ANTIBODIES

RELATED APPLICATION

This application claims priority to U.S. Provisional Application Serial No. 60/569,892, filed on May 10, 2004, the contents of which are incorporated by reference in its entirety.

BACKGROUND

Overly aggressive T cells often lead to unwanted immune responses, which, in turn, cause various disorders, e.g., autoimmune diseases, transplant rejection, allergic diseases, and T cell-derived cancers. Therefore, control of the aggressive T cells is critical in treating such disorders. The activity of these cells can be contained by immunosuppression or by induction of immunological tolerance. An alternative solution is induction of apoptosis, which is believed to be involved in removing unwanted cells, including overly aggressive T cells. See, e.g., Kabelitz et al. (1993) Immunol Today 14, 338-340; and Raff (1992) Nature 356, 397-399.

SUMMARY

This invention relates to antibodies and their derivatives that induce apoptosis upon binding to P-Selectin Glycoprotein Ligand-1 (PSGL-1) on activated T cells,

In one aspect, the invention features an immunoglobulin chain having three sequences that (i) contain, respectively, RSSQSIVHNDGNTYFE, KVSNRFS, and FQGSHVPLT (SEQ ID NOs: 1-3); (ii) contain, respectively, SFGMH, YINGGSSTIFYANAVKG, and YASYGGGAMDY (SEQ ID NOs: 4-6); (iii) contain, respectively, RASSTVNSTYLH, GSSNLAS, and QQYSGYPLT (SEQ ID NOs: 7-9); (iv) contain, respectively, AYYIH, VNPNTGGTSYNPKFKG, and SGSPYYRYDD (SEQ ID NOs: 10-12); (v) contain, respectively, RSSQSIVNSNGNTYLE, KVSNRFS, and FQGSHVPWT (SEQ ID NOs: 13-15); or (vi) contain, respectively, TNAMNWVRQAPGKGLE, TYYADSVKD, and GGSYWYFDV (SEQ ID NOs: 16-18).

Each of the just-described six sets of sequences corresponds to the three light or heavy chain complementarity determining regions (CDRs) of an antibody that binds to

PSGL-1, such as those of three mouse 15A7, 43B6, and 9F9 antibodies described in the examples below. Shown below are the light chains and heavy chain variable (V) regions of these three antibodies (SEQ ID NOs: 19-26, the CDRs are underlined and highlighted:

SEQ ID NO: 19 (PRT) and 27 (DNA) (Mouse 15A7 light chain V region):

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  1 ATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGCTTCCAGCAGTGAT
  1  M  K  L  P  V  R  L  L  V  L  M  F  W  I  P  A  S  S  S  D

 61 ATTTTGATGACCCAAACTCCACTGTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCAATA
 21  I  L  M  T  Q  T  P  L  S  L  P  V  S  L  G  D  Q  A  S  I

121 TCTTGCAGATCTAGTCAGAGCATTGTACATAATGATGGAAACACCTATTTTGAATGGTAC
 41  S  C  R  S  S  Q  S  I  V  H  N  D  G  N  T  Y  F  E  W  Y

181 CTGCAGAAACCAGGCCAGTCTCCAAAACTCCTGATCTACAAAGTTTCCAATCGATTTTCT
 61  L  Q  K  P  G  Q  S  P  K  L  L  I  Y  K  V  S  N  R  F  S

241 GGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACACATTTCACACTCAACATCAGC
 81  G  V  P  D  R  F  S  G  S  G  S  G  T  H  F  T  L  N  I  S

301 AGAGTGGAGGCTGAGGATCTGGGAATTTATTACTGCTTTCAAGGTTCATATGTTCCTCTC
101  R  V  E  A  E  D  L  G  I  Y  Y  C  F  Q  G  S  Y  V  P  L

361 ACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
121  T  F  G  A  G  T  K  L  E  L  K
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SEQ ID NO: 20 (PRT) and 28 (DNA) (Mouse 15A7 heavy chain V region):

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  1 ATGGACTCCAGGCTCAATTTAGTTTTCCTTGTCCTTATTTTAAAAGGTGTCCAGTGTGAT
  1  M  D  S  R  L  N  L  V  F  L  V  L  I  L  K  G  V  Q  C  D

 61 GTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGGAGGGTCCCGGAAACTCTCC
 21  V  Q  L  V  E  S  G  G  G  L  V  Q  P  G  G  S  R  K  L  S

121 TGTGCAGCCTCTGGATTCACTTTCAGTAGCTTTGGAATGCACTGGGTTCGTCAGGCTCCA
 41  C  A  A  S  G  F  T  F  S  S  F  G  M  H  W  V  R  Q  A  P

181 GAGAAGGGGCTGGAGTGGGTCGCATACATTAATGGTGGCAGTAGTACCATCTTCTATGCA
 61  E  K  G  L  E  W  V  A  Y  I  N  G  G  S  S  T  I  F  Y  A

241 AACGCAGTGAAGGGCCGATTCACCATCTCCAGAGACAATCCCAAGAATACCCTGTTCCTG
 81  N  A  V  K  G  R  F  T  I  S  R  D  N  P  K  N  T  L  F  L

301 CAAATGACCATTCTAAGGTCTGAGGACACGGCCATTTATTACTGTGGAAGGTATGCTAGT
101  Q  M  T  I  L  R  S  E  D  T  A  I  Y  Y  C  G  R  Y  A  S

361 TACGGAGGGGGTGCTATGGACTATTGGGGTCAAGGAACCTCAGTCACCGTCTCCTCA
121  Y  G  G  G  A  M  D  Y  W  G  Q  G  T  S  V  T  V  S  S
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SEQ ID NO: 21 (PRT) and 29 (DNA) (Mouse 43B6 light chain V region):

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  1 ATGGATTTTCTGGTGCAGATTTTCAGCTTCTTGCTAATCAGTGCCTCAGTTGCAATGTCC
  1  M  D  F  L  V  Q  I  F  S  F  L  L  I  S  A  S  V  A  M  S

 61 AGAGGAGAAAATGTGCTCACCCAGTCTCCAGCAATCATGTCTGCATCTCCAGGGGAAAAG
 21  R  G  E  N  V  L  T  Q  S  P  A  I  M  S  A  S  P  G  E  K

121 GTCACCATGACCTGCAGGGCCAGCTCAACTGTAAATTCCACTTACTTGCACTGGTTCCAG
 41  V  T  M  T  C  R  A  S  S  T  V  N  A  T  Y  L  H  W  F  Q

181 CAGAAGTCAGGTGCCTCCCCCAAACTCTGGATTTATGGCTCATCCAACTTGGCTTCTGGA
 61  Q  K  S  G  A  S  P  K  L  W  I  Y  G  S  S  N  L  A  S  G

241 GTCCCTGCTCGCTTCAGTGGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCAGT
 81  V  P  A  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I  S  S

301 GTGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGCAGTACAGTGGTTACCCACTCACG
101  V  E  A  E  D  A  A  T  Y  Y  C  Q  Q  Y  S  G  Y  P  L  T

361 TTCGGTGCTGGGACCACGCTGGAGCTGAAA
121  F  G  A  G  T  T  L  E  L  K
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SEQ ID NO: 22 (PRT) and 30 (DNA) (Mouse 43B6 heavy chain V region):

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  1 ATGGAATGGAGCTGGGTCTTTCTCTTCCTCCTGTCAGTCACTACAGGTGTCCACTCTGAG
  1  M  E  W  S  W  V  F  L  F  L  L  S  V  T  T  G  V  H  S  E

 61 GTCCAGCTGCAGCAGTCTGGACCTGACCTGGTGAAGCCTGGGGCTTTAGTGAAGATATCC
 21  V  Q  L  Q  Q  S  G  P  D  L  V  K  P  G  A  L  V  K  I  S

121 TGCAAGGCTTCTGGTTACTCATTCACTGCCTACTACATTCACTGGGTGAAGCAGAGCCAT
 41  C  K  A  S  G  Y  S  F  T  A  Y  Y  I  H  W  V  K  Q  S  H

181 GGAAAGAGCCTTGAGTGGATTGGACGTGTTAATCCTAATACTGGTGGTACTAGCTACAAC
 61  G  K  S  L  E  W  I  G  R  V  N  P  N  T  G  G  T  S  Y  N

241 CCGAAGTTCAAGGGCAAGGCCATATTAAATGTAGATAAGTCATCCAGCACAGCCTACATG
 81  P  K  F  G  K  K  A  I  L  N  V  D  K  S  S  S  T  A  Y  M

