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(54) Title: TIE1-BINDING LIGANDS

(57) Abstract: Tiel is a receptor tyrosine kinase protein that includes a transmembrane domain. Tiel is present on endothelial cells. This disclosure described antibodies that bind to Tiel, including ones that inhibit endothelial cell activity.

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TIE1-BINDING LIGANDS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Application Serial No. 60/494,713, filed on August 12, 2003, the contents of which are hereby incorporated by reference in its entirety.

BACKGROUND

The oxygen and nutrients supplied by the blood vessels are crucial for tissue development and function. Indeed, the cardiovascular system is the first organ system to develop in embryos. During organogenesis and the development tissues or tumors, the proximity of the growing cells to the circulatory system is ensured by the coordinated growth of blood vessels and organ parenchyma. It may be possible to prevent or treat diseases by modulating blood vessel development or angiogenesis.

Blood vessels are composed of an inner layer of endothelial cells and an outer layer of pericytes or smooth muscle cells. The first tubular structures are formed by endothelial cells that subsequently recruit pericytes and smooth muscle cells to ensheath them. The *de novo* formation of blood vessels from a dispersed population of mesodermally derived endothelial precursor cells is termed vasculogenesis. This primitive network undergoes successive morphogenetic events including sprouting, splitting and remodeling to generate the hierarchical vascular network from large to branched small vessels. These successive morphogenetic events are collectively called angiogenesis. Previous studies have identified a number of endothelial cell specific receptor tyrosine kinases (RTKs) and their cognate ligands, which mediate the vasculogenic and angiogenic development of blood vessels. Members of the vascular endothelial growth factor (VEGF) family and their receptors function during the formation of the initial embryonic vascular plexus, whereas angiopoietins (Angs) and their receptor, Tie2, as well as ephrins and their Eph receptors are implicated in the subsequent remodeling processes. See, e.g., Jones et al. (2001) *Nature Reviews* 2:257 for a review of receptors involved in angiogenic and lymphangiogenic responses.

Tie1 and Tie2 are RTKs that are expressed almost exclusively in endothelial cells and hematopoietic precursor cells. These two receptors are required for the normal development of vascular structures during embryogenesis. The two Tie receptors form a RTK subfamily since, unlike other RKT family members, they include extracellular EGF-homology domains. See, e.g., Partanen (1992) *Mol Cell Biol* 12:1698 and WO 93/14124. Targeted disruption of the Tie1 gene in mice results in a lethal phenotype characterized by extensive hemorrhage and defective microvessel integrity. See, e.g., Puri et al. (1995) *EMBO J* 14:5884. Tie2 null embryos have defects in vascular remodeling and maturation, resulting from improper recruitment of periendothelial supporting cells. At least three ligands, designated the angiopoietins (Ang), have been identified for Tie2, while the ligands for Tie1 are still unknown. Mice lacking Ang1 show defects in vascular development, which are reminiscent of but slightly less severe than those of mice lacking Tie2. Binding of Ang1 induces tyrosine phosphorylation of Tie2 and activation of its signaling pathways, but Ang2 has been reported to antagonize these effects in endothelial cells. Accordingly, transgenic overexpression of Ang2 disrupts blood vessels formation in mouse embryos. So far, four angiopoietins and many related proteins have been discovered, although none of them appears to binds Tie1.

SUMMARY

In one aspect, the invention features a protein (e.g., an isolated protein) that includes a heavy chain immunoglobulin variable domain sequence and a light chain immunoglobulin variable domain sequence. The protein ligand binds to Tie1 ectodomain. For example, the protein binds with an affinity K_D of less than 10^{-8} M, $5 \cdot 10^{-9}$ M, 10^{-9} M, 10^{-10} M, 10^{-11} M, or 10^{-12} M.

In one embodiment, one or more of the CDRs of the heavy and/or light chain variable domain sequence are human, primate, non-rodent (e.g., non-mouse or non-rat), or synthetic. In one embodiment, one or more of the framework regions of the heavy and/or light chain variable domain sequence are human, primate, or non-rodent (e.g., non-mouse or non-rat).

In one embodiment, the heavy chain includes one or more of the following properties:

i) a HC CDR1 that includes an amino acid sequence as follows:

(AGSR)-Y-(GVK)-M-(GSVF), (SEQ ID NO:117)

(AGSIMRH)-Y-(GVMK)-M-(GSVMFH) (SEQ ID NO:118), or

(AGSIMRNH)-Y-(AGTVMKPQ)-M-(AGSTVMYWFKH) (SEQ ID NO:119);

ii) a HC CDR2 that includes an amino acid sequence as follows:

5 X-I-Y-P-S-G-G-X-T-X-Y-A-D-S-V-K-G (SEQ ID NO:120), wherein X is any amino acid,

(GSV)-I-(SY)-P-S-G-G-(WQ)-T-(GY) (SEQ ID NO:121),

(GSV)-I-(SY)-P-S-G-G-(WNQ)-T-(GY) (SEQ ID NO:160)

(GSV)-I-(SY)-P-S-G-G-(WQ)-T-(GY)-Y-A-D-S-V-K-G (SEQ ID NO:122), or

10 (GSVW)-I-(SY)-P-S-G-G-(AGVMYWPQH)-T-(AGSTLVMYFKH)
(SEQ ID NO:123);

iii) a HC CDR3 that includes an amino acid sequence as follows:

V-(four or five residues)-F-D-(I/Y) (SEQ ID NO:124),

G-Y-G-P-I-A-P-G-L-D-Y (SEQ ID NO:125),

15 (GV)-N-Y-Y-(GYD)-S-(SD)-G-Y-G-P-I-A-P-G-L-D-Y (SEQ ID NO:126),

(GVD)-(AGLN)-(LYR)-(GSTLYH)-(GYD)-(AGSYFP)-(SFD)-(AGYD)-(IY)-
(GFD)-(YDP)-(IP)-A-P-G-L-D-Y (SEQ ID NO:127), or

VNYYDSSGYGPIAPGLDY (SEQ ID NO:128).

In one embodiment, the light chain includes one or more of the following

20 properties:

i) a LC CDR1 that includes an amino acid sequence as follows:

R-A-S-Q-S-(IV)-S-(SR)-X1-Y-L-(AN) (SEQ ID NO:129),

R-A-S-Q-S-V-S-S-X-L (SEQ ID NO:130),

R-A-S-Q-S-(IV)-S-(SR)-(SY)-(LY)-(ALN) (SEQ ID NO:131), or

15 R-A-S-(REQ)-(GSTRN)-(IV)-(GSTIRN)-(STIRH)-X1-(SYWNH)-(LV)-(ASN)
(SEQ ID NO:132), wherein X1 can be serine or absent;

ii) a LC CDR2 that includes an amino acid sequence as follows:

X-A-S-X-R-A-T (SEQ ID NO:133), wherein X can be any amino acid,

(AGD)-A-S-(STN)-R-A-T (SEQ ID NO:134),

0 (AGD)-A-S-(STN)-(LR)-(AEQ)-(ST) (SEQ ID NO:135), or

(AGTKDEH)-A-S-(STN)-(LR)-(AVEQ)-(ST) (SEQ ID NO:136); and

iii) a LC CDR3 that includes an amino acid sequence as follows:

Q-Q-(SYFR)-(GSYN)-S-(STYW)-(RP)-(LWRH)-(TIY) (SEQ ID NO:161),

Q-Q-(SYFR)-(GSYN)-S-(STYW)-(RP)-(LWR)-(TIY)-T (SEQ ID NO:137),

(LQ)-Q-(SYFR)-(GSYN)-(SKN)-(STYW)-(RP)-(LWR)-(TIY)-T (SEQ ID NO:138),

5 Q-Q-(YR)-(GS)-S-(SW)-P-R-X1-T (SEQ ID NO:139), wherein X1 is any amino acid or absent,

(LQ)-(LQ)-(SYFRD)-(GSYN)-(STRKN)-(STYWF)-(RP)-(ILMWRH)-(TIY)-(TI) (SEQ ID NO:140), or

0 (LQ)-(LRQ)-(SYFRD)-(GSYN)-(ASTRKN)-(STYWF)-(SVRP)-(STILMWRH)-(TIY)-(STI) (SEQ ID NO:141).

In one embodiment, the light chain includes one or more of the following properties:

i) a LC CDR1 that includes an amino acid sequence as follows:

S-X-(ND)-(IV)-(AG)-X1-X2-X3 (SEQ ID NO:142), or

5 T-(GR)-(ST)-S-X5-(ND)-(IV)-(AG)-X1-X2-X3-Y-X4-S (SEQ ID NO:143), wherein X1 is any amino acid (e.g., G or R), X2 is any amino acid (e.g., Y or N), X3 is any amino acid (e.g., F, N, or K), X4 is any amino acid (e.g., aliphatic, e.g., V or A);

ii) a LC CDR2 that includes an amino acid sequence as follows:

(DE)-V-N-N-R-P-S (SEQ ID NO:144)

0 (DE)-(VD)-(STDN)-(YRDN)-R-P-S (SEQ ID NO:145);

iii) a LC CDR3 that includes an amino acid sequence as follows:

(SQ)-S-(SY)-(ASID)-(GSR)-(ST)-(STRN)-(STYR)-(ATLY)-(SVWQ) (SEQ ID NO:146)

In one embodiment, the HC CDR2 includes an amino acid sequence as follows:

5 (GSVW)-I-(SY)-P-SG-G-(AGVMYWPQH)-T-(AGSTLVMYFKH)-Y-(AT)-D-S-V-K-G (SEQ ID NO:147) or (GSV)-I-(SY)-P-SG-G-(WQ)-T-(GY)-Y-(AT)-D-S-V-K-G (SEQ ID NO:148).

In one embodiment, the protein includes HC CDR1 and HC CDR2 sequences that are related to the corresponding CDR sequences of p-F3 and E3. For example, the protein includes the sequence MYGM (SEQ ID NO:149), at a position corresponding to HC CDR1. The sequence can be followed by a small amino acid, e.g., glycine, alanine, valine, or serine. In another example, the protein the sequence VISPSGGX₁TX₂YADSAVKG

(SEQ ID NO:150), at a position corresponding to HC CDR2. For example, X₁ can be a hydrophilic amino acid, e.g., glutamine or asparagine. For example, X₂ can be a small amino acid, e.g., glycine, alanine, valine, or serine.

In one embodiment, two or three of the CDRs of the HC variable domain sequence match motifs that also match a HC variable domain of an antibody described herein. Similarly, in one embodiment, two or three of the CDRs of the LC variable domain sequence match motifs that also match a LC variable domain of an antibody described herein. In still another embodiment, the matched motifs for the CDRs are based on a HC and a LC that are paired in an antibody described herein.

In one embodiment, the H1 and H2 hypervariable loops have the same canonical structure as an antibody described herein. In one embodiment, the L1 and L2 hypervariable loops have the same canonical structure as an antibody described herein.

In one embodiment, the HC CDR1 amino acid sequences have a length of at least 5 amino acids of which at least 3, 4, or 5 amino acids are identical to the CDR1 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the HC CDR2 amino acid sequences have a length of at least 15, 16, or 17 amino acids of which at least 10, 12, 14, 15, 16, or 17 amino acids are identical to the CDR2 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the HC CDR2 amino acid sequences have a length of at least 17 amino acids of which at least 14, 15, 16, or 17 amino acids are identical to the CDR2 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the HC CDR3 amino acid sequences have a length of at least 7 or 8 amino acids of which at least 5, 6, 7, or 8 amino acids are identical to the CDR3 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, two or three of the CDRs of the HC variable domain sequence match motifs described herein such that the motifs are a set of motifs that match a HC variable domain of a clone described herein, e.g., E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11,

s-G10, or s-H4. For example, the protein may include SEQ ID NO:118 and SEQ ID NO:160, e.g., motifs that match the E3 HC variable domain.

In one embodiment, the LC CDR1 amino acid sequences have a length of at least 10, 11, or 12 amino acids of which at least 7, 8, 9, 10, or 11 amino acids are identical to the CDR1 sequence of the LC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the LC CDR2 amino acid sequences have a length of at least 6 or 7 amino acids of which at least 5, 6, or 7 amino acids are identical to the CDR2 sequence of the LC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the LC CDR3 amino acid sequences have a length of at least of at least 8, 9, or 10 amino acids of which at least 7, 8, 9, or 10 amino acids are identical to the CDR3 sequence of the LC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, two or three of the CDRs of the LC variable domain sequence match motifs described herein such that the motifs are a set of motifs that match a LC variable domain of a clone described herein, e.g., E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. For example, the protein may include SEQ ID NO:132, SEQ ID NO:136, and SEQ ID NO:161, e.g., motifs that match the E3 LC variable domain.

In one embodiment, the amino acid sequence of the HC variable domain sequence is at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to the amino acid sequence of the HC variable domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, the amino acid sequence of the LC variable domain sequence is at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to the amino acid sequence of the LC variable domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, the amino acid sequences of the HC and LC variable domain sequences are at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to the amino acid sequences of the HC and LC variable domains of a clone selected from the group consisting of E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, and s-H4.

In one embodiment, the amino acid sequences of one or more framework regions (e.g., FR1, FR2, FR3, and/or FR4) of the HC and/or LC variable domain are at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to corresponding framework regions of the HC and LC variable domains of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, the light chain variable domain sequence is human or non-immunogenic in a human. In one embodiment, the heavy chain variable domain sequence is human or non-immunogenic in a human.

The protein can bind to cells that express Tie1, e.g., endothelial cells. In one embodiment, the protein does not substantially bind (e.g., does not detectably bind) to platelets (e.g., resting and/or activated platelets).

In one embodiment, the protein inhibits tube formation by HUVECs in vitro.

In one embodiment, the protein recognizes melanoma-associated structures in a histological section, e.g., not only melanoma tissue, but antigen in surrounding structures. In one embodiment, the protein does not stain blood vessels in normal skin in a histological section.

In one embodiment, the protein specifically binds to Tie1, e.g., it binds with at least a 10, 50, 100, 10^3 , or 10^4 fold preference for Tie1 relative to another human protein, e.g., Tie2, a natural protein other than Tie1 that has a Ig-like domain, an EGF-like domain, or fibronectin Type III repeat, or human serum albumin. In one embodiment, the protein binds to a domain of Tie1 described herein.

In another aspect, the invention features a protein (e.g., an isolated protein) that modulates activity of Tie1, e.g., the Tie1 receptor. For example, the protein is not naturally occurring. In one embodiment, the protein includes a HC and LC immunoglobulin variable domain sequence. In one embodiment, one or more of the CDRs of the heavy and/or light chain variable domain sequence are human, primate, non-rodent

(e.g., non-mouse or non-rat), or synthetic. In one embodiment, one or more of the framework regions of the heavy and/or light chain variable domain sequence are human, primate, or non-rodent (e.g., non-mouse or non-rat). In another embodiment, the protein is substantially free of an immunoglobulin variable domain, e.g., the protein includes a

5 peptide that independently interacts with Tie1.

In one embodiment, the protein activates an activity of the Tie1 protein.

In one embodiment, the protein includes the HC and LC immunoglobulin variable domains of the E3 antibody or domains that are at least 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical in the CDR regions. In one embodiment, the protein
10 competes with E3 for binding to Tie1 or binds to an epitope that overlaps an epitope that is recognized by E3 or that has at least one, two or three residues in common with an epitope that is recognized by E3.

In one embodiment, the activating protein enables IL-3 dependent cells that express a chimeric receptor including the Tie1 extracellular domain and the EpoR
15 intracellular domain to survive in the absence of IL-3.

In one embodiment, the protein can cause dimerization of Tie1. In one embodiment, the protein can cause auto-phosphorylation of the RTK domain of Tie1.

In one embodiment, the protein synergizes with the E3 antibody to activate an activity of Tie. In one embodiment, the protein includes the HC and LC immunoglobulin
20 variable domains of the G2 or C7 antibody or domains that are at least 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical in the CDR regions. In one embodiment, the protein competes with G2 or C7 for binding to Tie1 or binds to an epitope that overlaps an epitope that is recognized by G2 or C7 or that has at least one, two or three residues in common with an epitope that is recognized by G2 or C7.

5 In another embodiment, the protein antagonizes an activity of the Tie1 protein. For example, the protein can at least partially inhibit the ability of the E3 antibody to agonize the Tie protein. In one embodiment, the protein can at least partially inhibit the ability of the E3 antibody to enable IL-3 dependent cells that express a chimeric receptor including the Tie1 extracellular domain and the EpoR intracellular domain to survive in the absence
0 of IL-3.

In one embodiment, the HC and LC immunoglobulin variable domains of the protein include the amino acid sequences that are at least 70, 80, 85, 90, 91, 92, 93, 94, 95,

96, 97, 98, 99, or 100% identical to the amino acid sequences of respective immunoglobulin variable domains of B2 or D11.

In one embodiment, the Tie1 modulator protein includes the HC and LC immunoglobulin variable domains of an antibody selected from the group consisting of:
5 B2, D11, A2, A10, P-B1, P-B3, and P-C6 or immunoglobulin domains that are at least 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical in the CDR regions to the CDR regions of the respective antibodies. For example, the protein binds with an affinity K_D of less than 10^{-8} M, $5 \cdot 10^{-9}$ M, 10^{-9} M, 10^{-10} M, 10^{-11} M, or 10^{-12} M.

10 In one embodiment, the protein can at least partially inhibit the ability of a naturally occurring Tie1 binding protein from interacting with the Tie protein.

The protein can include other features described herein.

In another aspect, the invention features an antibody (e.g., an isolated antibody) that binds to the Tie1 ectodomain, but does not substantially bind to platelets, e.g., as detected by fluorescence activated cell sorting. For example, the antibody does not
15 substantially bind to activated platelets and/or resting platelets. In one embodiment, the antibody binds to endothelial cells. In one embodiment, the protein is a monoclonal antibody. The antibody can be provided in a preparation that is free of other Tie1-binding antibodies that have other specificities, e.g., free of Tie1 binding antibodies that bind to platelets. The antibody can include other features described herein.

20 In another aspect, the invention features a protein (e.g., an isolated protein) that preferentially binds to a Tie1 protein in a conformation stabilized by the E3 antibody relative to an endogenous Tie1 protein in an unstimulated state. In one embodiment, the protein includes immunoglobulin HC and LC domains. In another embodiment, the protein includes a peptide (e.g., of length less than 30, 28, 25, 22, 20, 18, 16, or 14 amino
25 acids) that independently binds to Tie1. For example, the peptide can include one, two, or three disulfide bonds. The protein can include other features described herein.

In another aspect, the invention features a protein (e.g., an isolated protein) that preferentially binds to a Tie1 protein in a dimeric conformation relative to a monomeric Tie1 protein. In one embodiment, the protein includes immunoglobulin HC and LC
30 domains. In another embodiment, the protein includes a peptide (e.g., of length less than 30, 28, 25, 22, 20, 18, 16, or 14 amino acids) that independently binds to Tie1. For

example, the peptide can include one, two, or three disulfide bonds. The protein can include other features described herein.

In another aspect, the invention features a protein (e.g., an isolated protein) that binds to an epitope of Tie1 ectodomain with a K_D of less than 2×10^{-7} M. The epitope overlaps, is within, or includes an epitope bound by E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4 or that includes at least one, two, or three residues in common. For example, the protein binds with an affinity K_D of less than 10^{-8} M, $5 \cdot 10^{-9}$ M, 10^{-9} M, 10^{-10} M, 10^{-11} M, or 10^{-12} M. In one embodiment, the protein includes immunoglobulin HC and LC domains. In another embodiment, the protein includes a peptide (e.g., of length less than 30, 28, 25, 22, 20, 18, 16, or 14 amino acids) that independently binds to Tie1. For example, the peptide can include one, two, or three disulfide bonds. The protein can include other features described herein.

In another aspect, the invention features a protein (e.g., an isolated protein) that competitively inhibits binding of E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4 to a Tie1 ectodomain. In one embodiment, the protein includes immunoglobulin HC and LC domains. In another embodiment, the protein includes a peptide (e.g., of length less than 30, 28, 25, 22, 20, 18, 16, or 14 amino acids) that independently binds to Tie1. For example, the peptide can include one, two, or three disulfide bonds. The protein can include other features described herein.

In another aspect, the invention features a protein (e.g., an isolated protein) that includes a heavy chain immunoglobulin variable domain and a light chain immunoglobulin variable domain and that antagonizes an activity of the Tie1 ectodomain. In one embodiment, CDR1 of the light chain variable domain includes: Q-S-X-S-S (SEQ ID NO:151) or R-A-S-Q-S-X-S-S-Y-L-A (SEQ ID NO:152), wherein X is any amino acid or optionally aliphatic, e.g., isoleucine or valine. In one embodiment, CDR2 of the light chain variable domain includes: A-S-X₁-R-X₂-T (SEQ ID NO:153) or D-A-S-X₁-R-X₂-T (SEQ ID NO:154), wherein X₁ is any amino acid or optionally a hydrophilic amino acid, e.g., serine or asparagine, and X₂ is any amino acid or optionally aliphatic or small aliphatic, e.g., alanine or valine. In one embodiment, CDR3 of the light chain variable domain includes: Q-R-S-X₂-W-P-R (SEQ ID NO:155) or X₁-Q-R-S-X₂-W-

P-R-T (SEQ ID NO:156), wherein X_1 is any amino acid or optionally leucine or glutamine, and X_2 is any amino acid or optionally lysine or serine.

In one embodiment, the protein competes with the B2 and/or D11 antibody for binding to Tie1 or competitively inhibits binding of B2 and/or D11 to Tie1.

5 In one embodiment, the protein antagonizes a Tie1 activity that is stimulated by the E3 antibody. In one embodiment, the protein inhibits dimerization of Tie1. The protein can include other features described herein.

In another aspect, the invention features an isolated, mono-specific protein including a heavy chain immunoglobulin variable domain sequence and a light chain
10 immunoglobulin variable domain sequence, wherein the protein binds to Tie1 ectodomain and includes a human or non-mouse constant domain (e.g., a human IgG1, 2, 3, or 4 constant domain. The protein can include other features described herein.

In another aspect, the invention features an isolated, human antibody that binds to a Tie1 ectodomain. The protein can include other features described herein.

15 In another aspect, the invention features an isolated antibody (e.g., an isolated antibody) that binds to a Tie1 ectodomain and contains less than 5, 4, 3, or 2 peptides (of between 6-9 amino acid length) that are non-human in origin or less than 5, 4, 3, or 2 peptides that are potential human T cell epitopes. In one embodiment, the antibody contains no peptide (of 6-9 amino acid length) that is non-human in origin or that is a
20 potential human T cell epitope.

In one embodiment, the antibody is obtained by a method that includes deimmunization. For example, the antibody is deimmunized, e.g., completely deimmunized. The protein can include other features described herein.

In another aspect, the invention features an isolated antibody that binds to a Tie1
25 ectodomain and that includes a modified Fc domain, e.g., a modified human Fc domain. For example, antibodies may include modifications, e.g., that alter Fc function. For example, the human IgG1 constant region can be mutated at one or more residues, e.g., one or more of residues 234 and 237, e.g., according to the number in US 5,648,260. Other exemplary modifications include those described in US 5,648,260. The protein can
0 include other features described herein.

In another aspect, the invention features an isolated protein that binds to the Tie1 receptor with an affinity K_D of less than 10^{-7} M, 10^{-8} M, $5 \cdot 10^{-9}$ M, 10^{-9} M, 10^{-10} M, 10^{-11} M, or 10^{-12} M. The protein can include other features described herein.

In another aspect, the invention features an isolated protein including a heavy chain immunoglobulin variable domain sequence and a light chain immunoglobulin variable domain sequence, wherein the protein binds to Tie1 ectodomain and, for example, includes at least one or more CDR's that are a non-primate CDR (e.g., a non-mouse or non-rabbit CDR) or a synthetic CDR. The protein can include other features described herein.

In another aspect, the invention features an isolated nucleic acid including a coding sequence that encodes a polypeptide including an immunoglobulin HC variable domain of an antigen binding protein that binds to Tie1. In one embodiment, the HC variable domain includes one or more of the following properties

i) a HC CDR1 that includes an amino acid sequence as follows:

(AGSR)-Y-(GVK)-M-(GSVF) (SEQ ID NO:117),

(AGSIMRH)-Y-(GVMK)-M-(GSVMFH) (SEQ ID NO:118), or

(AGSIMRNH)-Y-(AGTVMKPO)-M-(AGSTVMYWFKH) (SEQ ID NO:119);

ii) a HC CDR2 that includes an amino acid sequence as follows:

X-I-Y-P-S-G-G-X-T-X-Y-A-D-S-V-K-G (SEQ ID NO:120), wherein X is any

amino acid,

(GSV)-I-(SY)-P-S-G-G-(WQ)-T-(GY) (SEQ ID NO:121),

(GSV)-I-(SY)-P-S-G-G-(WQ)-T-(GY)-Y-A-D-S-V-K-G (SEQ ID NO:122), or

(GSVW)-I-(SY)-P-S-G-G-(AGVMYWPQH)-T-(AGSTLVMYFKH) (SEQ ID NO:123);

iii) a HC CDR3 that includes an amino acid sequence as follows:

V-(four or five residues)-F-D-(I/Y) (SEQ ID NO:124),

G-Y-G-P-I-A-P-G-L-D-Y (SEQ ID NO:125),

(GV)-N-Y-Y-(GYD)-S-(SD)-G-Y-G-P-I-A-P-G-L-D-Y (SEQ ID NO:126),

(GVD)-(AGLN)-(LYR)-(GSTLYH)-(GYD)-(AGSYFP)-(SFD)-(AGYD)-(IY)-

(GFD)-(YDP)-(IP)-A-P-G-L-D-Y (SEQ ID NO:127), or

VNYYDSSGYGPIAPGLDY (SEQ ID NO:128).

In one embodiment, the HC CDR1 amino acid sequences have a length of at least 5 amino acids of which at least 3, 4, or 5 amino acids are identical to the CDR1 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one

5 embodiment, the HC CDR2 amino acid sequences have a length of at least 15, 16, or 17 amino acids of which at least 10, 12, 14, 15, 16, or 17 amino acids are identical to the CDR2 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the HC CDR2 amino acid sequences have a length of at least 17
10 amino acids of which at least 14, 15, 16, or 17 amino acids are identical to the CDR2 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the HC CDR3 amino acid sequences have a length of at least of at least 7 or 8 amino acids of which at least 5, 6, 7, or 8 amino acids are identical to the CDR3
15 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, two or three of the CDRs of the HC variable domain sequence match motifs described herein such that the motifs are a set of motifs that match a HC variable domain of a clone described herein, e.g., E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6,
20 p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, the amino acid sequence of the HC variable domain sequence is at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to the amino acid sequence of the HC variable domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3,
25 p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, the nucleic acid further includes a second coding sequence that encodes a polypeptide including an immunoglobulin LC variable domain, e.g., a LC domain described herein. In one embodiment, the nucleic acid further includes a promoter
30 operably linked to the coding sequence.

In another aspect, the invention features an isolated nucleic acid including a coding sequence that encodes a polypeptide including an immunoglobulin LC variable domain of

an antigen binding protein that binds to Tie1. In one embodiment, the LC variable domain includes one or more of the following properties: i) a LC CDR1 that includes an amino acid sequence as follows:

R-A-S-Q-S-(IV)-S-(SR)-X1-Y-L-(AN) (SEQ ID NO:129),

5 R-A-S-Q-S-V-S-S-X-L (SEQ ID NO:130),

R-A-S-Q-S-(IV)-S-(SR)-(SY)-(LY)-(ALN) (SEQ ID NO:131), or

R-A-S-(REQ)-(GSTRN)-(IV)-(GSTIRN)-(STIRH)-X1-(SYWNH)-(LV)-(ASN)
(SEQ ID NO:132), wherein X1 can be serine or absent;

ii) a LC CDR2 that includes an amino acid sequence as follows:

10 X-A-S-X-R-A-T (SEQ ID NO:133), wherein X can be any amino acid,

(AGD)-A-S-(STN)-R-A-T (SEQ ID NO:134),

(AGD)-A-S-(STN)-(LR)-(AEQ)-(ST) (SEQ ID NO:135), or

(AGTKDEH)-A-S-(STN)-(LR)-(AVEQ)-(ST) (SEQ ID NO:136); and

iii) a LC CDR3 that includes an amino acid sequence as follows:

5 Q-Q-(SYFR)-(GSYN)-S-(STYW)-(RP)-(LWR)-(TIY)-T (SEQ ID NO:137),

(LQ)-Q-(SYFR)-(GSYN)-(SKN)-(STYW)-(RP)-(LWR)-(TIY)-T (SEQ ID
NO:138),

Q-Q-(YR)-(GS)-S-(SW)-P-R-X1-T (SEQ ID NO:139), wherein X1 is any amino
acid or absent,

0 (LQ)-(LQ)-(SYFRD)-(GSYN)-(STRKN)-(STYWF)-(RP)-(ILMWRH)-(TIY)-(TI)
(SEQ ID NO:140), or

(LQ)-(LRQ)-(SYFRD)-(GSYN)-(ASTRKN)-(STYWF)-(SVRP)-(STILMWRH)-
(TIY)-(STI) (SEQ ID NO:141); and

In one embodiment, the light chain includes one or more of the following
5 properties:

i) a LC CDR1 that includes an amino acid sequence as follows:

S-X-(ND)-(IV)-(AG)-X1-X2-X3 (SEQ ID NO:142), or

T-(GR)-(ST)-S-X5-(ND)-(IV)-(AG)-X1-X2-X3-Y-X4-S (SEQ ID NO:143),

wherein X1 is any amino acid (e.g., G or R), X2 is any amino acid (e.g., Y or N), X3 is
any amino acid (e.g., F, N, or K), X4 is any amino acid (e.g., aliphatic, e.g., V or A);

iii) a LC CDR2 that includes an amino acid sequence as follows:

(DE)-V-N-N-R-P-S (SEQ ID NO:144)

(DE)-(VD)-(STDN)-(YRDN)-R-P-S (SEQ ID NO:145);

v) a LC CDR3 that includes an amino acid sequence as follows:

(SQ)-S-(SY)-(ASID)-(GSR)-(ST)-(STRN)-(STYR)-(ATLY)-(SVWQ)

(SEQ ID NO:146)

5 In one embodiment, the HC CDR2 includes an amino acid sequence as follows:

(GSVW)-I-(SY)-P-SG-G-(AGVMYWPQH)-T-(AGSTLVMYFKH)-Y-(AT)-D-S-V-K-G

(SEQ ID NO:147) or (GSV)-I-(SY)-P-SG-G-(WQ)-T-(GY)-Y-(AT)-D-S-V-K-G

(SEQ ID NO:148).

In one embodiment, CDR1 of the light chain variable domain includes:

10 Q-S-X-S-S (SEQ ID NO:151) or R-A-S-Q-S-X-S-S-Y-L-A (SEQ ID NO:152), wherein X is any amino acid or optionally aliphatic, e.g., isoleucine or valine. In one embodiment, CDR2 of the light chain variable domain includes: A-S-X₁-R-X₂-T (SEQ ID NO:153) or D-A-S-X₁-R-X₂-T (SEQ ID NO:154), wherein X₁ is any amino acid or optionally a hydrophilic amino acid, e.g., serine or asparagine, and X₂ is any amino acid or optionally aliphatic or small aliphatic, e.g., alanine or valine. In one embodiment, CDR3 of the light chain variable domain includes: Q-R-S-X₂-W-P-R (SEQ ID NO:155) or X₁-Q-R-S-X₂-W-P-R-T (SEQ ID NO:156), wherein X₁ is any amino acid or optionally leucine or glutamine, and X₂ is any amino acid or optionally lysine or serine.

In one embodiment, the LC CDR1 amino acid sequences have a length of at least 10, 11, or 12 amino acids of which at least 7, 8, 9, 10, or 11 amino acids are identical to the CDR1 sequence of the LC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the LC CDR2 amino acid sequences have a length of at least 6 or 7 amino acids of which at least 5, 6, or 7 amino acids are identical to the CDR2 sequence of the LC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the LC CDR3 amino acid sequences have a length of at least 8, 9, or 10 amino acids of which at least 7, 8, 9, or 10 amino acids are identical to the CDR3 sequence of the LC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, the amino acid sequence of the LC variable domain sequence is at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to the amino acid sequence of the LC variable domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, the nucleic acid further includes a second coding sequence that encodes a polypeptide including an immunoglobulin HC variable domain, e.g., an HC domain described herein. In one embodiment, the nucleic acid further includes a promoter operably linked to the coding sequence.

In another aspect, the invention features a nucleic acid that includes one or more coding sequence that encodes one or more polypeptide chains that collectively include an immunoglobulin HC or LC variable domain of an antigen binding protein that binds to Tie1. In one embodiment, the nucleic acid segment encoding at least one of the variable domains hybridizes to a nucleic acid described herein, e.g., under stringent conditions (e.g., high stringency conditions). The nucleic acid can include other features described herein.

In another aspect, the invention features a host cell that contains a first nucleic acid sequence encoding a polypeptide including a HC variable domain of an antigen binding protein and a second nucleic acid sequence encoding a polypeptide including a LC variable domain of the antigen binding protein, wherein the antigen binding protein binds to Tie1 with a K_D of less than 2×10^{-7} M. In one embodiment, the HC or LC variable domain includes at least one human CDR. The antigen binding protein can include other features described herein.

In another aspect, the invention features a host cell that contains a first nucleic acid encoding a polypeptide including a HC variable region and a second nucleic acid encoding a polypeptide including a LC variable region, wherein the HC and the LC variable regions each include at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to respective amino acid sequences of the HC and LC variable domains of a clone selected from the group consisting of E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, and s-H4. The antigen binding protein can include other features described herein.

In another aspect, the invention features a pharmaceutical composition including a protein described herein that interacts with Tie1 and a pharmaceutically acceptable carrier.

In another aspect, the invention features a therapeutic composition including a protein described herein that interacts with Tie1 wherein the composition is sterile and
5 suitable for administration to a subject.

In another aspect, the invention features a method that includes: providing a signal-dependent or signal-responsive cell that expresses a chimeric receptor including the Tie1 extracellular domain and a heterologous intracellular sequence that can produce a signal; contacting a candidate compound to the cell; and evaluating a property of the cell
10 that is dependent on the signal. In one embodiment, the intracellular sequence includes at least a region of an intracellular sequence of the EpoR protein. The method can be used, e.g., to evaluate activity of a candidate compound, or a plurality of compounds.

In another aspect, the invention features a method that includes: providing an IL-3 dependent cell that expresses a chimeric receptor including the Tie1 extracellular domain
15 and the EpoR intracellular domain; contacting a candidate compound to the cell under conditions in which the concentration of IL-3 is not sufficient to sustain viability of the cell; and evaluating a property of the cell. The method can be used, e.g., to evaluate activity of a candidate compound, or a plurality of compounds. In one embodiment, the property is viability. In one embodiment, the evaluating includes an MTT assay. In one
0 embodiment, the method further includes administering the candidate compound to a subject. For example, the candidate compound includes a protein, e.g., a protein that includes an immunoglobulin variable domain.

In another aspect, the invention features method of identifying a compound that modulates Tie1 activity. The method includes: providing a plurality of candidate
5 compounds; and evaluating each compound of the plurality using a method described herein.

In another aspect, the invention features a culture cell that expresses a chimeric transmembrane protein including a region of the Tie1 extracellular domain and a heterologous intracellular sequence. In one embodiment, the intracellular sequence
10 includes a region of the EpoR intracellular domain. In one embodiment, the cell requires IL-3 or Tie1 for viability. For example, the cell is IL-3 dependent in the absence of the

chimeric transmembrane protein, but is viable in the presence of the E3 antibody and the absence of IL-3.

In another aspect, the invention features a preparation that includes the isolated mammalian cells (e.g., cells that expresses a chimeric transmembrane protein including a region of the Tie1 extracellular domain and a heterologous intracellular sequence) and a Tie1-binding ligand, wherein the Tie1-binding ligand is necessary to sustain viability of the cells.

In another aspect, the invention features a kit including: a Tie1-binding ligand and a culture cell that expresses a chimeric transmembrane protein including a region of the Tie1 extracellular domain and a heterologous intracellular sequence.

