune responses in a subject comprising administering to a subject in need of
20 treatment an effective amount of a PILAR agonist thereby stimulating immune responses in the subject.

13. The method of claim 12, wherein the subject has cancer.

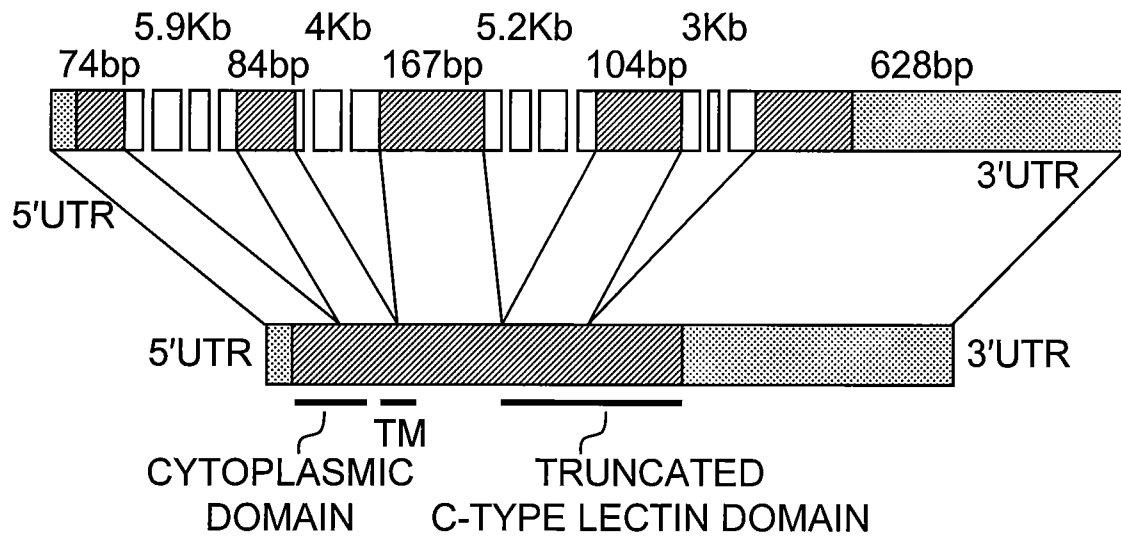
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14. A method for decreasing immune responses in a subject comprising administering to a subject in need of treatment an effective amount of a PILAR antagonist thereby decreasing immune responses in the subject.

30

15. The method of claim 14 wherein the subject has an autoimmune disease or inflammation.

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**FIG. 1A**

PILAR -----MINP-ELRD--GRADGFIHRIVPKLIQNWKIGLMCF*
CLEC2D -----MHDSNNVEKDITPSELPANPGCLHSKEHSIKATLIWRLFF-LIMF
CD69 MSSENCFAENSSSLHPESGQENDATSPHFSTRHEGSFQVPVLCVMNVVF

PILAR *LSIIITTVCIIMIATWS-----KHAKP*-----VACSGDWLGVRDKCFYFS*
CLEC2D LTIIVCGMVAALSAIRAN-----CHQEPSVCLQAACPESWIGFQRKCFYFS
CD69 ITILIIIALIALSVGQYNCPGQYTFSMPSDSHVSSCEDWVG YQRKCYFIS

PILAR ** * * ** ** * * * * *
DDTRNWTASKIFCSLQKAELAQIDTQEDMEFLKRYAGTDMHWIGLSRKQG
CLEC2D DDTKNWTSSQRFCDSDADLAQVESFQELNFLRLRYKGPSDHWIGLSREQG
CD69 TVKRSWTS AQNACSEHGATLAVIDSEKDMNFLKRYAGREEHWVGLKKEPG

PILAR *** ** * * * * *** *
DSWKWINGTTFNGWFEIIGNGSFAFLSADGVHSSRGFIDIKWICKSKPKYF
CLEC2D QPWKWINGTEWTRQFPILGAGECAYLNDKGASSARHYTERKWICKSKSDIH
CD69 HPWKWSNGKEFNWFWNTGSDKCVFLKNTEVSSMECEKNLYWICKNPKYK-

PILAR L 174 (SEQ ID NO:1)
CLEC2D V 191 (SEQ ID NO:3)
CD69 - 199 (SEQ ID NO:2)