301 GAGCTCCGCAGCCTGACATCTGAGGACTCTGCGGTCTATTACTGTGCAAGATCGGGATCC
101  E  L  R  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  S  G  S

361 CCCTACTATAGGTACGACGACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
121  P  Y  Y  R  Y  D  D  W  G  Q  G  T  T  L  T  V  S  S
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SEQ ID NO: 23 (PRT) and 31 (DNA) (Mouse 9F9 light chain V region):

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  1 ATGAAGTTGCCTGTTAGGCTGTTGGTGCTGATGTTCTGGATTCCTGCTTCCAGCAGTGAT
  1  M  K  L  P  V  R  L  L  V  L  M  F  W  I  P  A  S  S  S  D

 61 GTTTTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGATCAAGCCTCCATC
 21  V  L  M  T  Q  T  P  L  S  L  P  V  S  L  G  D  Q  A  S  I

121 TCTTGCAGATCTAGTCAGAGCATTGTAAATAGTAATGGAAACACCTATTTAGAATGGTAC
 41  S  C  R  S  S  Q  S  I  V  N  S  N  G  N  T  Y  L  E  W  Y

181 CTGCAGAAACCAGGCCAGTCTCCAAAGCTCCTGATCTACAAAGTTTCCAACCGATTTTCT
 61  L  Q  K  P  G  Q  S  P  K  L  L  I  Y  K  V  S  N  R  F  S

241 GGGGTCCCAGACAGGTTCAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGC
 81  G  V  P  D  R  F  S  G  S  G  S  G  T  D  F  T  L  K  I  S

301 AGAGTGGAGGCTGAGGATCTGGGAGTTTATTACTGCTTTCAAGGTTCACATGTTCCGTGG
101  R  V  E  A  E  D  L  G  V  Y  Y  C  F  Q  G  S  H  V  P  W

361 ACGTTCGGTGGAGGCACCAAGCTGGAAATCAAA
121  T  F  G  G  G  T  K  L  E  I  K
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SEQ ID NO: 24 (PRT) and 32 (DNA) (Mouse 9F9 heavy chain V region):

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  1 ATGCTGTTGGGGCTGAAGTGGGTTTTCTTTGTTGTTTTTTATCAAGGTGTGCATTGTGAG
  1  M  L  L  G  L  K  W  V  F  F  V  V  F  Y  Q  G  V  H  C  E

 61 GTGCAGCTTGTTGAGACTGGTGGAGGATTGGTGCAGCCTAAAGGGTCATTGAAACTCTCA
 21  V  Q  L  V  E  T  G  G  G  L  V  Q  P  K  G  S  L  K  L  S

121 TGTGCAGCCTCTGGATTCACCTTCAATACCAATGCCATGAACTGGGTCCGCCAGGCTCCA
 41  C  A  A  S  G  F  T  F  N  T  N  A  M  N  W  V  R  Q  A  P

181 GGAAAGGGTTTGGAATGGGTTGCTCGCATAAGAAGTAAAAGTAATAATTATGCAACATAT
 61  G  K  G  L  E  W  V  A  R  I  R  S  K  S  N  N  Y  A  T  Y

241 TATGCCGATTCAGTGAAAGACAGGTTCACCATCTCCAGAGATGATACACAAAGCATGATC
 81  Y  A  D  S  V  K  D  R  F  T  I  S  R  D  D  T  Q  S  M  I

301 TATCTGCAAATGAACAACTTGAAAACTGAGGACACAGGCATGTATTACTGTGTGAGAGGG
101  Y  L  Q  M  N  N  L  K  T  E  D  T  G  M  Y  Y  C  V  R  G

361 GGAAGCTACTGGTACTTCGATGTCTGGGGCGCAGGGACCACGGTCACCGTCTCCTCA
121  G  S  Y  W  Y  F  D  V  W  G  A  G  T  T  V  T  V  S  S
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As an antibody's antigen-binding specificity is determined by its light and heavy chain CDRs, the above-described CDRs can be used to generate antibody derivatives that retain the antigen-binding specificity. Examples of antibody derivatives include chimeric

antibodies, humanized antibodies, and their functional equivalents. Shown below are the light chain V region (SEQ ID NO: 25) and heavy chain V region (SEQ ID NO: 26) of a humanized 15A7 antibody, which include SEQ ID NOs: 1-3 and SEQ ID NOs: 4-6, respectively:

SEQ ID NO: 25 (humanized 15A7 light chain V region):

DIQMTQSPSSLSASVGDRVTITCRSSQSIVHNDGNTYFEWYQQKPGKAPKLLIYKVSNRFSGVPSRFSGSG
SGTHFTLTISSLQPEDFATYYCFQGSYVPLTFGQGTKVEIK

SEQ ID NO: 26 (humanized 15A7 heavy chain V region):

EVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAYINGGSSTIFYANAVKGRFTIS
RDNAKNTLYLQMNSLRAEDTAVYYCARYASYGGGAMDYWGQGTLVTVSS

This invention also features an isolated nucleic acid having a sequence that encodes one of the above-described immunoglobulin chains. The term "antibody" or "immunoglobulin chain" refers to an isolated polypeptide, i.e., a polypeptide that has been substantially separated from other proteins, lipids, and nucleic acids with which it is naturally associated. The polypeptide can constitute at least 50, 70, or 95% by dry weight of the purified preparation. An "isolated nucleic acid" refers to a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid. The term therefore covers, for example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule but is not flanked by both of the coding sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a CDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. The nucleic acid of this invention can be used to express a polypeptide of this invention. For this purpose, one can operatively link the nucleic acid to suitable regulatory sequences to generate an expression vector.

A vector refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked, and also capable of autonomous replication or integration into a host DNA. Examples include a plasmid, cosmid, and viral vector. A vector of this invention includes a nucleic acid in a form suitable for expression of the nucleic acid in a host cell. Preferably, the vector includes one or more regulatory sequences operatively linked to the nucleic acid sequence to be expressed. Examples of a regulatory sequence include promoters, enhancers, and other expression control elements (e.g., polyadenylation signals). Regulatory sequences also include those that direct constitutive expression of a nucleotide sequence, as well as tissue-specific regulatory and/or inducible sequences. The design of such an expression vector is based on considerations including the choice of the host cell to be transformed and the desired expression level. An expression vector can be introduced into host cells to produce a polypeptide of this invention. This invention also includes a host cell that contains the above-described nucleic acid. A host cell refers to a cell containing an exogenous coding sequence or non-coding sequence. An exogenous sequence can be introduced into a cell by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation. Suitable host cells include bacterial cells (e.g., E. coli, Bacillus subtilis, and Salmonella typhimurium), yeast cells (e.g., Saccharomyces cerevisiae and Schizosaccharomyces pombe), plant cells (e.g., Nicotiana tabacum and Gossypium hirsutum), and mammalian cells (e.g., murine hybridoma cells, CHO cells, and 3T3 fibroblasts).

To produce an immunoglobulin chain of this invention, one can place a host cell in a culture under conditions permitting expression of a polypeptide encoded by a nucleic acid described above, and isolate the polypeptide from the culture. Alternatively, a nucleic acid of this invention can be transcribed and translated in vitro, for example, using T7 promoter regulatory sequences and T7 polymerase.

Within the scope of this invention is an antibody. It is formed by a first immunoglobulin chain and a second immunoglobulin chain, which contain, respectively, the light chain CDRs and heavy chain CDRs of the mouse 15A7, 43B6, or 9F9 antibody mentioned above. Preferably, this antibody is formed by the light and heavy chains of 15A7.

Also within the scope of this invention is another antibody that (i) binds specifically to P-Selectin Glycoprotein Ligand 1 without interfering with binding between P-Selectin Glycoprotein Ligand 1 and P-Selectin and, (ii), upon binding to P-Selectin Glycoprotein Ligand 1 on an activated T cell, induces the death of the T cell. In one embodiment, this antibody binds specifically to human P-Selectin Glycoprotein Ligand 1.

Further within the scope of this invention is still another antibody that binds specifically to amino acid residues 115-126 of mature human P-Selectin Glycoprotein Ligand 1. Preferably, the antibody binds specifically to amino acid residues 117-123. More preferably, it binds specifically to amino acid residues 119-121, a consensus sequence among all tested epitopes. Indeed, mutation of one or more of these three amino acid residues abolishes antibody binding. In one example, this antibody, upon binding to P-Selectin Glycoprotein Ligand 1 on an activated T cell, induces the death of the activated T cell.