In another aspect, the invention features a method of evaluating a candidate compound. The method includes: providing a preparation that includes (i) a cell or membrane fraction that contains (a) an insoluble protein that includes a region of the Tie1 extracellular domain and a kinase domain and (b) ATP; (ii) a ligand that causes alters activity of the kinase domain; and (iii) the candidate compound; and evaluating phosphorylation state of the insoluble protein.

In another aspect, the invention features a method of evaluating a candidate compound. The method includes: providing a preparation that includes (i) a cell or membrane fraction that includes a Tie1 protein or a transmembrane protein that includes at least a region of the Tie1 extracellular domain and ATP; (ii) a ligand that causes autophosphorylation of Tie1 or the transmembrane protein; and (iii) the candidate compound; and evaluating phosphorylation state of the Tie1 protein.

In one embodiment, the ligand is an antibody. In one embodiment, the ligand includes the HC and LC immunoglobulin variable domains of the E3 antibody or domains that are at least 90% identical in the CDR regions. In one embodiment, the method further includes administering the candidate compound to a subject.

In another aspect, the invention features a method that includes: providing a preparation that includes (i) a cell or membrane fraction that includes a transmembrane protein that includes at least a region of the Tie1 extracellular domain and ATP; and (ii) a ligand that causes autophosphorylation of Tie1 or the transmembrane protein; and evaluating phosphorylation state of the transmembrane protein.

In another aspect, the invention features a method that includes: contacting a mammalian cell with a ligand that (i) can agonize Tie1 autophosphorylation and/or (ii) can enable an IL-3 dependent cell that expresses a chimeric receptor including the Tie1 extracellular domain and the EpoR intracellular domain to remain viable under conditions
5 in which the concentration of IL-3 is not sufficient to sustain viability of the cell; and evaluating the mammalian cell. In one embodiment, the cell expresses an endogenous Tie1 protein. In one embodiment, the cell is an endothelial cell. In one embodiment, the method further includes contacting the mammalian cell with a test compound, other than the ligand. For example, the ligand is an antibody. For example, the ligand includes the
10 HC and LC immunoglobulin variable domains of the E3 antibody or domains that are at least 90% identical in the CDR regions.

In another aspect, the invention features a method that includes: contacting a mammalian cell or fraction thereof with an agent that can modulate the activity of Tie1; and evaluating the mammalian cell or fraction thereof. In one embodiment, the agent is
15 contacted to the cell while the cell is living, and the evaluating includes isolating a fraction of the cell. In one embodiment, the agent is a protein, e.g., an antibody or a peptide. In one embodiment, the agent includes the HC and LC immunoglobulin variable domains of the E3 antibody or domains that are at least 90% identical in the CDR regions to the E3 antibody. In one embodiment, the agent includes the HC and LC immunoglobulin
20 variable domains of the B2 or D11 antibody or domains that are at least 90% identical in the CDR regions to the B2 or D11 antibody. In one embodiment, the agent includes the HC and LC immunoglobulin variable domains of the A2, A10, P-B1, P-B3, or P-C6 antibody or domains that are at least 70, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100% identical% identical in the CDR regions to the A2, A10, P-B1, P-B3, or P-C6
5 antibody. In one embodiment, the agent includes the HC and LC immunoglobulin variable domains of the G2 or C7 antibody or domains that are at least 90% identical in the CDR regions to the G2, or C7 antibody. The agent can include other features described herein.

In another aspect, the invention features a method of evaluating a test compound,.
25 The method includes evaluating interaction between an agent that can modulate the activity of Tie1 and a protein that includes at least a region of the Tie1 extracellular domain in the presence of the test compound. In one embodiment, the agent is a test

compound is a small organic compound with molecular weight less than 8000, 7000, 6000, 5000, or 3000 Daltons. For example, the evaluating includes contacting cells that include the protein that includes at least a region of the Tie1 extracellular domain with the agent in the presence of the test compound. In another example, the evaluating includes
5 forming a cell-free preparation that includes the protein that includes at least a region of the Tie1 extracellular domain, the agent, and the test compound.

In another aspect, the invention features an artificial protein complex that includes (i) a protein that includes a Tie1 extracellular domain and (ii) a ligand that can agonize or antagonize an activity of Tie1. In one embodiment, the ligand is an antibody (e.g., an
10 antibody described herein. For example, the ligand includes the HC and LC immunoglobulin variable domains of an antibody selected from the group consisting of: E3, B2, D11, A2, A10, P-B1, P-B3, P-C6, G2 and C7, or immunoglobulin domains that are at least 90% identical in the CDR regions to the CDR regions of the respective antibody. In one embodiment, the complex is present in a membrane fraction or on a
5 mammalian cell.

In another aspect the invention features a method that includes: administering a composition that includes a protein that interacts with Tie1 (e.g., a protein described herein) to a subject in an amount effective to modulate (e.g., reduce or increase) angiogenesis in the subject.

0 For example, the protein binds with an affinity K_D of less than 10^{-8} M, $5 \cdot 10^{-9}$ M, 10^{-9} M, 10^{-10} M, 10^{-11} M, or 10^{-12} M.

In one embodiment, one or more of the CDRs of the heavy and/or light chain variable domain sequence are human, primate, non-rodent (e.g., non-mouse or non-rat), or synthetic. In one embodiment, one or more of the framework regions of the heavy and/or
5 light chain variable domain sequence are human, primate, or non-rodent (e.g., non-mouse or non-rat).

In one embodiment, the heavy chain includes one or more of the following properties:

i) a HC CDR1 that includes an amino acid sequence as follows:

(AGSR)-Y-(GVK)-M-(GSVF), (SEQ ID NO:117)

(AGSIMRH)-Y-(GVMK)-M-(GSVMFH) (SEQ ID NO:118), or

(AGSIMRNH)-Y-(AGTVMKPQ)-M-(AGSTVMYWFKH) (SEQ ID NO:119);

ii) a HC CDR2 that includes an amino acid sequence as follows:

X-I-Y-P-S-G-G-X-T-X-Y-A-D-S-V-K-G (SEQ ID NO:120), wherein X is any amino acid,

(GSV)-I-(SY)-P-S-G-G-(WQ)-T-(GY) (SEQ ID NO:121),

5 (GSV)-I-(SY)-P-S-G-G-(WQ)-T-(GY)-Y-A-D-S-V-K-G (SEQ ID NO:122), or

(GSVW)-I-(SY)-P-S-G-G-(AGVMYWPQH)-T-(AGSTLVMYFKH) (SEQ ID NO:123);

iii) a HC CDR3 that includes an amino acid sequence as follows:

V-(four or five residues)-F-D-(I/Y) (SEQ ID NO:124),

10 G-Y-G-P-I-A-P-G-L-D-Y (SEQ ID NO:125),

(GV)-N-Y-Y-(GYD)-S-(SD)-G-Y-G-P-I-A-P-G-L-D-Y (SEQ ID NO:126),

(GVD)-(AGLN)-(LYR)-(GSTLYH)-(GYD)-(AGSYFP)-(SFD)-(AGYD)-(IY)-
(GFD)-(YDP)-(IP)-A-P-G-L-D-Y (SEQ ID NO:127), or

VNYYDSSGYGPIAPGLDY (SEQ ID NO:128).

15 In one embodiment, the light chain includes one or more of the following properties:

i) a LC CDR1 that includes an amino acid sequence as follows:

R-A-S-Q-S-(IV)-S-(SR)-X1-Y-L-(AN) (SEQ ID NO:129),

R-A-S-Q-S-V-S-S-X-L (SEQ ID NO:130),

20 R-A-S-Q-S-(IV)-S-(SR)-(SY)-(LY)-(ALN) (SEQ ID NO:131), or

R-A-S-(REQ)-(GSTRN)-(IV)-(GSTIRN)-(STIRH)-X1-(SYWNH)-(LV)-(ASN)
(SEQ ID NO:132), wherein X1 can be serine or absent;

ii) a LC CDR2 that includes an amino acid sequence as follows:

X-A-S-X-R-A-T (SEQ ID NO:133), wherein X can be any amino acid,

5 (AGD)-A-S-(STN)-R-A-T (SEQ ID NO:134),

(AGD)-A-S-(STN)-(LR)-(AEQ)-(ST) (SEQ ID NO:135), or

(AGTKDEH)-A-S-(STN)-(LR)-(AVEQ)-(ST) (SEQ ID NO:136); and

iii) a LC CDR3 that includes an amino acid sequence as follows:

Q-Q-(SYFR)-(GSYN)-S-(STYW)-(RP)-(LWR)-(TIY)-T (SEQ ID NO:137),

0 (LQ)-Q-(SYFR)-(GSYN)-(SKN)-(STYW)-(RP)-(LWR)-(TIY)-T (SEQ ID NO:138),

Q-Q-(YR)-(GS)-S-(SW)-P-R-X1-T (SEQ ID NO:139), wherein X1 is any amino acid or absent,

(LQ)-(LQ)-(SYFRD)-(GSYN)-(STRKN)-(STYWF)-(RP)-(ILMWRH)-(TIY)-(TI) (SEQ ID NO:140), or

5 (LQ)-(LRQ)-(SYFRD)-(GSYN)-(ASTRKN)-(STYWF)-(SVRP)-(STILMWRH)-(TIY)-(STI) (SEQ ID NO:141); and

In one embodiment, the light chain includes one or more of the following properties:

i) a LC CDR1 that includes an amino acid sequence as follows:

10 S-X-(ND)-(IV)-(AG)-X1-X2-X3 (SEQ ID NO:142), or

T-(GR)-(ST)-S-X5-(ND)-(IV)-(AG)-X1-X2-X3-Y-X4-S (SEQ ID NO:143), wherein X1 is any amino acid (e.g., G or R), X2 is any amino acid (e.g., Y or N), X3 is any amino acid (e.g., F, N, or K), X4 is any amino acid (e.g., aliphatic, e.g., V or A);

iii) a LC CDR2 that includes an amino acid sequence as follows:

5 (DE)-V-N-N-R-P-S (SEQ ID NO:144)

(DE)-(VD)-(STDN)-(YRDN)-R-P-S (SEQ ID NO:145);

v) a LC CDR3 that includes an amino acid sequence as follows:

(SQ)-S-(SY)-(ASID)-(GSR)-(ST)-(STRN)-(STYR)-(ATLY)-(SVWQ) (SEQ ID NO:146)

0 In one embodiment, the HC CDR2 includes an amino acid sequence as follows: (GSVW)-I-(SY)-P-SG-G-(AGVMYWPQH)-T-(AGSTLVMYFKH)-Y-(AT)-D-S-V-K-G (SEQ ID NO:147) or (GSV)-I-(SY)-P-SG-G-(WQ)-T-(GY)-Y-(AT)-D-S-V-K-G (SEQ ID NO:148).

In one embodiment, the HC CDR1 amino acid sequences have a length of at least 5 amino acids of which at least 3, 4, or 5 amino acids are identical to the CDR1 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the HC CDR2 amino acid sequences have a length of at least 15, 16, or 17 amino acids of which at least 10, 12, 14, 15, 16, or 17 amino acids are identical to the CDR2 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the HC CDR2 amino acid sequences have a length of at least 17

amino acids of which at least 14, 15, 16, or 17 amino acids are identical to the CDR2 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the HC CDR3 amino acid sequences have a length of at least of at least
 5 7 or 8 amino acids of which at least 5, 6, 7, or 8 amino acids are identical to the CDR3 sequence of the HC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

In one embodiment, the LC CDR1 amino acid sequences have a length of at least 10, 11, or 12 amino acids of which at least 7, 8, 9, 10, or 11 amino acids are identical to
 10 the CDR1 sequence of the LC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the LC CDR2 amino acid sequences have a length of at least 6 or 7 amino acids of which at least 5, 6, or 7 amino acids are identical to the CDR2 sequence of the LC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4,
 15 p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4. In one embodiment, the LC CDR3 amino acid sequences have a length of at least of at least 8, 9, or 10 amino acids of which at least 7, 8, 9, or 10 amino acids are identical to the CDR3 sequence of the LC of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-
 20 H4.

In one embodiment, the amino acid sequence of the HC variable domain sequence is at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to the amino acid sequence of the HC variable domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-
 15 H4.

In one embodiment, the amino acid sequence of the LC variable domain sequence is at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to the amino acid sequence of the LC variable domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-
 0 H4.

In one embodiment, the amino acid sequences of the HC and LC variable domain sequences are at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to the amino

acid sequences of the HC and LC variable domains of a clone selected from the group consisting of E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, and s-H4.

In one embodiment, the amino acid sequences of one or more framework regions
5 (e.g., FR1, FR2, FR3, and/or FR4) of the HC and/or LC variable domain are at least 70, 80, 85, 90, 92, 95, 97, 98, 99, or 100% identical to corresponding framework regions of the HC and LC variable domains of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

10 In one embodiment, the light chain variable domain sequence is human or non-immunogenic in a human. In one embodiment, the heavy chain variable domain sequence is human or non-immunogenic in a human.

The protein can bind to cells that express Tie1, e.g., endothelial cells. In one embodiment, the protein does not substantially bind (e.g., does not detectably bind) to
15 platelets.

In one embodiment, the protein specifically binds to Tie1, e.g., it binds with at least a 10, 50, 100, 10^3 , or 10^4 fold preference for Tie1 relative to another human protein, e.g., Tie2, a natural protein other than Tie1 that has a Ig-like domain, an EGF-like domain, or fibronectin Type III repeat, or human serum albumin. In one embodiment, the protein
20 binds to a domain of Tie1 described herein.

In one embodiment, the protein is delivered locally. In one embodiment, the protein is delivered systemically.

In one embodiment, the subject is in need of reduced angiogenesis, or identified as such. For example, the subject has an angiogenesis-related disorder. In another example,
25 the subject has a neoplastic disorder, e.g., a metastatic cancer. For example, the subject has an angiogenesis-dependent cancer or tumor.

In another embodiment, the subject has an inflammatory disorder, e.g., rheumatoid arthritis or psoriasis.

In one embodiment, the protein is administered in an amount effective to reduce
30 one or more of the following activities: sprouting, splitting and remodeling of blood vessels. In one embodiment, the protein is administered in an amount effective to reduce vasculogenesis or tubule formation.

In one embodiment, the method further includes, prior to the administering, identifying the subject as a subject in need of reduced angiogenesis. In one embodiment, the method further includes administering the protein continuously or in separate boluses. In one embodiment, the method further includes monitoring the subject during the course
5 of administration. For example, the monitoring includes imaging blood vessels (locally or throughout) the subject. For example, the imaging includes administering the same or different Tie1 binding ligand to the subject.

In another aspect the invention features a method that includes: administering a composition that includes a protein described herein (e.g., a protein that reduces a Tie1
10 activity) to a subject in an amount effective to reduce a Tie1 activity in the subject. The method can include other features described herein.

In another aspect the invention features a method that includes: administering a composition that includes a protein described herein (e.g., a protein that increase a Tie1 activity) to a subject in an amount effective to increase Tie1 activity in the subject. The
5 method can include other features described herein.

In one embodiment, the composition is administered locally, e.g., to a wound. For example, the subject is in need of treatment for wound healing (e.g., a burn, laceration, or surgery).

In one embodiment, the method includes administering a first antibody that
0 includes one or more CDRs of the E3 antibody and a second antibody that includes one or more CDRs of the G2 or C7 antibody.

In another aspect, the invention features a method that includes administering a composition that includes a protein described herein that modulates an activity of Tie1 to a subject in an amount effective to modulate Tie1 activity in the subject. In one
5 embodiment, the method includes administering first and second proteins that both interact with Tie1. For example, the first protein that agonizes Tie1 activity and second protein that antagonizes Tie1 activity is administered, e.g., to precisely titrate activity. For example, the first and second protein can be administered to different locations to inhibit angiogenesis in one area without affecting another area.

) In one embodiment, the first and second proteins are administered separately. In another embodiment, the first and second proteins are administered as a combination. The method can include other features described herein.

In another aspect the invention features a method that includes: administering a composition that includes a protein described herein (e.g., a protein that can modulate an activity of Tie1) to a subject in an amount effective to modulate endothelial cell activity in the subject. In one embodiment, the protein is delivered into the circulation.

5 In one embodiment, the composition is effective for sensitizing endothelial cells to a treatment, and providing a treatment to the subject that inhibits, kills, ablates, or otherwise arrests the sensitized endothelial cells.

In another aspect the invention features a method that includes: (i) contacting the sample (and optionally, a reference, e.g., control, sample) with a protein that binds to Tie1,
10 e.g., a protein described herein, under conditions that allow interaction of the Tie1-binding ligand and the Tie1 protein to occur; and (ii) detecting formation of a complex between the Tie1-binding ligand, and the sample (and optionally, the reference, e.g., control, sample).

In another aspect the invention features a method that includes: (i) administering to a subject (and optionally a control subject) an Tie1-binding ligand (e.g., an antibody or
15 antigen binding fragment thereof), under conditions that allow interaction of the Tie1-binding ligand and the Tie1 protein to occur; and (ii) detecting formation of a complex between the Tie1-binding ligand and a Tie1 molecule of the subject or detecting distribution of Tie1-binding ligand or at least one location of the Tie1-binding ligand in the subject. In one embodiment, the Tie1-binding ligand does not modulate the activity of
20 Tie1. The Tie1-binding ligand can be a protein described herein. In one embodiment, the ligand detects activated Tie1.

An antibody that binds to Tie1 is preferably monospecific, e.g., a monoclonal antibody, or antigen-binding fragment thereof. For example, the antibody can recognize Tie1 on a living cell, e.g., an endogenous Tie1 molecule or a Tie1 molecule that is
5 expressed from a heterologous nucleic acid. In one embodiment, the Tie1-binding ligand interacts with primary endothelial cells. The term "monospecific antibody" refers to an antibody that displays a single binding specificity and affinity for a particular target, e.g., epitope. This term includes a "monoclonal antibody" which refers to an antibody that is produced as a single molecular species, e.g., from a population of homogenous isolated
10 cells. A "monoclonal antibody composition" refers to a preparation of antibodies or fragments thereof of in a composition that includes a single molecular species of antibody.

In one embodiment, a monoclonal antibody is produced by a mammalian cell. One or more monoclonal antibody species may be combined.

The Tie1-binding antibodies can be full-length (e.g., an IgG (e.g., an IgG1, IgG2, IgG3, IgG4), IgM, IgA (e.g., IgA1, IgA2), IgD, and IgE) or can include only an antigen-binding fragment (e.g., a Fab, F(ab')₂ or scFv fragment), e.g., it does not include an Fc domain or a CH2, CH3, or CH4 sequence. The antibody can include two heavy chain immunoglobulins and two light chain immunoglobulins, or can be a single chain antibody. The antibodies can, optionally, include a constant region chosen from a kappa, lambda, alpha, gamma, delta, epsilon or a mu constant region gene. An Tie1-binding antibody can include a heavy and light chain constant region substantially from a human antibody, e.g., a human IgG1 constant region or a portion thereof. As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG1) that is encoded by heavy chain constant region genes.

In one embodiment, the antibody (or fragment thereof) is a recombinant or modified antibody, e.g., a chimeric, a humanized, a deimmunized, or an *in vitro* generated antibody. The term "recombinant" or "modified" human antibody, as used herein, is intended to include all antibodies that are prepared, expressed, created or isolated by recombinant means, such as antibodies expressed using a recombinant expression vector transfected into a host cell, antibodies isolated from a recombinant, combinatorial antibody library, antibodies isolated from an animal (e.g., a mouse) that is transgenic for human immunoglobulin genes or antibodies prepared, expressed, created or isolated by any other means that involves splicing of human immunoglobulin gene sequences to other DNA sequences. Such recombinant antibodies include humanized, CDR grafted, chimeric, deimmunized, *in vitro* generated antibodies, and may optionally include constant regions derived from human germline immunoglobulin sequences.

In one embodiment, the antibody binds to an epitope distinct from an epitope bound by known monoclonal antibodies that bind to Tie1, e.g., an antibody described in WO 95/26364, e.g., 3C4C7G6 and 10F11G6. In other embodiments, the antibody does not compete with known monoclonal antibodies that bind to Tie1, e.g., 3C4C7G6 and 10F11G6. In still other embodiments, the antibody does not compete with ligand described herein.

Also within the scope of the invention are antibodies or other ligands that bind overlapping epitopes of, or competitively inhibit the binding of the ligands disclosed

herein to Tie1. For example, the antibodies or other ligands bind overlapping epitopes of or competitively inhibit the binding of monospecific antibodies, e.g., E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4 to Tie1, or vice versa (e.g., the monospecific antibodies competitively inhibiting binding of the ligands). Overlapping epitopes can include at least one amino acid in common. Ligands that competitively inhibit binding of one another do not necessarily bind to overlapping epitopes. For example, they may inhibit binding by steric interference or by altering the conformation of Tie1.

Any combination of Tie1-binding ligands is within the scope of the invention, e.g., two or more antibodies that bind to different regions of Tie1, e.g., antibodies that bind to two different epitopes on the extracellular domain of Tie1, e.g., a bispecific antibody.

In one embodiment, the Tie1-binding antibody or antigen-binding fragment thereof includes at least one light or heavy chain immunoglobulin (or preferably, at least one light chain immunoglobulin and at least one heavy chain immunoglobulin). Preferably, each immunoglobulin includes a light or a heavy chain variable region having at least one, two and, preferably, three complementarity determining regions (CDR's) substantially identical to a CDR from an anti-Tie1 light or heavy chain variable region, respectively, i.e., from a variable region of an antibody described herein, e.g., E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

An Tie1-binding ligand described herein can be used alone, e.g., can be administered to a subject or used *in vitro* in non-derivatized or unconjugated forms. In other embodiments, the Tie1-binding ligand can be derivatized, modified or linked to another functional molecule, e.g., another polypeptide, protein, isotope, cell, or insoluble support. For example, the Tie1-binding ligand can be functionally linked (e.g., by chemical coupling, genetic fusion, non-covalent association or otherwise) to one or more other molecular entities, such as an antibody (e.g., if the ligand is an antibody to form a bispecific or a multi-specific antibody), a toxin, a radioisotope, a therapeutic (e.g., a cytotoxic or cytostatic) agent or moiety, among others. For example, the Tie1-binding ligand can be coupled to a radioactive ion (e.g., an α -, γ -, or β -emitter), e.g., iodine (^{131}I or ^{125}I), yttrium (^{90}Y), lutetium (^{177}Lu), actinium (^{225}Ac), rhenium (^{186}Re), or bismuth (^{212}Bi or ^{213}Bi).

In another aspect, the invention features a nucleic acid that includes a coding sequence that encodes a polypeptide comprising an immunoglobulin heavy or light chain variable domain that binds to Tie1, e.g., an immunoglobulin heavy or light chain variable domain described herein. For example, the nucleic acid can include a particular nucleic acid sequence described herein, a nucleic acid that is at least 75, 80, 85, 90, 95, 96, 97, 98, or 99% identical to a nucleic acid sequence described herein (e.g., a particular nucleic acid sequence), or a nucleic acid that specifically hybridizes (e.g., under conditions described herein) to a nucleic acid sequence described herein (e.g., a particular nucleic acid sequence), or fragments thereof (e.g., CDR-coding fragments).

A nucleic acid described herein can further include a promoter operably linked to the coding sequence. A nucleic acid can include a first and second coding sequence, e.g., wherein the first coding sequence encodes a polypeptide that includes an immunoglobulin heavy chain variable domain and the second coding sequence encodes a polypeptide that includes an immunoglobulin light chain variable domain.

In another aspect, the invention features a host cell that contains a first nucleic acid encoding a polypeptide comprising a heavy chain variable region and a second nucleic acid encoding a polypeptide comprising a light chain variable region. The heavy chain variable region and the light chain variable region can associate to form a Tie1 binding protein. These variable regions can have one or more properties described herein, e.g., at least 75, 80, 85, 90, 95, 96, 97, 98, or 99% identity to a sequence described herein. The invention also includes a method of providing a Tie1-binding antibody. The method can include providing a host cell described herein; and expressing said first and second nucleic acids in the host cell under conditions that allow assembly of said light and heavy chain variable regions to form an antigen binding protein that interacts with Tie1.

In another aspect, the invention provides compositions, e.g., pharmaceutical compositions, which include a pharmaceutically acceptable carrier, excipient or stabilizer, and at least one of the Tie1-binding ligands (e.g., antibodies or fragments thereof) described herein. In one embodiment, the compositions, e.g., the pharmaceutical compositions, include a combination of two or more of the aforesaid Tie1-binding ligands.

In another aspect, the invention features a kit that includes an Tie1-binding antibody (or fragment thereof), e.g., an Tie1-binding antibody (or fragment thereof) as described herein, for use alone or in combination with other therapeutic modalities, e.g., a

cytotoxic or labeling agent, e.g., a cytotoxic or labeling agent as described herein, along with instructions on how to use the Tie1 antibody or the combination of such agents to treat, prevent or detect a Tie1-related disorder, e.g., an endothelial cell related disorder, e.g., rheumatoid arthritis or metastatic cancer.

5 In another aspect, the protein ligand that binds to Tie1 is a polypeptide that is not an immunoglobulin. For example, the polypeptide can be of variable length, e.g., 4 to 100 amino acid residues in length, preferably 5 to 75, 6 to 50, or 7 to 40 amino acid residues in length, or more preferably 8 to 30 or 10 to 25 amino acid residues in length. In some
10 embodiments, the polypeptide includes non-standard or synthetic amino acid residues, e.g., norleucine, selenocysteine, pyrrolysine, etc. In some embodiments, the polypeptide includes cross-linking groups, e.g., two cysteine residues that can form a disulfide bond or some other type of chemical cross-linking moieties that can be used to cyclize the peptide. In other preferred embodiments, the polypeptide can be modified, e.g., using polyethylene glycol or fusion to a soluble protein, e.g., to increase the solubility or circulatory half-life
15 of the polypeptide.

In another aspect, the invention features a method of identifying a protein that specifically binds to Tie1. In preferred embodiments, the invention includes: providing a Tie1 antigen; providing a display library (e.g., a phage display library member); identifying a member present in the library, wherein the member expresses a protein that
20 specifically binds to the Tie1 antigen. The term "Tie1 antigen" refers to any antigenic fragment of Tie1 that is at least 8 amino acids in length. For example, a Tie1 antigen can include a fragment of the Tie1 ectodomain, e.g., a fragment that includes a folded protein domain such as a fragment described herein. In some embodiments, the Tie1 antigen is of human origin and includes, e.g., the extracellular domain of human Tie1 or a fragment
25 thereof (e.g., a fragment described herein. The Tie1 antigen can be a recombinant polypeptide optionally fused to another polypeptide, e.g., a Fc domain, or it can be a cell that expresses Tie1 on its surface (e.g., an endothelial cell). In other preferred embodiments, the Tie1 antigen has an activated conformation, e.g., the Tie1 antigen is a dimeric conformation or a conformation stabilized by the E3 antibody described herein.

30 The methods described here are, for example, applicable to libraries that are based on bacteriophage with a substantially complete genome (e.g., including a modified gene III) and to libraries that are based on bacteriophage particles that include a phagemid

nucleic acid. The terms "bacteriophage library member" and "phage" encompass members of both types of libraries. The term "bacteriophage particle" refers to a particle formed of bacteriophage coat proteins that packages a nucleic acid. The packaged nucleic acid can be a modified bacteriophage genome or a phagemid, e.g., a nucleic acid that
5 includes a bacteriophage origin of replication but lacks essential phage genes and cannot propagate in *E. coli* without help from "helper phage" or phage genes supplied *in trans*.

In other embodiments, the invention features a method of identifying a protein that specifically binds to Tie1. The method includes: providing a Tie1 antigen (e.g., an region of the Tie1 ectodomain); immunizing a non-human animal with the Tie1 antigen; and
10 isolating a cell that produces a immunoglobulin that interacts with Tie1. For example, the method can include producing hybridoma cells from the spleen of the animal (e.g., an immunized mouse); and identifying individual hybridoma cell lines expressing an antibody that specifically binds to the Tie1 antigen. For example, the

In preferred embodiments, the Tie1 antigen is of human origin and includes, e.g.,
15 the extracellular domain of human Tie1 or some fragment thereof, e.g., the HA binding domain of Tie1. The Tie1 antigen can be a recombinant polypeptide optionally fused to another polypeptide, e.g., a purification handle, or it can be a cell that expresses Tie1 (e.g., an endothelial cell) on its surface. In other preferred embodiments, the Tie1 antigen has an activated conformation, e.g., dimerized.

20 In preferred embodiments, the methods further include isolating a nucleic acid molecule from the identified phage or hybridoma, wherein the nucleic acid molecule encodes the polypeptide or antibody that specifically binds to the Tie1 antigen. The isolated nucleic acid molecules can be used to produce therapeutic agents, as described herein.

5 In another aspect, the invention features nucleic acids that encode proteins identified by the methods described herein. In preferred embodiments, the nucleic acids include sequences encoding a heavy and light chain immunoglobulin or immunoglobulin fragment described herein. For example, the invention features, a first and second nucleic acid encoding a heavy and light chain variable region, respectively, of an Tie1-binding
0 antibody molecule as described herein. Sequences encoding a heavy and light chain that function together can be present on separate nucleic acid molecules or on the same nucleic

acid molecule. In another aspect, the invention features host cells and vectors containing a nucleic acid described herein.

In yet another aspect, the invention features a method of producing an Tie1-binding antibody, or antigen-binding fragment thereof. The method includes: providing a
5 host cell that contains a first nucleic acid encoding a polypeptide comprising a heavy chain variable region, e.g., a heavy chain variable region as described herein; providing a second nucleic acid encoding a polypeptide comprising a light chain variable region, e.g., a light chain variable region as described herein; and expressing said first and second nucleic
10 acids in the host cell under conditions that allow assembly of said light and heavy chain variable regions to form an antigen binding protein that interacts with Tie1. The first and second nucleic acids can be linked or unlinked, e.g., expressed on the same or different vector, respectively. The first and second nucleic acids can be components of the same molecule or can reside on different molecules (e.g., different chromosomes or plasmids).

The host cell can be a eukaryotic cell, e.g., a mammalian cell, an insect cell, a yeast
5 cell, or a prokaryotic cell, e.g., *E. coli*. For example, the mammalian cell can be a cultured cell or a cell line. Exemplary mammalian cells include lymphocytic cell lines (e.g., NSO), Chinese hamster ovary cells (CHO), COS cells, oocyte cells, and cells from a transgenic animal, e.g., mammary epithelial cell. For example, nucleic acids encoding the antibodies described herein can be expressed in a transgenic animal. In one embodiment, the nucleic
0 acids are placed under the control of a tissue-specific promoter (e.g., a mammary specific promoter) and the antibody is produced in the transgenic animal. For example, the antibody molecule is secreted into the milk of the transgenic animal, such as a transgenic cow, pig, horse, sheep, goat or rodent. To produce a single chain antibody, the nucleic acid is configured to encode a single polypeptide that comprises both the heavy and light chain
5 variable domains.

Tie1 has been found to be overexpressed in association with a wide range of cancers. Targeting Tie1 on the tumor vasculature with Tie1-binding ligands (e.g., antibodies) can be used to inhibit, destroy, or otherwise antagonize the vasculature so that tumor growth and metastasis is reduced. The ligands can be, for example, associated with
a toxic payload or can mediate direct functional inhibition.

In another aspect, the invention features a method of inhibiting an activity of a cell, e.g., an endothelial cell, e.g., proliferation, adhesion, growth or survival of a cell, e.g., an

endothelial cell, e.g., an endothelial cell in the vicinity of a cancer, e.g., a tumor. Methods of the invention include contacting the cell with a Tie1-binding ligand, in an amount sufficient to inhibit the adhesion, migration, growth or proliferation of the cell. Methods of the invention can be used, for example, to treat or prevent a disorder, e.g., an inflammatory disorder (e.g., rheumatoid arthritis, lupus, restenosis, psoriasis, graft v. host response, or multiple sclerosis), or a cancerous disorder (e.g., a malignant or metastatic disorder), by administering to a subject (e.g., an experimental animal or a human patient) a Tie1-binding ligand in an amount effective to treat or prevent such disorder. a Tie1-binding ligand can also be used to treat or prevent stroke, heart disease, ulcers, scleroderma, infertility, and other diseases that are associated with insufficient angiogenesis.

A Tie1-binding ligand can be used to treat or prevent angiogenesis-related disorders, particularly angiogenesis-dependent cancers and tumors.

Angiogenesis-related disorders include, but are not limited to, solid tumors; blood born tumors such as leukemias; tumor metastasis; benign tumors (e.g., hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis); psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation.

"Angiogenesis-dependent cancers and tumors" are cancers tumors that require, for their growth (expansion in volume and/or mass), an increase in the number and density of the blood vessels supplying them with blood. In one embodiment a Tie1-binding ligand causes regression of such cancers and tumors. "Regression" refers to the reduction of tumor mass and size, e.g., a reduction of at least 2, 5, 10, or 25%.

In another aspect, the invention features a method of contacting a cell (in vitro, ex vivo, or in vivo), e.g., an endothelial cell, e.g., an endothelial cell in the vicinity of a cancer, e.g., a tumor. The method can include providing a ligand that interacts with Tie1, e.g., a ligand described herein, and contacting the cell with the ligand, in an amount sufficient to form at least one detectable ligand-cell complex. The ligand can include, for

example, a label or cytotoxic entity, e.g., an immunoglobulin Fc domain or a cytotoxic drug.

In another aspect, the invention features a method of treating, e.g., inhibiting, ablating or killing, a cell or impairing at least one activity of the cell. The method includes
5 providing an Tie1-binding ligand, e.g. a ligand described herein, and contacting the cell with the ligand, in an amount sufficient to impair at least one activity of the cell, inhibit, ablate or kill the cell. The contacting can be *in vitro* or *in vivo*. For example, the cell can be ae.g., an endothelial cell, e.g., an endothelial cell in the vicinity of a cancer, e.g., a tumor. The ligand can include a cytotoxic entity. Methods of the invention can be used,
10 for example, to treat or prevent a disorder, e.g., a endothelial cell-based disorder, a blood vessel disorder, wound healing, or a cancerous disorder (e.g., a malignant or metastatic disorder), by administering to a subject (e.g., an experimental animal or a human patient) an Tie1-binding ligand in an amount effective to treat or prevent such disorder.

The subject methods can be used on cells in culture, e.g. *in vitro* or *ex vivo*. For
15 example, an endothelial cell, e.g., an endothelial cell in cancer biopsy, can be cultured *in vitro* in culture medium and the contacting step can be effected by adding the Tie1-binding ligand to the culture medium. The method can be performed on cells (e.g., cancerous or metastatic cells) present in a subject, as part of an *in vivo* (e.g., therapeutic or prophylactic) protocol. For *in vivo* embodiments, the contacting step is effected in a subject and
20 includes administering the Tie1-binding ligand to the subject under conditions effective to permit both binding of the ligand to the cell, and the inhibition of adhesion, migration, growth or proliferation of the cell.

The method of the invention can be used to treat or prevent cancerous disorders, e.g., including but are not limited to, solid tumors, soft tissue tumors, and metastatic
5 lesions, particularly tumors that require a blood supply or angiogenesis. Examples of solid tumors include malignancies, e.g., sarcomas, adenocarcinomas, and carcinomas, of the various organ systems, such as those affecting lung, breast, lymphoid, gastrointestinal (e.g., colon), and genitourinary tract (e.g., renal, urothelial cells), pharynx, as well as adenocarcinomas which include malignancies such as most colon cancers, rectal cancer,
10 renal-cell carcinoma, liver cancer, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus. The subject can be a mammal, e.g., a

primate, preferably a higher primate, e.g., a human (e.g., a patient having, or at risk of, a disorder described herein, e.g., an endothelial cell-based disorder, e.g., cancer).

The Tie1-binding antibody or fragment thereof, e.g., an Tie1-binding antibody or fragment thereof as described herein, can be administered to the subject systemically (e.g., orally, parenterally, subcutaneously, intravenously, intramuscularly, intraperitoneally, intranasally, transdermally, or by inhalation), topically, or by application to mucous membranes, such as the nose, throat and bronchial tubes.