FIG. 1B

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CLEC2A    MINPELRDGRADGFIHRIVPKLIQNWKIGLMCFLSIIITTVCIIMIATW
INPE5792  MINPELRDGRADGFIHRIVPKLIQNWKIGLMCFLSIIITTVCIIMIATW
PILAR     MINPELRDGRADGFIHRIVPKLIQNWKIGLMCFLSIIITTVCIIMIATW
          *****

CLEC2A    SKHAKPVACSGDWLGVRDKCFYFSDDTRNWTASKIFCSLQKAELAQIDT
INPE5792  SKHAKPVACSGDWLGVRDKCFYFSDDTRNWTASKIFCSLQKAELAQIDT
PILAR     SKHAKPVACSGDWLGVRDKCFYFSDDTRNWTASKIFCSLQKAELAQIDT
          *****

CLEC2A    QEDMEFLKRYAGTDMHWIGLSRKQGDSWKWTNGTTFNGWP-----SNS
INPE5792  QEDMEFLKRYAGTDMHWIGLSRKQGDSWKWTNGTTFNGWP-----SNS
PILAR     QEDMEFLKRYAGTDMHWIGLSRKQGDSWKWTNGTTFNGWFEIIGNGSFA
          *****          * :

CLEC2A    KWSCN--WSLR-----QWLLLLLGPLR- 160 (SEQ ID NO:5)
INPE5792  KWSCN--WSLR-----QWLLLLLGPLR- 160 (SEQ ID NO:4)
PILAR     FLSADGVHSSRGFIDIKWICSKPKYFL 174 (SEQ ID NO:1)
          *.:      * *      :*:

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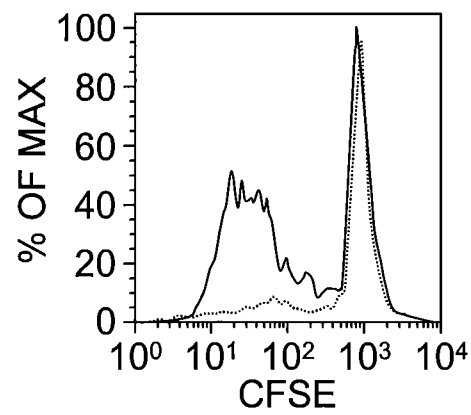
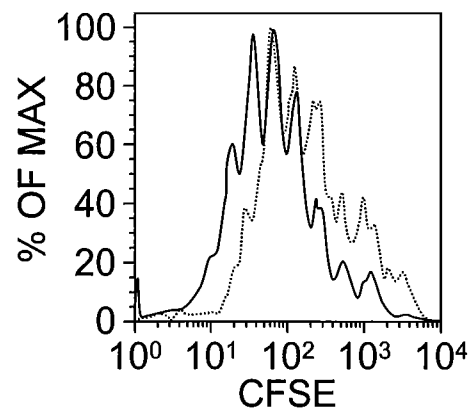
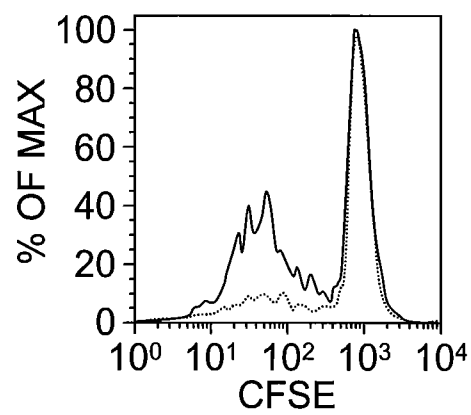
FIG. 1C