In one embodiment, one of the two antibodies mentioned immediately above is formed by a light chain and a heavy chain that contain, respectively, SEQ ID NOs: 1-3 and SEQ ID NOs: 4-6 (e.g., SEQ ID NOs: 19 and 20, or SEQ ID NOs: 25 and 26).

In a further aspect, the invention features a method of inducing death of an activated T cell. The method includes contacting one of the three antibodies described above with an activated T cell, in which binding of the antibody to the activated T cell induces cell death.

The invention also features a method of modulating a T cell-mediated immune response in a subject. The method includes (1) identifying a subject having or as being at risk of having a condition related to an excessive T cell-mediated immune response and (2) administering to the subject an effective amount of one of the three antibodies described above. An "excessive T cell-mediated immune response" refers to a response caused by an excessive level of activated T cells. An excessive level refers to (1) a level higher than a normal level, and (2) a level higher than desired in an individual, even though it is not greater than a normal level. Examples of the condition include an inflammatory disease, an autoimmune disease, an allergic disease, or a T cell cancer, as

well as the situation in which a subject has received or is contemplated to receive an allogeneic or xenogeneic transplant.

The details of one or more embodiments of the invention are set forth in the accompanying description below. Other features, objects, and advantages of the invention will be apparent from the detailed description.

DETAILED DESCRIPTION

This invention is based, at least in part, on an unexpected discovery that activated T cells can be induced to undergo apoptosis and be depleted by binding of antibodies or their derivatives to PSGL-1 on the activated cells. The antibodies and derivatives are useful for treating conditions associated with an excessive or unwanted T cell-mediated immune response or T cell proliferation.

Accordingly, the invention features polypeptides that contain immunoglobulin light or heavy chain CDRs of anti-PSGL-1 antibodies, as well as nucleic acids encoding them. Both the immunoglobulin chains and nucleic acids can be used to make the above-mentioned antibodies and derivatives.

An immunoglobulin chain of the invention can be obtained as a synthetic polypeptide or a recombinant polypeptide. To prepare a recombinant polypeptide, a nucleic acid encoding it can be linked to another nucleic acid encoding a fusion partner, e.g., Glutathione-S-Transferase (GST), 6x-His epitope tag, M13 Gene 3 protein, or an immunoglobulin heavy chain constant region. The resultant fusion nucleic acid can be introduced to a cell for protein expression. The fusion protein can be isolated from the host cell by methods well known in the art. The isolated fusion protein can be further treated, e.g., by enzymatic digestion, to remove the fusion partner and obtain the recombinant polypeptide of interest. Alternatively, an immunoglobulin chain can be obtained from a suitable host cell by activating endogenous expression of a nucleic acid encoding the chain.

The amino acid composition of an immunoglobulin chain of the invention may vary without disrupting the ability of forming an antibody capable of binding to PSGL-1. For example, such a variant can contain one or more conservative amino acid substitutions. A "conservative amino acid substitution" is one in which the amino acid

residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a polypeptide is preferably replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of a polypeptide of this invention, such as by saturation mutagenesis, and the resultant mutants can be screened for the ability of forming an antibody capable of binding to PSGL-1 to identify variants of this invention as described below in the examples. Thus, as an example, the term "an immunoglobulin chain containing SEQ ID NO: 19" covers immunoglobulin chains containing variants of SEQ ID NO: 19.

The above-described immunoglobulin chains and variants can be used to make an antibody of this invention or its derivatives. An "antibody" includes intact molecules as well as fragments thereof, such as Fab, $F(ab')_2$, Fv, scFv (single chain antibody), and dAb (domain antibody; Ward, et. al. (1989) Nature, 341, 544). A derivative of an antibody refers to a protein or a protein complex having a polypeptide variant of this invention. An antibody or derivative of this invention can be made by co-expressing corresponding light and heavy chain CDRs-containing polypeptides in a suitable host cell as described in the examples below. Alternatively, they can be made by methods known in the art of making monoclonal and polyclonal antibodies and fragments. See, e.g., Harlow and Lane, (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, New York.

To make an antibody of this invention, PSGL-1 or its antigenic fragment can be coupled to a carrier protein, such as KLH, mixed with an adjuvant, and injected into a host animal. Antibodies produced in that animal can then be purified by peptide affinity chromatography. Commonly employed host animals include rabbits, mice, guinea pigs,

and rats. Various adjuvants that can be used to increase the immunological response depend on the host species and include Freund's adjuvant (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Useful human adjuvants include BCG (bacille Calmette-Guerin) and Corynebacterium parvum.

Polyclonal antibodies, heterogeneous populations of antibody molecules, are present in the sera of the immunized subjects. Monoclonal antibodies, homogeneous populations of antibodies to a particular antigen, can be prepared using standard hybridoma technology. See, e.g., Kohler et al. (1975) Nature 256, 495; Kohler et al. (1976) Eur. J. Immunol. 6, 511; Kohler et al. (1976) Eur. J. Immunol. 6, 292; and Hammerling et al. (1981) Monoclonal Antibodies and T Cell Hybridomas, Elsevier, N.Y. In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described in U.S. Patent No. 4,376,110; the human B-cell hybridoma technique (Kosbor et al. (1983) Immunol Today 4, 72; Cole et al. (1983) Proc. Natl. Acad. Sci. USA 80, 2026) and the EBV-hybridoma technique (Cole et al. (1983) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD, and any subclass thereof. The hybridoma producing the monoclonal antibodies of the invention may be cultivated in vitro or in vivo. The ability to produce high titers of monoclonal antibodies in vivo makes it a particularly useful method of production.

In addition, techniques developed for the production of "chimeric antibodies" can be used. See, e.g., Morrison et al. (1984) Proc. Natl. Acad. Sci. USA 81, 6851; Neuberger et al. (1984) Nature 312, 604; and Takeda et al. (1984) Nature 314, 452. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Alternatively, techniques described for the production of single chain antibodies (U.S. Patent Nos. 4,946,778 and 4,704,692) can be adapted to produce a phage library of single chain Fv antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the

Fv region via an amino acid bridge. Moreover, antibody fragments can be generated by known techniques. For example, such fragments include, but are not limited to, $F(ab')_2$ fragments that can be produced by pepsin digestion of an antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of $F(ab')_2$ fragments. Antibodies can also be humanized by methods described in the examples below or known in the art. For example, monoclonal antibodies with a desired binding specificity can be commercially humanized (Scotgene, Scotland; and Oxford Molecular, Palo Alto, Calif.). Fully humanized antibodies, such as those expressed in transgenic animals are within the scope of the invention (see, e.g., Green et al. (1994) Nature Genetics 7, 13; and U.S. Patent Nos. 5,545,806 and 5,569,825).

Also within the scope of this invention is a method of inducing death of activated T cells, e.g., by contacting activated T cells with an antibody of the invention in vitro, and by administering to a subject in need thereof an effective amount of the antibody. Subjects to be treated can be identified as having or being at risk for having a condition related to an excessive or unwanted T cell-mediated immune response, e.g., patients suffering from autoimmune diseases, transplant rejection, allergic diseases, or T cell-derived cancers. This method can be performed alone or in conjunction with other drugs or therapy.

The term "treating" refers to administration of a composition to a subject with the purpose to cure, alleviate, relieve, remedy, prevent, or ameliorate a disorder, the symptom of the disorder, the disease state secondary to the disorder, or the predisposition toward the disorder. An "effective amount" is an amount of the composition that is capable of producing a medically desirable result in a treated subject.

Exemplary diseases to be treated include diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, and psoriatic arthritis), multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythematosus, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjogren's Syndrome, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, type I diabetes, inflammatory bowel diseases, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum

leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves' disease, sarcoidosis, primary biliary cirrhosis, uveitis posterior, interstitial lung fibrosis, graft-versus-host disease, cases of transplantation (including transplantation using allogeneic or xenogeneic tissues) such as bone marrow transplantation, liver transplantation, or the transplantation of any organ or tissue, allergies such as atopic allergy, AIDS, and T cell neoplasms such as leukemias or lymphomas.