The methods can further include the step of monitoring the subject, e.g., for a reduction in one or more of: a reduction in tumor size; reduction in cancer markers, e.g., levels of cancer specific antigen; reduction in the appearance of new lesions, e.g., in a bone scan; a reduction in the appearance of new disease-related symptoms; or decreased or stabilization of size of soft tissue mass; or any parameter related to improvement in clinical outcome. The subject can be monitored in one or more of the following periods: prior to beginning of treatment; during the treatment; or after one or more elements of the treatment have been administered. Monitoring can be used to evaluate the need for further treatment with the same Tie1-binding ligand or for additional treatment with additional agents. Generally, a decrease in one or more of the parameters described above is indicative of the improved condition of the subject. Information about the monitoring can be recorded, e.g., in electronic or digital form.

The Tie1-binding ligand can be used alone in unconjugated form to thereby inhibit adhesion, migration, or extravasation of the Tie1-expressing cells, or ablate or kill the Tie1-expressing cells. If the ligand is an antibody, the ablation or killing can be mediated, e.g., by an antibody-dependent cell killing mechanisms such as complement-mediated cell lysis and/or effector cell-mediated cell killing. In other embodiments, the Tie1-binding ligand can be bound (e.g., physically associated, either directly or indirectly, covalently or non-covalently) to a substance, e.g., a cytotoxic agent or moiety, effective to kill or ablate the Tie1-expressing cells. For example, the Tie1-binding ligand can be coupled to a radioactive ion (e.g., an α -, γ -, or β -emitter), e.g., iodine (^{131}I or ^{125}I), yttrium (^{90}Y), lutetium (^{177}Lu), actinium (^{225}Ac), or bismuth (^{213}Bi). The methods and compositions of the invention can be used in combination with other therapeutic modalities. In one embodiment, the methods of the invention include administering to the subject an Tie1-binding ligand, e.g., an Tie1-binding antibody or fragment thereof, in combination with a

cytotoxic agent, in an amount effective to treat or prevent said disorder. The ligand and the cytotoxic agent can be administered simultaneously or sequentially. In other embodiments, the methods and compositions of the invention are used in combination with surgical and/or radiation procedures.

5 In another aspect, the invention features methods for detecting the presence of a Tie1 protein or a cell expressing Tie1 (e.g., an endothelial cell) in a sample, *in vitro* (e.g., a biological sample, a tissue biopsy, e.g., a cancerous lesion). The subject method can be used to evaluate, e.g., diagnose or stage a disorder described herein, e.g., a cancerous disorder. The method includes: (i) contacting the sample (and optionally, a reference, e.g., control sample) with an Tie1-binding ligand, as described herein, under conditions that
10 allow interaction of the Tie1-binding ligand and the Tie1 protein to occur; and (ii) detecting formation of a complex between the Tie1-binding ligand, and the sample (and optionally, the reference, e.g., control, sample). Formation of the complex is indicative of the presence of Tie1 protein (e.g., activated Tie1 protein), and can indicate the suitability or need for a treatment described herein. For example, a statistically significant change in the formation of the complex in the sample relative to the reference sample, e.g., the
5 control sample, is indicative of the presence of Tie1 (e.g., activated Tie1) in the sample.

In yet another aspect, the invention provides a method for detecting the presence of Tie1 (e.g., activated Tie1) *in vivo* (e.g., *in vivo* imaging in a subject). The subject method
10 can be used to evaluate, e.g., diagnose, localize, or stage a disorder described herein, e.g., a cancerous disorder. The method includes: (i) administering to a subject (and optionally a control subject) an Tie1-binding ligand (e.g., an antibody or antigen binding fragment thereof), under conditions that allow interaction of the Tie1-binding ligand and the Tie1 protein to occur; and (ii) detecting formation of a complex between the ligand and Tie1, wherein a statistically significant change in the formation of the complex in the subject
15 relative to the reference, e.g., the control subject or subject's baseline, is indicative of the presence of the Tie1. The presence of activated Tie1 in particular locations within a subject can be indicative of an endothelial-cell related disorder, e.g., an angiogenesis-related disorder, e.g., a cancer, e.g., metastatic cancer, or other angiogenesis-related disorder described herein.

In other embodiments, a method of diagnosing or staging, a disorder as described herein (e.g., an inflammatory or cancerous disorder), is provided. The method includes:

(i) identifying a subject having, or at risk of having, the disorder; (ii) obtaining a sample of a tissue or cell affected with the disorder; (iii) contacting said sample or a control sample with an Tie1-binding ligand, under conditions that allow interaction of the binding agent and the Tie1 protein to occur, and (iv) detecting formation of a complex. A statistically significant increase in the formation of the complex between the ligand with respect to a reference sample, e.g., a control sample, is indicative of the disorder or the stage of the disorder. For example, the finding of activated Tie1 on tumor cells located in a solid tumor can indicate that the tumor is progressing into a metastatic tumor.

Preferably, the Tie1-binding ligand used in the *in vivo* and *in vitro* diagnostic methods is directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound binding agent. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials. In one embodiment, the Tie1-binding ligand is coupled to a radioactive ion, e.g., indium (^{111}In), iodine (^{131}I or ^{125}I), yttrium (^{90}Y), actinium (^{225}Ac), bismuth (^{213}Bi), sulfur (^{35}S), carbon (^{14}C), tritium (^3H), rhodium (^{188}Rh), or phosphorous (^{32}P). In another embodiment, the ligand is labeled with an NMR contrast agent.

The invention also provides polypeptides and nucleic acids that encompass a range of amino acid and nucleic acid sequences, e.g., sequences described herein or sequences related to those described herein. For example, the invention features nucleic acids that encodes each of the polypeptides described herein. The nucleic acid can include the cognate codons or any set of codons that can be translated to produce the respective polypeptide. Such polypeptides include individual subunits of a multi-chain protein, e.g., an antibody that includes a plurality of different polypeptide chains. The nucleic acid may also be a nucleic acid fragment or vector that is not expressed, but includes a sequence encoding at least a part of an immunoglobulin variable region (e.g., including a CDR described herein) or a complement thereof. Such nucleic acids can be used to prepare useful constructs, cells, and proteins. In addition, the invention features a host cell that includes a nucleic acid described herein. The cell can express a protein described herein, e.g., on its surface.

As used herein, the term "antibody" refers to a protein that includes at least one immunoglobulin variable domain or immunoglobulin variable domain sequence. For example, an antibody can include a heavy (H) chain variable region (abbreviated herein as

VH), and a light (L) chain variable region (abbreviated herein as VL). In another example, an antibody includes two heavy (H) chain variable regions and two light (L) chain variable regions. The term “antibody” encompasses antigen-binding fragments of antibodies (e.g., single chain antibodies, Fab fragments, F(ab')₂, a Fd fragment, a Fv fragments, and dAb fragments) as well as complete antibodies.

The VH and VL regions can be further subdivided into regions of hypervariability, termed “complementarity determining regions” (“CDR”), interspersed with regions that are more conserved, termed “framework regions” (FR). The extent of the framework region and CDR's has been precisely defined (see, Kabat, E.A., *et al.* (1991) *Sequences of Proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242, and Chothia, C. *et al.* (1987) *J. Mol. Biol.* 196:901-917). Kabat definitions are used herein. Each VH and VL is typically composed of three CDR's and four FR's, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4.

An “immunoglobulin domain” refers to a domain from the variable or constant domain of immunoglobulin molecules. Immunoglobulin domains typically contain two β -sheets formed of about seven β -strands, and a conserved disulphide bond (see, e.g., A. F. Williams and A. N. Barclay 1988 *Ann. Rev Immunol.* 6:381-405). The canonical structures of hypervariable loops of an immunoglobulin variable can be inferred from its sequence, as described in Chothia *et al.* (1992) *J. Mol. Biol.* 227:799-817; Tomlinson *et al.* (1992) *J. Mol. Biol.* 227:776-798); and Tomlinson *et al.* (1995) *EMBO J.* 14(18):4628-38.

As used herein, an “immunoglobulin variable domain sequence” refers to an amino acid sequence which can form the structure of an immunoglobulin variable domain. For example, the sequence may include all or part of the amino acid sequence of a naturally-occurring variable domain. For example, the sequence may omit one, two or more N- or C-terminal amino acids, internal amino acids, may include one or more insertions or additional terminal amino acids, or may include other alterations. In one embodiment, a polypeptide that includes immunoglobulin variable domain sequence can associate with another immunoglobulin variable domain sequence to form a target binding structure (or “antigen binding site”), e.g., a structure that interacts with Tie1, e.g., binds to or inhibits Tie1.

The VH or VL chain of the antibody can further include all or part of a heavy or light chain constant region, to thereby form a heavy or light immunoglobulin chain, respectively. In one embodiment, the antibody is a tetramer of two heavy immunoglobulin chains and two light immunoglobulin chains, wherein the heavy and light immunoglobulin chains are inter-connected by, e.g., disulfide bonds. The heavy chain constant region includes three domains, CH1, CH2 and CH3. The light chain constant region includes a CL domain. The variable region of the heavy and light chains contains a binding domain that interacts with an antigen. The constant regions of the antibodies typically mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system. The term "antibody" includes intact immunoglobulins of types IgA, IgG, IgE, IgD, IgM (as well as subtypes thereof). The light chains of the immunoglobulin may be of types kappa or lambda. In one embodiment, the antibody is glycosylated. An antibody can be functional for antibody-dependent cytotoxicity and/or complement-mediated cytotoxicity.

One or more regions of an antibody can be human or effectively human. For example, one or more of the variable regions can be human or effectively human. For example, one or more of the CDRs can be human, e.g., HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, and LC CDR3. Each of the light chain CDRs can be human. HC CDR3 can be human. One or more of the framework regions can be human, e.g., FR1, FR2, FR3, and FR4 of the HC or LC. In one embodiment, all the framework regions are human, e.g., derived from a human somatic cell, e.g., a hematopoietic cell that produces immunoglobulins or a non-hematopoietic cell. In one embodiment, the human sequences are germline sequences, e.g., encoded by a germline nucleic acid. One or more of the constant regions can be human or effectively human. In another embodiment, at least 70, 75, 80, 85, 90, 92, 95, or 98% of the framework regions (e.g., FR1, FR2, and FR3, collectively, or FR1, FR2, FR3, and FR4, collectively) or the entire antibody can be human or effectively human. For example, FR1, FR2, and FR3 collectively can be at least 70, 75, 80, 85, 90, 92, 95, 98, or 99% identical to a human sequence encoded by a human germline V segment of a locus encoding a light or heavy chain sequence.

All or part of an antibody can be encoded by an immunoglobulin gene or a segment thereof. Exemplary human immunoglobulin genes include the kappa, lambda,

alpha (IgA1 and IgA2), gamma (IgG1, IgG2, IgG3, IgG4), delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Full-length immunoglobulin "light chains" (about 25 Kd or 214 amino acids) are encoded by a variable region gene at the NH₂-terminus (about 110 amino acids) and a kappa or lambda constant region gene at the COOH--terminus. Full-length immunoglobulin "heavy chains" (about 50 Kd or 446 amino acids), are similarly encoded by a variable region gene (about 116 amino acids) and one of the other aforementioned constant region genes, e.g., gamma (encoding about 330 amino acids).

The term "antigen-binding fragment" of a full length antibody (or simply "antibody portion," or "fragment"), as used herein, refers to one or more fragments of a full-length antibody that retain the ability to specifically bind to a target of interest. Examples of binding fragments encompassed within the term "antigen-binding fragment" of a full length antibody include (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CH1 domains; (ii) a F(ab')₂ fragment, a bivalent fragment including two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR) that retains functionality. Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules known as single chain Fv (scFv). See *e.g.*, Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883.

Antibody fragments can be obtained using any appropriate technique including conventional techniques known to those with skill in the art. The term "monospecific antibody" refers to an antibody that displays a single binding specificity and affinity for a particular target, e.g., epitope. This term includes a "monoclonal antibody" or "monoclonal antibody composition," which as used herein refer to a preparation of antibodies or fragments thereof of single molecular composition. As used herein, "isotype" refers to the antibody class (e.g., IgM or IgG1) that is encoded by heavy chain constant region genes.

In one embodiment, the HC or LC of an antibody includes sequences that correspond to an amino acid sequence encoded by a human germline sequence, e.g., the framework regions and/or in the CDRs. For example, the antibody can include sequences from the human DP47 antibody. In one embodiment, one or more codons for the antibody
5 are altered relative to the germline nucleic acid sequence, but are chosen to encode the same amino acid sequence. Codons can be selected, e.g., to optimize expression in a particular system, create restriction enzyme sites, create a silent fingerprint, etc.

In one embodiment, CDR2 of the antibody HC includes at least 11, 12, 13, 14, or 15 amino acid positions that are identical to the amino acids found in CDR2 of DP47.

10 A “humanized” immunoglobulin variable region is an immunoglobulin variable region that includes sufficient number of human framework amino acid positions such that the immunoglobulin variable region does not elicit an immunogenic response in a normal human. Descriptions of “humanized” immunoglobulins include, for example, US 6,407,213 and US 5,693,762.

5 An “effectively human” immunoglobulin variable region is an immunoglobulin variable region that includes a sufficient number of human framework amino acid positions such that the immunoglobulin variable region does not elicit an immunogenic response in a normal human. An “effectively human” antibody is an antibody that includes a sufficient number of human amino acid positions such that the antibody does
0 not elicit an immunogenic response in a normal human.

The term “Tie1 ectodomain” refers to any extracellular region of a Tie1 protein, e.g., a region that includes about amino acids 25-759 of SEQ ID NO:2. Other exemplary regions are regions that include one or more EGF domains (e.g., 214-256, 258-303, 303-345, 214-303, 258-345, or 214-345 of SEQ ID NO:2); one or more IG-Like C2 domains
5 (e.g., 43-105, 43-426, 372-426); one or more Fibronectin Type III repeats (e.g., 446-540, 543-639, 643-744, 446-639, 543-744, or 446-744 of SEQ ID NO:2); and combinations thereof.

As used herein, “binding affinity” refers to the apparent association constant or K_a . The K_a is the reciprocal of the dissociation constant (K_d). A ligand may, for example,
) have a binding affinity of at least 10^5 , 10^6 , 10^7 or 10^8 M^{-1} for a particular target molecule. Higher affinity binding of a ligand to a first target relative to a second target can be indicated by a higher K_a (or a smaller numerical value K_d) for binding the first target than

the K_a (or numerical value K_d) for binding the second target. In such cases the ligand has specificity for the first target relative to the second target. Differences in binding affinity (e.g., for specificity or other comparisons) can be at least 1.5, 2, 5, 10, 50, 100, or 1000-fold. For example, a Tie1 binding ligand may preferentially bind to Tie at least 1.5, 2, 5, 10, 50, 100, or 1000-fold better than to another antigen, e.g., Tie2, EGF, fibronectin, or human serum albumin. A Tie1 binding ligand may also be species-specific or species-general (e.g., can bind to a Tie1 protein from more than one species).

Binding affinity can be determined by a variety of methods including equilibrium dialysis, equilibrium binding, gel filtration, ELISA, surface plasmon resonance, or spectroscopy (e.g., using a fluorescence assay). These techniques can be used to measure the concentration of bound and free ligand as a function of ligand (or target) concentration. The concentration of bound ligand ([Bound]) is related to the concentration of free ligand ([Free]) and the concentration of binding sites for the ligand on the target where (N) is the number of binding sites per target molecule by the following equation:

$$[\text{Bound}] = N \cdot [\text{Free}] / ((1/K_a) + [\text{Free}])$$

Although quantitative measurements of K_a are routine, it is not always necessary to make an exact determination of K_a , though, since sometimes it is sufficient to obtain a qualitative measurement of affinity, e.g., determined using a method such as ELISA or FACS analysis, is proportional to K_a , and thus can be used for comparisons, such as determining whether a higher affinity is, e.g., 2, 5, 10, 20, or 50 fold higher than a reference. Binding affinity is typically evaluated in 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005 % (v/v) surfactant P20.

An "isolated composition" refers to a composition that is removed from at least 90% of at least one component of a natural sample from which the isolated composition can be obtained. Compositions produced artificially or naturally can be "compositions of at least" a certain degree of purity if the species or population of species of interests is at least 5, 10, 25, 50, 75, 80, 90, 95, 98, or 99% pure on a weight-weight basis.

An "epitope" refers to the site on a target compound that is bound by a ligand, e.g., a polypeptide ligand or an antigen-binding ligand (e.g., a Fab or antibody). In the case where the target compound is a protein, for example, an epitope may refer to the amino acids that are bound by the ligand. Overlapping epitopes include at least one common amino acid residue.

As used herein, the term “substantially identical” (or “substantially homologous”) is used herein to refer to a first amino acid or nucleotide sequence that contains a sufficient number of identical or equivalent (e.g., with a similar side chain, e.g., conserved amino acid substitutions) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have similar activities. In the case of antibodies, the second antibody has the same specificity and has at least 50% of the affinity of the same.

Sequences similar or homologous (e.g., at least about 85% sequence identity) to the sequences disclosed herein are also part of this application. In some embodiment, the sequence identity can be about 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or higher. Alternatively, substantial identity exists when the nucleic acid segments will hybridize under selective hybridization conditions (e.g., highly stringent hybridization conditions), to the complement of the strand. The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form.

Calculations of “homology” or “sequence identity” between two sequences (the terms are used interchangeably herein) are performed as follows. The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid “identity” is equivalent to amino acid or nucleic acid “homology”). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch ((1970) *J. Mol. Biol.* 48:444-453) algorithm which has been
5 incorporated into the GAP program in the GCG software package, using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or
10 80 and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that should be used if the practitioner is uncertain about what parameters should be applied to determine if a molecule is within a sequence identity or homology limitation of the invention) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

15 As used herein, the term "homologous" is synonymous with "similarity" and means that a sequence of interest differs from a reference sequence by the presence of one or more amino acid substitutions (although modest amino acid insertions or deletions) may also be present. Presently preferred means of calculating degrees of homology or similarity to a reference sequence are through the use of BLAST algorithms (available
20 from the National Center of Biotechnology Information (NCBI), National Institutes of Health, Bethesda MD), in each case, using the algorithm default or recommended parameters for determining significance of calculated sequence relatedness. The percent identity between two amino acid or nucleotide sequences can also be determined using the algorithm of E. Meyers and W. Miller ((1989) *CABIOS*, 4:11-17) which has been
25 incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

As used herein, the term "hybridizes under low stringency, medium stringency, high stringency, or very high stringency conditions" describes conditions for hybridization and washing. Guidance for performing hybridization reactions can be found in *Current*
0 *Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Aqueous and nonaqueous methods are described in that reference and either can be used. Specific hybridization conditions referred to herein are as follows: 1) low stringency hybridization

conditions in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by two washes in 0.2X SSC, 0.1% SDS at least at 50°C (the temperature of the washes can be increased to 55°C for low stringency conditions); 2) medium stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60°C; 3) high stringency hybridization conditions in 6X SSC at about 45°C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65°C; and 4) very high stringency hybridization conditions are 0.5M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2X SSC, 1% SDS at 65°C.

It is understood that the ligands described herein may have mutations relative to a ligand described herein (e.g., a conservative or non-essential amino acid substitutions), which do not have a substantial effect on the polypeptide functions. Whether or not a particular substitution will be tolerated, i.e., will not adversely affect desired biological properties, such as binding activity can be determined as described in Bowie, et al. (1990) *Science* 247:1306-1310. 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). It is possible for framework and CDR amino acid residues to include one or more conservative substitutions.

A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of the binding agent, e.g., the antibody, without abolishing or more preferably, without substantially altering a biological activity, whereas an "essential" amino acid residue results in such a change.

Generally, where "X" is used to represent an amino acid residue, any amino acid (e.g., any of the twenty naturally occurring amino acids) can be used at that position, or at least a subset thereof (e.g., any of the nineteen non-cysteine amino acids).

The terms "polypeptide" or "peptide" (which may be used interchangeably) refer to a polymer of three or more amino acids linked by a peptide bond, e.g., between 3 and

30, 12 and 60, or 30 and 300, or over 300 amino acids in length. The polypeptide may include one or more unnatural amino acids. Typically, the polypeptide includes only natural amino acids. A "protein" can include one or more polypeptide chains.

Accordingly, the term "protein" encompasses polypeptides. A protein or polypeptide can also include one or more modifications, e.g., a glycosylation, amidation, phosphorylation, and so forth. The term "small peptide" can be used to describe a polypeptide that is between 3 and 30 amino acids in length, e.g., between 8 and 24 amino acids in length.

Statistical significance can be determined by any art known method. Exemplary statistical tests include: the Students T-test, Mann Whitney U non-parametric test, and Wilcoxon non-parametric statistical test. Some statistically significant relationships have a P value of less than 0.05, or 0.02. Particular ligands may show a difference, e.g., in specificity or binding, that are statistically significant (e.g., P value < 0.05 or 0.02).

Other features and advantages of the instant invention will become more apparent from the following detailed description and claims. Embodiments of the invention can include any combination of features described herein. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates a bivariate FACS plot showing labelling with the platelet specific marker CD42 with Tie1 and labelling with the E3 antibody. Only a background number of CD42 positive cells are labelled by the E3 antibody.

FIGs. 2A, 2B, 3A, and 3B are plots of the number of branching points versus antibody concentration comparing germlined E3 (3A and 3B) with parental E3 (2A and 2B)

DETAILED DESCRIPTION

This disclosure provides, inter alia, methods for identifying proteins that bind to Tie1 and Tie1 binding proteins. The identified protein may be, for example, a small peptide (e.g., a cyclic or linear peptide, e.g., of between 7 and 25 amino acids), a polypeptide (e.g., a polypeptide of at least 20 amino acids), or a multi-chain protein (e.g.,

including at least two peptides or polypeptides). An example of a multi-chain protein is an antibody that has separate heavy and light chains. An example of a polypeptide is a single chain antibody.

Tie1 is a receptor tyrosine kinase protein that includes a transmembrane domain.

- 5 Tie1 is present almost exclusively on endothelial cells. Accordingly, a Tie1 binding ligand can be used, e.g., to specifically recognize or target an endothelial cell. Some Tie1 binding ligands can also be used to agonize or antagonize endothelial cells. In some embodiments, these Tie1-binding ligands have an affinity for particular structural features (e.g., a feature listed below), a combination of features listed below, and/or an epitope that
- 10 includes at least one amino acid in a structural feature listed below:

Key	From	To	Length	Description
SIGNAL	1	24	24	POTENTIAL.
CHAIN	25	1138	1114	TYROSINE-PROTEIN KINASE RECEPTOR TIE1.
DOMAIN	25	759	735	EXTRACELLULAR (POTENTIAL) .
TRANSMEM	760	784	25	POTENTIAL.
DOMAIN	785	1138	354	CYTOPLASMIC (POTENTIAL) .
DOMAIN	43	105	63	IG-LIKE C2-TYPE 1.
DOMAIN	214	256	43	EGF-LIKE 1.
DOMAIN	258	303	46	EGF-LIKE 2.
DOMAIN	305	345	41	EGF-LIKE 3.
DOMAIN	372	426	55	IG-LIKE C2-TYPE 2.
DOMAIN	446	540	95	FIBRONECTIN TYPE-III 1.
DOMAIN	543	639	97	FIBRONECTIN TYPE-III 2.
DOMAIN	643	744	102	FIBRONECTIN TYPE-III 3.
DOMAIN	839	1118	280	PROTEIN KINASE.
NP_BIND	845	853	9	ATP (BY SIMILARITY) .
BINDING	870	870		ATP (BY SIMILARITY) .
ACT_SITE	979	979		BY SIMILARITY.
CARBOHYD	83	83		N-LINKED (GLCNAC...) (POTENTIAL) .
CARBOHYD	161	161		N-LINKED (GLCNAC...) (POTENTIAL) .
CARBOHYD	503	503		N-LINKED (GLCNAC...) (POTENTIAL) .
CARBOHYD	596	596		N-LINKED (GLCNAC...) (POTENTIAL) .

CARBOHYD	709	709	N-LINKED (GLCNAC...) (<i>POTENTIAL</i>).
MOD_RES	1007	1007	PHOSPHORYLATION (AUTO-) (<i>BY SIMILARITY</i>).

The sequence is relative to the amino acid sequence provided in SEQ ID NO:2 (Example 1, below).

5 One method for identifying proteins that bind to Tie1 includes: providing a library and selecting from the library one or more members that encode a protein that binds to the Tie1 antigen or a fragment thereof (e.g., the extracellular domain, an EGF domain, a fibronectin repeat, or an Ig-superfamily domain (e.g., a Ig-like C2-type 2 domain). The selection can be performed in a number of ways. For example, the library can be a display
10 library.

The Tie1 can be tagged and recombinantly expressed. The Tie1 is purified and attached to a support, e.g., to affinity beads, or paramagnetic beads or other magnetically responsive particles.

The Tie1 can also be expressed on the surface of a cell. Members of the display
15 library that specifically bind to the cell, e.g., only if the Tie1 is activated, can be selected.

Tie1 Agonists and Antagonists

In one embodiment, a Tie1-binding ligand can modulate a Tie1 activity. For example, the Tie1-binding ligand is a Tie1 agonist or antagonist.

Tie1 agonists can be used, e.g., to stimulate an activity of an endothelial cell. For
20 example, a Tie1 agonist can be used to increase phosphatidylinositol 3-kinase (PI3 kinase) activity in an endothelial cell and/or Akt kinase activity. Kontos et al. suggest that the cytoplasmic domain of Tie1 can associate with the p85 subunit of PI3 kinase and activate PI3 kinase activity. Kontos (2002) Mol. Cell. Biol. 22:1704-1713. The Tie cytoplasmic domain may also associate with a protein tyrosine phosphatase Shp2. See,
5 e.g., Marron et al. (2000) Adv. Exp. Med. Biol. 476:35-46. A Tie1 agonist may also increase dimerization, and/or tyrosine phosphorylation (e.g., as a result of auto-phosphorylation) of the Tie1 cytoplasmic domain, e.g., the tyrosine in the motif YVN at about amino acid 1117.

Tie1-binding ligand can be evaluated for agonist activity in a cell assay (e.g., in the
0 BaF3 cell assay as described below in Example 2). An exemplary cell assay uses a growth

factor dependent cell in which a chimeric receptor that includes the Tie1 ectodomain fused to the intracellular domain of the growth factor receptor is expressed. Cells are evaluated for ability to grow in the absence of the essential growth factor, but in the presence of a test compound, e.g., a Tie1-binding ligand. If the Tie1-binding ligand agonizes Tie1, a signalling activity of the Tie1 chimera can substitute for stimulation by the required growth factor thorough its cognate receptor. Thus, survival of the cell in the absence of the required growth factor can be used as an indication that the Tie1-binding ligand agonizes Tie1.

Tie1 antagonists can be used, e.g., to reduce an activity of an endothelial cell. For example, a Tie1 antagonists can be used to decrease phosphatidylinositol 3-kinase (PI3 kinase) activity in an endothelial cell, Shp2 activity, and/or Akt kinase activity. A Tie1 antagonists may also reduce dimerization, and/or tyrosine phosphorylation (e.g., as a result of auto-phosphorylation) of the Tie1 cytoplasmic domain, e.g., the tyrosine in the motif YVN at about amino acid 1117.

Tie1-binding ligand can be evaluated for antagonist activity in a cell assay. For example, the antagonist can be assayed for ability to prevent a Tie1 agonist (such as the E3 antibody) to stimulate Tie1 in a cell assay described herein. (e.g., the BaF3 cell assay as described below in Example 2).

Display Libraries

A number of methods can be used to identify proteins that bind to Tie1. In one embodiment, a display library is used to identify proteins that bind to Tie1. A display library is a collection of entities; each entity includes an accessible protein component and a recoverable component that encodes or identifies the protein component. The protein component can be of any length, e.g. from three amino acids to over 300 amino acids. In a selection, the protein component of each member of the library is probed with Tie1 protein and if the protein component binds to Tie1, the display library member is identified, e.g., by retention on a support.

Retained display library members are recovered from the support and analyzed. The analysis can include amplification and a subsequent selection under similar or dissimilar conditions. For example, positive and negative selections can be alternated.

The analysis can also include determining the amino acid sequence of the protein component and purification of the protein component for detailed characterization.

A variety of formats can be used for display libraries. Examples include the following.

- 5 **Phage Display.** One format utilizes viruses, particularly bacteriophages. This format is termed "phage display." The protein component is typically covalently linked to a bacteriophage coat protein. The linkage results from translation of a nucleic acid encoding the protein component fused to the coat protein. The linkage can include a flexible peptide linker, a protease site, or an amino acid incorporated as a result of suppression of a stop codon. Phage display is described, for example, in Ladner *et al.*, U.S. Patent No. 5,223,409; Smith (1985) *Science* 228:1315-1317; WO 92/18619; WO 91/17271; WO 92/20791; WO 92/15679; WO 93/01288; WO 92/01047; WO 92/09690; WO 90/02809; de Haard *et al.* (1999) *J. Biol. Chem* 274:18218-30; Hoogenboom *et al.* (1998) *Immunotechnology* 4:1-20; Hoogenboom *et al.* (2000) *Immunol Today* 2:371-8; 10 Fuchs *et al.* (1991) *Bio/Technology* 9:1370-1372; Hay *et al.* (1992) *Hum Antibod Hybridomas* 3:81-85; Huse *et al.* (1989) *Science* 246:1275-1281; Griffiths *et al.* (1993) *EMBO J* 12:725-734; Hawkins *et al.* (1992) *J Mol Biol* 226:889-896; Clackson *et al.* (1991) *Nature* 352:624-628; Gram *et al.* (1992) *PNAS* 89:3576-3580; Garrard *et al.* (1991) *Bio/Technology* 9:1373-1377; Rebar *et al.* (1996) *Methods Enzymol.* 267:129-49; 15 Hoogenboom *et al.* (1991) *Nuc Acid Res* 19:4133-4137; and Barbas *et al.* (1991) *PNAS* 88:7978-7982.

Phage display systems have been developed for filamentous phage (phage f1, fd, and M13) as well as other bacteriophage (e.g. T7 bacteriophage and lambdoid phages; see, e.g., Santini (1998) *J. Mol. Biol.* 282:125-135; Rosenberg *et al.* (1996) *Innovations* 25 6:1-6; Houshmet *et al.* (1999) *Anal Biochem* 268:363-370). The filamentous phage display systems typically use fusions to a minor coat protein, such as gene III protein, and gene VIII protein, a major coat protein, but fusions to other coat proteins such as gene VI protein, gene VII protein, gene IX protein, or domains thereof can also been used (see, e.g., WO 00/71694). In one embodiment, the fusion is to a domain of the gene III protein, 30 e.g., the anchor domain or "stump," (see, e.g., U.S. Patent No. 5,658,727 for a description of the gene III protein anchor domain). It is also possible to physically associate the protein being displayed to the coat using a non-peptide linkage, e.g., a non-covalent bond

or a non-peptide covalent bond. For example, a disulfide bond and/or c-fos and c-jun coiled-coils can be used for physical associations (see, e.g., Cramer *et al.* (1993) *Gene* 137:69 and WO 01/05950).

The valency of the protein component can also be controlled. Cloning of the
5 sequence encoding the protein component into the complete phage genome results in multivariant display since all replicates of the gene III protein are fused to the protein component. For reduced valency, a phagemid system can be utilized. In this system, the nucleic acid encoding the protein component fused to gene III is provided on a plasmid, typically of length less than 7000 nucleotides. The plasmid includes a phage origin of
0 replication so that the plasmid is incorporated into bacteriophage particles when bacterial cells bearing the plasmid are infected with helper phage, e.g. M13K01. The helper phage provides an intact copy of gene III and other phage genes required for phage replication and assembly. The helper phage has a defective origin such that the helper phage genome is not efficiently incorporated into phage particles relative to the plasmid that has a wild
5 type origin.

Bacteriophage displaying the protein component can be grown and harvested using standard phage preparatory methods, e.g. PEG precipitation from growth media.

After selection of individual display phages, the nucleic acid encoding the selected protein components, by infecting cells using the selected phages. Individual colonies or plaques can be picked, the nucleic acid isolated and sequenced.

Cell-based Display. In still another format the library is a cell-display library. Proteins are displayed on the surface of a cell, e.g., a eukaryotic or prokaryotic cell. Exemplary prokaryotic cells include *E. coli* cells, *B. subtilis* cells, and spores (see, e.g., Lu *et al.* (1995) *Biotechnology* 13:366). Exemplary eukaryotic cells include yeast (e.g., *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Hansenula*, or *Pichia pastoris*). Yeast surface display is described, e.g., in Boder and Wittrup (1997) *Nat. Biotechnol.* 15:553-557 and WO 03/029456, which describes a yeast display system that can be used to display immunoglobulin proteins such as Fab fragments and the use of mating to generate combinations of heavy and light chains.

In one embodiment, variegate nucleic acid sequences are cloned into a vector for yeast display. The cloning joins the variegated sequence with a domain (or complete) yeast cell surface protein, e.g., Aga2, Aga1, Flo1, or Gas1. A domain of these proteins

can anchor the polypeptide encoded by the variegated nucleic acid sequence by a transmembrane domain (e.g., Flo1) or by covalent linkage to the phospholipid bilayer (e.g., Gas1). The vector can be configured to express two polypeptide chains on the cell surface such that one of the chains is linked to the yeast cell surface protein. For example, the two chains can be immunoglobulin chains.

In one embodiment, nucleic acids encoding immunoglobulin heavy chains that have been mutagenized based on an initial Tie1-binding immunoglobulin are introduced into yeast cells of one cell type, and nucleic acids encoding immunoglobulin light chains that have been mutagenized based on an initial Tie1-binding immunoglobulin are introduced into yeast cells of the other cell type. These two populations of cells can be combined to form diploid yeast that each express an immunoglobulin heavy and light chain. The yeast cells can be selected and/or screened for cells that bind to Tie1, e.g., bind with improved affinity.

Ribosome Display. RNA and the polypeptide encoded by the RNA can be physically associated by stabilizing ribosomes that are translating the RNA and have the nascent polypeptide still attached. Typically, high divalent Mg^{2+} concentrations and low temperature are used. See, e.g., Mattheakis *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:9022 and Hanes *et al.* (2000) *Nat Biotechnol.* 18:1287-92; Hanes *et al.* (2000) *Methods Enzymol.* 328:404-30; and Schaffitzel *et al.* (1999) *J Immunol Methods.* 231(1-2):119-35.

Polypeptide-Nucleic Acid Fusions. Another format utilizes polypeptide-nucleic acid fusions. Polypeptide-nucleic acid fusions can be generated by the in vitro translation of mRNA that include a covalently attached puromycin group, e.g., as described in Roberts and Szostak (1997) *Proc. Natl. Acad. Sci. USA* 94:12297-12302, and U.S. Patent No. 6,207,446. The mRNA can then be reverse transcribed into DNA and crosslinked to the polypeptide.

Other Display Formats. Yet another display format is a non-biological display in which the protein component is attached to a non-nucleic acid tag that identifies the polypeptide. For example, the tag can be a chemical tag attached to a bead that displays the polypeptide or a radiofrequency tag (see, e.g., U.S. Patent No. 5,874,214).

Display technology can also be used to obtain ligands, e.g., antibody ligands, particular epitopes of a target. This can be done, for example, by using competing non-target molecules that lack the particular epitope or are mutated within the epitope, e.g.,

with alanine. Such non-target molecules can be used in a negative selection procedure as described below, as competing molecules when binding a display library to the target, or as a pre-elution agent, e.g., to capture in a wash solution dissociating display library members that are not specific to the target.

5 **Iterative Selection.** In one preferred embodiment, display library technology is used in an iterative mode. A first display library is used to identify one or more ligands for a target. These identified ligands are then varied, e.g., using a mutagenesis method, to form a second display library. Higher affinity ligands are then selected from the second library, e.g., by using higher stringency or more competitive binding and washing
10 conditions.

In some implementations, the mutagenesis is targeted to regions known or likely to be at the binding interface. If, for example, the identified ligands are antibodies, then mutagenesis can be directed to the CDR regions of the heavy or light chains as described herein. Further, mutagenesis can be directed to framework regions near or adjacent to the
15 CDRs, e.g., framework regions, particular within ten, five, or three amino acids of a CDR junction.. In the case of antibodies, mutagenesis can also be limited to one or a few of the CDRs, e.g., to make precise step-wise improvements.