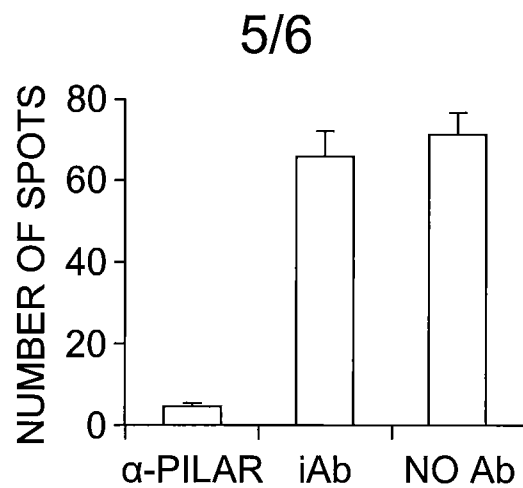
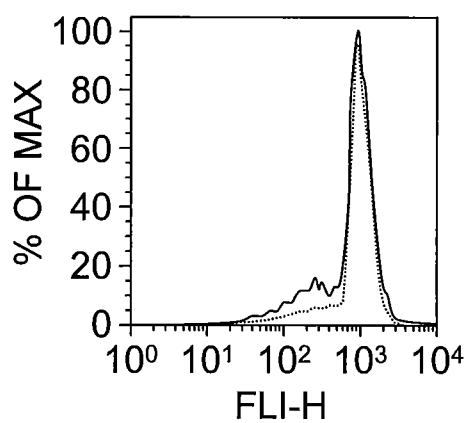
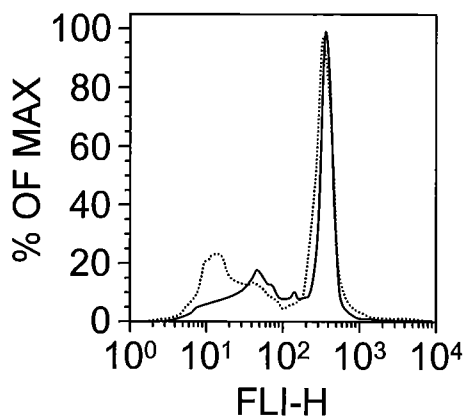
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L I Q N W K I G L M C F L S I I I T T
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V C I I M I A T W S K H A K P V A C S
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G D W L G V R D K C F Y F S D D T R N
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W T A S K I F C S L Q K A E L A Q I D
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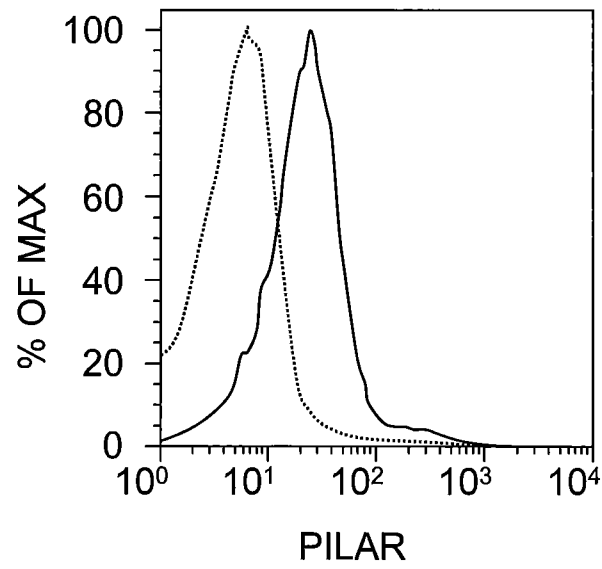
FIG. 1D

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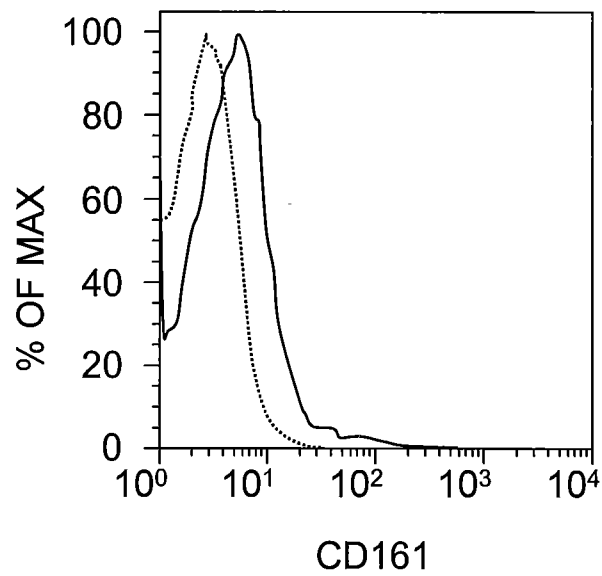
**FIG. 2A****FIG. 2B****FIG. 2C**

*FIG. 3A**FIG. 3B**FIG. 3C*

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PILAR
FIG. 4A



CD161
FIG. 4B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US08/59879

A. CLASSIFICATION OF SUBJECT MATTER

IPC: C07H 21/04(2006.01);C07K 14/705(2006.01),16/28(2006.01);A61K 38/16(2006.01)

USPC: 536/23.1;530/350,387.1;514/12

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)

U.S. : 536/23.1;530/350,387.1;514/12

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NCBI Accession No. EF127467 (Huarte-Sobrino et al.), 20 November 2006.	1-3
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Y		4-15



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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

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Date of the actual completion of the international search

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Date of mailing of the international search report

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