In one in vivo approach, a therapeutic composition (e.g., a composition containing an antibody of the invention) is administered to the subject. Generally, the antibody is suspended in a pharmaceutically-acceptable carrier (e.g., physiological saline) and administered orally or by intravenous infusion, or injected or implanted subcutaneously, intramuscularly, intrathecally, intraperitoneally, intrarectally, intravaginally, intranasally, intragastrically, intratracheally, or intrapulmonarily.

The dosage required depends on the choice of the route of administration; the nature of the formulation; the nature of the subject's illness; the subject's size, weight, surface area, age, and sex; other drugs being administered; and the judgment of the attending physician. Suitable dosages are in the range of 0.01-100.0 mg/kg. Variations in the needed dosage are to be expected in view of the variety of compositions available and the different efficiencies of various routes of administration. For example, oral administration would be expected to require higher dosages than administration by intravenous injection. Variations in these dosage levels can be adjusted using standard empirical routines for optimization as is well understood in the art. Encapsulation of the composition in a suitable delivery vehicle (e.g., polymeric microparticles or implantable devices) may increase the efficiency of delivery, particularly for oral delivery.

Also within the scope of this invention is a pharmaceutical composition that contains a pharmaceutically-acceptable carrier and an effective amount of an antibody of the invention. The pharmaceutical composition can be used to treat diseases described above. The pharmaceutically-acceptable carrier includes a solvent, a dispersion medium,

a coating, an antibacterial and antifungal agent, and an isotonic and absorption delaying agent.

The pharmaceutical composition of the invention can be formulated into dosage forms for different administration routes utilizing conventional methods. For example, it can be formulated in a capsule, a gel seal, or a tablet for oral administration. Capsules can contain any standard pharmaceutically-acceptable materials such as gelatin or cellulose. Tablets can be formulated in accordance with conventional procedures by compressing mixtures of the composition with a solid carrier and a lubricant. Examples of solid carriers include starch and sugar bentonite. The composition can also be administered in a form of a hard shell tablet or a capsule containing a binder, e.g., lactose or mannitol, a conventional filler, and a tableting agent. The pharmaceutical composition can be administered via the parenteral route. Examples of parenteral dosage forms include aqueous solutions, isotonic saline or 5% glucose of the active agent, or other well-known pharmaceutically-acceptable excipient. Cyclodextrins, or other solubilizing agents well known to those familiar with the art, can be utilized as pharmaceutical excipients for delivery of the therapeutic agent.

The efficacy of a composition of this invention can be evaluated both in vitro and in vivo. See, e.g., the examples below. Briefly, the composition can be tested for its ability to induce death of activated T cells in vitro. For in vivo studies, the composition can be injected into an animal (e.g., a mouse model) and its therapeutic effects are then accessed. Based on the results, an appropriate dosage range and administration route can be determined.

The specific examples below are to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. Without further elaboration, it is believed that one skilled in the art can, based on the description herein, utilize the present invention to its fullest extent. All publications recited herein are hereby incorporated by reference in their entirety.

EXAMPLE 1: Mouse Monoclonal Antibodies 15A7, 43B6, and 9F9

Generation of anti-PSGL-1 antibodies

Standard techniques were used to generate mouse monoclonal antibodies that specifically bound to human PSGL-1 (hCD162). More specifically, mice were immunized with membrane fraction of PHA-activated human T cells and sacrificed to generate hybridoma cell lines. Supernatants from resultant hybridoma cell lines were screened for binding to CHO cells that stably expressed hCD162. Those lines producing antibodies that bound to hCD162-expressing CHO cells, but not the parental CHO cells, were identified, subcloned, and further analyzed as described below.

Among the lines identified were m152-15A7, m166-43B6, and m128-9F9. They produced IgGl antibodies 15A7, 43B6, and 9F9, respectively. Immunoblotting assay showed that these three antibodies pulled down from lysate of activated T-cells a protein that could be detected by anti-hCD162 antibody (kpl-1, PharMingen, San Diego, CA).

The just-described three antibodies were tested for their abilities to induce apoptosis of activated T cells. Culture supernatants containing monoclonal antibodies secreted by the three hybridoma cell lines were respectively incubated with either non-activated human T cells (Day 0) or in vitro activated human T cells (Day 7) for 6 hours. The cells were then stained with annexin V and subjected to FACS analysis. CD3-positive cells were gated to ensure counting of either in vitro activated human T cells or resting human T cells. The apoptotic cells were annexin V staining-positive. Table 1 summarizes the percentage of apoptotic T cells among all of the T cells scanned.

Table 1 Percentage of apoptotic T cells

	Untreated	**Anti-myc**	**m128-9F9**	**Untreated**	**Anti-myc**	**m152-15A7**	**M166-43136**
Day 0	4.17	6.67	5.82	18.18	15.52	5.23	6.57
Day 7	12.63	13.36	28.71	24.18	23.08	51.66	49.44

These result indicates that mouse 15A7, 43B6, and 9F9 antibodies (1) are hCD162-specific and (2) can bind to human activated T cells and induce apoptosis of activated T cells, but not resting human T cells.

Apoptosis assay was also conducted on PHA-activated human peripheral blood mononuclear cells (PBMC). It was found that the antibodies only induced apoptosis in activated T cells, but not in resting T cells, B cells, or in neutrophils.

It is known that T cell-depleting antibodies, such as anti-CD3, are able to induce production of soluble factors. Therapy using such antibodies usually results in a deleterious cytokine syndrome. To test if anti-PSGL-1 antibody also caused cytokine-associated side effects, freshly isolated human PBMC were cultured with 15A7 for 24, 48, or 72 hours. The levels of cytokines in the supernatant were then determined. Considerable amounts of IL-2, TNF-α, and IFN-γ were produced in PHA-activated PBMC (positive control), while levels of these cytokines from 15A7-treated cells were not detectable. These results supported that anti-PSGL-1 has no or little effect on resting peripheral blood cells, in both aspects of apoptotic induction and cell activation.

Since the above-described antibodies selectively induce apoptosis of activated T cells without causing adverse effects on resting T or other immune cells, administration of them to a subject is unlikely to result in lymphopenia or broad immunodeficiency like anti-CD3 or immunosuppressant does.

Epitope mapping of anti-CD162 antibodies

To map the binding epitopes of mouse 15A7, 43B6, and 9179 on human CD162, a series of fusion proteins covering various regions of human CD 162 were expressed and purified. Interactions between the fusion proteins and these monoclonal antibodies were examined by Sandwich enzyme-linked immunosorbent assay (ELISA).

Briefly, fragments covering various regions of human CD162 gene were expressed as fusion proteins with human immunoglobulin gamma 1 heavy chain constant region in E. *coli*. cDNA encoding the human immunoglobulin gamma 1 heavy chain constant region was amplified by PCR with primers having a *Bgl*II site and *a BamH*I site. The PCR product was cut by *Bgl*II and *BamHI,* and subcloned into a pET-32a vector (Novagen) that had been digested by the same enzymes. Then, cDNAs encoding various regions of hCD162 were amplified by PCR with primers having an *Nde*I site at the 5' end and a *Bgl*II site at the 3' end. The PCR products were cut by the corresponding enzymes and in frame fused to the sequence encoding the human immunoglobulin gamma 1 heavy

chain constant region in the pET-32a vector. Primers used in each construction are listed in Table 2, and the sequences of the primers are listed in Table 3.

Table 2. Names of primers used in each experiment.

For amplifying sequences encoding:	Forward primer	Reverse primer
E. coli expressed hCD162 fragments		
42-119	AB1001	AB1005
42-80	AB1001	AB1008
61-99	AB1003	AB1009
81-119	Ab1004	AB1005
42-70	AB1001	AB1007
42-60	AB1001	AB1006
50-80	AB1002	AB1008
50-70	AB1002	Ab1007
42-319	AB1001	Ab1010
115-126	AB1022	AB1023
115-126EtoR	AB1024	AB1025
V region of cDNAs		
light chain	AB1058	AB1059
heavy chain	AB1058	AB1060
Mammalian expressed hCD162 fragments		
1-119	AB1011	AB1013
1-319	AB1011	AB1012
110-319	AB1058	AB1059
94-148	AB1020	AB1021
119-222	AB1018	AB1019
174-269	AB1016	AB1017
214-317	AB1014	AB1015
Chimeric chains		
15A7 light chain	AB1030	AB1031
15A7 heavy chain	AB1032	AB1033
9F9 light chain	AB1026	A131027
9F9 heavy chain	AB1028	AB1029
43B6 light chain	AB1034	AB1035
43B6 heavy chain	AB1036	AB1037
Humanized chains		
15A7 light chain	AB1048	AB1057
15A7 light chain 1st pair	AD1049	AB1050
15A7 light chain 2nd pair	AB1051	AB1052
15A7 light chain 3rd pair	AB1053	AB1054
15A7 light chain 4th pair	AB1055	Ab1056
15A7 heavy chain	AB1038	AB1047
15A7 heavy chain 1st pair	AB1039	AB1040
15A7 heavy chain 2nd pair	AB1041	AB1042

15A7 heavy chain 3rd pair	AB1043	AB1044
15A7 heavy chain 4th pair	AB1045	AB1046

Table 3. Primer sequences.