Some exemplary mutagenesis techniques include: error-prone PCR (Leung *et al.* (1989) *Technique* 1:11-15), recombination (see, e.g., USSN 10/279,633), DNA shuffling
20 using random cleavage (Stemmer (1994) *Nature* 389-391; termed “nucleic acid shuffling”), RACHITT™ (Coco *et al.* (2001) *Nature Biotech.* 19:354), site-directed mutagenesis (Zoller *et al.* (1987) *Nucl Acids Res* 10:6487-6504), cassette mutagenesis (Reidhaar-Olson (1991) *Methods Enzymol.* 208:564-586) and incorporation of degenerate oligonucleotides (Griffiths *et al.* (1994) *EMBO J* 13:3245).

25 In one example of iterative selection, the methods described herein are used to first identify a protein ligand from a display library that binds a Tie1 with at least a minimal binding specificity for a target or a minimal activity, e.g., an equilibrium dissociation constant for binding of greater than 1 nM, 10 nM, or 100 nM. The nucleic acid sequence encoding the initial identified protein ligand are used as a template nucleic acid for the
30 introduction of variations, e.g., to identify a second protein ligand that has enhanced properties (e.g., binding affinity, kinetics, or stability) relative to the initial protein ligand.

Off-Rate Selection. Since a slow dissociation rate can be predictive of high affinity, particularly with respect to interactions between polypeptides and their targets, the methods described herein can be used to isolate ligands with a desired kinetic dissociation rate (i.e. reduced) for a binding interaction to a target.

- 5 To select for slow dissociating ligands from a display library, the library is contacted to an immobilized target. The immobilized target is then washed with a first solution that removes non-specifically or weakly bound biomolecules. Then the immobilized target is eluted with a second solution that includes a saturation amount of free target, i.e., replicates of the target that are not attached to the particle. The free target
0 binds to biomolecules that dissociate from the target. Rebinding is effectively prevented by the saturating amount of free target relative to the much lower concentration of immobilized target.

- The second solution can have solution conditions that are substantially physiological or that are stringent. Typically, the solution conditions of the second
5 solution are identical to the solution conditions of the first solution. Fractions of the second solution are collected in temporal order to distinguish early from late fractions. Later fractions include biomolecules that dissociate at a slower rate from the target than biomolecules in the early fractions.

Further, it is also possible to recover display library members that remain bound to the target even after extended incubation. These can either be dissociated using chaotropic conditions or can be amplified while attached to the target. For example, phage bound to the target can be contacted to bacterial cells.

Selecting and Screening for Specificity. "Selection" refers to a process in which many members of a display library are allowed to contact the target and those that bind are recovered and propagated. The selection can be from a library having numerous members, e.g., more than 10^{10} members. "Screening" refers to a process in which isolated members of the library are tested singly for binding to the target. Through automation, thousands of candidates may be screened in a highly parallel process. The display library selection methods described herein can include a selection process that discards display library members that bind to a non-target molecule. Examples of non-target molecules include, e.g., extracellular domains of molecules that include an immunoglobulin super-family domain or an EGF domain and receptor tyrosine kinases other than Tie1, e.g., Tie2. In

one implementation, a so-called “negative selection” step is used to discriminate between the target and related non-target molecule and a related, but distinct non-target molecules. The display library or a pool thereof is contacted to the non-target molecule. Members of the sample that do not bind the non-target are collected and used in subsequent selections for binding to the target molecule or even for subsequent negative selections. The negative selection step can be prior to or after selecting library members that bind to the target molecule.

In another implementation, a screening step is used. After display library members are isolated for binding to the target molecule, each isolated library member is tested for its ability to bind to a non-target molecule (e.g., a non-target listed above). For example, a high-throughput ELISA screen can be used to obtain this data. The ELISA screen can also be used to obtain quantitative data for binding of each library member to the target. The non-target and target binding data are compared (e.g., using a computer and software) to identify library members that specifically bind to Tie1.

The display library selection and screening methods described herein can include a selection or screening process that selects for display library members that bind to specific sites on the target molecule. For example, elution with high concentration of an antibody described herein can be used to select for phage that bind to an epitope that is near or overlaps with the epitope bound by the antibody used for elution. Accordingly, one can screen for a phage that binds to the E3-binding site of Tie1 by performing ELISAs with and without E3 antibody in the buffer.

Selection and screening for Tie1-binding antibodies:

The following provides one exemplary method for identifying antibodies that bind to Tie1 using a phagemid Fab library. For example, three rounds of selection can be performed with decreasing amounts of target protein (e.g., 100, 50 and 50 μ g for first, second, and third rounds, respectively). The target is immobilized on streptavidin coated magnetic beads (Dynal). The library is depleted against streptavidin coated magnetic beads prior to each round of selection and optionally against an unrelated protein which may include a common purification handle. For example, if the target is produced as a fusion to a Fc domain, the library can be depleted against soluble Trail-Fc (a commercially available Fc fusion protein). The depletion process removes Fc binders.

Each round of selection can include, e.g., two cycles of streptavidin magnetic bead depletion, a cycle of binding of phage to Tie1-coated beads, ten cycles of washes, elution of bound phage, and propagation of enriched phage for the next round. Phage bound to Tie1-coated beads after ten washes can be directly amplified or eluted before

5 amplification. After three rounds of selection, individual clones may be grown in 96-well microtiter plates and individually screened for Tie1 binding activity by phage ELISA.

ELISAs can include evaluations of binding to Tie1, specificity controls, and unrelated controls. Isolates can be DNA fingerprinted to determine the diversity emerging from the selection process. For example, positive isolates can be PCR amplified with the

10 oligonucleotide primers M13-reverse and geneIII-forward (see, e.g., Marks *et al.* (1991), *J. Mol. Biol.* 222:581). The products can be analyzed by *Bst*NI fingerprinting.

An exemplary method for performing ELISA's with phage that display a ligand is as follows. Individual clones can be grown and rescued as described previously (Marks *et al.* (1991), *J. Mol. Biol.* 222:581). For ELISAs, 96-well Immulon 2 HB plates (Thermo

15 Labsystems) are coated with 1 µg/well ImmunoPure™ streptavidin (Pierce) in PBS and incubated overnight at 4°C. After three washes with PBS, 100 µL of biotinylated Tie1 protein is allowed to bind to the immobilized streptavidin for 30-60 minutes at room temperature. Then, Tie1-coated wells are blocked with 300 µL of 2% milk/1x PBS/0.05% Tween (2% MPBST) for two hours at 37°C. The wells are incubated with 100 µL of

20 phage culture supernatant that had been blocked with 2% MPBST for one hour at room temperature. The wells are washed five times with 1xPBS/Tween 0.1% (PBST), and incubated with 100 µL of anti-M13-HRP secondary antibody at a 1:5,000 dilution for one hour at room temperature. The wells are washed five times with PBST before developing with TMB-solution and read at 630 nm.

25 For the cell ELISAs, cells are washed once in PBS and resuspended at a concentration of 1×10^6 to 2×10^6 cells/mL of PBS. A final concentration of $1-2 \times 10^5$ cells per well of a 96-well tissue culture plate (Falcon, VWR) can be used. The cells are fixed by adding an equal volume of 0.2% glutaraldehyde (Sigma-Aldrich) and incubating at 37°C for 12 minutes. They are then washed three times with PBS using an automated

30 plate washer (Bio-Tek Instruments, Inc.) and blocked with 200 µL of 2% MPBST for one hour at room temperature. The rest of the ELISA procedure can be performed as described above except that 1xPBS/Tween 0.05% is used for the washes and incubations.

Germlining Antibodies

It is possible to modify an antibody that binds Tie1, e.g., an antibody described herein, in order to make the variable regions of the antibody more similar to one or more
5 germline sequences. For example, an antibody can include one, two, three or more amino acid substitutions, e.g., in a framework or CDR region, to make it more similar to a reference germline sequence. One exemplary germlining method can include: identifying one or more germline sequences that are similar (e.g., most similar in a particular
0 database) to the sequence of the isolated antibody. Then mutations (at the amino acid level) can be made in the isolated antibody, either incrementally, in combination, or both. For example, a nucleic acid library that includes sequences encoding some or all possible germline mutations is made. The mutated antibodies are then evaluated, e.g., to identify an antibody that has one or more additional germline residues relative to the isolated antibody and that is still useful (e.g., has a functional activity). In one embodiment, as
5 many germline residues are introduced into an isolated antibody as possible.

In one embodiment, mutagenesis is used to substitute or insert one or more germline residues into a CDR region. For example, the germline CDR residue can be from a germline sequence that is similar (e.g., most similar) to the variable region being modified. After mutagenesis, activity (e.g., binding or other functional activity) of the antibody can be evaluated to determine if the germline residue or residues are tolerated. Similar mutagenesis can be performed in the framework regions.

Selecting a germline sequence can be performed in different ways. For example, a germline sequence can be selected if it meets a predetermined criteria for selectivity or similarity, e.g., at least a certain percentage identity, e.g., at least 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 99.5% identity. The selection can be performed using at least 2, 3, 5, or 10 germline sequences. In the case of CDR1 and CDR2, identifying a similar germline sequence can include selecting one such sequence. In the case of CDR3, identifying a similar germline sequence can include selecting one such sequence, but may including using two germline sequences that separately contribute to the amino-terminal portion and the carboxy-terminal portion. In other implementations more than one or two germline sequences are used, e.g., to form a consensus sequence.

In one embodiment, with respect to a particular reference variable domain sequence, e.g., a sequence described herein, a related variable domain sequence has at least 30, 40, 50, 60, 70, 80, 90, 95 or 100% of the CDR amino acid positions that are not identical to residues in the reference CDR sequences, residues that are identical to residues at corresponding positions in a human germline sequence (i.e., an amino acid sequence encoded by a human germline nucleic acid).

In one embodiment, with respect to a particular reference variable domain sequence, e.g., a sequence described herein, a related variable domain sequence has at least 30, 50, 60, 70, 80, 90 or 100% of the FR regions are identical to FR sequence from a human germline sequence, e.g., a germline sequence related to the reference variable domain sequence.

Accordingly, it is possible to isolate an antibody which has similar activity to a given antibody of interest, but is more similar to one or more germline sequences, particularly one or more human germline sequences. For example, an antibody can be at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.5% identical to a germline sequence in a region outside the CDRs (e.g., framework regions). Further an antibody can include at least 1, 2, 3, 4, or 5 germline residues in a CDR region, the germline residue being from a germline sequence of similar (e.g., most similar) to the variable region being modified. Germline sequences of primary interest are human germline sequences. The activity of the antibody (e.g., the binding activity) can be within a factor of 100, 10, 5, 2, 0.5, 0.1, and 0.001 of the original antibody.

Exemplary germline reference sequences for V κ include: O12/O2, O18/O8, A20, A30, L14, L1, L15, L4/18a, L5/L19, L8, L23, L9, L24, L11, L12, O11/O1, A17, A1, A18, A2, A19/A3, A23, A27, A11, L2/L16, L6, L20, L25, B3, B2, A26/A10, and A14. See, e.g., Tomlinson et al. (1995) EMBO J. 14(18):4628-3.

A germline reference sequence for the HC variable domain can be based on a sequence that has particular canonical structures, e.g., 1-3 structures in the H1 and H2 hypervariable loops. The canonical structures of hypervariable loops of an immunoglobulin variable domain can be inferred from its sequence, as described in Chothia et al. (1992) *J. Mol. Biol.* 227:799-817; Tomlinson et al. (1992) *J. Mol. Biol.* 227:776-798; and Tomlinson et al. (1995) EMBO J. 14(18):4628-38. Exemplary sequences with a 1-3 structure include: DP-1, DP-8, DP-12, DP-2, DP-25, DP-15, DP-7,

DP-4, DP-31, DP-32, DP-33, DP-35, DP-40, 7-2, hv3005, hv3005f3, DP-46, DP-47, DP-58, DP-49, DP-50, DP-51, DP-53, and DP-54.

Diversity

Display libraries and other libraries include variation at one or more positions in the displayed polypeptide. The variation at a given position can be synthetic or natural. For some libraries, both synthetic and natural diversity are included.

Synthetic Diversity. Libraries can include regions of diverse nucleic acid sequence that originate from artificially synthesized sequences. Typically, these are formed from degenerate oligonucleotide populations that include a distribution of nucleotides at each given position. The inclusion of a given sequence is random with respect to the distribution. One example of a degenerate source of synthetic diversity is an oligonucleotide that includes NNN wherein N is any of the four nucleotides in equal proportion.

Synthetic diversity can also be more constrained, e.g., to limit the number of codons in a nucleic acid sequence at a given trinucleotide to a distribution that is smaller than NNN. For example, such a distribution can be constructed using less than four nucleotides at some positions of the codon. In addition, trinucleotide addition technology can be used to further constrain the distribution.

So-called "trinucleotide addition technology" is described, e.g., in Wells *et al.* (1985) *Gene* 34:315-323, US 4,760,025 and US 5,869,644. Oligonucleotides are synthesized on a solid phase support, one codon (i.e., trinucleotide) at a time. The support includes many functional groups for synthesis such that many oligonucleotides are synthesized in parallel. The support is first exposed to a solution containing a mixture of the set of codons for the first position. The unit is protected so additional units are not added. The solution containing the first mixture is washed away and the solid support is deprotected so a second mixture containing a set of codons for a second position can be added to the attached first unit. The process is iterated to sequentially assemble multiple codons. Trinucleotide addition technology enables the synthesis of a nucleic acid that at a given position can encode a number of amino acids. The frequency of these amino acids can be regulated by the proportion of codons in the mixture. Further the choice of amino acids at the given position is not restricted to quadrants of the codon table as is the case if

mixtures of single nucleotides are added during the synthesis. Synthetic oligonucleotides including randomized or spiked codons can be also be used for producing a library for an affinity maturation selection.

Natural Diversity. Libraries can include regions of diverse nucleic acid sequence that originate (or are synthesized based on) from different naturally-occurring sequences. An example of natural diversity that can be included in a display library is the sequence diversity present in immune cells (see also below). Nucleic acids are prepared from these immune cells and are manipulated into a format for polypeptide display.

Antibody Display Libraries

In one embodiment, the display library presents a diverse pool of proteins, each of which includes an immunoglobulin domain, e.g., an immunoglobulin variable domain. Display libraries are particular useful, for example for identifying human or “humanized” antibodies that recognize human antigens. Such antibodies can be used as therapeutics to treat human disorders such as endothelial-related disorders, e.g., metastatic cancer. Since the constant and framework regions of the antibody are human, these therapeutic antibodies may avoid themselves being recognized and targeted as antigens. The constant regions are also optimized to recruit effector functions of the human immune system. The *in vitro* display selection process surmounts the inability of a normal human immune system to generate antibodies against self-antigens.

A typical antibody display library displays a polypeptide that includes a VH domain and a VL domain. An “immunoglobulin domain” refers to a domain from the variable or constant domain of immunoglobulin molecules. Immunoglobulin domains typically contain two β -sheets formed of about seven β -strands, and a conserved disulphide bond (see, e.g., A. F. Williams and A. N. Barclay 1988 *Ann. Rev Immunol.* 6:381-405). The canonical structures of hypervariable loops of an immunoglobulin variable can be inferred from its sequence, as described in Chothia et al. (1992) *J. Mol. Biol.* 227:799-817; Tomlinson et al. (1992) *J. Mol. Biol.* 227:776-798; and Tomlinson et al. (1995) *EMBO J.* 14(18):4628-38. The display library can display the antibody as a Fab fragment (e.g., using two polypeptide chains) or a single chain Fv (e.g., using a single polypeptide chain). Other formats can also be used.

As in the case of the Fab and other formats, the displayed antibody can include a constant region as part of a light or heavy chain. In one embodiment, each chain includes one constant region, e.g., as in the case of a Fab. In other embodiments, additional constant regions are displayed.

- 5 Antibody libraries can be constructed by a number of processes (see, e.g., de Haard *et al.* (1999) *J. Biol. Chem* 274:18218-30; Hoogenboom *et al.* (1998) *Immunotechnology* 4:1-20. and Hoogenboom *et al.* (2000) *Immunol Today* 21:371-8). Further, elements of each process can be combined with those of other processes. The processes can be used such that variation is introduced into a single immunoglobulin domain (e.g., VH or VL) or
- 0 into multiple immunoglobulin domains (e.g., VH and VL). The variation can be introduced into an immunoglobulin variable domain, e.g., in the region of one or more of CDR1, CDR2, CDR3, FR1, FR2, FR3, and FR4, referring to such regions of either and both of heavy and light chain variable domains. In one embodiment, variation is introduced into all three CDRs of a given variable domain. In another preferred
- 5 embodiment, the variation is introduced into CDR1 and CDR2, e.g., of a heavy chain variable domain. Any combination is feasible. In one process, antibody libraries are constructed by inserting diverse oligonucleotides that encode CDRs into the corresponding regions of the nucleic acid. The oligonucleotides can be synthesized using monomeric nucleotides or trinucleotides. For example, Knappik *et al.* (2000) *J. Mol. Biol.* 296:57-86
- 1 describe a method for constructing CDR encoding oligonucleotides using trinucleotide synthesis and a template with engineered restriction sites for accepting the oligonucleotides.

In another process, an animal, e.g., a non-human animal, e.g., a rodent, is immunized with the Tie1. The animal is optionally boosted with the antigen to further stimulate the response. Then spleen cells are isolated from the animal, and nucleic acid encoding VH and/or VL domains is amplified and cloned for expression in the display library. The non-human animal can include one or more human immunoglobulin gene sequences. For example, the animal can include a complete human immunoglobulin locus. The animal may also have an inactivated endogenous immunoglobulin locus.

In yet another process, antibody libraries are constructed from nucleic acid amplified from naïve germline immunoglobulin genes (e.g., human genes). The amplified nucleic acid includes nucleic acid encoding the VH and/or VL domain. Sources of

immunoglobulin-encoding nucleic acids are described below. Amplification can include PCR, e.g., with primers that anneal to the conserved constant region, or another amplification method.

5 Nucleic acid encoding immunoglobulin domains or fragments thereof can be obtained from the immune cells of, e.g., a human, a primate, mouse, rabbit, camel, or rodent. In one example, the cells are selected for a particular property. B cells at various stages of maturity can be selected. In another example, the B cells are naïve.

0 In one embodiment, fluorescent-activated cell sorting (FACS) is used to sort B cells that express surface-bound IgM, IgD, or IgG molecules. Further, B cells expressing different isotypes of IgG can be isolated. In another preferred embodiment, the B or T cell is cultured *in vitro*. The cells can be stimulated *in vitro*, e.g., by culturing with feeder cells or by adding mitogens or other modulatory reagents, such as antibodies to CD40, CD40 ligand or CD20, phorbol myristate acetate, bacterial lipopolysaccharide, concanavalin A, phytohemagglutinin or pokeweed mitogen.

5 In still another embodiment, the cells are isolated from a subject that has an immunological disorder, e.g., systemic lupus erythematosus (SLE), rheumatoid arthritis, vasculitis, Sjogren syndrome, systemic sclerosis, or anti-phospholipid syndrome. The subject can be a human, or an animal, e.g., an animal model for the human disease, or an animal having an analogous disorder. In yet another embodiment, the cells are isolated
) from a transgenic non-human animal that includes a human immunoglobulin locus.

In one preferred embodiment, the cells have activated a program of somatic hypermutation. Cells can be stimulated to undergo somatic mutagenesis of immunoglobulin genes, for example, by treatment with anti-immunoglobulin, anti-CD40, and anti-CD38 antibodies (see, e.g., Bergthorsdottir *et al.* (2001) *J Immunol.* 166:2228). In another embodiment, the cells are naïve.

The nucleic acid encoding an immunoglobulin variable domain can be isolated from a natural repertoire by the following exemplary method. First, RNA is isolated from the immune cell. Full length (i.e., capped) mRNAs are separated (e.g. by dephosphorylating uncapped RNAs with calf intestinal phosphatase). The cap is then removed with tobacco acid pyrophosphatase and reverse transcription is used to produce the cDNAs.

The reverse transcription of the first (antisense) strand can be done in any manner with any suitable primer. See, e.g., de Haard *et al.* (1999) *J. Biol. Chem* 274:18218-30. The primer binding region can be constant among different immunoglobulins, e.g., in order to reverse transcribe different isotypes of immunoglobulin. The primer binding
5 region can also be specific to a particular isotype of immunoglobulin. Typically, the primer is specific for a region that is 3' to a sequence encoding at least one CDR. In another embodiment, poly-dT primers may be used (and may be preferred for the heavy-chain genes).

A synthetic sequence can be ligated to the 3' end of the reverse transcribed strand.

- 0 The synthetic sequence can be used as a primer binding site for binding of the forward primer during PCR amplification after reverse transcription. The use of the synthetic sequence can obviate the need to use a pool of different forward primers to fully capture the available diversity.

The variable domain-encoding gene is then amplified, e.g., using one or more
i rounds. If multiple rounds are used, nested primers can be used for increased fidelity. The amplified nucleic acid is then cloned into a display library vector.

Any method for amplifying nucleic acid sequences may be used for amplification. Methods that maximize and do not bias diversity are preferred. A variety of techniques can be used for nucleic acid amplification. The polymerase chain reaction (PCR; U.S. Patent Nos. 4,683,195 and 4,683,202, Saiki, *et al.* (1985) *Science* 230, 1350-1354) utilizes cycles of varying temperature to drive rounds of nucleic acid synthesis. Transcription-based methods utilize RNA synthesis by RNA polymerases to amplify nucleic acid (U.S. Pat. No 6,066,457; U.S. Pat. No 6,132,997; U.S. Pat. No 5,716,785; Sarkar *et al.*, *Science* (1989) 244: 331-34 ; Stofler *et al.*, *Science* (1988) 239: 491). NASBA (U.S. Patent Nos. 5,130,238; 5,409,818; and 5,554,517) utilizes cycles of transcription, reverse-transcription, and RNaseH-based degradation to amplify a DNA sample. Still other amplification methods include rolling circle amplification (RCA; U.S. Patent Nos. 5,854,033 and 6,143,495) and strand displacement amplification (SDA; U.S. Patent Nos. 5,455,166 and 5,624,825).

Secondary Screening Methods

After selecting candidate display library members that bind to a target, each candidate display library member can be further analyzed, e.g., to further characterize its binding properties for the target. Similarly candidate ligands (e.g., by immunization, etc.)
5 obtained by other methods can also be analyzed. Each candidate ligand can be subjected to one or more secondary screening assays. The assay can be for a binding property, a catalytic property, a physiological property (e.g., cytotoxicity, renal clearance, immunogenicity), a structural property (e.g., stability, conformation, oligomerization state) or another functional property. The same assay can be used repeatedly, but with varying
10 conditions, e.g., to determine pH, ionic, or thermal sensitivities.

As appropriate, the assays can use the display library member directly, a recombinant polypeptide produced from the nucleic acid encoding a displayed polypeptide, or a synthetic peptide synthesized based on the sequence of a displayed polypeptide. Exemplary assays for binding properties include the following.

5 **ELISA.** Proteins encoded by a display library can also be screened for a binding property using an ELISA assay. For example, each protein is contacted to a microtitre plate whose bottom surface has been coated with the target, e.g., a limiting amount of the target. The plate is washed with buffer to remove non-specifically bound polypeptides. Then the amount of the protein bound to the plate is determined by probing the plate with
0 an antibody that can recognize the polypeptide, e.g., a tag or constant portion of the polypeptide. The antibody is linked to an enzyme such as alkaline phosphatase, which produces a colorimetric product when appropriate substrates are provided. The protein can be purified from cells or assayed in a display library format, e.g., as a fusion to a filamentous bacteriophage coat. Alternatively, cells (e.g., live or fixed) that express the
5 target molecule, e.g., Tie1, can be plated in a microtitre plate and used to test the affinity of the peptides/antibodies present in the display library or obtained by selection from the display library.

In another version of the ELISA assay, each polypeptide of a diversity strand library is used to coat a different well of a microtitre plate. The ELISA then proceeds
10 using a constant target molecule to query each well.

Cell Binding Assays. Tie1-binding proteins can be evaluated for their ability to interact with one or more cell types, e.g., endothelial cells or platelets. Fluorescent

activated cell sorting (FACS) is one exemplary method for testing an interaction between a protein and a cell. The Tie1 binding protein is labeled directly or indirectly with a fluorophore, before or after, binding to the cells, and then cells are counted in a FACS sorter.

5 For example, the following method can be used to evaluate whether a Tie1 binding protein interacts with platelets or other cell types.

Isolation of Platelets. Human blood can be obtained from informed healthy volunteers. For example, venous blood is collected into one-sixth volume of ACD (2.5 g of sodium citrate, 1.5 g citric acid, and 2.5 g glucose in 100 ml dH₂O). The blood is
10 centrifuged at 800×g for 15 min at room temperature and the platelet-rich plasma is removed and incubated for 60 min at 37°C. in the presence of 1 mM acetylsalicylic acid followed by centrifugation at 1000.times.g for 10 min at room temperature. The platelet pellet can be resuspended at a density of 2·10⁸ cells/ml with HEPES-buffered Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 3 mM NaH₂PO₄, 5 mM glucose, 10
15 mM HEPES pH 7.4, 0.2% bovine serum albumin, and 0.05 U/mL apyrase). See also, e.g., Kornecki et al. (1990) J Biol Chem. 265:10,042-10,048 and Naik et al. (1995) Biochem J. 310:155-162).

FACS. For FACS analysis of platelets. Cells can be resuspended in 0.1% BSA/PBS (4 × 10⁵ cells/sample) in the presence of PGE1 (1 mg/mL) and incubated with
20 the Tie1 ligand (e.g., at about 5 µg/mL) or with a control. After a 1-hour incubation at 22°C, the cells are washed with 0.1% BSA/PBS, treated with 50 µL 1/100 diluted FITC-labeled secondary antibody, incubated for 30 minutes on ice, washed, and resuspended in 0.1% BSA/PBS. The samples are analyzed using an Immunocytometry Systems flow cytometer (FAC Sort, Becton Dickinson, San Jose, CA). See also, e.g., Malgorzata et al.
25 (2000) Blood, Vol. 95 No. 8 (April 15 pp. 2600-2609).

In addition, it is possible to evaluate platelets by Westerns analysis of SDS-page separated proteins from isolated platelets and by immunoprecipitation. Still other methods involve binding cells to surfaces to which the Tie1 binding ligand is attached (e.g., coated to).

30 Other cell types can be prepared for FACS by methods known in the art.

Homogeneous Binding Assays. The binding interaction of candidate polypeptide with a target can be analyzed using a homogenous assay, i.e., after all components of the

assay are added, additional fluid manipulations are not required. For example, fluorescence resonance energy transfer (FRET) can be used as a homogenous assay (see, for example, Lakowicz *et al.*, U.S. Patent No. 5,631,169; Stavrianopoulos, *et al.*, U.S. Patent No. 4,868,103). A fluorophore label on the first molecule (e.g., the molecule
5 identified in the fraction) is selected such that its emitted fluorescent energy can be absorbed by a fluorescent label on a second molecule (e.g., the target) if the second molecule is in proximity to the first molecule. The fluorescent label on the second molecule fluoresces when it absorbs to the transferred energy. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, the
10 spatial relationship between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. A binding event that is configured for monitoring by FRET can be conveniently measured through standard fluorometric detection means well known in the art (e.g., using a fluorimeter). By titrating the amount of the first or second binding
15 molecule, a binding curve can be generated to estimate the equilibrium binding constant.

Another example of a homogenous assay is Alpha Screen (Packard Bioscience, Meriden CT). Alpha Screen uses two labeled beads. One bead generates singlet oxygen when excited by a laser. The other bead generates a light signal when singlet oxygen diffuses from the first bead and collides with it. The signal is only generated when the two
20 beads are in proximity. One bead can be attached to the display library member, the other to the target. Signals are measured to determine the extent of binding.

The homogenous assays can be performed while the candidate polypeptide is attached to the display library vehicle, e.g., a bacteriophage.

Surface Plasmon Resonance (SPR). The binding interaction of a molecule
5 isolated from a display library and a target can be analyzed using SPR. SPR or Biomolecular Interaction Analysis (BIA) detects biospecific interactions in real time, without labeling any of the interactants. Changes in the mass at the binding surface (indicative of a binding event) of the BIA chip result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)).
0 The changes in the refractivity generate a detectable signal, which are measured as an indication of real-time reactions between biological molecules. Methods for using SPR are described, for example, in U.S. Patent No. 5,641,640; Raether (1988) *Surface*

Plasmons Springer Verlag; Sjolander and Urbaniczky (1991) *Anal. Chem.* 63:2338-2345; Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705 and on-line resources provide by BIAcore International AB (Uppsala, Sweden).

Information from SPR can be used to provide an accurate and quantitative measure of the equilibrium dissociation constant (K_d), and kinetic parameters, including K_{on} and K_{off} , for the binding of a biomolecule to a target. Such data can be used to compare different biomolecules. For example, proteins encoded by nucleic acid selected from a library of diversity strands can be compared to identify individuals that have high affinity for the target or that have a slow K_{off} . This information can also be used to develop structure-activity relationships (SAR). For example, the kinetic and equilibrium binding parameters of matured versions of a parent protein can be compared to the parameters of the parent protein. Variant amino acids at given positions can be identified that correlate with particular binding parameters, e.g., high affinity and slow K_{off} . This information can be combined with structural modeling (e.g., using homology modeling, energy minimization, or structure determination by crystallography or NMR). As a result, an understanding of the physical interaction between the protein and its target can be formulated and used to guide other design processes.

Protein Arrays. Proteins identified from the display library can be immobilized on a solid support, for example, on a bead or an array. For a protein array, each of the polypeptides is immobilized at a unique address on a support. Typically, the address is a two-dimensional address. Protein arrays are described below (see, e.g., Diagnostics). It is also possible to use a protein array to evaluate any plurality of proteins, e.g., for interaction with Tie1.

Cellular Assays. Candidate proteins can be selected from a library by transforming the library into a host cell; the library could have been previously identified from a display library. For example, the library can include vector nucleic acid sequences that include segments that encode the polypeptides and that direct expression, e.g., such that the proteins are produced within the cell, secreted from the cell, or attached to the cell surface. The cells can be screened or selected for proteins that bind to the Tie1, e.g., as detected by a change in a cellular phenotype or a cell-mediated activity. For example, in the case of an antibody that binds to Tie1, the activity may be autophosphorylation,

activation of PI3 Kinase, activation of AKT, or a change in endothelial cell activity (e.g., proliferation).

In another embodiment, the library of cells is in the form of a cellular array. The cellular array can likewise be screened for any appropriate detectable activity.

- 5 In other embodiments, competition binding assays are used to identify proteins that are compete with a reference ligand for binding to Tie1. Similarly, epitope mapping can be used to identify proteins that bind to a particular epitope of Tie. Fragments and mutants of Tie1 can be also be used in the ligand-identification process, e.g., in one or more of characterization, screening, or immunization.

10 Methods for Obtaining Tie1-binding antibodies

In addition to the use of display libraries, other methods can be used to obtain a Tie1-binding antibody or in combination with the use of display libraries. For example, the Tie1 ectodomain or a region thereof can be used as an antigen in a non-human animal, e.g., a rodent.

- 5 In one embodiment, the non-human animal includes at least a part of a human immunoglobulin gene. For example, it is possible to engineer mouse strains deficient in mouse antibody production with large fragments of the human Ig loci. Using the hybridoma technology, antigen-specific Mabs derived from the genes with the desired specificity may be produced and selected. See, e.g., XenoMouse™, Green et al. Nature
0 Genetics 7:13-21 (1994), U.S. 20030070185, WO 96/34096, published Oct. 31, 1996, and PCT Application No. PCT/US96/05928, filed Apr. 29, 1996.

- In another embodiment, a monoclonal antibody is obtained from the non-human animal, and then modified, e.g., humanized or deimmunized. Winter describes a CDR-grafting method that may be used to prepare the humanized antibodies of the present
5 invention (UK Patent Application GB 2188638A, filed on March 26, 1987; Winter US 5,225,539. All of the CDRs of a particular human antibody may be replaced with at least a portion of a non-human CDR or only some of the CDRs may be replaced with non-human CDRs. It is only necessary to replace the number of CDRs required for binding of the humanized antibody to a predetermined antigen.

- 9 Humanized antibodies can be generated by replacing sequences of the Fv variable region that are not directly involved in antigen binding with equivalent sequences from

human Fv variable regions. General methods for generating humanized antibodies are provided by Morrison, S. L., 1985, *Science* 229:1202-1207, by Oi et al., 1986, *BioTechniques* 4:214, and by Queen et al. US 5,585,089, US 5,693,761 and US 5,693,762. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of immunoglobulin Fv variable regions from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from a hybridoma producing an antibody against a predetermined target, as described above. The recombinant DNA encoding the humanized antibody, or fragment thereof, can then be cloned into an appropriate expression vector.

A Tie1-binding antibody may also be modified by specific deletion of human T cell epitopes or "deimmunization" by the methods disclosed in WO 98/52976 and WO 00/34317, the contents of which are specifically incorporated by reference herein. Briefly, the heavy and light chain variable regions of an antibody can be analyzed for peptides that bind to MHC Class II; these peptides represent potential T-cell epitopes (as defined in WO 98/52976 and WO 00/34317). For detection of potential T-cell epitopes, a computer modeling approach termed "peptide threading" can be applied, and in addition a database of human MHC class II binding peptides can be searched for motifs present in the V_H and V_L sequences, as described in WO 98/52976 and WO 00/34317. These motifs bind to any of the 18 major MHC class II DR allotypes, and thus constitute potential T cell epitopes. Potential T-cell epitopes detected can be eliminated by substituting small numbers of amino acid residues in the variable regions, or preferably, by single amino acid substitutions. As far as possible conservative substitutions are made, often but not exclusively, an amino acid common at this position in human germline antibody sequences may be used. Human germline sequences are disclosed in Tomlinson, I.A. *et al.* (1992) *J. Mol. Biol.* 227:776-798; Cook, G. P. *et al.* (1995) *Immunol. Today* Vol. 16 (5): 237-242; Chothia, D. *et al.* (1992) *J. Mol. Bio.* 227:799-817. The V BASE directory provides a comprehensive directory of human immunoglobulin variable region sequences (compiled by Tomlinson, I.A. *et al.* MRC Centre for Protein Engineering, Cambridge, UK). After the deimmunizing changes are identified, nucleic acids encoding V_H and V_L can be constructed by mutagenesis or other synthetic methods (e.g., de novo synthesis, cassette replacement, and so forth). Mutagenized variable sequence can, optionally, be fused to a human constant region, e.g., human IgG1 or κ constant regions.

In some cases a potential T cell epitope will include residues which are known or predicted to be important for antibody function. For example, potential T cell epitopes are usually biased towards the CDRs. In addition, potential T cell epitopes can occur in framework residues important for antibody structure and binding. Changes to eliminate these potential epitopes will in some cases require more scrutiny, e.g., by making and testing chains with and without the change. Where possible, potential T cell epitopes that overlap the CDRs were eliminated by substitutions outside the CDRs. In some cases, an alteration within a CDR is the only option, and thus variants with and without this substitution should be tested. In other cases, the substitution required to remove a potential T cell epitope is at a residue position within the framework that might be critical for antibody binding. In these cases, variants with and without this substitution should be tested. Thus, in some cases several variant deimmunized heavy and light chain variable regions were designed and various heavy/light chain combinations tested in order to identify the optimal deimmunized antibody. The choice of the final deimmunized antibody can then be made by considering the binding affinity of the different variants in conjunction with the extent of deimmunization, i.e., the number of potential T cell epitopes remaining in the variable region. Deimmunization can be used to modify an antibody that includes a non-human sequence, e.g., a murine antibody or other non-human monoclonal antibody. Deimmunization can be used to modify an antibody isolated from a display library.

Endothelial Cell Assays

A Tie1-binding ligand or a candidate ligand can be characterized using a cellular assay, e.g., to evaluate a change in a cellular phenotype or other activity when the ligand is contacted to the cell. Typically the cell expresses a protein that includes at least part of the ectodomain of Tie. In some embodiments, the cell expresses a full-length, mature Tie1 protein.

Endothelial cell proliferation. A candidate Tie1-binding ligand can be tested for endothelial proliferation inhibiting activity using a biological activity assay such as the bovine capillary endothelial cell proliferation assay, the chick CAM assay, the mouse corneal assay, and evaluating the effect of the ligand on implanted tumors. The chick

CAM assay is described, e.g., by O'Reilly, et al. in "Angiogenic Regulation of Metastatic Growth" Cell, vol. 79 (2), Oct. 21, 1994, pp. 315-328. Briefly, three day old chicken embryos with intact yolks are separated from the egg and placed in a petri dish. After three days of incubation a methylcellulose disc containing the protein to be tested is

5 applied to the CAM of individual embryos. After 48 hours of incubation, the embryos and CAMs are observed to determine whether endothelial growth has been inhibited. The mouse corneal assay involves implanting a growth factor-containing pellet, along with another pellet containing the suspected endothelial growth inhibitor, in the cornea of a mouse and observing the pattern of capillaries that are elaborated in the cornea.

10 Angiogenesis. Angiogenesis may be assayed, e.g., using various human endothelial cell systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of
15 cells through membranes in presence or absence of angiogenesis enhancer or suppressors; and tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel™ (Becton Dickinson).

Cell adhesion. Cell adhesion assays measure adhesion of cells to purified adhesion proteins or adhesion of cells to each other, in presence or absence of candidate Tie1
20 binding ligands. Cell-protein adhesion assays measure the ability of agents to modulate the adhesion of cells to purified proteins. For example, recombinant proteins are produced, diluted to 2.5 g/mL in PBS, and used to coat the wells of a microtiter plate. The wells used for negative control are not coated. Coated wells are then washed, blocked with 1% BSA, and washed again. Compounds are diluted to 2.times. final test concentration and added to
5 the blocked, coated wells. Cells are then added to the wells, and the unbound cells are washed off. Retained cells are labeled directly on the plate by adding a membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

Cell-cell adhesion assays can be used to measure the ability of candidate Tie1
3 binding ligands to modulate binding of cells to each other. These assays can use cells that naturally or recombinantly express an adhesion protein of choice. In an exemplary assay, cells expressing the cell adhesion protein are plated in wells of a multiwell plate together

with other cells (either more of the same cell type, or another type of cell to which the cells adhere). The cells that can adhere are labeled with a membrane-permeable fluorescent dye, such as BCECF, and allowed to adhere to the monolayers in the presence of candidate ligands. Unbound cells are washed off, and bound cells are detected using a fluorescence plate reader. High-throughput cell adhesion assays have also been described. See, e.g., Falsey J R et al., *Bioconjug Chem.* May-June 2001;12(3):346-53.

Tubulogenesis. Tubulogenesis assays can be used to monitor the ability of cultured cells, generally endothelial cells, to form tubular structures on a matrix substrate, which generally simulates the environment of the extracellular matrix. Exemplary substrates include Matrigel™ (Becton Dickinson), an extract of basement membrane proteins containing laminin, collagen IV, and heparin sulfate proteoglycan, which is liquid at 4°C. and forms a solid gel at 37°C. Other suitable matrices comprise extracellular components such as collagen, fibronectin, and/or fibrin. Cells are stimulated with a pro-angiogenic stimulant, and their ability to form tubules is detected by imaging. Tubules can generally be detected after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Tube formation assays are well known in the art (e.g., Jones M K et al., 1999, *Nature Medicine* 5:1418-1423). These assays have traditionally involved stimulation with serum or with the growth factors FGF or VEGF. In one embodiment, the assay is performed with cells cultured in serum free medium. In one embodiment, the assay is performed in the presence of one or more pro-angiogenic agents, e.g., inflammatory angiogenic factors such as TNF- α , or FGF, VEGF, phorbol myristate acetate (PMA), TNF-alpha, ephrin, etc.

Cell Migration. An exemplary assay for endothelial cell migration is the human microvascular endothelial (HMVEC) migration assay. See, e.g., Tolsma et al. (1993) *J. Cell Biol* 122, 497-511. Migration assays are known in the art (e.g., Paik J H et al., 2001, *J Biol Chem* 276:11830-11837). In one example, cultured endothelial cells are seeded onto a matrix-coated porous lamina, with pore sizes generally smaller than typical cell size. The lamina is typically a membrane, such as the transwell polycarbonate membrane (Corning Costar Corporation, Cambridge, Mass.), and is generally part of an upper chamber that is in fluid contact with a lower chamber containing pro-angiogenic stimuli. Migration is generally assayed after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Migration is assessed as the number of cells that

crossed the lamina, and may be detected by staining cells with hemotoxylin solution (VWR Scientific.), or by any other method for determining cell number. In another exemplary set up, cells are fluorescently labeled and migration is detected using fluorescent readings, for instance using the Falcon HTS FluoroBlok (Becton Dickinson).

- 5 While some migration is observed in the absence of stimulus, migration is greatly increased in response to pro-angiogenic factors. The assay can be used to test the effect of a Tie1-binding ligand on endothelial cell migration.

Sprouting assay. An exemplary sprouting assay is a three-dimensional in vitro angiogenesis assay that uses a cell-number defined spheroid aggregation of endothelial
10 cells ("spheroid"), embedded in a collagen gel-based matrix. The spheroid can serve as a starting point for the sprouting of capillary-like structures by invasion into the extracellular matrix (termed "cell sprouting") and the subsequent formation of complex anastomosing networks (Korff and Augustin, 1999, J Cell Sci 112:3249-58). In an exemplary experimental set-up, spheroids are prepared by pipetting 400 human umbilical
5 vein endothelial cells into individual wells of a nonadhesive 96-well plates to allow overnight spheroidal aggregation (Korff and Augustin: J Cell Biol 143: 1341-52, 1998). Spheroids are harvested and seeded in 900 µl of methocel-collagen solution and pipetted into individual wells of a 24 well plate to allow collagen gel polymerization. Test agents are added after 30 min by pipetting 100 µl of 10-fold concentrated working dilution of the
0 test substances on top of the gel. Plates are incubated at 37°C for 24 h. Dishes are fixed at the end of the experimental incubation period by addition of paraformaldehyde. Sprouting intensity of endothelial cells can be quantitated by an automated image analysis system to determine the cumulative sprout length per spheroid.

- In some embodiments, a Tie1 binding ligand as a statistically significant effect on
5 an assay described herein, e.g., a cellular assay described herein.

Ligand Production

- Standard recombinant nucleic acid methods can be used to express a protein ligand that binds to Tie1. See, for example, the techniques described in Sambrook & Russell, *Molecular Cloning: A Laboratory Manual*, 3rd Edition, Cold Spring Harbor Laboratory,
1 N.Y. (2001) and Ausubel *et al.*, *Current Protocols in Molecular Biology* (Greene Publishing Associates and Wiley Interscience, N.Y. (1989). Generally, a nucleic acid

sequence encoding the protein ligand is cloned into a nucleic acid expression vector. If the protein includes multiple polypeptide chains, each chain can be cloned into an expression vector, e.g., the same or different vectors, that are expressed in the same or different cells. Methods for producing antibodies are also provided below.

5 **Antibody Production.** Some antibodies, e.g., Fabs, can be produced in bacterial cells, e.g., *E. coli* cells. For example, if the Fab is encoded by sequences in a phage display vector that includes a suppressible stop codon between the display entity and a bacteriophage protein (or fragment thereof), the vector nucleic acid can be shuffled into a bacterial cell that cannot suppress a stop codon. In this case, the Fab is not fused to the
10 gene III protein and is secreted into the media.

Antibodies can also be produced in eukaryotic cells. In one embodiment, the antibodies (e.g., scFv's) are expressed in a yeast cell such as *Pichia* (see, e.g., Powers *et al.* (2001) *J Immunol Methods*. 251:123-35), *Hansenula*, or *Saccharomyces*.

In one embodiment, antibodies are produced in mammalian cells. Preferred
15 mammalian host cells for expressing the clone antibodies or antigen-binding fragments thereof include Chinese Hamster Ovary (CHO cells) (including dhfr- CHO cells, described in Urlaub and Chasin (1980) *Proc. Natl. Acad. Sci. USA* 77:4216-4220, used with a DHFR selectable marker, e.g., as described in Kaufman and Sharp (1982) *Mol. Biol.*
159:601-621), lymphocytic cell lines, e.g., NS0 myeloma cells and SP2 cells, COS cells,
20 and a cell from a transgenic animal, e.g., a transgenic mammal. For example, the cell is a mammary epithelial cell.

In addition to the nucleic acid sequence encoding the immunoglobulin domain, the recombinant expression vectors may carry additional sequences, such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and selectable
25 marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced (see e.g., U.S. Patents Nos. 4,399,216, 4,634,665 and 5,179,017). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin or methotrexate, on a host cell into which the vector has been introduced. Preferred selectable marker genes include the dihydrofolate reductase
30 (DHFR) gene (for use in *dhfr*⁻ host cells with methotrexate selection/amplification) and the *neo* gene (for G418 selection).

In an exemplary system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into *dhfr*- CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to enhancer/promoter regulatory elements (e.g., derived from SV40, CMV, adenovirus and the like, such as a CMV enhancer/AdMLP promoter regulatory element or an SV40 enhancer/AdMLP promoter regulatory element) to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the antibody from the culture medium. For example, some antibodies can be isolated by affinity chromatography with a Protein A or Protein G.

For antibodies that include an Fc domain, the antibody production system preferably synthesizes antibodies in which the Fc region is glycosylated. For example, the Fc domain of IgG molecules is glycosylated at asparagine 297 in the CH2 domain. This asparagine is the site for modification with biantennary-type oligosaccharides. It has been demonstrated that this glycosylation is required for effector functions mediated by Fcγ receptors and complement C1q (Burton and Woof (1992) *Adv. Immunol.* 51:1-84; Jefferis *et al.* (1998) *Immunol. Rev.* 163:59-76). In a preferred embodiment, the Fc domain is produced in a mammalian expression system that appropriately glycosylates the residue corresponding to asparagine 297. The Fc domain can also include other eukaryotic post-translational modifications.

Antibodies can also be produced by a transgenic animal. For example, U.S. 5,849,992 describes a method of expressing an antibody in the mammary gland of a transgenic mammal. A transgene is constructed that includes a milk-specific promoter and nucleic acids encoding the antibody of interest and a signal sequence for secretion. The milk produced by females of such transgenic mammals includes, secreted-therein, the

antibody of interest. The antibody can be purified from the milk, or for some applications, used directly.

It is also possible to produce antibodies that bind to Tie1 by immunization, e.g., using an animal, e.g., with natural, human, or partially human immunoglobulin loci. Non-human antibodies can also be modified to include substitutions for human immunoglobulin sequences, e.g., consensus human amino acid residues at particular positions, e.g., at one or more of the following positions (preferably at least five, ten, twelve, or all): (in the FR of the variable domain of the light chain) 4L, 35L, 36L, 38L, 43L, 44L, 58L, 46L, 62L, 63L, 64L, 65L, 66L, 67L, 68L, 69L, 70L, 71L, 73L, 85L, 87L, 98L, and/or (in the FR of the variable domain of the heavy chain) 2H, 4H, 24H, 36H, 37H, 39H, 43H, 45H, 49H, 58H, 60H, 67H, 68H, 69H, 70H, 73H, 74H, 75H, 78H, 91H, 92H, 93H, and/or 103H (according to the Kabat numbering). See, e.g., U.S. 6,407,213.

Tie1 production. Method for producing Tie1 ectodomain protein, Tie1 protein, or Tie1 liposomes are known in the art. See, e.g., WO 93/14124.

Biotinylation Methods. A variety of methods are available to biotinylate proteins, e.g., an immunoglobulin protein or a target protein. For example, the protein can be incubated with a 5-fold molar excess of sulfo-NHS-SS-biotin in 50 mM HEPES, pH 8.0, 100 mM NaCl overnight at 4°C. Free biotin is removed by buffer exchange into PBS, 0.01% Tween 20, e.g., using a Biomax device with a 10 kDa molecular weight cut-off membrane or by dialysis. The number of biotin molecules incorporated per mole of protein can be determined using the HABA assay as described by the manufacturer (Pierce).

Pharmaceutical Compositions

In another aspect, the invention provides compositions, e.g., pharmaceutically acceptable compositions, which include an Tie1-binding ligand, e.g., an antibody molecule, other polypeptide or peptide identified as binding to Tie1, or described herein, formulated together with a pharmaceutically acceptable carrier. As used herein, "pharmaceutical compositions" encompass labeled ligands (e.g., for in vivo imaging) as well as therapeutic compositions.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and

absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of administration, the active compound, *i.e.*, protein ligand may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

A “pharmaceutically acceptable salt” refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19).

Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanolic acids, hydroxy alkanolic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chlorprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

The compositions of this invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (*e.g.*, injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The preferred form depends on the intended mode of administration and therapeutic application. Typical preferred compositions are in the form of injectable or infusible solutions, such as compositions similar to those used for administration of humans with antibodies. The preferred mode of administration is parenteral (*e.g.*, intravenous, subcutaneous, intraperitoneal, intramuscular). In a preferred embodiment, the Tie1-binding ligand is administered by intravenous infusion or injection. In another preferred embodiment, the Tie1-binding ligand is administered by intramuscular or subcutaneous injection.

The phrases “parenteral administration” and “administered parenterally” as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular,

intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Pharmaceutical compositions typically must be sterile and stable under the conditions of manufacture and storage. A pharmaceutical composition can also be tested to insure it meets regulatory and industry standards for administration. For example, endotoxin levels in the preparation can be tested using the Limulus amebocyte lysate assay (e.g., using the kit from Bio Whittaker lot # 7L3790, sensitivity 0.125 EU/mL) according to the USP 24/NF 19 methods. Sterility of pharmaceutical compositions can be determined using thioglycollate medium according to the USP 24/NF 19 methods. For example, the preparation is used to inoculate the thioglycollate medium and incubated at 35°C for 14 or more days. The medium is inspected periodically to detect growth of a microorganism.

The composition can be formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the active compound (*i.e.*, the ligand) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

The anti-Tie1 protein ligands of the invention can be administered by a variety of methods known in the art, although for many applications, the preferred route/mode of administration is intravenous injection or infusion. For example, for therapeutic applications, the Tie1-binding ligand can be administered by intravenous infusion at a rate

of less than 30, 20, 10, 5, or 1 mg/min to reach a dose of about 1 to 100 mg/m² or 7 to 25 mg/m². The route and/or mode of administration will vary depending upon the desired results. In certain embodiments, the active compound may be prepared with a carrier that will protect the compound against rapid release, such as a controlled release formulation, including implants, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known. *See, e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

In certain embodiments, the ligand may be orally administered, for example, with an inert diluent or an assimilable edible carrier. The compound (and other ingredients, if desired) may also be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the subject's diet. For oral therapeutic administration, the compounds may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. To administer a compound of the invention by other than parenteral administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation.

Pharmaceutical compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a pharmaceutical composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants and modules useful in the invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug

delivery system. Of course, many other such implants, delivery systems, and modules are also known.

In certain embodiments, the compounds of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, e.g., U.S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may include one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, e.g., V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685).

Dosage regimens are adjusted to provide the optimum desired response (e.g., a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

An exemplary, non-limiting range for a therapeutically or prophylactically effective amount of an antibody of the invention is 0.1-20 mg/kg, more preferably 1-10 mg/kg. The Tiel-binding antibody can be administered by intravenous infusion at a rate of less than 30, 20, 10, 5, or 1 mg/min to reach a dose of about 1 to 100 mg/m² or about 5 to 30 mg/m². For ligands smaller in molecular weight than an antibody, appropriate amounts can be proportionally less. It is to be noted that dosage values may vary with the type and severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or

supervising the administration of the compositions, and that dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

The pharmaceutical compositions of the invention may include a “therapeutically effective amount” or a “prophylactically effective amount” of an Tie1-binding ligand of the invention. A “therapeutically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the composition may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the protein ligand to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the composition are outweighed by the therapeutically beneficial effects. A “therapeutically effective dosage” preferably inhibits a measurable parameter, e.g., inflammation or tumor growth rate by at least about 20%, more preferably by at least about 40%, even more preferably by at least about 60%, and still more preferably by at least about 80% relative to untreated subjects. The ability of a compound to inhibit a measurable parameter, e.g., cancer, can be evaluated in an animal model system predictive of efficacy in human tumors. Alternatively, this property of a composition can be evaluated by examining the ability of the compound to inhibit, such inhibition *in vitro* by assays known to the skilled practitioner.

A “prophylactically effective amount” refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

Also within the scope of the invention are kits including the protein ligand that binds to Tie1 and instructions for use, e.g., treatment, prophylactic, or diagnostic use. In one embodiment, the instructions for diagnostic applications include the use of the Tie1-binding ligand (e.g., antibody or antigen-binding fragment thereof, or other polypeptide or peptide) to detect Tie1, *in vitro*, e.g., in a sample, e.g., a biopsy or cells from a patient having an inflammatory disorder or a cancer or neoplastic disorder, or *in vivo*. In another embodiment, the instructions for therapeutic applications include suggested dosages and/or modes of administration in a patient with a cancer or neoplastic disorder. The kit can further contain a least one additional reagent, such as a diagnostic or therapeutic agent,

e.g., a diagnostic or therapeutic agent as described herein, and/or one or more additional Tie1-binding ligands, formulated as appropriate, in one or more separate pharmaceutical preparations.

Stabilization and Retention

5 In one embodiment, an Tie1-binding ligand (e.g., a Tie1-binding antibody described herein) is physically associated with a moiety that improves its stabilization and/or retention in circulation, e.g., in blood, serum, lymph, or other tissues.

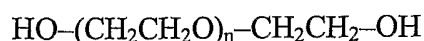
For example, an Tie1-binding ligand can be associated with a polymer, e.g., a substantially non-antigenic polymers, such as polyalkylene oxides or polyethylene oxides. 10 Suitable polymers will vary substantially by weight. Polymers having molecular number average weights ranging from about 200 to about 35,000 are usually selected for the purposes of the present invention. Molecular weights of from about 1,000 to about 15,000 are preferred and 2,000 to about 12,500 are particularly preferred.

For example, an Tie1-binding ligand can be conjugated to a water soluble polymer, 15 e.g., hydrophilic polyvinyl polymers, e.g. polyvinylalcohol and polyvinylpyrrolidone.. A non-limiting list of such polymers include polyalkylene oxide homopolymers such as polyethylene glycol (PEG) or polypropylene glycols, polyoxyethylenated polyols, copolymers thereof and block copolymers thereof, provided that the water solubility of the block copolymers is maintained. Additional useful polymers include polyoxyalkylenes 20 such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (e.g. polymannuronic acid, or alginic acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including 5 homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextrane sulfate, dextran, dextrans, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, e.g. hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; heparin or heparon.

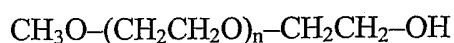
0 Other compounds can also be attached to the same polymer, e.g., a cytotoxin, a label, or another targeting agent, e.g., another Tie1-binding ligand or an unrelated ligand.

Mono-activated, alkoxy-terminated polyalkylene oxides (PAO's), e.g., monomethoxy-terminated polyethylene glycols (mPEG's); C₁₋₄ alkyl-terminated polymers; and bis-activated polyethylene oxides (glycols) can be used for crosslinking. See, e.g., U.S. 5,951,974.

- 5 In its most common form poly(ethylene glycol), PEG, is a linear or branched polyether terminated with hydroxyl groups and having the general structure:



- PEG can be synthesized by anionic ring opening polymerization of ethylene oxide initiated by nucleophilic attack of a hydroxide ion on the epoxide ring. Particularly useful
10 for polypeptide modification is monomethoxy PEG, mPEG, having the general structure:



For further description, see, e.g., Roberts et al. (2002) *Advanced Drug Delivery Reviews* 54:459-476.

- In one embodiment, the polymer prior to cross-linking need not be, but preferably
15 is, water soluble. Generally, after crosslinking, the product is water soluble, e.g., exhibits a water solubility of at least about 0.01 mg/ml, and more preferably at least about 0.1 mg/ml, and still more preferably at least about 1 mg/ml. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if the conjugate is intended to be
20 administered by such routes.

- In one embodiment, the polymer contains only a single group which is reactive. This helps to avoid cross-linking of protein molecules. However, it is within the scope herein to maximize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or ion exchange chromatography to recover substantially
25 homogenous derivatives. In other embodiments, the polymer contains two or more reactive groups for the purpose of linking multiple ligands to the polymer backbone. Again, gel filtration or ion exchange chromatography can be used to recover the desired derivative in substantially homogeneous form.

- The molecular weight of the polymer can range up to about 500,000 D, and
0 preferably is at least about 20,000 D, or at least about 30,000 D, or at least about 40,000 D. The molecular weight chosen can depend upon the effective size of the conjugate to be achieved, the nature (e.g. structure, such as linear or branched) of the

polymer, and the degree of derivatization.

The covalent crosslink can be used to attach an Tie1-binding ligand to a polymer, for example, crosslinking to the N-terminal amino group and epsilon amino groups found on lysine residues, as well as other amino, imino, carboxyl, sulfhydryl, hydroxyl or other hydrophilic groups. The polymer may be covalently bonded directly to the Tie1-binding ligand without the use of a multifunctional (ordinarily bifunctional) crosslinking agent. Covalent binding to amino groups is accomplished by known chemistries based upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, activated succinimidyl esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylchloroformate or P-nitrophenylchloroformate activated PEG.) Carboxyl groups can be derivatized by coupling PEG-amine using carbodiimide. Sulfhydryl groups can be derivatized by coupling to maleimido-substituted PEG (e.g. alkoxy-PEG amine plus sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) WO 97/10847 or PEG-maleimide commercially available from Shearwater Polymers, Inc., Huntsville, Ala.). Alternatively, free amino groups on the ligand (e.g. epsilon amino groups on lysine residues) can be thiolated with 2-imino-thiolane (Traut's reagent) and then coupled to maleimide-containing derivatives of PEG, e.g., as described in Pedley et al., Br. J. Cancer, 70: 1126-1130 (1994).

Functionalized PEG polymers that can be attached to an Tie1-binding ligand are available, e.g., from Shearwater Polymers, Inc. (Huntsville, Ala.). Such commercially available PEG derivatives include, e.g., amino-PEG, PEG amino acid esters, PEG-hydrazide, PEG-thiol, PEG-succinate, carboxymethylated PEG, PEG-propionic acid, PEG amino acids, PEG succinimidyl succinate, PEG succinimidyl propionate, succinimidyl ester of carboxymethylated PEG, succinimidyl carbonate of PEG, succinimidyl esters of amino acid PEGs, PEG-oxycarbonylimidazole, PEG-nitrophenyl carbonate, PEG tresylate, PEG-glycidyl ether, PEG-aldehyde, PEG vinylsulfone, PEG-maleimide, PEG-orthopyridyl-disulfide, heterofunctional PEGs, PEG vinyl derivatives, PEG silanes, and PEG phospholides. The reaction conditions for coupling these PEG derivatives may vary depending on the Tie1-binding ligand, the desired degree of PEGylation, and the PEG derivative utilized. Some factors involved in the choice of PEG derivatives include: the

desired point of attachment (such as lysine or cysteine R-groups), hydrolytic stability and reactivity of the derivatives, stability, toxicity and antigenicity of the linkage, suitability for analysis, etc. Specific instructions for the use of any particular derivative are available from the manufacturer.

- 5 The conjugates of an Tie1-binding ligand and a polymer can be separated from the unreacted starting materials, e.g., by gel filtration or ion exchange chromatography, e.g., HPLC. Heterologous species of the conjugates are purified from one another in the same fashion. Resolution of different species (e.g. containing one or two PEG residues) is also possible due to the difference in the ionic properties of the unreacted amino acids. See,
10 e.g., WO 96/34015.

Treatments

- Protein ligands that bind to Tie1 (e.g., those described herein) have therapeutic and prophylactic utilities. For example, these ligands can be administered to cells in culture, e.g. *in vitro* or *ex vivo*, or in a subject, e.g., *in vivo*, to treat, prevent, and/or diagnose a
5 variety of disorders, such as endothelial cell disorders, blood vessel development disorders, wound healing, inflammatory diseases and cancers, particularly metastatic cancers.

- As used herein, the term "treat" or "treatment" is defined as the application or administration of an Tie1-binding antibody, alone or in combination with one or more
0 other agents (e.g., a second agent) to a subject, e.g., a patient, or application or administration of the agent to an isolated tissue or cell, e.g., cell line, from a subject, e.g., a patient, who has a disorder (e.g., a disorder as described herein), a symptom of a disorder or a predisposition toward a disorder, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disorder, the symptoms of the disorder or
5 the predisposition toward the disorder. Treating a cell refers to the inhibition of growth or activity, ablation, killing of a cell *in vitro* or *in vivo*, or otherwise reducing capacity of a cell, e.g., an aberrant cell, to mediate a disorder, e.g., a disorder as described herein (e.g., a cancerous disorder).

- In one embodiment, "treating a cell" refers to a reduction in the activity and/or
1 proliferation of a cell, e.g., a hyperproliferative cell. Such reduction does not necessarily indicate a total elimination of the cell, but a reduction, e.g., a statistically significant

reduction, in the activity or the number of the cell. An example of a reduction in activity is a reduction in migration of the cell or a reduction in cell differentiation.

As used herein, an amount of an Tie1-binding ligand effective to treat a disorder, or a “therapeutically effective amount” refers to an amount of the ligand which is effective, upon single or multiple dose administration to a subject, in treating a cell, e.g., an endothelial cell (e.g., a Tie1-expressing endothelial cell) or cancer cell (particularly a metastatic cell thereof), or in prolonging curing, alleviating, relieving or improving a subject with a disorder as described herein beyond that expected in the absence of such treatment. As used herein, “inhibiting the growth” of the neoplasm refers to slowing, interrupting, arresting or stopping its growth and metastases and does not necessarily indicate a total elimination of the neoplastic growth.

As used herein, an amount of an Tie1-binding ligand effective to prevent a disorder, or a “a prophylactically effective amount” of the ligand refers to an amount of an Tie1-binding ligand, e.g., an Tie1-binding antibody described herein, which is effective, upon single- or multiple-dose administration to the subject, in preventing or delaying the occurrence of the onset or recurrence of a disorder, e.g., an endothelial cell-related disorder, a blood vessel development disorder, an inflammatory disease or a cancer.

The terms “induce”, “inhibit”, “potentiate”, “elevate”, “increase”, “decrease” or the like, e.g., which denote quantitative differences between two states, refer to a difference, e.g., a statistically significant difference, between the two states. For example, “an amount effective to inhibit the proliferation of the Tie1-expressing hyperproliferative cells” means that the rate of growth of the cells will be different, e.g., statistically significantly different, from the untreated cells.

As used herein, the term “subject” is intended to include human and non-human animals. Preferred human animals include a human patient having a disorder characterized by abnormal cell proliferation or cell differentiation. The term “non-human animals” of the invention includes all vertebrates, e.g., non-mammals (such as chickens, amphibians, reptiles) and mammals, such as non-human primates, sheep, dog, cow, pig, etc.

In one embodiment, the subject is a human subject. Alternatively, the subject can be a mammal expressing a Tie1-like antigen with which an antibody of the invention cross-reacts. A protein ligand of the invention can be administered to a human subject for

therapeutic purposes (discussed further below). Moreover, an Tie1-binding ligand can be administered to a non-human mammal expressing the Tie1-like antigen to which the ligand binds (e.g., a primate, pig or mouse) for veterinary purposes or as an animal model of human disease. Regarding the latter, such animal models may be useful for evaluating the therapeutic efficacy of the ligand (e.g., testing of dosages and time courses of administration).

In one embodiment, the invention provides a method of treating (e.g., inhibiting, ablating or killing) a cell (e.g., a non-cancerous cell, e.g., a normal, benign or hyperplastic cell, or a cancerous cell, e.g., a malignant cell, e.g., cell found in a solid tumor, a soft tissue tumor, or a metastatic lesion (e.g., a cell found in renal, urothelial, colonic, rectal, pulmonary, breast or hepatic, cancers and/or metastasis)). Methods of the invention include the steps of contacting the cell with an Tie1-binding ligand, e.g., an Tie1-binding antibody described herein, in an amount sufficient to treat, e.g., inhibit, ablate or kill, the cell.

The subject method can be used on cells in culture, e.g. *in vitro* or *ex vivo*. For example, cancerous or metastatic cells (e.g., renal, urothelial, colon, rectal, lung, breast, ovarian, prostatic, or liver cancerous or metastatic cells) can be cultured *in vitro* in culture medium, e.g., with endothelial cells, and the contacting step can be effected by adding the Tie1-binding ligand to the culture medium. The method can be performed on cells (e.g., cancerous or metastatic cells) present in a subject, as part of an *in vivo* (e.g., therapeutic or prophylactic) protocol. For *in vivo* embodiments, the contacting step is effected in a subject and includes administering the Tie1-binding ligand to the subject under conditions effective to permit both binding of the ligand to the cell and the treating, e.g., the killing or ablating of the cell.

The method can be used to treat a cancer. As used herein, the terms "cancer", "hyperproliferative", "malignant", and "neoplastic" are used interchangeably, and refer to those cells an abnormal state or condition characterized by rapid proliferation or neoplasm. The terms include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. "Pathologic hyperproliferative" cells occur in disease states characterized by malignant tumor growth.

The common medical meaning of the term “neoplasia” refers to “new cell growth” that results as a loss of responsiveness to normal growth controls, e.g. to neoplastic cell growth. A “hyperplasia” refers to cells undergoing an abnormally high rate of growth. However, as used herein, the terms neoplasia and hyperplasia can be used interchangeably, as their context will reveal, referring generally to cells experiencing abnormal cell growth rates. Neoplasias and hyperplasias include “tumors,” which may be benign, premalignant or malignant. In one embodiment, reduction in Tie1 activity by a Tie1-binding ligand can reduce or prevent angiogenesis near and around the tumor, thereby reducing or preventing tumor growth. In another embodiment, the neoplasia includes endothelial cells that are proliferating abnormally. A Tie1-binding ligand can be used to modulate the cells of the neoplasia themselves.

Examples of cancerous disorders include, but are not limited to, solid tumors, soft tissue tumors, and metastatic lesions. Examples of solid tumors include malignancies, e.g., sarcomas, adenocarcinomas, and carcinomas, of the various organ systems, such as those affecting lung, breast, lymphoid, gastrointestinal (e.g., colon), and genitourinary tract (e.g., renal, urothelial cells), pharynx, prostate, ovary as well as adenocarcinomas which include malignancies such as most colon cancers, rectal cancer, renal-cell carcinoma, liver cancer, non-small cell carcinoma of the lung, cancer of the small intestine and so forth. Metastatic lesions of the aforementioned cancers can also be treated or prevented using the methods and compositions of the invention.

The subject method can be useful in treating malignancies of the various organ systems, such as those affecting lung, breast, lymphoid, gastrointestinal (e.g., colon), and genitourinary tract, prostate, ovary, pharynx, as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus. Exemplary solid tumors that can be treated include: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangi endotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma,

papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, testicular tumor, lung carcinoma, small cell lung carcinoma, non-small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, endependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma.

The term "carcinoma" is recognized by those skilled in the art and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, e.g., which include malignant tumors composed of carcinomatous and sarcomatous tissues. An "adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures.

The term "sarcoma" is recognized by those skilled in the art and refers to malignant tumors of mesenchymal derivation.

The subject method can also be used to inhibit the proliferation of hyperplastic/neoplastic cells of hematopoietic origin, e.g., arising from myeloid, lymphoid or erythroid lineages, or precursor cells thereof. For instance, the invention contemplates the treatment of various myeloid disorders including, but not limited to, acute promyeloid leukemia (APML), acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML) (reviewed in Vaickus, L. (1991) *Crit Rev. in Oncol./Hematol.* 11:267-97). Lymphoid malignancies which may be treated by the subject method include, but are not limited to acute lymphoblastic leukemia (ALL), which includes B-lineage ALL and T-lineage ALL, chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HLL) and Waldenstrom's macroglobulinemia (WM). Additional forms of malignant lymphomas contemplated by the treatment method of the invention include, but are not limited to, non-Hodgkin's lymphoma and variants thereof, peripheral T-cell lymphomas, adult T-cell leukemia/lymphoma (ATL), cutaneous T-cell lymphoma (CTCL), large granular lymphocytic leukemia (LGF) and Hodgkin's disease. As Tie1 has

been shown to be upregulated in acute myelogenous leukaemia and myelodysplastic syndrome (Verstovsek et al., 2001, Leuk, Lymphoma), B cell chronic lymphocytic leukaemia (Aguayo et al, 2001. Leukemia Research), ligands that interact with Tie1 can be used to detect, treat, or prevent these diseases.

5 Methods of administering Tie1-binding ligands are described in “Pharmaceutical Compositions”. Suitable dosages of the molecules used will depend on the age and weight of the subject and the particular drug used. The ligands can be used as competitive agents to inhibit, reduce an undesirable interaction, e.g., between a natural or pathological agent and the Tie1.

10 In one embodiment, the Tie1-binding ligands are used to inhibit (e.g., inhibit at least one activity of, reduce proliferation, migration, growth or viability) of a cell, e.g., an endothelial cell *in vivo*. The ligands can be used by themselves or conjugated to an agent, e.g., a cytotoxic drug, radioisotope. This method includes: administering the ligand alone or attached to a cytotoxic drug, to a subject requiring such treatment.

15 The terms “cytotoxic agent” and “cytostatic agent” and “anti-tumor agent” are used interchangeably herein and refer to agents that have the property of inhibiting the growth or proliferation (e.g., a cytostatic agent), or inducing the killing, of hyperproliferative cells, e.g., an aberrant cancer cell. In cancer therapeutic embodiment, the term “cytotoxic agent” is used interchangeably with the terms “anti-cancer” or “anti-tumor” to mean an
20 agent, which inhibits the development or progression of a neoplasm, particularly a solid tumor, a soft tissue tumor, or a metastatic lesion.

Nonlimiting examples of anti-cancer agents include, e.g., anti-microtubule agents, topoisomerase inhibitors, antimetabolites, mitotic inhibitors, alkylating agents, intercalating agents, agents capable of interfering with a signal transduction pathway,
25 agents that promote apoptosis, radiation, and antibodies against other tumor-associated antigens (including naked antibodies, immunotoxins and radioconjugates). Examples of the particular classes of anti-cancer agents are provided in detail as follows:
antitubulin/antimicrotubule, e.g., paclitaxel, vincristine, vinblastine, vindesine, vinorelbin, taxotere; topoisomerase I inhibitors, e.g., topotecan, camptothecin, doxorubicin, etoposide,
30 mitoxantrone, daunorubicin, idarubicin, teniposide, amsacrine, epirubicin, merbarone, piroxantrone hydrochloride; antimetabolites, e.g., 5-fluorouracil (5-FU), methotrexate, 6-mercaptopurine, 6-thioguanine, fludarabine phosphate, cytarabine/Ara-C, trimetrexate,

gemcitabine, acivicin, alanosine, pyrazofurin, N-Phosphoracetyl-L-Asparate=PALA, pentostatin, 5-azacitidine, 5-Aza 2'-deoxycytidine, ara-A, cladribine, 5 - fluorouridine, FUDR, tiazofurin, N-[5-[N-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-N-methylamino]-2-thenoyl]-L-glutamic acid; alkylating agents, e.g., cisplatin, carboplatin, mitomycin C, BCNU=Carmustine, melphalan, thiotepa, busulfan, chlorambucil, plicamycin, dacarbazine, ifosfamide phosphate, cyclophosphamide, nitrogen mustard, uracil mustard, pipobroman, 4-ipomeanol; agents acting via other mechanisms of action, e.g., dihydrolenperone, spiromustine, and desipeptide; biological response modifiers, e.g., to enhance anti-tumor responses, such as interferon; apoptotic agents, such as actinomycin D; and anti-hormones, for example anti-estrogens such as tamoxifen or, for example antiandrogens such as 4'-cyano-3-(4-fluorophenylsulphonyl)-2-hydroxy-2-methyl-3'-(trifluoromethyl) propionanilide.