Name	Sequence	NO:
AB1001	cccgggacCATATGcaggccaccgaatatgagtacc	39
AB1002	tatgagCATATGgattatgatttcctgccagaaacgg	40
AB1003	aaacggagCATATGgaaatgctgaggaacagcactgacacc	41
AB1004	aacccctCATATGaccactgtggagcctgctgcaaggcg	42
AB1005	gtggtcAGATCTtccatagctgctgaatccgtggacagg	43
AB1006	GTTCCTCAGATCTTCTGGAGGCTCCGTTTCTGGCAGG	44
AB1007	AGGCCCAAGATCTGGAGTGGTGTCAGTGCTGTTCCTC	45
AB1008	ggctccAGATCTgtagactcaggggttccaggccc	46
AB1009	gtggtcAGATCTgtgactgcccctcctgcatccaggcc	47
AB1010	GCCAGCAGATCTTGCTTCACAGAGATGTGGTCTGGGG	48
AB1011	cgcggatccatgcctctgcaactcctcctgttgc	49
AB1012	GCCAGCCTCGAGCTTCACAGAGATGTGGTCTGGGG	50
AB1013	GGTCTGctcgagCATAGCTGCTGAATCCGTGGACAGGTTC	51
AB1058	agacaggccaccgaagggaacctgtccacg	52
AB1059	cgtggacaggttcccttcggtggcctgtct	53
AB1014	ccgctcgagcgccaagattaggatggc	54
AB1015	cgggatccactcaaaccacagccatgg	55
AB1016	ccgctcgagtggtagtaggttccatgg	56
AB1017	cgggatcaactcaacccacaggcctg	57
AB1018	ctgtgcctcgagggctgtggtttgagtg	58
AB1019	cgggatccatggagatacagaccactcaac	59
AB1020	cgggatccgatgcaggaggggcagtcac	60
AB1021	ggccgtcactcgagttgtctgtgcctc	61
AB1022	TatgGATTCAGCAGCTATGGAGATACAGACCACTCAACCAgcA	62
AB1023	GATCTgcTGGTTGAGTGGTCTGTATCTCCATAGCTGCTGAATCCA	63
AB1024	TatgGATTCAGCAGCTATGCGGATACAGACCACTCAACCAgcA	64
AB1025	GATCTgcTGGTTGAGTGGTCTGTATCCGCATAGCTGCTGAATCCA	65
AB1026	CTAGTCTAGATGACCCAAACTCCACTCTCCC	66
AB1027	CTAGTCTAGAATTAGGAAAGTGCACTTAGCATCAGCCCGTTTGATTTCC	67
AB1028	TAACATtctagATGCTGTTGGGGCTGAAGTGGG	68
AB1029	GGATAGTCTAGAGGTTGTGAGGACTCACCTGAGGAGACGGTGACCGTGG	69
AB1030	CTAGTCTAGATGGAGACAGACACACTCCTGTTATGGG	70
AB1031	CTAGTCTAGAATTAGGAAAGTGCACTTTTTCCAGCTTGGTCCCCCCTCC	71
AB1032	CTAGTCTAGATGGACTCCAGGCTCAATTTAGTTTTCC	72
AB1033	CTAGTCTAGAGGTTGTGAGGACTCACCTGAGGAGACGGTGACTGAGGttcc	73
AB1034	CTAGTCTAGATGGATTTTCTGGTGCAGATTTTCAGC	74
AB1035	CTAGTCTAGAATTAGGAAAGTGCACTTAGCATCAGCCCGTTTCAGCTCC	75
AB1036	CTAGTCTAGATGGAATGGAGCTGGGTCTTTCTC	76
AB1037	CTAGTCTAGAGGTTGTGAGGACTCACCAGCTTCCAGTGGATAGACTGATGG	77
AB1038	TCTATCTAGATGAACTTCGGGTCCAGCTTGATTTTCCTTGTCCTTGTTTTAAAAGGTGTCCAGTG	78
AB1039	CCTTGTTTTAAAAGGTGTCCAGTGTGAAGTGCAACTGGTGGAGTCTGGGGGAGGCTTAGTGCAGCCTGG	79
AB1040	CTGAAAGTGAATCCAGAGGCTGCACAGGAGAGTCTCAAGCTTCCTCCAGGCTGCACTAAGCCTCC	80
AB1041	GCCTCTGGATTCACTTTCAGTAGCTTTGGAATGCACTGGGTTCGCCAGGCTCCAGGGAAGGGACTCGAG	81

AB1042	GCATAGAAGATGGTACTACTGCCACCATTAATGTATGCGACCCACTCGAGTCCCTTCCCTGGAGCC	82
AB1043	GTAGTACCATCTTCTATGCAAACGCAGTGAAGGGCCGATTCACCATCTCCAGAGATAATGCC	83
AB1044	CCTCAGCCCTCAGAGAATTCATTTGCAGGTACAGGGTGTTCTTGGCATTATCTCTGGAGATGG	84
AB1045	GAATTCTCTGAGGGCTGAGGACACGGCCGTGTATTACTGTGCAAGATATGCTAGTTACGGAGG	85
AB1046	CTGTGACCAGGGTGCCTTGGCCCCAATAGTCCATAGCACCCCCTCCGTAACTAGCATATC	86
AB1047	ACCCTCTAGAGGTTGTGAGGACTCACCTGAGGAGACTGTGACCAGGGTGCCTTGGCC	87
AB1048	TCTATCTAGATGGAGACAGACACAATCCTGCTATGGGTGCTGCTGCTCTGGGTTCCAGGC	88
AB1049	GCTGCTCTGGGTTCCAGGCTCCACTGGTGACATTCAGATGACCCAATCTCCGAGCTCTTTG	89
AB1050	GATCTGCAGGTGATAGTGACCCTATCCCCTACAGACGCAGACAAAGAGCTCGGAGATTGG	90
AB1051	CACTATCACCTGCAGATCTAGTCAGAGCATTGTACATAATGATGGAAACACCTATTTTGAATG	91
AB1052	GATGAGAAGCTTGGGTGCCTTTCCTGGTTTCTGTTGGTACCATTCAAAATAGGTGTTTC	92
AB1053	GCACCCAAGCTTCTCATCTATAAAGTTTCCAATCGATTTTCTGGTGTCCCATCCAGGTTTAGTGGC	93
AB1054	GCAGAGAAGAGATGGTGAGGGTGAAGTGTGTCCCAGACCCACTGCCACTAAACCTGGATGG	94
AB1055	CTCACCATCTCTTCTCTGCAGCCGGAGGATTTCGCAACCTATTACTGTTTTCAAG	95
AB1056	CCTTGGTGCCTTGACCGAACGTGAGAGGAACATATGAACCTTGAAAACAGTAATAGG	96
AB1057	ACCCTCTAGAATTAGGAAAGTGCACTTACGTTTGATTTCCACCTTGGTGCCTTGACCG	97
AB1058	TATATCTAGAATTCCCCCCCCCCCCCCCCC	98
AB1059	TATAGAGCTCAAGCTTGGATGGTGGGAAGATGGATACAGTTGGTGC	99
AB1060	TATAGAGCTCAAGCTTCCAGTGGATAGAC(C/A/T)GATGGGG(C/G)TGT(C/T)GTTTTGGC	100

The above-described expression constructs were transformed into *Escherichia coli* strain BL21 (DE3). The transformed cells were harvested after 6 hours of IPTG (2 mM) induction and resuspended in PBS. After the cells were sonicated and spun down at 14,000g for 10 minutes, the resultant supernatants were collected for purification of the fusion proteins. More specifically, the supernatants were first incubated with protein G or protein A beads for 3 hours at 4°C. The beads were then spun down at 3,000g and washed with washing buffer I (0.05% Triton X-100, 50 mM Tris-HCl, pH 8.5, 400 mM NaCl, 1mM $CaCl_2$ and 1mg/ml OVA) and washing buffer II (0.05% Triton X-100, 50 mM Tris-HCl, pH 8.5 and 150 mM of NaCl) for 5 times each. Bound proteins were then eluted with an elution buffer containing 0.1M of glycine-HCl, pH 2.7 and neutralized with 1 M Tris-HCl, pH 8.6. All purified fusion proteins were quantified by Bio-Rad protein assay (Bio-Rad Laboratories, Cat. No. 500-0006) and verified by SDSPAGE.

A sandwich ELISA was conducted to study the interaction between the hCD162 fragments and each of 15A7, 9F9, and 43B6. 96-well microtiter plates were coated with goat anti-human IgG (Southern Biotechnology, Cat. No. 2040-01) antibody (2 μg/ml,50 μl/well) overnight at 4°C. Plates were blocked by incubation with 0.25% of BSA in PBS (150 μl/well) for 1 hour at 37°C. The blocked plates were then incubated with

fusion proteins containing various fragments of human CD162 (2 μg/ml) for 2 hours at room temperature. After washing 4 times with PBS containing 0.05% of Tween 20 (PBST), the plates were incubated with testing antibodies (2 μg/ml) for 1.5 hours at room temperature. After incubation, the plates were washed 4 times with PBST. 50 μl of 1 to 3000 diluted goat anti-mouse IgG conjugated with alkaline phosphatase (Southern Biotechnology, Cat. No. 1031-04) was then added to each well and the plates were incubated for 1 hour at 37°C. Enzyme reaction was carried out by adding 50 μl of an alkaline phosphatase substrate solution (1 alkaline phosphatase substrate tablet dissolved in 5 ml of substrate buffer containing 0.012 M of Na_2CO_3, 0.16 M of $NaHCO_3$ and 1 mM of $MgCl_2$ at pH 8.6), and absorbance at 405 nm was determined.

It was found that 43B6 and 9F9 were able to interact with all fusion proteins containing residues 50 to 60 of mature human CD162, indicating that epitopes of 43B6 and 9179 were located between residues 50-60. Unlike 9179 and 43136, 15A7 only bound to the fusion protein covering residues 42 to 319, but not the fusion protein covering residues 42-119, indicating that the epitope of 15A7 was located between residues 119 to 319. The location of the epitope of 15A7 was then narrowed down to between residues 115 to 126. Change of one amino acid at position 120 (Glu →Arg) diminished interaction between 15A7 and the fusion protein, indicating that the primary contacting domain of 15A7 on human CD 162 is located at or adjacent to position 120, and the residue Glu is essential for the interaction.

Fusion proteins covering various human CD 162 regions were also expressed in mammalian cells and were tested for their interaction with 15A7. Fragments covering these regions were expressed as fusion proteins with human immunoglobulin gamma 1 heavy chain constant region in mammalian cells. First, the cDNA encoding human immunoglobulin gamma 1 heavy chain constant region was inserted into a pcDNA3 vector (Invitrogen). Second, cDNAs encoding various regions of hCD162 were amplified by PCR with primers introducing *a BamHI* site at the 5' end and an *Xho*I site at the 3' end. These PCR products were cut by the corresponding enzymes and subcloned into the human immunoglobulin gamma 1 heavy chain constant region-containing pcDNA3 vector. The name and sequence for each primer are listed in Tables 2 and 3 above.

The just-described mammalian expression vectors were transiently transfected into COS-7 cells by Lipofectamine 2000 (Invitrogen, Cat. No. 11668-027) following manufacturer's guide. The transfected cells were grown in ultra low-Ig medium (Invitrogen, Cat. No. 16250-078). The expressed proteins were purified and subjected to Sandwich ELISA in the same manner described above.

The ELISA results show that only the fusion proteins containing residues 94 to 148 were able to interact with 15A7. These results are consistent with the idea that the epitope of 15A7 is located between residues 115 to 126.

All of the above results indicate that the epitopes of 9F9, 43B6, and 15A7 are protein-dependent, instead of carbohydrate modification-dependent, since all three antibodies bind bacterially expressed fusion proteins. They also indicate that, although 15A7, 9F9, and 43B6 show similar properties in term of binding specificity and function of inducing apoptosis in activated T cells, they function through different domains of human CD 162 and behave differently.

EXAMPLE 2: Chimeric Antibodies 15A7, 43B6, and 9F9

Cloning of light and heavy chain variable regions of anti-CD162 antibodies

cDNAs encoding the light and heavy chain variable regions (V_L and V_H) of antibodies 15A7, 43B6, and 9F9 were amplified by an anchored PCR method. The 3' primers hybridized to the C regions and the 5' primers hybridized to G tails attached to the cDNA using terminal deoxytransferase. The PCR fragments were cloned into a pCRII vector (Invitrogen). Several independent clones for each chain were sequenced and compared. A sequence represented by the majority of the independent clones was picked. The translated amino acid sequence was then analyzed to confirm that the chosen sequence possessed the characteristics of typical mouse light or heavy chain V region, and belonged to a specific subtype. The complementarity determining regions (CDRs) were then identified by comparing the translated amino acid sequences with consensus sequence of each subtype. The name and sequence for each primer used are listed in Tables 2 and 3 above. The deduced amino acid sequences of the light and heavy chain V regions of 15A7, 43B6, and 9F9 (SEQ ID NOs: 19-24) are shown in Summary.

Chimeric Antibodies

To generate vectors for expressing chimeric antibodies, cDNAs encoding the V_L and V_H regions of 15A7, 43B6, and 9F9 were amplified by PCR using primers to include the 5' signal peptide sequence and the 3' splice donor signal. The primers also introduced *Xba*I sites at both ends of the PCR products, which were then cut by *Xba*I enzyme and ligated into *Xba*I-digested pVK, pVgl, pVg2, or pVg4 vector. More specifically, the V_L region cDNAs of 15A7, 43136, and 9F9 were subcloned into the plasmid pVk. This plasmid contained a CMV promoter, and a sequence encoding the human light chain constant region. The V_H region cDNAs of 15A7, 43B6 and 9179 were subcloned into plasmids pVgl, pVg2, or pVg4. Each of the three plasmids had a CMV promoter. They also contained, respectively, the human heavy chain constant regions of IgG1, IgG2, and IgG4.

Each of the above-described light chain-encoding plasmids was co-transfected with a heavy chain-encoding plasmid into COS-7 cells. The supernatants of the transfected cells were collected. Chimeric antibodies in the supernatants were analyzed for the ability to bind to human CD 162 and to induce apoptosis of activated T cells.

It was found that all chimeric antibodies made from 15A7, 43B6, and 9F9 bound to Sp2/0 transfectants stably expressing human CD162, but not to parental Sp2/0 cells, indicating that they retained the human CD162-binding ability specificity. Furthermore, it was found that the chimeric antibodies induced apoptosis in T cells that had been activated for 7 days, indicating that they retained this function of their mouse counterparts as well.

Humanized Antibodies

Mouse 15A7 was used to make humanized antibodies by grafting its CDRs onto a human framework. To retain binding affinity and specificity, it is essential to conserve the V region conformation when grafting the CDRs onto the human framework. To select a proper framework donor, the amino acid sequences of mouse 15A7 light and heavy chain V regions were compared with those of 50 mouse antibodies that had been humanized.