Since the Tie1-binding ligands recognize Tie1-expressing endothelial cells and can bind to endothelial cells that are associated with (e.g., in proximity of or intermingled with) cancer cells, e.g., cancerous lung, liver, colon, breast, ovarian, epidermal, laryngeal, and cartilage cells, and particularly metastatic cells thereof, Tie1-binding ligands can be used to inhibit (e.g., inhibit at least one activity, reduce growth and proliferation, or kill) any such cells to which the ligands bind. Reducing endothelial cell activity near a cancer can indirectly inhibit (e.g., inhibit at least one activity, reduce growth and proliferation, or kill) the cancer cells which may be dependent on the endothelial cells for nutrients, growth signals and so forth.

Alternatively, the ligands bind to cells in the vicinity of the cancerous cells, but are sufficiently close to the cancerous cells to directly or indirectly inhibit (e.g., inhibit at least one activity, reduce growth and proliferation, or kill) the cancers cells. Thus, the Tie1-binding ligands (e.g., modified with a toxin, e.g., a cytotoxin) can be used to selectively inhibit (e.g., kill or ablate cells in cancerous tissue (including the cancerous cells themselves and endothelial cells associated with or invading the cancer).

The ligands may be used to deliver a variety of cytotoxic drugs including therapeutic drugs, a compound emitting radiation, molecules of plants, fungal, or bacterial origin, biological proteins, and mixtures thereof. The cytotoxic drugs can be intracellularly acting cytotoxic drugs, such as short-range radiation emitters, including, for example, short-range, high-energy α -emitters, as described herein.

Enzymatically active toxins and fragments thereof are exemplified by diphtheria toxin A fragment, non-binding active fragments of diphtheria toxin, exotoxin A (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, α -sacrin, certain *Aleurites fordii* proteins, certain Dianthin proteins, *Phytolacca americana* proteins (PAP, PAPII and PAP-S), *Morodica charantia* inhibitor, curcin, crotin, *Saponaria officinalis* inhibitor, gelonin, mitogillin, restrictocin, phenomycin, and enomycin. Procedures for preparing enzymatically active polypeptides of the immunotoxins are described in W084/03508 and W085/03508, and in the appended Examples below.

Examples of cytotoxic moieties that can be conjugated to the antibodies include adriamycin, chlorambucil, daunomycin, methotrexate, neocarzinostatin, and platinum.

In the case of polypeptide toxins, recombinant nucleic acid techniques can be used to construct a nucleic acid that encodes the ligand (or a protein component thereof) and the cytotoxin (or a protein component thereof) as translational fusions. The recombinant nucleic acid is then expressed, e.g., in cells and the encoded fusion polypeptide isolated.

Procedures for conjugating protein ligands (e.g., antibodies) with the cytotoxic agents have been previously described. Procedures for conjugating chlorambucil with antibodies are described by Flechner (1973) *European Journal of Cancer*, 9:741-745; Ghose et al. (1972) *British Medical Journal*, 3:495-499; and Szekerke, et al. (1972) *Neoplasma*, 19:211-215. Procedures for conjugating daunomycin and adriamycin to antibodies are described by Hurwitz, E. et al. (1975) *Cancer Research*, 35:1175-1181 and Arnon et al. (1982) *Cancer Surveys*, 1:429-449. Procedures for preparing antibody-ricin conjugates are described in U.S. Patent No. 4,414,148 and by Osawa, T., et al. (1982) *Cancer Surveys*, 1:373-388 and the references cited therein. Coupling procedures as also described in EP 86309516.2.

To kill or ablate normal, benign hyperplastic, or cancerous cells, a first protein ligand is conjugated with a prodrug which is activated only when in close proximity with a prodrug activator. The prodrug activator is conjugated with a second protein ligand, preferably one which binds to a non-competing site on the target molecule. Whether two protein ligands bind to competing or non-competing binding sites can be determined by conventional competitive binding assays. Drug-prodrug pairs suitable for use in the practice of the invention are described in Blakely et al., (1996) *Cancer Research*, 56:3287-3292.

Alternatively, the Tie1-binding ligand can be coupled to high energy radiation emitters, for example, a radioisotope, such as ^{131}I , a γ -emitter, which, when localized at the tumor site, results in a killing of several cell diameters. See, e.g., S.E. Order, "Analysis, Results, and Future Prospective of the Therapeutic Use of Radiolabeled Antibody in Cancer Therapy", *Monoclonal Antibodies for Cancer Detection and Therapy*, R.W. Baldwin et al. (eds.), pp 303-316 (Academic Press 1985). Other suitable radioisotopes include α -emitters, such as ^{212}Bi , ^{213}Bi , and ^{211}At , and β -emitters, such as ^{186}Re and ^{90}Y . Moreover, Lu^{117} may also be used as both an imaging and cytotoxic agent.

Radioimmunotherapy (RIT) using antibodies labeled with ^{131}I , ^{90}Y , and ^{177}Lu is under intense clinical investigation. There are significant differences in the physical characteristics of these three nuclides and as a result, the choice of radionuclide is very critical in order to deliver maximum radiation dose to the tumor. The higher beta energy particles of ^{90}Y may be good for bulky tumors. The relatively low energy beta particles of ^{131}I are ideal, but *in vivo* dehalogenation of radioiodinated molecules is a major disadvantage for internalizing antibody. In contrast, ^{177}Lu has low energy beta particle with only 0.2-0.3 mm range and delivers much lower radiation dose to bone marrow compared to ^{90}Y . In addition, due to longer physical half-life (compared to ^{90}Y), the tumor residence times are higher. As a result, higher activities (more mCi amounts) of ^{177}Lu labeled agents can be administered with comparatively less radiation dose to marrow. There have been several clinical studies investigating the use of ^{177}Lu labeled antibodies in the treatment of various cancers. (Mulligan T et al. (1995) *Clin Cancer Res.* 1:1447-1454; Meredith RF, et al. (1996) *J Nucl Med* 37:1491-1496; Alvarez RD, et al. (1997) *Gynecologic Oncology* 65: 94-101).

The Tie1-binding ligands can be used directly *in vivo* to eliminate antigen-expressing cells via natural complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC). The protein ligands of the invention, can include complement binding effector domain, such as the Fc portions from IgG1, -2, or -3 or corresponding portions of IgM which bind complement. In one embodiment, a population of target cells is *ex vivo* treated with a binding agent of the invention and appropriate effector cells. The treatment can be supplemented by the addition of complement or serum containing complement. Further, phagocytosis of target cells coated with a protein ligand of the invention can be improved by binding of complement proteins.

In another embodiment target, cells coated with the protein ligand which includes a complement binding effector domain are lysed by complement.

Also encompassed by the invention is a method of killing or ablating which involves using the anti-Tie1 ligand for prophylaxis. For example, these materials can be
5 used to prevent or delay development or progression of cancers.

Use of the therapeutic methods of the invention to treat cancers has a number of benefits. Since the protein ligands specifically recognize Tie1, other tissue is spared and high levels of the agent are delivered directly to the site where therapy is required. Treatment in accordance with the invention can be effectively monitored with clinical
0 parameters. Alternatively, these parameters can be used to indicate when such treatment should be employed.

Tie1-binding ligands of the invention can be administered in combination with one or more of the existing modalities for treating cancers, including, but not limited to: surgery; radiation therapy, and chemotherapy.

5 Diagnostic Uses

Protein ligands that bind to Tie1 (e.g., antibodies, e.g., an antibody described herein) have *in vitro* and *in vivo* diagnostic, therapeutic and prophylactic utilities.

In one aspect, the invention provides a diagnostic method for detecting the presence of a Tie1, *in vitro* (e.g., a biological sample, such as tissue, biopsy, e.g., a
0 cancerous tissue) or *in vivo* (e.g., *in vivo* imaging in a subject).

The method includes: (i) contacting a sample with Tie1-binding ligand; and (ii) detecting formation of a complex between the Tie1-binding ligand and the sample. The method can also include contacting a reference sample (e.g., a control sample) with the ligand, and determining the extent of formation of the complex between the ligand and the
5 sample relative to the same for the reference sample. A change, e.g., a statistically significant change, in the formation of the complex in the sample or subject relative to the control sample or subject can be indicative of the presence of Tie1 in the sample.

Another method includes: (i) administering the Tie1-binding ligand to a subject; and (iii) detecting formation of a complex between the Tie1-binding ligand, and the subject. The detecting can include determining location or time of formation of the complex.

The Tie1-binding ligand can be directly or indirectly labeled with a detectable substance to facilitate detection of the bound or unbound antibody. Suitable detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials and radioactive materials.

5 Complex formation between the Tie1-binding ligand and Tie1 can be detected by measuring or visualizing either the ligand bound to the Tie1 or unbound ligand. Conventional detection assays can be used, e.g., an enzyme-linked immunosorbent assays (ELISA), a radioimmunoassay (RIA) or tissue immunohistochemistry. Further to labeling the Tie1-binding ligand, the presence of Tie1 can be assayed in a sample by a competition
10 immunoassay utilizing standards labeled with a detectable substance and an unlabeled Tie1-binding ligand. In one example of this assay, the biological sample, the labeled standards and the Tie1 binding agent are combined and the amount of labeled standard bound to the unlabeled ligand is determined. The amount of Tie1 in the sample is inversely proportional to the amount of labeled standard bound to the Tie1 binding agent.

15 Fluorophore and chromophore labeled protein ligands can be prepared. Since antibodies and other proteins absorb light having wavelengths up to about 310 nm, the fluorescent moieties should be selected to have substantial absorption at wavelengths above 310 nm and preferably above 400 nm. A variety of suitable fluorescers and chromophores are described by Stryer (1968) *Science*, 162:526 and Brand, L. et al. (1972)
20 *Annual Review of Biochemistry*, 41:843-868. The protein ligands can be labeled with fluorescent chromophore groups by conventional procedures such as those disclosed in U.S. Patent Nos. 3,940,475, 4,289,747, and 4,376,110. One group of fluorescers having a number of the desirable properties described above is the xanthene dyes, which include the fluoresceins and rhodamines. Another group of fluorescent compounds are the
25 naphthylamines. Once labeled with a fluorophore or chromophore, the protein ligand can be used to detect the presence or localization of the Tie1 in a sample, e.g., using fluorescent microscopy (such as confocal or deconvolution microscopy).

Histological Analysis. Immunohistochemistry can be performed using the protein ligands described herein. For example, in the case of an antibody, the antibody can
30 synthesized with a label (such as a purification or epitope tag), or can be detectably labeled, e.g., by conjugating a label or label-binding group. For example, a chelator can be attached to the antibody. The antibody is then contacted to a histological preparation,

e.g., a fixed section of tissue that is on a microscope slide. After an incubation for binding, the preparation is washed to remove unbound antibody. The preparation is then analyzed, e.g., using microscopy, to identify if the antibody bound to the preparation. The method can be used to evaluate an endothelial cell or tissue formed by endothelial cells,

5 e.g., blood vessels.

The antibody (or other polypeptide or peptide) can be unlabeled at the time of binding. After binding and washing, the antibody is labeled in order to render it detectable.

Protein Arrays. The Tie1-binding ligand can also be immobilized on a protein
10 array. The protein array can be used as a diagnostic tool, e.g., to screen medical samples (such as isolated cells, blood, sera, biopsies, and the like). Of course, the protein array can also include other ligands, e.g., that bind to Tie1 or to other target molecules, such as hyaluronic acid.

Methods of producing polypeptide arrays are described, e.g., in De Wildt *et al.*
15 (2000) *Nat. Biotechnol.* 18:989-994; Lueking *et al.* (1999) *Anal. Biochem.* 270:103-111; Ge (2000) *Nucleic Acids Res.* 28, e3, I-VII; MacBeath and Schreiber (2000) *Science* 289:1760-1763; WO 01/40803 and WO 99/51773A1. Polypeptides for the array can be spotted at high speed, e.g., using commercially available robotic apparatus, e.g., from Genetic MicroSystems or BioRobotics. The array substrate can be, for example,
20 nitrocellulose, plastic, glass, e.g., surface-modified glass. The array can also include a porous matrix, e.g., acrylamide, agarose, or another polymer.

For example, the array can be an array of antibodies, e.g., as described in De Wildt, *supra*. Cells that produce the protein ligands can be grown on a filter in an arrayed format. Polypeptide production is induced, and the expressed polypeptides are immobilized to the
25 filter at the location of the cell.

A protein array can be contacted with a labeled target to determine the extent of binding of the target to each immobilized polypeptide from the diversity strand library. If the target is unlabeled, a sandwich method can be used, e.g., using a labeled probe, to detect binding of the unlabeled target.

30 Information about the extent of binding at each address of the array can be stored as a profile, e.g., in a computer database. The protein array can be produced in replicates and used to compare binding profiles, e.g., of a target and a non-target. Thus, protein

arrays can be used to identify individual members of the diversity strand library that have desired binding properties with respect to one or more molecules.

FACS. (Fluorescent Activated Cell Sorting). The Tie1-binding ligand can be used to label cells, e.g., cells in a sample (e.g., a patient sample). The ligand is also
5 attached (or attachable) to a fluorescent compound. The cells can then be sorted using fluorescent activated cell sorted (e.g., using a sorter available from Becton Dickinson Immunocytometry Systems, San Jose CA; see also U.S. 5,627,037; 5,030,002; and 5,137,809). As cells pass through the sorter, a laser beam excites the fluorescent compound while a detector counts cells that pass through and determines whether a
10 fluorescent compound is attached to the cell by detecting fluorescence. The amount of label bound to each cell can be quantified and analyzed to characterize the sample.

The sorter can also deflect the cell and separate cells bound by the ligand from those cells not bound by the ligand. The separated cells can be cultured and/or characterized.

In vivo Imaging. In still another embodiment, the invention provides a method for
15 detecting the presence of a Tie1-expressing cancerous tissues *in vivo*. The method includes (i) administering to a subject (e.g., a patient having a cancer or neoplastic disorder) an Tie1-binding antibody, conjugated to a detectable marker; (ii) exposing the subject to a means for detecting said detectable marker to the Tie1-expressing tissues or
20 cells. For example, the method can be used visualize blood vessels or the location of endothelial cells, e.g., Tie1-expressing endothelial cells. The subject can be imaged, e.g., by NMR or other tomographic means.

Examples of labels useful for diagnostic imaging in accordance with the invention include radiolabels such as ^{131}I , ^{111}In , ^{123}I , $^{99\text{m}}\text{Tc}$, ^{32}P , ^{125}I , ^3H , ^{14}C , and ^{188}Rh , fluorescent
25 labels such as fluorescein and rhodamine, nuclear magnetic resonance active labels, positron emitting isotopes detectable by a positron emission tomography ("PET") scanner, chemiluminescers such as luciferin, and enzymatic markers such as peroxidase or phosphatase. Short-range radiation emitters, such as isotopes detectable by short-range detector probes can also be employed. The protein ligand can be labeled with such
30 reagents using known techniques. For example, see Wensel and Meares (1983) *Radioimmunoimaging and Radioimmunotherapy*, Elsevier, New York for techniques

relating to the radiolabeling of antibodies and D. Colcher et al. (1986) *Meth. Enzymol.* 121: 802-816.

A radiolabeled ligand of this invention can also be used for *in vitro* diagnostic tests. The specific activity of a isotopically-labeled ligand depends upon the half-life, the isotopic purity of the radioactive label, and how the label is incorporated into the antibody.

Procedures for labeling polypeptides with the radioactive isotopes (such as ^{14}C , ^3H , ^{35}S , ^{125}I , ^{32}P , ^{131}I) are generally known. For example, tritium labeling procedures are described in U.S. Patent No. 4,302,438. Iodinating, tritium labeling, and ^{35}S labeling procedures, e.g., as adapted for murine monoclonal antibodies, are described, e.g., by

Goding, J.W. (*Monoclonal antibodies : principles and practice : production and application of monoclonal antibodies in cell biology, biochemistry, and immunology* 2nd ed. London ; Orlando : Academic Press, 1986. pp 124-126) and the references cited therein. Other procedures for iodinating polypeptides, such as antibodies, are described by Hunter and Greenwood (1962) *Nature* 144:945, David et al. (1974) *Biochemistry*

13:1014-1021, and U.S. Patent Nos. 3,867,517 and 4,376,110. Radiolabeling elements which are useful in imaging include ^{123}I , ^{131}I , ^{111}In , and $^{99\text{m}}\text{Tc}$, for example. Procedures for iodinating antibodies are described by Greenwood, F. et al. (1963) *Biochem. J.* 89:114-123; Marchalonis, J. (1969) *Biochem. J.* 113:299-305; and Morrison, M. et al. (1971) *Immunochemistry* 289-297. Procedures for $^{99\text{m}}\text{Tc}$ -labeling are described by

Rhodes, B. et al. in Burchiel, S. et al. (eds.), *Tumor Imaging: The Radioimmunochemical Detection of Cancer*, New York: Masson 111-123 (1982) and the references cited therein. Procedures suitable for ^{111}In -labeling antibodies are described by Hnatowich, D.J. et al. (1983) *J. Immunol. Methods*, 65:147-157, Hnatowich, D. et al. (1984) *J. Applied Radiation*, 35:554-557, and Buckley, R. G. et al. (1984) *F.E.B.S.* 166:202-204.

In the case of a radiolabeled ligand, the ligand is administered to the patient, is localized to the tumor bearing the antigen with which the ligand reacts, and is detected or "imaged" *in vivo* using known techniques such as radionuclear scanning using e.g., a gamma camera or emission tomography. See e.g., A.R. Bradwell et al., "Developments in Antibody Imaging", *Monoclonal Antibodies for Cancer Detection and Therapy*, R.W.

Baldwin et al., (eds.), pp 65-85 (Academic Press 1985). Alternatively, a positron emission transaxial tomography scanner, such as designated Pet VI located at Brookhaven National Laboratory, can be used where the radiolabel emits positrons (e.g., ^{11}C , ^{18}F , ^{15}O , and ^{13}N).

MRI Contrast Agents. Magnetic Resonance Imaging (MRI) uses NMR to visualize internal features of living subject, and is useful for prognosis, diagnosis, treatment, and surgery. MRI can be used without radioactive tracer compounds for obvious benefit. Some MRI techniques are summarized in EP-A-0 502 814. Generally, the differences related to relaxation time constants T1 and T2 of water protons in different environments are used to generate an image. However, these differences can be insufficient to provide sharp high resolution images.

The differences in these relaxation time constants can be enhanced by contrast agents. Examples of such contrast agents include a number of magnetic agents paramagnetic agents (which primarily alter T1) and ferromagnetic or superparamagnetic (which primarily alter T2 response). Chelates (e.g., EDTA, DTPA and NTA chelates) can be used to attach (and reduce toxicity) of some paramagnetic substances (e.g., Fe^{+3} , Mn^{+2} , Gd^{+3}). Other agents can be in the form of particles, e.g., less than 10 μm to about 10 nm in diameter). Particles can have ferromagnetic, antiferromagnetic or superparamagnetic properties. Particles can include, e.g., magnetite (Fe_3O_4), $\gamma\text{-Fe}_2\text{O}_3$, ferrites, and other magnetic mineral compounds of transition elements. Magnetic particles may include: one or more magnetic crystals with and without nonmagnetic material. The nonmagnetic material can include synthetic or natural polymers (such as sepharose, dextran, dextrin, starch and the like

The Tie1-binding ligands can also be labeled with an indicating group containing of the NMR-active ^{19}F atom, or a plurality of such atoms inasmuch as (i) substantially all of naturally abundant fluorine atoms are the ^{19}F isotope and, thus, substantially all fluorine-containing compounds are NMR-active; (ii) many chemically active polyfluorinated compounds such as trifluoroacetic anhydride are commercially available at relatively low cost, and (iii) many fluorinated compounds have been found medically acceptable for use in humans such as the perfluorinated polyethers utilized to carry oxygen as hemoglobin replacements. After permitting such time for incubation, a whole body MRI is carried out using an apparatus such as one of those described by Pykett (1982) *Scientific American*, 246:78-88 to locate and image cancerous tissues.

Information obtained from evaluating an Tie1-binding ligand, e.g., a ligand described herein, can be recorded on machine-compatible media, e.g., computer readable or computer accessible media. The information can be stored as a computer

representation, e.g., in a database (e.g., in the case of imaging using a ligand, a database of images for one or a plurality of subjects). The term "computer representation" refers to information which is in a form that can be manipulated by a computer. The act of storing a computer representation refers to the act of placing the information in a form suitable for
5 manipulation by a computer.

Also within the scope of the invention are kits including the protein ligand that binds to Tie1 and instructions for diagnostic use, e.g., the use of the Tie1-binding ligand (e.g., antibody or antigen-binding fragment thereof, or other polypeptide or peptide) to detect Tie1, *in vitro*, e.g., in a sample, e.g., a biopsy or cells from a patient having a cancer
0 or neoplastic disorder, or *in vivo*, e.g., by imaging a subject. The kit can further contain a least one additional reagent, such as a label or additional diagnostic agent. For *in vivo* use the ligand can be formulated as a pharmaceutical composition.

The following invention is further illustrated by the following examples (commencing on the following page), which should not be construed as limiting.

EXAMPLES

Example 1:

An exemplary Tiel amino acid sequence is as follows:

5 MVWRVPPFLLPILFLASHVGAADVLTLLANLRLTDPQRFFLTCVSGEAGAGRGSDAWGPP
 LLEKDDRIVRTPPGPPLRLARNGSHQVTLRGFSKPSDLVGVFSCVGGAGARRTRVIYVH
 NSPGAHLPLDKVTHTVNKGDTAVLSARVHKEKQTDVIWKSNGSYFYTLDWHEAQDGRFLL
 QLPNVQPPSSGIYSATYLEASPLGSAFFRLIVRGCGAGRWGPGCTKECPGCLHGGVCHDH
 DGEVCVPPGFTGTRCEQACREGRFGQSCQECPGISGCRGLTFCLPDPYGCSCSGSWRGS
 10 QCQEACAPGHFGADCRLLQCQCQNGGTCDRFSGCVCPSGWHGVHCEKSDRIPQILNMASEL
 EFNLETMPRINCAAAGNPPFVRGSIELRKPDGTVLLSTKAIVEPEKTTAEFEVPRVLAD
 SGFWECRVSTSGGQDSRRFKVNVKVPVPLAAPRLLTQSRQLVVSPLVSFSGDGPISTV
 RLHYRPQDSTMDWSTIVVDPSENVTLMNLRPKTGYSVRVQLSRPGEGGEGAWGPPTLMTT
 DCPEPLLQPWLEGWHVEGTDRLRVSWSLPLVPGPLVGDGFLRLWDGTRGQERRENVSSP
 15 QARTALLTGLTPGTHYQLDVQLYHCTLLGPASPPAHVLLPPSGPPAPRHLHAQALSDSEI
 QLTWKHPEALPGPI SKYVVEVQVAGGAGDPLWIDVDRPEETSTIIRGLNASTRYLFRMRA
 SIQGLGDWSNTVEESTLGNGLQAEQPVQESRAAEGLDQQLILAVVGSVSATCLTILAAAL
 LTLVCIRRSCLHRRRTFTYQSGSGEETILQFSSGTLTLTRRPKLQPEPLSYPVLEWEDIT
 FEDLIGEGNFGQVIRAMIKKDGKMNAAIKMLKEYASENDRDFAGELEVLCCKLGHHPNI
 20 INLLGACKNRGYLYIAIEYAPYGNLLDFLRKSRLVETDPAFAREHGTASTLSSRQLLRFA
 SDAANGMQYLSEKQFIHRDLAARNVLVGENLASKIADFGLSRGEEVYVKKTMGRPLPVRWM
 AIESLNYSVYTTKSDVWSFGVLLWEIVSLGGTPYCGMTCAELYEKLPPQGYRMEQPRNCDD
 EVYELMRQCWRDRPYERPPFAQIALQLGRMLEARKAYVNMSLFENFTYAGIDATAEEA

(SEQ ID NO:2)

25 An exemplary nucleic acid sequence that encodes Tiel is as follows:

atggtctggc	gggtgcccc	tttcttgctc	cccatcctct	tcttggtctc	tcattgtggc	60
gcggcggtgg	acctgacgct	gctggccaac	ctgcggctca	cggaccccc	gcgcttcttc	120
ctgacttgcg	tgtctgggga	ggccggggcg	gggaggggct	cggacgcctg	gggcccggcc	180
ctgctgctgg	agaaggacga	ccgtatcgctg	cgcaccccg	ccggggccacc	cctgcgcctg	240
gcgcgcaacg	gttcgcacca	ggtcacgctt	cgcggcttct	caaagccctc	ggacctcgctg	300
ggcgtcttct	cctgcgtggg	cgggtgctgg	gcgcggcgca	cgcgcgtcat	ctacgtgcac	360
aacagccctg	gagccacact	gcttcacag	aaggtcacac	acactgtgaa	caaaggtgac	420
accgctgtac	tttctgcacg	tgtgcacaag	gagaagcaga	cagacgtgat	ctggaagagc	480
aacggatcct	acttctacac	cctggactgg	catgaagccc	aggatggg	gttcctgctg	540
35 cagctcccaa	atgtgcagcc	accatcgagc	ggcatctaca	gtgccactta	cctggaagcc	600
agccccctgg	gcagcgcctt	cttctcgctc	atcgtgcggg	gttggtgggg	tgggcgctgg	660
gggcccaggct	gtaccaagga	gtgcccagg	tgcctacatg	gaggtgtctg	ccacgacct	720
gacggcgaat	gtgtatgccc	ccctggcttc	actggcacc	gctgtgaaca	ggcctgcaga	780
gagggccgtt	ttgggcagag	ctgcccagg	cagtgcacc	gcataatcagg	ctgcccgggg	840
40 ctcaccttct	gcctcccaga	cccctatggc	tgctcttgctg	gatctggctg	gagaggaagc	900
cagtgcgaag	aagcttgctg	ccctggctcat	tttggggctg	attgccgact	ccagtgccag	960
tgtcagaatg	gtggcacttg	tgaccggttc	agtgtgtgtg	tctgcccctc	tgggtggcat	1020
ggagtgcact	gtgagaagtc	agaccggatc	ccccagatcc	tcaacatggc	ctcagaactg	1080
gagttcaact	tagagacgat	gccccggatc	aactgtgcag	ctgcagggaa	ccccttcccc	1140
45 gtgcccgggca	gcataagact	acgcaagcca	gacggcactg	tgctcctgtc	caccaaggcc	1200
attgtggagc	cagagaagac	cacagctgag	ttcgaggtgc	cccgtttggt	tcttgccggac	1260
agtgggttct	gggagtgccg	tgtgtccaca	tctggcggcc	aagacagccg	gcgcttcaag	1320
gtcaatgtga	aagtgccttc	cgtgcccctg	gctgcacctc	ggctcctgac	caagcagagc	1380
cgccagcttg	tggctctccc	gctggtctcg	ttctctgggg	atggacccat	ctccactgtc	1440
50 cgcttcgact	accggcccca	ggacagctacc	atggactggt	cgaccattgt	ggtggacccc	1500
agtgagaacg	tcaggttaat	gaacctgagg	ccaaagacag	gatacagtg	tcgtgtgcag	1560
ctgagccggc	caggggaagg	aggagagggg	gcctgggggc	ctcccacctc	catgaccaca	1620
gactgtcctg	agcctttgtt	gcagccgtgg	ttggagggct	ggcatgtgga	aggcactgac	1680
cggctgcgag	tgagctggtc	cttgcccttg	gtgcccgggc	cactggtggg	cgacggtttc	1740

	ctgctgcgcc	tgtgggacgg	gacacggggg	caggagcggc	gggagaacgt	ctcatcccc	1800
	cagggccgca	ctgccctcct	gacgggactc	acgcctggca	cccactacca	gctggatgtg	1860
	cagctctacc	actgcaccct	cctgggcccg	gcctcgcccc	ctgcacacgt	gcttctgccc	1920
	cccagtgggc	ctccagcccc	ccgacacctc	cacgcccagg	ccctctcaga	ctccgagatc	1980
5	cagctgacat	ggaagcacc	ggaggctctg	cctgggccaa	tatccaagta	cgttgtggag	2040
	gtgcaggtgg	ctgggggtgc	aggagaccca	ctgtggatag	acgtggacag	gcctgaggag	2100
	acaagcacca	tcatccgtgg	cctcaacgcc	agcacgcgct	acctcttccg	catgcggggc	2160
	agcattcagg	ggctcgggga	ctggagcaac	acagtagaag	agtccaccct	gggcaacggg	2220
	ctgcaggctg	agggcccagt	ccaagagagc	cgggcagctg	aagagggcct	ggatcagcag	2280
10	ctgatccctg	cggtgggtgg	ctccgtgtct	gccacctgcc	tcaccatcct	ggccgccctt	2340
	ttaaccctgg	tgtgcatccg	cagaagctgc	ctgcatcgga	gacgcacctt	cacctaccag	2400
	tcaggctcgg	gcgaggagac	catactgcag	ttcagctcag	ggaccttgac	acttaccggg	2460
	cggccaaaac	tgcagcccga	gcccctgagc	taccagtgct	tagagtggga	ggacatcacc	2520
	tttgaggacc	tcatcgggga	ggggaacttc	ggccaggtca	tccgggccat	gatcaagaag	2580
15	gacgggctga	agatgaacgc	agccatcaaa	atgctgaaa	agtatgcctc	tgaaaatgac	2640
	catcgtagct	ttgcggggaga	actggaagtt	ctgtgcaaat	tggggcatca	ccccaacatc	2700
	atcaacctcc	tggggggcctg	taagaaccga	ggttacttgt	atatcgctat	tgaatatgcc	2760
	ccctacggga	acctgctaga	ttttctgctg	aaaagccggg	tcctagagac	tgaccagct	2820
	tttgctcgag	agcatgggac	agcctctacc	cttagctccc	ggcagctgct	gcgtttcgcc	2880
20	agtgatgcgg	ccaatggcat	gcagtacctg	agtgagaagc	agttcatcca	cagggaacctg	2940
	gctgcccggga	atgtgctggt	cggagagaac	ctagctcca	agattgcaga	cttcggcctt	3000
	tctcggggag	aggaggttta	tgtgaagaag	acgatggggc	gtctccctgt	gcgctggatg	3060
	gccattgagt	ccctgaacta	cagtgtctat	accaccaaga	gtgatgtctg	gtcctttgga	3120
	gtccttcttt	gggagatagt	gagccttgga	ggtacacctt	actgtggcat	gacctgtgcc	3180
25	gagctctatg	aaaagctgcc	ccagggctac	cgcattggagc	agcctcgaaa	ctgtgacgat	3240
	gaagtgtacg	agctgatgcg	tcagtgtctg	cgggaccgtc	cctatgagcg	accccccttt	3300
	gcccagattg	cgctacagct	aggccgcagt	ctggaagcca	ggaaggccta	tgtgaacatg	3360
	tcgctgtttg	agaacttcac	ttacgcgggc	attgatgcca	cagctgagga	ggcctga	3417

30 (SEQ ID NO:1)

Example 2: Selection and Primary Screening

We have used phage display to select Tie1-specific antibodies from a very large phage library that displays immunoglobulins as Fab fragments. To isolate antibodies
 35 specific to Tie1, a phage displayed Fab antibody library was selected against the Tie1 extracellular domain fused to human Fc or to a histidine purification tag.

Selection in solution was done using biotin labelled antigen which was captured on streptavidin coated magnetic beads (M-280-DYNAL). Selection on cells expressing Tie1
 40 was performed using a Kingfisher automated magnetic bead capture device. Selection on immobilized antigen was performed using Tie1-Fc coated onto immunotubes.

Several selection strategies were used :

Strategy 1 : Round 1 (500mM biotin labelled Tie1/magnetic beads), Round 2 (1 x 10⁷ Tie1 expressing cells/Kingfisher), Round 3 (1 x 10⁷ Tie1 expressing cells/Kingfisher)

Strategy 2 : Round 1 (500mM biotin labelled Tie1/magnetic beads), Round 2 (1 x 10⁷ Tie1 expressing cells/Kingfisher), (300mM biotin labelled Tie1/magnetic beads)
 45

Strategy 3 : Round 1 (Tie1 Fc coated immunotubes at 5 μ g/ml), Round 2 (Tie1-Fc coated immunotubes), Round 3 (Tie1 Fc coated immunotubes plus depletion with human IgG)

Library members recovered from the selection strategies were tested for antigen binding in phage ELISA. Each isolate was tested for binding to coated Tie1 Fc. Strategy 1 did not identify any binding clones whereas strategy 2 identified 13 positive clones ($n = 95$). Strategy 3 identified 86 binding clones ($n = 95$).

Sequence analysis of the selected clones were grouped on the basis of the CDR3 selected of the heavy chain and resulted in 23 different antibodies with unique VH-CDR3 sequences.

We reformatted the selected Fabs as completely human antibodies by recloning the VH and VL coding sequences from the display library vector into two vectors of a mammalian expression vector system. These vectors contain the human kappa constant domain and the human gamma-1 heavy chain constant region. The vectors were co-transfected into mammalian CHO-K1 cells for expression and production of the corresponding complete IgGs. These antibodies were characterized using several assays as described below, including:

1. Western blotting and immunoprecipitation of Tie1 transfected cells and primary human endothelial cells;
2. Immunofluorescence of Tie1 transfected cells and primary human endothelial cells;
3. Stimulation and inhibition of Tie1 in Ba/F3 cells and primary human endothelial cells; and
4. Immunostaining of human tissues.

We identified 23 antibodies that interact with Tie1. See also Table 1, below.

After sequence confirmation of the reformatted clones they were used in a transient transfection of Hek293T cells. After growth the IgG was purified from culture supernatants using a protein A column. The quality of purified IgG1 was determined using SDS-PAGE.

The specificity of the Tie1 specific IgG's can be determined in a whole cell ELISA on mouse lung microvascular endothelial cells (LEII) and LEII-Tie1 cells transfected with a Tie1 expression construct. Cells are seeded into 96 well plates at a density of 10,000

cells/well and were fixed using 4% paraformaldehyde. Staining and detection of binding of IgG1 to LE11 cells are detected using standard labelling with a HRP conjugated rabbit anti human HRP and TMB staining. Binding of purified IgG1 to LEII-Tie1 transfected cells can also be corrected for Tie1 protein that is expressed endogenously. Alternatively
5 cells that have little or no endogenous Tie1 can be used for the analysis.

At least one of the binding antibodies – E3 – functions as a Tie1 activating antibody in the BaF3 cell bioassay. We studied Tie1 phosphorylation in response to E3 IgG treatment in transiently transfected COS1 cells and human primary endothelial cells. Our results indicate that E3 IgG activates the Tie1 receptor. E3 can be used, instead of
10 possible natural ligands to characterize several functions of Tie1 *in vitro* and *in vivo*. The region of Tie1 which interacts with E3 can be the target for small molecular weight compounds for Tie1 activation or inhibition. Although E3 functions in one particular Tie1 activating assay, E3 and other positives in this assay may also have inhibitory effect as to other functions or in other contexts. For example, E3 can inhibit tube formation by
15 HUVEC cells. See below.

In addition, we found two antibodies that inhibit the survival effect conferred by E3 in the BaF3 cell bioassay. These two antibodies may inhibit dimerization of Tie1 induced by E3 in the BaF3 assay. Two antibodies, B2 and D11, completely blocked the viability of Tie1/EpoR cells when used in combination with E3. Thus, B2 and D11
20 antibodies can be used to inhibit Tie1 *in vivo*.

Methods

Cell culture

COS1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), glutamine and antibiotics. The murine
25 Ba/F3 pre-B lymphocytes were cultured in DMEM supplemented with 10% FCS, glutamine, antibiotics and 2 ng/ml interleukin-3 (Calbiochem). Human dermal microvascular endothelial cells (HDMVECs), obtained from PromoCell (Heidelberg, Germany) were cultured in endothelial cell medium provided by the supplier and used at passages 4-7.