It was found that a mouse antibody, mDREG-55, had high sequence homology to mouse 15A7 V region in both light and heavy chains. Listed below is a sequence alignment of mouse 15A7 against this mDREG-55 antibody (CDRs are highlighted):

```
Light chain alignment:
mDREG-55        DIVLTQSPASLSVSLGERASISCKASQSVDY-DGDSYMNWYQQKPGQPPKLLIYAASNLES
                DI++TQ+P SL VSLG++ASISC++SQS+ + DG++Y  WY QKPGQ PKLLIY  SN  S
m15A7           DILMTQTPLSLPVSLGDQASISCRSSQSIVHNDGNTYFEWYLQKPGQSPKLLIYKVSNRFS

mDREG-55        GIPARFSGSGSGTDFTLNIHPVEEEDAATYYCQQSNEDPWTFGGGTKLEIK          (SEQ ID NO: 33)
                G+P RFSGSGSGT FTLNI  VE ED    YYC Q + P TF GGTKLE+K
m15A7           GVPDRFSGSGSGTHFTLNISRVEAEDLGIYYCFQGSYVPLTFGAGTKLELK          (SEQ ID NO: 34)

Heavy chain alignment:
MDREG-55        EVKLVESGGGLVKPGGSLKLSCAASGFTFSTYAMSWVRQTPEKRLEWVASISTGGST-YYPDSVKG
                +V+LVESGGGLV+PGGS KLSCAASGFTFS++ M WVRQ PEK LEWVA I+ G ST +Y ++VKG
m15A7           DVQLVESGGGLVQPGGSRKLSCAASGFTFSSFGMHWVRQAPEKGLEWVAYINGGSSTIFYANAVKG

MDREG-55        RFTISRDNARNILYLQMSSLRSEDTAMYYCAR--DY-DGYFDYWGQGTTLTVSS       (SEQ ID NO: 35)
                RFTISRDN +N L+LQM+ LRSEDTA+YYC R   Y  G  DYWGQGT++TVSS
m15A7           RFTISRDNPKNTLFLQMTILRSEDTAIYYCGRYASYGGGAMDYWGQGTSVTVSS       (SEQ ID NO: 36)
```

Mouse DREG-55 is a monoclonal IgG1 antibody against L-selectin. The sequences of mouse 15A7 V_L and V_H regions were respectively 64.3% (framework only: 73.8%) and 70% (framework only: 81.6%) homologous to those of mouse DREG55. Humanized DREG-55 (HuDREG-55) had been constructed using framework sequences of V_L and V_H regions from a human antibody Gal. Therefore, to humanize mouse 15A7, the framework sequences of human Gal light and heavy chains were used to replace the counter parts of mouse 15A7.

The humanized 15A7 light and heavy variable regions were each assembled by 4 pairs of synthetic oligonucleotides (~ 80 bases in length). The oligonucleotides of each pair were overlapped by around 20 nucleotides. Nucleotide sequences were selected and synthesized to encode the protein sequences of the humanized variable regions including signal peptides. The assembling and amplification of the genes were conducted in four steps: (1) the four pairs of complementary oligonucleotides were annealed and extended with Klenow fragment in 4 separate reactions; (2) the resulting 4 dsDNA fragments were mixed pair wise, denatured, reannealed, and extended in two separate reactions; (3) the resulting two dsDNA fragments were mixed, denatured, reannealed, and extended to create the final full-length dsDNA; and (4) the resulting DNA was amplified by PCR

with primers to introduce an *Xba*I site at both ends. The PCR fragment was then cut by *Xba*I and inserted into the respective *Xba*I-digested pVK and pVg4 vectors. Then, at positions where the interactions between CDR and the framework were considered important, the Gal's residues were changed back into those of the mouse 15A7 (i.e., I62V and D74H). Listed below are alignments of mouse 15A7 and humanized 15A7 (Hul5A7) against mDREG-55, in which V62 and H74 are underlined.

Light chain alignment:

hDREG-55	DIQMTQSPSSLSASVGDRVTITCKASQSVDY-DGDSYMNWYQQKPGKAPKLLIYAASNLES	
mouse 15A7	DILMTQTPLSLPVSLGDQASISCRSSQSIVHNDGNTYFEWYLQKPGQSPKLLIYKVSNRFS	
Hu15A7	DIQMTQSPSSLSASVGDRVTITCRSSQSIVHNDGNTYFEWYQQKPGKAPKLLIYKVSNRFS	
hDREG-55	GIPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQSNEDPWTFGQGTKVEIK	(SEQ ID NO: 37)
m15A7	GVPDRFSGSGSGTHFTLNISRVEAEDLGIYYCFQGSYVPLTFGAGTKLELK	(SEQ ID NO: 34)
Hu15A7	GVPSRFSGSGSGTHFTLTISSLQPEDFATYYCFQGSYVPLTFGQGTKVEIK	(SEQ ID NO: 25)

Heavy chain alignment:

hDREG-55	EVQLVESGGGLVQPGGSLRLSCAASGFTFSTYAMSWVRQAPGKGLEWVASISTGGST-YYPDSVKG	
m15A7	DVQLVESGGGLVQPGGSRKLSCAASGFTFSSFGMHWVRQAPEKGLEWVAYINGGSSTIFYANAVKG	
Hu15A7	EVQLVESGGGLVQPGGSLRLSCAASGFTFSSFGMHWVRQAPGKGLEWVAYINGGSSTIFYANAVKG	
hDREG-55	RFTISRDNAKNTLYLQMNSLRAEDTAVYYCAR--DY-DGYFDYWGQGTLVTVSS	(SEQ ID NO: 38)
m15A7	RFTISRDNPKNTLFLQMTILRSEDTAIYYCGRYASYGGGAMDYWGQGTSVTVSS	(SEQ ID NO: 36)
Hu15A7	RFTISRDNAKNTLYLQMNSLRAEDTAVYYCARYASYGGGAMDYWGQGTLVTVSS	(SEQ ID NO: 26)

Plasmids thus obtained encoded humanized 15A7 heavy and light chains. These plasmids were then co-transfected into COS-7 cells. The exhausted supernatants from cultured cells were then collected. Humanized 15A7 in the supernatants was tested for its ability to bind to CHO transfectants stably expressing hCD162 and to induce apoptosis in the T cells activated for 7 days. The results show that it retains these abilities.

Preparation of Chimeric and Humanized Antibodies

Cells producing humanized and chimeric antibodies were generated. More specifically, Sp2/0 cells (Sp2/0-Agl4; ATCC CRL 1581) were stably transfected with the appropriate plasmids by electroporation using a Gene Pulser apparatus (Bio-Rad Laboratories) at 360 V and 25 μF capacitance according to the manufacturer's instructions. Before transfection, the plasmids were linearized by digesting with *BamH*I enzyme. All transfections were performed using 10^7 cells in PBS and 20 μg each of

plasmid DNA. The cells from each transfection were plated into two 96-well tissue culture plates. After 48 hours, a selective medium (DMEM 10% FBS/hypoxanthine/thymidine media supplement) and 1μg/ml mycophenolic acid was applied. Antibody-producing cells were screened and isolated by examining the presence of antibody in the culture supernatant by ELISA.

Isolated cells were cultured in serum-free or low-Ig medium, and the cultured supernatant was collected. Antibodies were purified by passage over a column of staphylococcal protein A-Sepharose CL-4B. After washing 5 times each with washing buffer I (0.05% Triton X-100, 50mM Tris-HCl, pH 8.5, 400 mM NaCl, 1 mM $CaC1_2$ and 1 mg/ml OVA) and washing buffer II (0.05% Triton X-100, 50 mM Tris-HCl, pH 8.5 and 150 mM of NaCl), the bound antibodies were eluted with an elution buffer containing 0.1 M of glycine-HCl, pH 2.7, and neutralized with 1 M Tris-HCl, pH 8.6.

Affinity measurements

Binding affinities of the above-described mouse, chimeric, and humanized 15A7 antibodies were determined by competitive binding.

Mouse 15A7 was biotinylated by an EZ-Link Sulfo-NHS-Biotin system (Pierce Biotechnology, Cat. No. 21217). Briefly, 0.5 mg (3.3×10^{-6} nmoles) of mouse 15A7 was dissolved in 187 μl of PBS and mixed with 6.8×10^{-5} nmoles of Sulfo-NHS-Biotin. The mixture was then incubated on ice for 2 hours before free biotins were removed by dialyzing at 4°C overnight against PBS. The Biotin-labeled mouse 15A7 thus obtained was stored at 4°C until use.

Sp2/0 transfectants stably expressing human CD162 were used as source of human CD 162 antigen. Biotin-labeled mouse 15A7 was used as tracer. Increasing amounts of competitor antibodies (mouse, chimeric, or humanized 15A7) were mixed with 35 ng of Biotin-labeled mouse 15A7 and incubated with 1×10^5 CD162-expressing Sp2/0 cells for 1.5 hours at 4°C with constant shaking. After washing, secondary antibody, Streptavidin-PE (Becton Dickinson Immunocytometry System Inc. Cat. No. 349023) was added to the mixture. After incubating for 45 minutes at 4°C, the cells were washed again, resuspended in 300 μl of PBS-1% of FBS, and subjected to FACS analysis.