Western blotting and immunoprecipitation

COS1 cells were transfected with pcDNA3-Tie1-V5 (1 µg DNA per 10 cm cell culture plate) using FUGENE 6 (Roche) according to manufacturer's instruction and incubated for 48 h before stimulation. For immunoprecipitation, Tie1 transfected cells and HMVEC cells were lysed in DOC-RIPA lysis buffer (50mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton-X-100, 0.1% SDS, 1% DOC, 10mM EDTA) supplemented with aprotinin, leupeptin, PMSF and sodium vanadate. Immunoprecipitation was carried out from equal amount of cell lysates by incubating with polyclonal anti-human Tie1 antibodies (R&D), monoclonal anti-V5 antibodies (Invitrogen) or altogether 23 anti-Tie1 antibodies (1 µg/ml) for 1 to 2 h followed by incubation with protein G-Sepharose (Amersham Pharmacia Biotech AB) for 1 h. The immunoprecipitates were washed twice with PBS-T and twice with PBS, followed by elution with the Laemmli buffer and separation in 8% SDS-PAGE. The blots were probed with the 23 anti-Tie1 antibodies (5 µg/ml) and subsequently anti-human Fc antibodies conjugated with HRP.

Immunofluorescence staining

COS1 cells on the glass coverslips were transiently transfected with pcDNA3-Tie1-V5 (the V5-epitope was added to the 3' terminus of pcDNA3-Tie1) (1 µg DNA per 10 cm cell culture plate) using FUGENETM 6 (Roche) according to manufacturer's instruction and incubated for 48 h before staining. Cells were fixed in 4% paraformaldehyde for 10 min at 4°C. If required, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. Unspecific binding sites were blocked by incubation with 1% BSA in PBS for 30 min. The cells were then stained with anti-Tie1 antibodies (5 µg/ml) and anti-V5 antibodies for 1 h at room temperature, followed by incubation with FITC- conjugated anti-human antibodies (DAKO, 40 µg/ml) and TRITC-conjugated anti-mouse antibodies (DAKO, 15 µg/ml) for 30 min. Hoechst 33258 fluorochrome (Sigma, 0.5 µg/ml) was used for the staining of the nuclei.

Ba/F3 bioassay

To generate Tie1-EpoR expressing Ba/F3 cells for the bioassay, Ba/F3 pre-B cells were stably transfected with a nucleic acid that expresses chimeric receptor containing the extracellular domain of human Tie1 fused with the transmembrane and cytoplasmic

domains of the mouse erythropoietin receptor. The nucleic acid used was a Tie1-EpoR chimeric cDNA in a pEF-BOS expression vector. The nucleic acid encoding the chimeric receptor was constructed by cloning the PCR amplified extracellular part of human Tie1 (bp 37-2316 of X60975) as EcoRI-BglIII fragment into mEpoR-pcDNA vector. The cDNA encoding for the chimeric receptor consisting of the extracellular part of Tie1 fused with the transmembrane and intracellular domains of EpoR was subcloned into the pEF-BOS expression vector. Vector was linearized and co-transfected into Ba/F3 cells with pcDNA3.1(+) Zeo vector (Invitrogen). Stable cell pools were generated by selection with 250 µg/ml Zeocin. The expression of Tie1/EpoR fusion protein in several clones was analyzed by Western blotting with an antibody against EpoR.

To perform the assays, Ba/F3 cells expressing the Tie1-EpoR chimera were split in 96-well microtiter plates at 50 000 cells/well in the presence of the indicated concentrations of anti-Tie1 antibodies. As controls, Zeocin resistant pools not expressing the Tie1-EpoR were used. After 48 h, the viability of the cells was determined by adding MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma), 0.5 mg/ml), followed by further 2 h of culture, addition of an equal volume of cell lysis solution (10% SDS, 10 mM HCl) and incubation overnight at 37°C. Absorbance was measured at 540 nm.

Tie1 phosphorylation assay

COS1 cells were transfected with pcDNA3-Tie1-V5. After 24 h of transfection, the cells were serum starved for 8 h and then treated with E3 IgG. For the Tie1 phosphorylation assay, HDMVECs were cultured on 10 cm dishes to near confluence, starved (8-16 h) in serum free medium and stimulated as indicated. After the stimulations, the cells were lysed in lysis buffer (RIPA-DOC: 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton-X-100, 0.1% SDS, 0.5% DOC, 10 mM EDTA, supplemented with aprotinin, leupeptin, PMSF and sodium vanadate). Clarified lysates from transfected COS1 cells or HDMVECs were immunoprecipitated with anti-V5 or anti-Tie1 B9, respectively. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted using the anti-phosphotyrosine and anti-Tie1 (R&D systems) antibodies.

Immunostaining of human tissues

- To evaluate reactivity of anti-Tie1 antibodies in immunohistochemistry, 5 μ m cryosections of human kidney and lung were dried at room temperature for 30 min and fixed with cold acetone for 10 min. Slides were washed with PBS and treated with 0.03 %
- 5 H₂O₂ in PBS for 15 min to reduce endogenous peroxidase activity. TNB (30 min at room temperature) was used to block non-specific binding and sections were incubated with Tie1 antibodies at concentration of 10 μ g/ml overnight at + 4°C. After several washings with PBS, biotinylated anti human antibody (1:300, Zymed) was added to the tissues. Signal was amplified by using a TSA kit and detected with AEC staining.

10 **Results**

Western blotting, immunoprecipitation and immunofluorescence of Tie1 transfected cells and primary human endothelial cells (see Table 1).

Table 1: Assay Summary

Clone	WB: Tie1-transfected	WB: HDMEC	IP: Tie1-transfected	IP: HDMEC	IF: Tie1-transfected	IF: HDMEC	BaF3 assay
E3	+	+	ND	-	+	+	+
G2	+	+	++	+	+	++	-
A2	+	+	++	+	+	++	-
A10	+	+	++	+	+	+	-
B2	+	+	+	-	+	+	-
B9	+	+	++	++	++	+	-
C2	+	+	++	++	+	++	-
C7	+	+	++	+	+	+	-
C10	+	+	++	++	++	+	-
D11	+	+	+	-	+	++	-
E11	+	+	++	+	+	++	-
G10	+	+	++	+	++	+	-
H1	+	+	++	+	++	+	-
H4	+	+	++	+	+	+	-
P-A1	+	+	++	++	+	++	-
P-A10	+	+	++	-	+	+	-
P-B1	+	+	+	-	weak	+	-
P-B3	+	+	-	-	+	+	-
P-C6	+	+	+	-	+	++	-
P-D12	+	+	+	-	+	+	-
P-F3	+	+	-	-	++	++	-
P-F4	+	+	++	-	cross	++	-
P-G3	+	+	++	+	+	+	-
PH1	-	-	-	-	-	-	-

To confirm the binding ability of the 23 selected anti-Tie1 antibodies, we first performed western blotting and immunoprecipitation using COS1 cells transfected with pcDNA3-Tie1-V5 (V5 tagged) and primary endothelial cells. Next, to find out if the anti-Tie1 antibodies recognize Tie1 in living cells, those cells were studied by immunofluorescence staining. All the antibodies analyzed recognized both transfected and endogenous Tie1, although differences were detected in the binding affinity as shown in Table 1.

10 ***Stimulation and inhibition of Tie1 in Tie1-EpoR transfected Ba/F3 cells and human primary endothelial cells***

Although no ligand for Tie1 has been identified, we used the following efficient screening method for Tie1-binding ligands. Interleukin-3 dependent pre-B-lymphocyte (Ba/F3) cells were transfected with a construct that expresses a Tie1-EpoR fusion protein. Since Ba/F3 cells are IL-3 dependent, they die unless IL-3 is provided. However, Tie-EpoR receptor expressing Ba/F3 cells can survive and proliferate if the medium contains a Tie1-binding ligand, either a natural ligand or an artificial mimetic. Cell survival can be quantitated, e.g., by colorimetric MTT-assay, which measures mitochondrial activity.

The results from the BaF3 cell assays indicated that, of the 23 different monoclonal antibodies tested, only E3 IgG was able to promote survival of Tie1-EpoR cells whereas the viability of EpoR Ba/F3 cells used as a control was not affected by E3 IgG. The IgG part of the immunoglobulin molecule was needed for the survival effect of E3 IgG, as the E3 Fab fragment had no effect on the viability of Tie1-EpoR cells. A concentration of 50 ng/ml of E3 IgG gave almost maximal viability in Tie1-EpoR cell survival assays.

To test if the E3 IgG binding to the extracellular region of Tie1 induces autophosphorylation of Tie1, the Tie1 receptor phosphorylation level in response to E3 IgG treatment was studied in transiently transfected COS1 cells and human primary endothelial cells. COS1 cells were transfected with an expression vector containing a V5-tagged full length Tie1 cDNA, and, after serum starvation, the cells were treated with E3 IgG (200 ng/ml). Cell lysates were extracted at several time points and Tie1 was immunoprecipitated with anti-V5 followed by western blotting using anti-phosphotyrosine and anti-Tie1 antibodies. The results indicated that Tie1 is tyrosine phosphorylated after

10 to 30 min of E3 IgG stimulation. To determine if E3 IgG induces Tie1 phosphorylation in primary endothelial cells, HDMVEC cells were serum starved and stimulated with several concentrations of E3 for 60 min. Tie1 was then immunoprecipitated from cell lysates and subjected to anti-phosphotyrosine blotting analysis, which showed receptor phosphorylation following E3 IgG stimulation at 50-200 ng/ml. Also higher concentrations of E3 (500-1000 ng/ml) induced Tie1 phosphorylation but the response was more rapid and was most prominent after 5 min of stimulation.

To study the kinetics of E3 IgG induced Tie1 activation, cells were stimulated with E3 IgG (200 ng/ml) and receptor phosphorylation was studied at various time points. Tie1 phosphorylation was highest 15-30 min after E3 IgG treatment but phosphorylation persisted for up to 1 h.

To determine if any of the other monoclonal antibodies tested inhibit the survival effect of E3 IgG in Tie1-EpoR Ba/F3 assay, antibodies were studied in combination with E3 IgG. A concentration of 100 ng/ml of E3 IgG together with 100 (1:1) or 500 (1:5) ng/ml of the other antibodies were used and the viability of Tie1-EpoR cells was measured. The results from both combinations of E3 IgG and the test antibody (in 1:1 and 1:5 ratios) were similar and indicated that two of the 23 antibodies (B2 and D11) blocked completely the survival effect of E3 IgG (Table 2). Several antibodies (A2, A10, P-B1, P-B3 and P-C6) inhibited the viability effect of E3 IgG to some extent and two of the antibodies (G2 and C7) promoted the survival of Tie1/EpoR Ba/F3 cells in combination with E3 IgG.

Table 2: BaF3 Assay

Treatment	Average MTT activity in BaF3 cell assay	St. Dev.
IL3, 2	0.48	0.038
0	0.01	0.003
E3	0.61	0.032
E3+G2	0.74	0.034
E3+A2	0.38	0.016
E3+A10	0.52	0.011
E3+B2	0.00	0.001
E3+B9	0.65	0.011
E3+C2	0.62	0.016
E3+C7	0.84	0.086
E3+C10	0.67	0.004
E3+D11	0.01	0.003
E3+E11	0.61	0.048
E3+G10	0.65	0.010
E3+H1	0.60	0.017
E3+H4	0.65	0.026
E3+PA1	0.60	0.035
E3+PA10	0.65	0.041
E3+PB1	0.47	0.011
E3+P-B3	0.51	0.007
E3+PC6	0.35	0.008
E3+PD12	0.60	0.038
E3+PF3	0.55	0.032
E3+PF4	0.56	0.009
E3+PG3	0.77	0.030
E3+PH1	0.63	0.040

Immunostaining of human tissues

The anti-Tie1 antibodies react with human Tie1 in cultured cells. It is also possible
5 to determine whether they could stain human tissue samples from lung and kidney as well
as from tumors by using biotinylated anti-Tie1 antibodies and detecting bound antibodies
using labeled streptavidin or avidin.

In one embodiment, Tie ligands (such as the Tie1 antibodies described herein can
be produced from gene-based vectors, such as transgenes or via adenoviral delivery.

10

Example 3: Exemplary Sequences

The following are sequences of exemplary immunoglobulin variable domains:

Translation of Reverse_Complement_VH-PA1-Tie1-phagemid.TXT(1-396)

Universal code

Total amino acid number: 132, MW=14169

5 Max ORF: 1-396, 132 AA, MW=14169

```

1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1      E V Q L L E S G G G L V Q P G G S L R L

10 61      TCTTGCGCTGCTTCCGGATTCACTTTCTCTATTTACAAGATGTCTTGGGTTCGCCAAGCT
21      S C A A S G F T F S I Y K M S W V R Q A

121     CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCCAGACTAAGTAT
41      P G K G L E W V S S I Y P S G G Q T K Y

15 181     GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61      A D S V K G R F T I S R D N S K N T L Y

241     TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
20 81      L Q M N S L R A E D T A V Y Y C A R V N

301     TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
101     Y Y D S S G Y G P I A P G L D Y W G Q G

25 361     ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGC (SEQ ID NO:3)
121     T L V T V S S A S T K G (SEQ ID NO:4)

```

Translation of VL-PA1-Tie1-phagemid.TXT(1-311)

Universal code

30 Total amino acid number: 103, MW=11169

Max ORF: 1-309, 103 AA, MW=11169

```

1      CAAGACATCCAGATGACCCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCC
1      Q D I Q M T Q S P G T L S L S P G E R A

35 61      ACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAG
21      T L S C R A S Q S V S S S Y L A W Y Q Q

121     AAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATC
40 41      K P G Q A P R L L I Y G A S S R A T G I

181     CCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTG
61      P D R F S G S G S G T D F T L T I S R L

15 241     GAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGGTAGCTCCCGGTGGACGTTTC
81      E P E D F A V Y Y C Q Q Y G S S R W T F

301     GGCCAAGGGAC (SEQ ID NO:5)
101     G Q G (SEQ ID NO:6)

50

```

Translation of Reverse_Complement_VH-PA5-Tie1-phagemid.TXT(1-396)

Universal code

Total amino acid number: 132, MW=14309

Max ORF: 1-396, 132 AA, MW=14309

```

5      1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
      1      E V Q L L E S G G G L V Q P G G S L R L

      61      TCTTGCCTGCTTCCGGATTCACTTTCTCTTATTACCTTATGTATTGGGTTTCGCCAAGCT
10     21      S C A A S G F T F S Y Y L M Y W V R Q A

      121     CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCTGGACTGTTTAT
      41      P G K G L E W V S S I Y P S G G W T V Y

      15     181    GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
      61      A D S V K G R F T I S R D N S K N T L Y

      241     TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
20     81      L Q M N S L R A E D T A V Y Y C A R V N

      301     TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
      101     Y Y D S S G Y G P I A P G L D Y W G Q G

      361     ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGC (SEQ ID NO:7)
25     121     T L V T V S S A S T K G (SEQ ID NO:8)

```

Translation of VL-PA5-Tie1-phagemid.TXT(1-308)

Universal code

30 Total amino acid number: 101, MW=10801

Max ORF: 85-306, 74 AA, MW=7983

```

      1      CAAGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTCCGAGACAGAGTC
35     1      Q D I Q M T Q S P S S L S A S V G D R V

      61      ACCATCACTTGCCGGGCAAGTTAGAGCATTAGCACCTCTTTAAATTGGTATCAGCAAAAA
      21      T I T C R A S * S I S T S L N W Y Q Q K

      121     TCAGGGAAAGCCCCTAAGCTCCTGATATATGCTGCATCCAGTTTGCAAAGTGAAGTCCCA
40     41      S G K A P K L L I Y A A S S L Q S E V P

      181     TCAAGGTTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCACCAGTCTGCAA
      61      S R F S G S G S G T D F T L T I T S L Q

      15     241     CCTGAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTACCCCTCCGACTTTTCGGC
      81      P E D F A T Y Y C Q Q S Y S T P P T F G

      301     CAAGGGAC (SEQ ID NO:9)
50     101     Q G (SEQ ID NO:10)

```

Translation of VH-PA6-Tie1-phagemid.TXT(1-439)

Universal code

Total amino acid number: 146, MW=15647

Max ORF: 1-438, 146 AA, MW=15647

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L

61 TCTTGCGCTGCTTCCGGATTCACTTTCTCTATGTACGTTATGAAGTGGGTTCGCCAAGCT
10 21 S C A A S G F T F S M Y V M K W V R Q A

121 CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCTATACTCGTTAT
41 P G K G L E W V S S I Y P S G G Y T R Y

15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y

241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
81 L Q M N S L R A E D T A V Y Y C A R V N

20 301 TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
101 Y Y D S S G Y G P I A P G L D Y W G Q G

361 ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCTAGCACCC
25 121 T L V T V S S A S T K G P S V F P L A P

421 TCCTCCAAGAGCACCTCTG (SEQ ID NO:11)
141 S S K S T S (SEQ ID NO:12)

30

Translation of VL-PA6-Tie1-phagemid.TXT(1-311)

Universal code

Total amino acid number: 103, MW=11056

Max ORF: 1-309, 103 AA, MW=11056

35 1 CAAGACATCCAGATGACCCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCC
1 Q D I Q M T Q S P G T L S L S P G E R A

61 ACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAG
40 21 T L S C R A S Q S V S S S Y L A W Y Q Q

121 AAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATC
41 K P G Q A P R L L I Y G A S S R A T G I

45 181 CCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTG
61 P D R F S G S G S G T D F T L T I S R L

241 GAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGGTAGCTCCCTATTCACTTTC
81 E P E D F A V Y Y C Q Q Y G S S L F T F

50 301 GGCCCTGGGAC (SEQ ID NO:13)
101 G P G (SEQ ID NO:14)

55

Translation of VH-PA10-Tie1-phagemid.TXT(1-439)

Universal code

Total amino acid number: 146, MW=15499

Max ORF: 1-438, 146 AA, MW=15499

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGGCGCTGCTTCCGGATTCACTTTCTCTTCTTACAAGATGGGTTGGGTTCCGCAAGCT
10 21 S C A A S G F T F S S Y K M G W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTTGGATCTATCCTTCTGGTGGCGGTACTACTTAT
41 P G K G L E W V S W I Y P S G G G T T Y
15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
81 L Q M N S L R A E D T A V Y Y C A R V N
20 301 TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
101 Y Y D S S G Y G P I A P G L D Y W G Q G
361 ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCTAGCACCC
25 121 T L V T V S S A S T K G P S V F P L A P
421 TCCTCCAAGAGCACCTCTG (SEQ ID NO:15)
141 S S K S T S (SEQ ID NO:16)

30

Translation of VL-PA10-Tie1-phagemid.TXT(1-311)

Universal code

Total amino acid number: 103, MW=11110

Max ORF: 1-309, 103 AA, MW=11110

35
1 CAAGACATCCAGATGACCCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCC
1 Q D I Q M T Q S P G T L S L S P G E R A
61 ACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGGTACCAGCAG
40 21 T L S C R A S Q S V S S S Y L A W Y Q Q
121 AAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATC
41 K P G Q A P R L L I Y G A S S R A T G I
45 181 CCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTG
61 P D R F S G S G S G T D F T L T I S R L
241 GAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGGTAGCTCACCGTGGACGTTT
81 E P E D F A V Y Y C Q Q Y G S S P W T F
50 301 GGCCAAGGGAC (SEQ ID NO:17)
101 G Q G (SEQ ID NO:18)

55

Translation of VH-PB1-Tie1-phagemid.TXT(1-446)

Universal code

Total amino acid number: 148, MW=15809

Max ORF: 1-444, 148 AA, MW=15809

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCCTGCTTCCGGATTCACTTTCTCTCGTTACCCTATGGTTTGGGTTCCGCAAGCT
10 21 S C A A S G F T F S R Y P M V W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTCTCCTTCTGGTGGCCAGACTTTTTAT
41 P G K G L E W V S V I S P S G G Q T F Y
15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGGGGTC
81 L Q M N S L R A E D T A V Y Y C A R G V
20 301 CTCACCACCGCTTTTGTATATCTGGGGCCAAGGGACAATGGTCACCGTCTCAAGCGCCTCC
101 L T T A F D I W G Q G T M V T V S S A S
361 ACCAAGGGCCCCATCGGTCTTCCCGCTAGCACCTCCTCCAAAGCACCTCTGGGGGCACAG
25 121 T K G P S V F P L A P S S K A P L G A Q
421 CGGCCCTGGGCTGCCTGGTCAAGGAC (SEQ ID NO:19)
141 R P W A A W S R (SEQ ID NO:20)

30

Translation of VL-PB1-Tie1-phagemid.TXT(1-308)

Universal code

Total amino acid number: 102, MW=11057

Max ORF: 1-306, 102 AA, MW=11057

35
1 CAAGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTGGGAGACAGAGTC
1 Q D I Q M T Q S P S S L S A S V G D R V
61 ACCATCACTTGCCGGGCAAGTCAGAACATTAACAGCTATTTAAATTGGTATCAGCAGAAA
40 21 T I T C R A S Q N I N S Y L N W Y Q Q K
121 CCAGGGCAAGCCCCCTAAACTCCTGATCTATGCTGCCTCCAATTTGGAAACTGCGGTCCCA
41 P G Q A P K L L I Y A A S N L E T A V P
45 181 TCAAGGTTTCAGCGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGTAGCCTGCAG
61 S R F S G S G S G T D F T L T I S S L Q
241 CCTGAAGATTTTGCAACTTATTATTGTCAACAATTTAATACTTACCCTCTCACTTTTCGGC
81 P E D F A T Y Y C Q Q F N T Y P L T F G
50 301 GGAGGGAC (SEQ ID NO:21)
101 G G (SEQ ID NO:22)

55

Translation of VH-PB3-Tie1-phagemid.TXT(1-393)

Universal code

Total amino acid number: 131, MW=13931

Max ORF: 1-393, 131 AA, MW=13931

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L

61 TCTTGCCTGCTTCCGGATTCACTTTCTCTCGTTACGGTATGCATTGGGTTCGCCAAGCT
10 21 S C A A S G F T F S R Y G M H W V R Q A

121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTCTCCTTCTGGTGGCATGACTTATTAT
41 P G K G L E W V S V I S P S G G M T Y Y

15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACACTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N T K N T L Y

241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTGGGA
81 L Q M N S L R A E D T A V Y Y C A R V G

20 301 GCTACCGGGCCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCAAGCGCCTCC
101 A T G P F D I W G Q G T M V T V S S A S

361 ACCAAGGGCCCATCGGTCTTCCCGCTAGCACCC (SEQ ID NO:23)
25 121 T K G P S V F P L A P (SEQ ID NO:24)

Translation of VL-PB3-Tie1-phagemid.TXT(1-308)

Universal code

30 Total amino acid number: 102, MW=11032

Max ORF: 1-306, 102 AA, MW=11032

1 CAAGACATCCAGATGACCCAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCC
1 Q D I Q M T Q S P A T L S L S P G E R A

35 61 ACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCACCTACTTAGCCTGGTACCAACAGAAA
21 T L S C R A S Q S V S T Y L A W Y Q Q K

121 CCTGGCCAGGCTCCCAGGCTTCTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCA
40 41 P G Q A P R L L I Y D A S N R A T G I P

181 GGCAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAG
61 G R F S G S G S G T D F T L T I S S L E

45 241 GCTGAAGACTTTGCAGTTTATTACTGTGTCAGCAGCGTAGCAGCTGGCCGATCACCTTCGGC
81 A E D F A V Y Y C Q Q R S S W P I T F G

301 CAAGGGAC (SEQ ID NO:25)
101 Q G (SEQ ID NO:26)
50

Translation of VH-PC6-Tie1-phagemid.TXT(1-429)

Universal code

Total amino acid number: 143, MW=14727

Max ORF: 1-429, 143 AA, MW=14727

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGGCGCTGCTTCCGGATTCACTTTCTCTCATTACGGTATGACTTGGGTTCGCCAAGCT
10 21 S C A A S G F T F S H Y G M T W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTCTCCTTCTGGTGGCCAGACTGGTTAT
41 P G K G L E W V S V I S P S G G Q T G Y
15 181 GCTGACTCCGTTAAAGGTGCGTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGGGTGGTGGC
81 L Q M N S L R A E D T A V Y Y C A G G G
20 301 TACGCAGCCTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCAAGCGCCTCCACC
101 Y A A F D Y W G Q G T L V T V S S A S T
361 AAGGGCCCATCGGTCTTCCCGCTAGCACCTCCTCCAAGAGCACCTCTGGGGGCACAGCG
25 121 K G P S V F P L A P S S K S T S G G T A
421 GCCCTGGGC (SEQ ID NO:27)
141 A L G (SEQ ID NO:28)

30

Translation of VL-PC6-Tie1-phagemid.TXT(1-308)

Universal code

Total amino acid number: 102, MW=11014

Max ORF: 1-306, 102 AA, MW=11014

35
1 CAAGACATCCAGATGACCCAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCC
1 Q D I Q M T Q S P A T L S L S P G E R A
61 ACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCTACTTAGCCTGGTACCAACAGAAA
40 21 T L S C R A S Q S V S S Y L A W Y Q Q K
121 CCTGGCCAGGCTCCCAGGCTCCTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCA
41 P G Q A P R L L I Y D A S N R A T G I P
45 181 GCCAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAG
61 A R F S G S G S G T D F T L T I S S L E
241 CCTGAAGATTTTGCAGTTTATTACTGTCAGCAGCGTAGCAACTGGCCTCTCACTTTTCGGC
81 P E D F A V Y Y C Q Q R S N W P L T F G
50 301 GGAGGGAC (SEQ ID NO:29)
101 G G (SEQ ID NO:30)

55

Translation of Reverse_Complement_VH-PD6-Tie1-phagemid.TXT(1-396)

Universal code

Total amino acid number: 132, MW=14217

Max ORF: 1-396, 132 AA, MW=14217

```

5      1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
      1      E V Q L L E S G G G L V Q P G G S L R L

      61      TCTTGCGCTGCTTCCGGATTCACTTTCTCTGCTTACCGTATGGAGTGGGTTCGCCAAGCT
10     21      S C A A S G F T F S A Y R M E W V R Q A

      121     CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCATTACTTATTAT
      41      P G K G L E W V S S I Y P S G G I T Y Y

15     181     GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACTCTAAGAATACTCTCTAC
      61      A D S V K G R F T I S R D N S K N T L Y

      241     TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
20     81      L Q M N S L R A E D T A V Y Y C A R V N

      301     TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
      101     Y Y D S S G Y G P I A P G L D Y W G Q G

      361     ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGC (SEQ ID NO:31)
25     121     T L V T V S S A S T K G (SEQ ID NO:32)

```

Translation of VL-PD6-Tie1-phagemid.TXT(1-308)

Universal code

30 Total amino acid number: 101, MW=10731

Max ORF: 115-306, 64 AA, MW=6731

```

      1      CAAGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTT
      1      Q D I Q M T Q S P S S L S A S V G D R V

35     61      ACCATCACTTGCCGGGCAAGTCAGGGCATTACCACTTATTTAGGCTGGTATTAGCAGAAA
      21      T I T C R A S Q G I T T Y L G W Y * Q K

      121     CCAGGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCACTTTGCAAAGTGGGGTCCCA
40     41      P G K A P K L L I Y A A S T L Q S G V P

      181     GCAAAGTTCAGCGGCAGTGGATCTGGGACACTTTTCACTCTCACCATCAGCGGTCTGCAA
      61      A K F S G S G S G T L F T L T I S G L Q

45     241     CCTGAAGATTCTGCAACTTACTACTGTCACCAGAGTTACAATACCCCTTGGACGTTTCGGC
      81      P E D S A T Y Y C H Q S Y N T P W T F G

      301     CAAGGGAC (SEQ ID NO:33)
50     101     Q G (SEQ ID NO:34)

```

Translation of VH-PD10-Tie1-phagemid.TXT(1-412)

Universal code

Total amino acid number: 136, MW=14313

Max ORF: 181-411, 77 AA, MW=8216

5
1 GAAGTTC AATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCGCTGCTTCCGGATTCACCTTCTCTGGTTACGGTATGCATTGGGTTGCGCAAGCT
10 21 S C A A S G F T F S G Y G M H W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTCTCCTTCTGGTGGCCAGACTTGGTAG
41 P G K G L E W V S V I S P S G G Q T W *
15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGGCGGG
81 L Q M N S L R A E D T A V Y Y C A R G G
20 301 ACCAGTAACCCACTGT'TTTACTGGGGCCAGGGAACCCTGGTCACCGTCTCAAGCGCCTCC
101 T S N P L F Y W G Q G T L V T V S S A S
361 ACCAAGGGCCCATCGGTCTTCCCGCTAGCACCCCTCCTCCAAGAGCACCTCTG (SEQ ID
25 NO:35)121 T K G P S V F P L A P S S K S T S (SEQ
ID NO:36)

Translation of VL-PD10-Tie1-phagemid.TXT(1-308)

30 Universal code

Total amino acid number: 102, MW=11069

Max ORF: 1-306, 102 AA, MW=11069

35 1 CAAGACATCCAGATGACCCAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAAAGAGCC
1 Q D I Q M T Q S P A T L S L S P G E R A
61 ACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCTACTTAGCCTGGTACCAACAGAAA
21 T L S C R A S Q S V S S Y L A W Y Q Q K
40 121 CCTGGCCAGGCTCCCAGGCTCCTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCA
41 P G Q A P R L L I Y D A S N R A T G I P
181 GCCAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAG
61 A R F S G S G S G T D F T L T I S S L E
45 241 CCTGAAGATTTTGCAGTTTATTACTGTCAGCAGCGTAGCAACTGGCCTCCGACTTTTGGC
81 P E D F A V Y Y C Q Q R S N W P P T F G
301 CAGGGGAC (SEQ ID NO:37)
50 101 Q G (SEQ ID NO:38)

Translation of VH-PD12-Tie1-phagemid.TXT(1-444)

Universal code

Total amino acid number: 148, MW=15535

Max ORF: 1-444, 148 AA, MW=15535

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCGCTGCTTCCGGATTCACTTTCTCTGGTTACGGTATGCATTGGGTTCCGCAAGCT
10 21 S C A A S G F T F S G Y G M H W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTCTCCTTCTGGTGGCCAGACTTCTTAT
41 P G K G L E W V S V I S P S G G Q T S Y
15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGATAGG
81 L Q M N S L R A E D T A V Y Y C A R D R
20 301 CAGTATTACTATGGTTCCGGGAGTCTTGACTACTGGGGCCAGGGAACCCCTGGTCACCGTC
101 Q Y Y Y G S G S L D Y W G Q G T L V T V
361 TCAAGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCTAGCACCCCTCCTCCAAGAGCACC
25 121 S S A S T K G P S V F P L A P S S K S T
421 TCTGGGGGCACAGCGGCCCTGGGC (SEQ ID NO:39)
141 S G G T A A L G (SEQ ID NO:40)

30

Translation of VL-PD12-Tie1-phagemid.TXT(1-308)

Universal code

Total amino acid number: 102, MW=11060

Max ORF: 1-306, 102 AA, MW=11060

35
1 CAAGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTC
1 Q D I Q M T Q S P S S L S A S V G D R V
61 ACCGTCACTTGCCGGGCAAGTCAGAGCATTAGCAGTTATTTAAATTGGTATCAGCAGAAA
40 21 T V T C R A S Q S I S S Y L N W Y Q Q K
121 CCAGGGAAAGCCCCTAAACTCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCA
41 P G K A P K L L I Y A A S S L Q S G V P
45 181 TCAAGGTTCACTGGCGGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAA
61 S R F S G G G S G T D F T L T I S S L Q
241 CCTGAAGATTTTGCAACTTATTTCTGTCTACAAGATTACAAATACCCGTGGACGTTTCGGC
50 81 P E D F A T Y F C L Q D Y K Y P W T F G
301 CAAGGGAC (SEQ ID NO:41)
101 Q G (SEQ ID NO:42)

55

Translation of Reverse_Complement_VH-PF3-Tie1-phagemid.TXT(1-375)

Universal code

Total amino acid number: 125, MW=13201

Max ORF: 1-375, 125 AA, MW=13201

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCCTGCTTCCGGATTCACTTTCTCTATGTACGGTATGGGTGGGTTCGCCAAGCT
10 21 S C A A S G F T F S M Y G M G W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTCTCCTTCTGGTGGCCAGACTGCTTAT
41 P G K G L E W V S V I S P S G G Q T A Y
15 181 GCTGACTCCGTTAAAGGTGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTGGCC
81 L Q M N S L R A E D T A V Y Y C A R V A
20 301 TTGCTCCTGGGCCACGCTTTTGATATCTGGGGCCAAGGGACAATGGTCACCGTCTCAAGC
101 L L L G H A F D I W G Q G T M V T V S S
361 GCCTCCACCAAGGGC (SEQ ID NO:43)
25 121 A S T K G (SEQ ID NO:44)

Translation of VL-PF3-Tie1-phagemid.TXT(1-308)

Universal code

30 Total amino acid number: 102, MW=11162

Max ORF: 1-306, 102 AA, MW=11162

1 CAAGACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATCTTTAGGAGACAGAGTC
1 Q D I Q M T Q S P S T L S A S L G D R V
35 61 ACCATCACTTGCCGGGCCAGTGAGAGTATTAGTAGGTGGTGGCCTGGTATCAGCAGAAA
21 T I T C R A S E S I S R W L A W Y Q Q K
121 CCAGGGAAAGCCCCTAAGCTCCTGATGTATGAGGCATCCACTTTAGAAAGTGGGGTCCCA
40 41 P G K A P K L L M Y E A S T L E S G V P
181 TCAAGGTTACCGGCACTGGATCTGGGACAGAATTCACTCTCACCATCAGCAGCCTGCAG
61 S R F T G T G S G T E F T L T I S S L Q
45 241 CCCGATGATTTTGCAACTTATTACTGTCTCAGCAGCGTAGCAACTGGCCCCCTCACTTTTCGGC
81 P D D F A T Y Y C Q Q R S N W P L T F G
301 GGAGGGAC (SEQ ID NO:45)
101 G G (SEQ ID NO:46)
50

Translation of VH-PF4-Tie1-phagemid.TXT(1-429)

Universal code

Total amino acid number: 143, MW=14996

Max ORF: 1-429, 143 AA, MW=14996

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCGCTGCTTCCGGATTCACTTTTCTCTGCTTACATGATGTCTTGGGTTTCGCCAAGCT
10 21 S C A A S G F T F S A Y M M S W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCTATACTTATTAT
41 P G K G L E W V S S I Y P S G G Y T Y Y
15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGGCTTA
81 L Q M N S L R A E D T A V Y Y C A R G L
20 301 CGGGGAGGTCTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCAAGCGCCTCCACC
101 R G G P D Y W G Q G T L V T V S S A S T
361 AAGGGCCCATCGGTCTTCCCGCTAGCACCCCTCCTCCAAGAGCACCTCTGGGGGCACAGCG
25 121 K G P S V F P L A P S S K S T S G G T A
421 GCCCTGGGC (SEQ ID NO:47)
141 A L G (SEQ ID NO:48)

30

Translation of VL-PF4-Tie1-phagemid.TXT(1-308)

Universal code

Total amino acid number: 102, MW=10966

Max ORF: 1-306, 102 AA, MW=10966

35
1 CAAGACATCCAGATGACCCAGTCTCCTTCCACCCTGTCTGCATATGTAGGAGACAGTGTCT
1 Q D I Q M T Q S P S T L S A Y V G D S V
61 ACCATCACTTGCCGGGCCAGTCAGAGTGTGAGAAGGTCGTTGGCCTGGTATCAGCAGAGA
40 21 T I T C R A S Q S V R R S L A W Y Q Q R
121 CCAGGGAAAGCCCCAAGTCCCTCATCTATAAGGCGTCTACTTTAGAGACTGGGGTCCCA
41 P G K A P K S L I Y K A S T L E T G V P
45 181 CCAAGGTTTCAGCGGCAGTGGATCTGGGACAGAATTCACCTCTCACCATCAGCAGCCTGCAG
61 P R F S G S G S G T E F T L T I S S L Q
241 CCTGAAGATTCTGCAATTTATTACTGCCAACAATATGGTAGTTTTCGCTCACTTTCGGC
81 P E D S A I Y Y C Q Q Y G S F P L T F G
50 301 GGAGGGAC (SEQ ID NO:49)
101 G G (SEQ ID NO:50)

55

Translation of VH-PG3-Tie1-phagemid.TXT(1-441)