It was found that the half-maximum competing concentration of mouse 15A7 was 3.72 μg/ml while those of chimeric and humanized 15A7 were around 5.71 μg/ml and 4.51 μg/ml, respectively. These results indicate that the affinities of mouse, chimeric, and humanized 15A7 are comparable. In other words, the binding affinity (Ka) for mouse 15A7 is $4.03 \times 10^7\ M^{-1}$ while those for chimeric and humanized 15A7 are $2.62 \times 10^7\ M^{-1}$ and $3.33 \times 10^7\ M^{-1}$, respectively.

Competition analysis

Competition analysis was conducted to study interaction among the above-described three mouse antibodies, PSGL-1, and P-selectin.

P-selectin is a major high-affinity ligand for PSGL-1 on most leukocytes. In order to investigate whether the three antibodies prevent binding of P-selectin to PSGL-1, binding of purified human P-selectin to activated T cells was measured in the presence of the three antibodies. KPL-1, known to block interaction of P-selectin and PSGL-1, was used as a positive control.

Human PBMC were activated with 1 % PHA for 2 days and maintained in IL-2-containing medium for 3 days. The cells were incubated with titrated 9F9, 15A7, 43B6, KPL-1 (an PSGL-1 antagonist), or a control antibody (9E10) for 30 minutes, followed by the addition of recombinant human P-selectin (1.25 μg/ml). Binding of P-selectin to activated T cells was measured by anti-P-selectin-FITC analyzed on FRCS.

Consistent with previous reports, KPL-1 almost completely abolished P-selectin's binding to activate T cells at a low concentration (0.31 μg/ml). 43B6 blocked binding of P-selectin to activated T cells as effectively as KPL-1 did, whereas a higher concentration of 9F9 was required to achieve the same effect. Indeed, 0.08 μg/ml KPL or 43B6 was needed to abolish 50% of the binding. In contrast, 5 μg/ml 9F9 was required. Moreover, 15A7 did not have any inhibitory effect on P-selectin binding even at 20 μg/ml. Surprisingly, it enhanced binding of P-selectin to PSGL-1. These results indicate that 15A7 and P-selectin bind to different motifs of PSGL-1 on activated T cells.

The fact that 15A7 did not compete with P-selectin for PSGL-1 indicates that in vivo administration of 15A7 is not supposed to affect innate immunity by interfering P-selectin-dependent recruitment of leukocytes.

It has been reported that PSGL-1 is expressed at low levels on platelets. The effects of 15A7 antibodies on platelets were examined. It was found that the antibodies did not enhance or inhibit aggregation of human platelets.

EXAMPLE 3: Hamster Monoclonal Antibody TAB4 against mouse PSGL-1

A monoclonal antibody against mouse PSGL-l, TAB4, was prepared in the manner similar to the method described in Example 1. It induced T cell apoptosis in vitro and depleted T cells in vivo. To determine if it interfered with binding between mouse PSGL-1 and mouse P-selectin, competition analysis was performed in the manner similar to the method described in Example 2. It was found that TAB4 did not inhibit mouse P-selectin binding to mouse PSGL-1 even at a concentration as high as 20 μg/ml.

EXAMPLE 4: Mouse Monoclonal Antibodies 4B7, 5C4, 12E7, 14B3, 17E5, and 18D12

Additional monoclonal antibodies against human PSGL-l, 4B7, 5C4, 12E7, 14B3, 17E5, and 18D12, were characterized. Upon binding to an activated T cell, they all induced death of the activated T cells. Competition analysis was conducted in the manner described in Example 2 to determine if they blocked interaction between PSGL-1 and P-selectin. It was found that these antibodies have little, if any, inhibitory effect on human P-selectin binding to human PSGL-1, even at the highest concentration tested (5 μg/ml).

OTHER EMBODIMENTS

All of the features disclosed in this specification may be combined in any combination. Each feature disclosed in this specification may be replaced by an alternative feature serving the same, equivalent, or similar purpose. Thus, unless expressly stated otherwise, each feature disclosed is only an example of a generic series of equivalent or similar features.

From the above description, one skilled in the art can easily ascertain the essential characteristics of the present invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, other embodiments are also within the scope of the invention.

CLAIMS:

1. An isolated immunoglobulin chain comprising three amino acid sequences selected from (i) SEQ ID NOs: 1-3; or (ii) SEQ ID Nos: 4-6.

2. The immunoglobulin chain of claim 1, wherein the immunoglobulin chain comprises an amino acid sequence selected from SEQ ID NOs: 19, 20, 25 or 26.

3. The immunoglobulin chain of claim 2, wherein the immunoglobulin chain comprises an amino acid sequence selected from SEQ 113 NOs: 25 or 26.

4. An isolated antibody, comprising a first immunoglobulin chain of claim 1 and a second immunoglobulin chain also of claim 1, wherein the first chain, which is a light chain, and the second chain, which is a heavy chain, comprise, respectively,

SEQ ID NOs: 1-3 and SEQ ID NOs: 4-6.

5. The antibody of claim 4, wherein the light chain and the heavy chain comprise, respectively, SEQ ID NOs: 19 and 20, or SEQ ID NOs: 25 and 26.

6. The antibody of claim 5, wherein the light chain and the heavy chain comprise, respectively, SEQ ID NOs: 25 and 26.

7. An isolated antibody that binds specifically to human P-Selectin Glycoprotein Ligand 1 without interfering with binding between P-Selectin Glycoprotein Ligand 1 and P-Selectin, wherein the antibody, upon binding to P-Selectin Glycoprotein Ligand T-cell, induces death of the activated T-cell and wherein the antibody includes a light chain and a heavy chain comprising, respectively, SEQ ID NOs: 1-3 and SEQ ID NOs: 4-6.

8. The antibody of claim 7, wherein the light chain and the heavy chain comprise, respectively, SEQ ID NOs: 25 and 26.

9. An isolated antibody that binds specifically to amino acid residues 115-126 of human P-Selectin Glycoprotein Ligand 1.

10. The antibody of claim 9, wherein the antibody binds specifically to amino acid residues 117-123 of human P-Selectin Glycoprotein Ligand 1.

11. The antibody of claim 10, wherein the antibody binds specifically to amino acid residues 119-121 of human P-Selectin Glycoprotein Ligand 1.

12. The antibody of claim 9, wherein the antibody includes a light chain and a heavy chain comprising, respectively, SEQ ID NOs: 1-3 and SEQ ID NOs: 4-6.

13. The antibody of claim 9, wherein the antibody, upon binding to P-Selectin Glycoprotein Ligand 1 on an activated T-cell, induces death of the activated T-cell.

14. The antibody of claim 13, wherein the antibody includes a light chain and a heavy chain comprising, respectively, SEQ ID NOs: 1-3 and SEQ ID NOs: 4-6.

15. A method of inducing death of an activated T cell, comprising contacting an antibody of any one of claims 4 to 14 with an activated T cell, wherein binding of the antibody to the activated T cell induces death of the activated T cell.

16. Use of an antibody of any one of claims 4 to 14 in the preparation of a medicament for modulating a T cell-mediated immune response in a subject, wherein the subject has or is at risk of having a condition related to an excessive T cell-mediated immune response.

17. The use of claim 16, wherein the condition is an inflammatory disease, an autoimmune disease, an allergic disease, or a T cell cancer.

18. The use of claim 16, wherein the condition is rejection of an allogeneic or xenogeneic transplant.

19. An isolated nucleic acid comprising a sequence encoding the immunoglobulin chain of claim 1 or claim 2.

20. A vector comprising the nucleic acid of claim 19.

21. An isolated host cell comprising a nucleic acid encoding SEQ ID NOs: 1-3, or SEQ ID NOs: 4-6.

22. An isolated host cell comprising a nucleic acid encoding SEQ ID NO: 19, 20, 25 or 26.

23. The host cell of claim 21 or claim 22, wherein the cell is bacterial cell, a yeast cell, a plant cell, an insect cell, or a mammalian cell.

24. The host cell of claim 23, wherein the mammalian cell is a hybridoma cell.

25. An antibody according to any one of claims 4 to 14, wherein the antibody is a chimeric antibody.

26. An antibody according to any one of claims 4 to 14, wherein the antibody is a humanized antibody.

27. An isolated antibody that binds specifically to human P-Selectin Glycoprotein Ligand 1 (PSOL-1), wherein the antibody comprises (1) a light chain comprising a variable region having the amino acid sequence of SEQ ID NO: 19 , and (ii) a heavy chain comprising a variable region having the amino acid sequence of SEQ ID NO: 20.