Universal code

Total amino acid number: 147, MW=15647

Max ORF: 1-441, 147 AA, MW=15647

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGGCGCTGCTTCCGGATTCACCTTCTCTCATTACATGATGGTTTGGGTTCGCCAAGCT
10 21 S C A A S G F T F S H Y M M V W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCTGGACTTATTAT
41 P G K G L E W V S S I Y P S G G W T Y Y
15 181 GCTGACTCCGTTAAAGGTCGCTTCACCTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGGCTGGAC
81 L Q M N S L R A E D T A V Y Y C A R L D
20 301 TACGGTGGTAATTCCGCCTACTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCA
101 Y G G N S A Y F D Y W G Q G T L V T V S
361 AGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCTAGCACCTCCTCCAAGAGCACCTCT
25 121 S A S T K G P S V F P L A P S S K S T S
421 GGGGGCACACGGGCCCTGGGC (SEQ ID NO:51)
141 G G T A A L G (SEQ ID NO:52)

30

Translation of VL-PG3-Tie1-phagemid.TXT(1-327)

Universal code

Total amino acid number: 109, MW=11792

Max ORF: 1-327, 109 AA, MW=11792

35 1 CAGAGCGTCTTGACTCAGCCGCACTCTGTGTGCGCCTCTCCGGGGAAGACGGTAACCATC
1 Q S V L T Q P H S V S A S P G K T V T I
61 TCCTGCACCCGCAGCAGTGGCAACATTGCCAGCAACTTTGTCCAGTGGTACCAACAGCGC
40 21 S C T R S S G N I A S N F V Q W Y Q Q R
121 CCGGGCAGTGTCCCCACCACTGTGATTTATGAAGATGACCGAAGACCCTCTGGGGTCCCT
41 P G S V P T T V I Y E D D R R P S G V P
45 181 GATCGCTTTTCTGGCTCCATCGACAGTTCTTCCAACCTCTGCTTTCTCAGCATCTCTGGA
61 D R F S G S I D S S S N S A F L S I S G
241 CTGAAGACTGAGGACGAGGCAGACTATTACTGTCTCAGTCTCATGATCGTACCACCGTGCT
81 L K T E D E A D Y Y C Q S H D R T T R A
50 301 TGGGTGTTTCGGCGGAGGGACCAAGCTG (SEQ ID NO:53)
101 W V F G G G T K L (SEQ ID NO:54)

55

Translation of VH-SA2-Tie1-phagemid.TXT(1-413)

Universal code

Total amino acid number: 137, MW=14682

Max ORF: 1-411, 137 AA, MW=14682

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L

10
61 TCTTGCGCTGCTTCCGGATTCACTTTCTCTCGTTACACTATGATGTGGGTTGCGCAAGCT
21 S C A A S G F T F S R Y T M M W V R Q A

121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGGTATCTATCCTTCTGGTGGCGTTACTCTTTAT
41 P G K G L E W V S G I Y P S G G V T L Y

15
181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y

241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
81 L Q M N S L R A E D T A V Y Y C A R V N

20
301 TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
101 Y Y D S S G Y G P I A P G L D Y W G Q G

361 ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCT (SEQ ID
25 NO:55)
121 T L V T V S S A S T K G P S V F P (SEQ ID
NO:56)

Translation of VL-SA2-Tie1-phagemid.TXT(1-339)

30 Universal code

Total amino acid number: 113, MW=12358

Max ORF: 1-339, 113 AA, MW=12358

1 CACAGTGCACAAGACATCCAGATGACCCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGG
15 1 H S A Q D I Q M T Q S P G T L S L S P G

61 GAAAGAGCCACACTCTCCTGCAGGGCCAGTCGGAGTGTGATCATCAGCTACGTAGCCTGG
21 E R A T L S C R A S R S V I I S Y V A W

10
121 TACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGAGCGTCCACCAGGGCC
41 Y Q Q K P G Q A P R L L I Y G A S T R A

181 ACTGGCATCCCAGACAGGTTCACTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATC
15 61 T G I P D R F S G S G S G T D F T L T I

241 AGCAGACTGGAGCCTGAAGACTTTGCAGTGTATTTCTGTCAGCTTTATGGTAGGTACCA
81 S R L E P E D F A V Y F C Q L Y G R S P

301 CGGATCATCTTCGGCCAAGGGACACGACTGGAGATTAAA (SEQ ID NO:57)
0 101 R I I F G Q G T R L E I K (SEQ ID NO:58)

Translation of VH-SA10-Tie1-phagemid.TXT(1-369)

Universal code

Total amino acid number: 123, MW=13314

Max ORF: 1-369, 123 AA, MW=13314

```

5      1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
      1      E V Q L L E S G G G L V Q P G G S L R L

      61      TCTTGCCTGCTTCCGGATTCACTTTCTCTAATTACGTTATGGTTTGGGTTTCGCCAAGCT
10     21      S C A A S G F T F S N Y V M V W V R Q A

      121     CCTGGTAAAGGTTTGGAGTGGGTTTCTGGTATCTATCCTTCTGGTGGCCATACTAAGTAT
      41      P G K G L E W V S G I Y P S G G H T K Y

15     181     GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
      61      A D S V K G R F T I S R D N S K N T L Y

      241     TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
      81      L Q M N S L R A E D T A V Y Y C A R V N

10     301     TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
      101     Y Y D S S G Y G P I A P G L D Y W G Q G

      361     ACCCTGGTC (SEQ ID NO:59)
15     121     T L V (SEQ ID NO:60)

```

Translation of VL-SA10-Tie1-phagemid.TXT(1-339)

Universal code

0 Total amino acid number: 113, MW=12445

Max ORF: 1-339, 113 AA, MW=12445

```

      1      CACAGTGCACAAGACATCCAGATGACCCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGG
      1      H S A Q D I Q M T Q S P G T L S L S P G

5     61      GAAAGAGCCACCCTCTTCTGCAGGGCCAGTCAGCGTGTTACCAGCAACTCCTTGGCCTGG
      21      E R A T L F C R A S Q R V T S N S L A W

      121     TACCAGCAGAGACCTGGCCAGGCTCCAGGCTCCTCATCTATGATGCATCCACCAGGGCC
0     41      Y Q Q R P G Q A P R L L I Y D A S T R A

      181     ACTGGCATCCCAGACCGCTTCAGTGGCAGTGGGTCTGGGGAGGGACTTCACTCTCACCATC
      61      T G I P D R F S G S G S G R D F T L T I

5     241     AGCAGACTGGAGCCTGAAGATTTTGCAGTTTATTACTGTCAGCGATATGGTAGTTTCAGTG
      81      S R L E P E D F A V Y Y C Q R Y G S S V

      301     TTGTACTCTTTTGGCCAGGGGACGAAGTTGGAAATCACA (SEQ ID NO:61)
      101     L Y S F G Q G T K L E I T (SEQ ID NO:62)
)

```

Translation of VH-SB2-Tie1-phagemid.TXT(1-383)

Universal code

Total amino acid number: 127, MW=13611

Max ORF: 1-381, 127 AA, MW=13611

```

5      1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
      1      E V Q L L E S G G G L V Q P G G S L R L

      61      TCTTGGCGCTGCTTCCGGATTCACTTTCTCTATTTACGGTATGGCTTGGGTTCGCCAAGCT
10     21      S C A A S G F T F S I Y G M A W V R Q A

      121     CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTCTCCTTCTGGTGGCCAGACTTTTTAT
      41      P G K G L E W V S V I S P S G G Q T F Y

15     181     GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
      61      A D S V K G R F T I S R D N S K N T L Y

      241     TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTTTAC
      81      L Q M N S L R A E D T A V Y Y C A R V Y

20     301     TACTACGGTATGGACGTCTGGGGCCAAGGGACCACGGTCACCGTCTCAAGCGCCTCCACC
      101     Y Y G M D V W G Q G T T V T V S S A S T

      361     AAGGGCCCATCGGTCTTCCCGCT (SEQ ID NO:63)
25     121     K G P S V F P (SEQ ID NO:64)

```

Translation of VL-SB2-Tie1-phagemid.TXT(1-333)

Universal code

30 Total amino acid number: 111, MW=12221

Max ORF: 1-333, 111 AA, MW=12221

```

      1      CACAGTGCACAAGACATCCAGATGACCCAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGG
      1      H S A Q D I Q M T Q S P A T L S L S P G

35     61      GAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCTACTTAGCCTGGTAC
      21      E R A T L S C R A S Q S V S S Y L A W Y

      121     CAACAAAAACCTGGCCAGGCTCCCAGGCTCCTCATTATGATGCATCCAACAGGGCCACT
40     41      Q Q K P G Q A P R L L I Y D A S N R A T

      181     GGCATCCCAGCCAGGTTCACTGGCAGTGGGTCTGAGACAGACTTCACTCTCACCATCAGC
      61      G I P A R F S G S G S E T D F T L T I S

45     241     AGCCTAGAGCCTGAAGATTTTGCAGTTTATTACTGTGAGCAGCGTAGCAAGTGGCCTCGG
      81      S L E P E D F A V Y Y C Q Q R S K W P R

      301     ACTTTTGGCCAGGGGACCAAGCTGGAGATCAAA (SEQ ID NO:65)
50     101     T F G Q G T K L E I K (SEQ ID NO:66)

```

Translation of VH-SB9-Tie1-phagemid.TXT(1-413)

Universal code

Total amino acid number: 137, MW=14778

Max ORF: 1-411, 137 AA, MW=14778

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGGCGCTGCTTCCGGATTCACTTTCTCTTCTTACGTTATGATGTGGGTTCGCCAAGCT
10 21 S C A A S G F T F S S Y V M M W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGGTATCTATCCTTCTGGTGGCTGGACTTATTAT
41 P G K G L E W V S G I Y P S G G W T Y Y
15 181 ACTGACTCCGTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 T D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
81 L Q M N S L R A E D T A V Y Y C A R V N
20 301 TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
101 Y Y D S S G Y G P I A P G L D Y W G Q G
361 ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCT (SEQ ID
25 NO:67)
121 T L V T V S S A S T K G P S V F P (SEQ ID
NO:68)

30 Translation of VL-SB9-Tie1-phagemid.TXT(1-336)

Universal code

Total amino acid number: 112, MW=12010

Max ORF: 1-336, 112 AA, MW=12010

35 1 CACAGTGCACAAGACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTTGGA
1 H S A Q D I Q M T Q S P S S L S A S V G
61 GATAGAGTCACCATCACTTGCCGGGCAAGTCAGAGTGTGAGCAGTCATTTAAGTTGGTTT
40 21 D R V T I T C R A S Q S V S S H L S W F
121 CAGCAGAGACCAGGGAAAGCCCCAACCTCCTGATCTATCATGCATCCAGTTTGCAAAGT
41 Q Q R P G K A P N L L I Y H A S S L Q S
181 GGGGTCCCATCAAGGTTTCAGTGGCAGTGGGTCTGGGACAGATTTACGCTCACCATCAGC
45 61 G V P S R F S G S G S G T D F T L T I S
241 AGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTGAGCAGAGTTACGCTACTTCTCG
81 S L Q P E D F A T Y Y C Q Q S Y A T S S
50 301 ATCACCTTCGGCCAGGGGACACGACTGGACATTAAA (SEQ ID NO:69)
101 I T F G Q G T R L D I K (SEQ ID NO:70)

Translation of VH-SC2-Tie1-phagemid.TXT(1-413)

Universal code

Total amino acid number: 137, MW=14650

Max ORF: 1-411, 137 AA, MW=14650

```

5      1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
      1      E V Q L L E S G G G L V Q P G G S L R L

      61      TCTTGGCGCTGCTTCCGGATTCACTTTCTCTCGTTACAAGATGAAGTGGGTTCCGCAAGCT
0      21      S C A A S G F T F S R Y K M K W V R Q A

      121     CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTATCCTTCTGGTGGCGGTACTGGTTAT
      41      P G K G L E W V S V I Y P S G G G T G Y

5      181     GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
      61      A D S V K G R F T I S R D N S K N T L Y

      241     TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
      81      L Q M N S L R A E D T A V Y Y C A R V N
10

      301     TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
      101     Y Y D S S G Y G P I A P G L D Y W G Q G

      361     ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCT (SEQ ID
15 NO:71)
      121     T L V T V S S A S T K G P S V F P (SEQ ID
      NO:72)

```

30 Translation of VL-SC2-Tie1-phagemid.TXT(1-428)

Universal code

Total amino acid number: 142, MW=15044

Max ORF: 1-426, 142 AA, MW=15044

```

35      1      CACAGTGCACAGAGCGTCTTGACTCAGCCTGACTCCGTGTCTGGGTCTCCTGGAGAGTCG
      1      H S A Q S V L T Q P D S V S G S P G E S

      61      ATCACCATCTCCTGCACTGGAAGCAGCAGAGACGTTGGTGGTTATAACTATGTCTCCTGG
40      21      I T I S C T G S S R D V G G Y N Y V S W

      121     TACCAACAACACCCAGGCAAAGCCCCCAAACCTCTTGCTTTATGATGTCACTTATCGGCCC
      41      Y Q Q H P G K A P K L L L Y D V T Y R P

      181     TCAGGGATTTCTGGTCGCTTCTCTGGCTCCAAGTCTGGCGACACGGCCTCCCTGACCATC
45      61      S G I S G R F S G S K S G D T A S L T I

      241     TCTGGGCTCCGGACTGAGGACGAGGCTGATTATTACTGCAGCTCATCTATAGGCACCAGG
      81      S G L R T E D E A D Y Y C S S S I G T R

50      301     ACTTATGTCTTCGGAAGTGGGACCAAGGTCACCGTCCTACGTCAGCCCAAGGCCAACCCC
      101     T Y V F G S G T K V T V L R Q P K A N P

      361     ACTGTCACTCTGTTCCCGCCCTCCTCTGAGGAGCTCCAAGCCAACAAGGCCACACTAGTG
55      121     T V T L F P P S S E E L Q A N K A T L V

      421     TGTCTGAT (SEQ ID NO:73)
      141     C L (SEQ ID NO:74)

```

Translation of VH-SC7-Tie1-phagemid.TXT(1-386)

Universal code

Total amino acid number: 128, MW=13785

Max ORF: 1-384, 128 AA, MW=13785

```

5      1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
      1      E V Q L L E S G G G L V Q P G G S L R L

      61      TCTTGCGCTGCTTCCGGATTCACCTTTCTCTCGTTACGTTATGTATTGGGTTCCGCAAGCT
10     21      S C A A S G F T F S R Y V M Y W V R Q A

      121      CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTATCCTTCTGGTGGCGCTACTTATTAT
      41      P G K G L E W V S V I Y P S G G A T Y Y

      181      GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
15     61      A D S V K G R F T I S R D N S K N T L Y

      241      TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGACGGGGA
      81      L Q M N S L R A E D T A V Y Y C A R R G

20     301      AGTAGTGGTGGCTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCAAGCGCCTCC
      101      S S G A F D Y W G Q G T L V T V S S A S

      361      ACCAAGGGCCCCATCGGTCTTCCCCGCT (SEQ ID NO:75)
25     121      T K G P S V F P (SEQ ID NO:76)

```

Translation of VL-SC7-Tie1-phagemid.TXT(1-434)

Universal code

30 Total amino acid number: 144, MW=15027

Max ORF: 1-432, 144 AA, MW=15027

```

      1      CACAGTGCACAGAGCGTCTTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCG
      1      H S A Q S V L T Q P A S V S G S P G Q S

35     61      ATCACCATCTCCTGCACTGGAACCAGCAGTGACATTGGTCGTTATAACTATGCCTCCTGG
      21      I T I S C T G T S S D I G R Y N Y A S W

      121      TACCAACAACGCCCAGGCAAATCCCCCAAACCTCCTGATTTATGAGGTCAGTGATCGGCCC
40     41      Y Q Q R P G K S P K L L I Y E V S D R P

      181      TCAGGGGTTTCTAATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGATCATC
      61      S G V S N R F S G S K S G N T A S L I I

45     241      TCTGGGCTCCAGGCTGAGGACGAGGCTGATTATTACTGCAGCTCATATTCAAGCACCAAC
      81      S G L Q A E D E A D Y Y C S S Y S S T N

      301      AGTCTCCAAGTGGTATTTCGGCGGAGGGACCAAGCTGAGCGTCCTAGGTCAAGCCAAGGCT
50     101      S L Q V V F G G G T K L S V L G Q P K A

      361      GCCCCCTCGGTCACTCTGTTCCCGCCCTCCTCTGAGGAGCTTCAAGCCAACAAGGCCACA
      121      A P S V T L F P P S S E E L Q A N K A T

      421      CTGGTGTGTCTCAT (SEQ ID NO:77)
55     141      L V C L (SEQ ID NO:78)

```

Translation of VH-SC10-Tie1-phagemid.TXT(1-413)

Universal code

Total amino acid number: 137, MW=14688

Max ORF: 1-411, 137 AA, MW=14688

```
1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
5  1      E V Q L L E S G G G L V Q P G G S L R L

61     TCTTGCGCTGCTTCCGGATTCACTTTCTCTGCTTACGGTATGTCTTGGGTTCGCCAAGCT
21     S C A A S G F T F S A Y G M S W V R Q A

10  121    CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTATCCTTCTGGTGGCTGGACTTATTAT
41     P G K G L E W V S V I Y P S G G W T Y Y

181    GCTGACTCCGTAAAGGTGCGTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61     A D S V K G R F T I S R D N S K N T L Y

15  241    TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
81     L Q M N S L R A E D T A V Y Y C A R V N

301    TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
20  101     Y Y D S S G Y G P I A P G L D Y W G Q G

361    ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCT (SEQ ID
NO:79)

121    T L V T V S S A S T K G P S V F P (SEQ ID
25 NO:80)
```

Translation of VL-SC10-Tie1-phagemid.TXT(1-336)

Universal code

30 Total amino acid number: 112, MW=12256

Max ORF: 1-336, 112 AA, MW=12256

```
1      CACAGTGCACAAGACATCCAGATGACCCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGG
35  1      H S A Q D I Q M T Q S P G T L S L S P G

61     GAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGG
21     E R A T L S C R A S Q S V S S S Y L A W

121    TACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCC
10  41     Y Q Q K P G Q A P R L L I Y G A S S R A

181    ACTGGCATCCCAGACAGGTTCACTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATC
61     T G I P D R F S G S G S G T D F T L T I

15  241    AGCAGACTGGAGCCTGAAGATTTTGCACTGTATTACTGTCAGCAGTATAATAACTGGCCT
81     S R L E P E D F A V Y Y C Q Q Y N N W P

301    CGGACGTTTCGCCAAGGGACCAAGGTGGAAATCAAA (SEQ ID NO:81)
10  101     R T F G Q G T K V E I K (SEQ ID NO:82)
```

Translation of VH-SD11-Tie1-phagemid.TXT(1-395)

Universal code

Total amino acid number: 131, MW=14005

Max ORF: 1-393, 131 AA, MW=14005

```

1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1      E V Q L L E S G G G L V Q P G G S L R L
61     TCTTGCGCTGCTTCCGGATTCACTTTCTCTGGTTACGCTATGTGGTGGGTTTCGCCAAGCT
21     S C A A S G F T F S G Y A M W W V R Q A
121    CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTCTCCTTCTGGTGGCGCTACTGCTTAT
41     P G K G L E W V S S I S P S G G A T A Y
181    GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61     A D S V K G R F T I S R D N S K N T L Y
241    TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGATGCG
81     L Q M N S L R A E D T A V Y Y C A R D A
301    GGGAGTTATTATTGGGGCTGGTTCGACCCCTGGGGCCAGGGAACCCTGGTCCACCGTCTCA
101    G S Y Y W G W F D P W G Q G T L V T V S
361    AGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCT (SEQ ID NO:83)
5 121    S A S T K G P S V F P (SEQ ID NO:84)

```

Translation of VL-SD11-Tie1-phagemid.TXT(1-333)

Universal code

0 Total amino acid number: 111, MW=12194

Max ORF: 1-333, 111 AA, MW=12194

```

1      CACAGTGCACAAGACATCCAGATGACCCAGTCTCCAGCCACCTTGTCTTTGTCTCCAGGG
5 1      H S A Q D I Q M T Q S P A T L S L S P G
61     GAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTATTAGCAGCTACTTAGCCTGGTAC
21     E R A T L S C R A S Q S I S S Y L A W Y
121    CAACAGAAACCTGGCCAGCCTCCCAGGCTCCTCATCTATGATGCATCCAGCAGGGTTACT
10 41     Q Q K P G Q P P R L L I Y D A S S R V T
181    GGCATCCCAGCCAGGTTTCAGTGGCAGTGGCTTTGGGACAGACTTCACTCTCACCATTAGT
61     G I P A R F S G S G F G T D F T L T I S
15 241    AGCCTGGAGCCTGAAGATTTTGCAGTTTATTACTGTCTCCAGCGTAGCAGCTGGCCCCGA
81     S L E P E D F A V Y Y C L Q R S S W P R
301    ACTTTTGGCCAGGGGACCAAGCTGGAGATCAAA (SEQ ID NO:85)
50 101    T F G Q G T K L E I K (SEQ ID NO:86)

```

Translation of VH-SE11-Tie1-phagemid.TXT(1-413)

Universal code

Total amino acid number: 137, MW=14670

Max ORF: 1-411, 137 AA, MW=14670

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCCTGCTTCCGGATTCACTTTCTCTGGTTACGTTATGTTTTGGGTTCGCCAAGCT
10 21 S C A A S G F T F S G Y V M F W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGGTATCTATCCTTCTGGTGGCTGGACTGTTTAT
41 P G K G L E W V S G I Y P S G G W T V Y
15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGTCAAT
81 L Q M N S L R A E D T A V Y Y C A R V N
20 301 TACTATGATAGTAGTGGTTACGGTCCTATAGCTCCTGGACTTGACTACTGGGGCCAGGGA
101 Y Y D S S G Y G P I A P G L D Y W G Q G
361 ACCCTGGTCACCGTCTCAAGCGCCTCCACCAAGGGCCCATCGGTCTTCCCGCT (SEQ ID
25 NO:87)
121 T L V T V S S A S T K G P S V F P (SEQ ID
NO:88)

30 Translation of VL-SE11-Tie1-phagemid.TXT(1-333)

Universal code

Total amino acid number: 111, MW=11962

Max ORF: 1-333, 111 AA, MW=11962

35 1 CACAGTGCACAAGACATCCAGATGACCCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGG
1 H S A Q D I Q M T Q S P G T L S L S P G
61 GAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGG
40 21 E R A T L S C R A S Q S V S S S Y L A W
121 TACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCC
41 Y Q Q K P G Q A P R L L I Y G A S S R A
181 ACTGGCATCCCAGACAGGTTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATC
45 61 T G I P D R F S G S G S G T D F T L T I
241 AGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTGAGCAATATGGTAGCTCACGG
81 S R L E P E D F A V Y Y C Q Q Y G S S R
50 301 ACGTTCGGCCAAGGGACCAACGTGGAAATCAAA (SEQ ID NO:89)
101 T F G Q G T N V E I K (SEQ ID NO:90)

Translation of VH-SG4-Tie1-phagemid.TXT(1-395)

Universal code

Total amino acid number: 131, MW=14168

Max ORF: 1-393, 131 AA, MW=14168

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCCTGCTTCCGGATTCACTTTCTCTTCTTACATGATGACTTGGGTTCGCCAAGCT
10 21 S C A A S G F T F S S Y M M T W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCTATACTTATTAT
41 P G K G L E W V S S I Y P S G G Y T Y Y
15 181 GCTGACTCCGTTAAAGGTGCGTTCACTATCTCTAGAGACAACTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGGAGGG
81 L Q M N S L R A E D T A V Y Y C A R G G
20 301 TATGGCGACTCGTCATTTTTTTTTTGGTACTGAGGGCCAGGGAACCCTGGTCACCGTCTCA
101 Y G D S S F F F D Y W G Q G T L V T V S
361 AGCGCTCCACCAAGGGCCCATCGGTCTTCCCGCT (SEQ ID NO:91)
25 121 S A S T K G P S V F P (SEQ ID NO:92)

Translation of VL-SG4-Tie1-phagemid.TXT(1-333)

Universal code

30 Total amino acid number: 111, MW=11832

Max ORF: 1-333, 111 AA, MW=11832

1 CACAGTGCACAAGACATCCAGATGACCCAGTCTCCAGCCACCCTGTCTGTGTCTCCAGGG
1 H S A Q D I Q M T Q S P A T L S V S P G
35 61 GAAGGAGCCACCCTCTCTTGCAGGGCCAGTCGGAGTGTGGCAGCAACTTAGCCTGGTAC
21 E G A T L S C R A S R S V G S N L A W Y
121 CAGCAGAAGCCTGGCCAGGCTCCCAGGCTCCTCATCTATGATGCATCCACCAGGGCCACT
40 41 Q Q K P G Q A P R L L I Y D A S T R A T
181 GGTATCCCCGCCAGGTTCACTGGCAGTGGGTCTGGGACAAAGTTCACTCTCACCATCAGC
61 G I P A R F S G S G S G T K F T L T I S
45 241 AGCCTCCAGTCTGAAGATTTTGCAGTTTATTACTGTGTCAGCAGCGTAGCAATTGGCCTCTC
81 S L Q S E D F A V Y Y C Q Q R S N W P L
301 ACTTTCGGCGGAGGGACCAAGGTGGAGATCAAA (SEQ ID NO:93)
101 T F G G G T K V E I K (SEQ ID NO:94)
50

Complete sequence VH-SG9 not known

5

10 Translation of VL-SG9-Tiel-phagemid.TXT(1-428)
Universal code
Total amino acid number: 142, MW=14993
Max ORF: 1-426, 142 AA, MW=14993

15 1 CACAGTGCACAGAGCGTCTTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCG
1 H S A Q S V L T Q P A S V S G S P G Q S

61 ATCACCATCTCCTGCACTGGAACCAGCAGTGACGTTGGTGATGATAACTATGTCTCCTGG
21 I T I S C T G T S S D V G D D N Y V S W
20
121 TACCAACAACACCCAGACAAAGCCCCCAAACATCATGATTTATGAGGTCAGTTATCGGCCC
41 Y Q Q H P D K A P K L M I Y E V S Y R P

181 TCAGGGGTTTCTAATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGACCATC
25 61 S G V S N R F S G S K S G N T A S L T I

241 TCTGGGCTCCAGACTGAGGACGAGGCTGATTATTATTGCGGCTCATATCGCGTCAGCAGC
81 S G L Q T E D E A D Y Y C G S Y R V S S

30 301 TCCTATGTCTTCGGAACTGGGACCAAGGTCACCGTCCTAGGTCAGCCCAAGGCCAACCCC
101 S Y V F G T G T K V T V L G Q P K A N P

361 ACTGTCACTCTGTTCCCGCCCTCCTCTGAGGAGCTCCAAGCCAACAAGGCCACACTAGTG
121 T V T L F P P S S E E L Q A N K A T L V
35
421 TGTCTGAT (SEQ ID NO:95)
141 C L (SEQ ID NO:96)

40

Translation of VH-SG10-Tie1-phagemid.TXT(1-363)

Universal code

Total amino acid number: 121, MW=13390

Max ORF: 1-363, 121 AA, MW=13390

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCCTGCTTCCGGATTCACCTTCTCTCGTTACAAGATGTTTTGGGTTCGCCAAGCT
10 21 S C A A S G F T F S R Y K M F W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTATCCTTCTGGTGGCCCTACTATGTAT
41 P G K G L E W V S V I Y P S G G P T M Y
15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGGGATG
81 L Q M N S L R A E D T A V Y Y C A R G M
20 301 GTCCGTGGATATAGTGGCTACGATTACCCTTTCTTGGACTACTGGGGCCAGGGAACCCCTG
101 V R G Y S G Y D Y P F L D Y W G Q G T L
361 GTC (SEQ ID NO:97)
25 121 V (SEQ ID NO:98)

Translation of VL-SG10-Tie1-phagemid.TXT(1-333)

Universal code

30 Total amino acid number: 111, MW=11981

Max ORF: 1-333, 111 AA, MW=11981

1 CACAGTGCACAAGACATCCAGATGACCCAGTCTCCATCTTCCCTGTCTGCATCTGTAGGG
1 H S A Q D I Q M T Q S P S S L S A S V G
35 61 GACAGAGTCACCATCACTTGCCGAGCAAGTCAGACCATTAGCAGCTATTTAAATTGGTAT
21 D R V T I T C R A S Q T I S S Y L N W Y
121 CAGCAGAAGCCAGGGAAAGCCCCTAAGCTCCTGATCTATGCTGCATCCAGTTTGCAAAGT
40 41 Q Q K P G K A P K L L I Y A A S S L Q S
181 GGGGTCCCATCAAGGTTCAAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGC
61 G V P S R F S G S G S G T D F T L T I S
45 241 AGTCTGCAACCTGAAGATTTTGCAACTTACTACTGTCAACAGAGTTACAGTACCCCTCGT
81 S L Q P E D F A T Y Y C Q Q S Y S T P R
301 ACGTTTCGGCCAAGGGACCAAGGTGGAAATCAAA (SEQ ID NO:99)
50 101 T F G Q G T K V E I K (SEQ ID NO:100)

Translation of VH-SH1-Tiel-phagemid.TXT(1-386)

Universal code

Total amino acid number: 128, MW=13767

Max ORF: 1-384, 128 AA, MW=13767

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCCTGCTTCCGGATTCACTTTCTCTGCTTACCAGATGGTTTGGGTTGCGCAAGCT
10 21 S C A A S G F T F S A Y Q M V W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCTGGACTTATTAT
41 P G K G L E W V S S I Y P S G G W T Y Y
15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGGCACG
81 L Q M N S L R A E D T A V Y Y C A R G T
20 301 CACCTCCCGGGGGTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCAAGCGCCTCC
101 H L P G V D Y W G Q G T L V T V S S A S
361 ACCAAGGGCCCATCGGTCTTCCCGCT (SEQ ID NO:101)
25 121 T K G P S V F P (SEQ ID NO:102)

Translation of VL-SH1-Tiel-phagemid.TXT(1-339)

Universal code

30 Total amino acid number: 113, MW=12225

Max ORF: 1-339, 113 AA, MW=12225

1 CACAGTGCACAAGACATCCAGATGACCCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGG
1 H S A Q D I Q M T Q S P G T L S L S P G
35 61 GAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGCAGCTACTTAGCCTGG
21 E R A T L S C R A S Q S V S S S Y L A W
121 TACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCC
40 41 Y Q Q K P G Q A P R L L I Y G A S S R A
181 ACTGGCATCCCAGACAGGTTCACTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATC
61 T G I P D R F S G S G S G T D F T L T I
45 241 AGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGTATGGTAGCTCCCCC
81 S R L E P E D F A V Y Y C Q Q Y G S S P
301 ATGTACACTTTTGGCCAGGGGACCAAGCTGGAGATCAAA (SEQ ID NO:103)
101 M Y T F G Q G T K L E I K (SEQ ID NO:104)
50

Translation of VH-SH4-Tie1-phagemid.TXT(1-339)

Universal code

Total amino acid number: 113, MW=12481

Max ORF: 1-339, 113 AA, MW=12481

5
1 GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
1 E V Q L L E S G G G L V Q P G G S L R L
61 TCTTGCGCTGCTTCCGGATTCACTTTCTCTTCTTACAAGATGGGTTGGGTTCCGCAAGCT
10 21 S C A A S G F T F S S Y K M G W V R Q A
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCTGGACTCATTAT
41 P G K G L E W V S S I Y P S G G W T H Y
15 181 GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
61 A D S V K G R F T I S R D N S K N T L Y
241 TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCAAGAGTACTA
81 L Q M N S L R A E D T A V Y Y C A R V L
20 301 CTACACTACTTTGACTACTGGGGCCAGGGAACCCTGGTC (SEQ ID NO:105)
101 L H Y F D Y W G Q G T L V (SEQ ID NO:106)

25 Translation of VL-SH4-Tie1-phagemid.TXT(1-415)

Universal code

Total amino acid number: 138, MW=14287

Max ORF: 1-414, 138 AA, MW=14287

30 1 CACAGTGCACAGAGCGTCTTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCG
1 H S A Q S V L T Q P A S V S G S P G Q S
61 ATCACCATCTCCTGCACTGGAACCAGCAGTGACGTTGGTGGTTATAAATATGTCTCCTGG
35 21 I T I S C T G T S S D V G G Y K Y V S W
121 TACCAACAGCACCCAGGCAAAGCCCCAAACTCATTATTTCTGACGTCAATAATCGGCCC
41 Y Q Q H P G K A P K L I I S D V N N R P
181 TCAGGGGTTTCTGATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGACCATC
40 61 S G V S D R F S G S K S G N T A S L T I
241 TCTGGGCTCCAGGCTGAGGACGACGGTGATTATTACTGCAGTTCCTACGCAAGTAGTTCC
81 S G L Q A E D D G D Y Y C S S Y A S S S
45 301 TATACAAGCAGTACCACTTGGGTGTTCCGGCGGGGGACCAAGCTGACCGTCCTAGGTCAG
101 Y T S S T T W V F G G G T K L T V L G Q
361 CCCAAGGCTGCCCCCTTGGTCACTCTGTTCCACCTCCTCTGAGGAGCTTCAAG (SEQ
ID NO:107)
50 121 P K A A P L V T L F P P S S E E L Q (SEQ
ID NO:108)

Translation of Reverse_Complement_K2086117(1-471)

Universal code

Total amino acid number: 157, MW=16967

Max ORF: 1-471, 157 AA, MW=16967

5

T-G2-Tiel-heavy

```

10      -----Fr1-----
      1      GAAGTTCAATTGTTAGAGTCTGGTGGCGGTTCTTGTTTCAGCCTGGTGGTTCTTTACGTCTT
      1      E V Q L L E S G G G L V Q P G G S L R L

      -----Fr1-----|...CDR1.....|-----Fr2-----
15      61      TCTTGCCTGCTTCCGGATTCACTTTCTCTTCTTACAAGATGGGTTGGGTTGCGCAAGCT
      21      S C A A S G F T F S S Y K M G W V R Q A

      -----Fr2-----|.....CDR2.....
20      121     CCTGGTAGAGGTTTGAGTGGGTTTCTTCTATCTATCCTTCTGGTGGCTGGACTCATTAT
      41      P G R G L E W V S S I Y P S G G W T H Y

      .....CDR2.....|-----Fr3-----
      181     GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACCTCTAAGAATACTCTCTAC
      61      A D S V K G R F T I S R D N S K N T L Y

25      -----Fr3-----|.CDR3.
      241     TTGCAGATGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCAAGAGTACTA
      81      L Q M N S L R A E D T A V Y Y C A R V L

30      .....CDR3.....|-----Fr4-----
      301     CTACACTACTTTGACTACTGGGGCCAGGGAACCCCTGGTCACCGTCTCAAGCGCCTCCACC
      101     L H Y F D Y W G Q G T L V T V S S A S T

35      361     AAGGGCCCATCGGTCTTCCCCCTGGCACCCCTCCTCCAAGAGCACCTCGGGGGCACAGCGG
      121     K G P S V F P L A P S S K S T S G A Q R

40      421     CCCTGGGCTGCCTGGTCAAGGACTACTTCCCGGATACCGGTGACGGTGTC (SEQ ID
      NO:109)
      141     P W A A W S R T T S R D T G D G V (SEQ ID
      NO:110)

```

Translation of C3-G2_pUCrev(1-325)
 Universal code
 Total amino acid number: 108, MW=11191
 Max ORF: 1-324, 108 AA, MW=11191

5

T-G2-Tie1-lambda-light
 2a2.272A12/DPL11

10

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-----Fr1-----
1  CAGAGCGTCTTGACTCAGCCTGCCTCCGTGTCTGGGTCTCCTGGACAGTCGATCACCATC
15 1  Q S V L T Q P A S V S G S P G Q S I T I

-Fr1-|.....CDR1.....|----Fr2-----
61 TCCTGCACTGGAACCAGCAGTGACGTTGGTGGTTATAAATATGTCTCCTGGTACCAACAG
21  S C T G T S S D V G G Y K Y V S W Y Q Q

-----Fr2-----|.....CDR2.....|----Fr3--
121 CACCCAGGCAAAGCCCCAACTCATTATTTCTGACGTCAATAATCGGCCCTCAGGGGTT
41  H P G K A P K L I I S D V N N R P S G V

-----Fr3-----
181 TCTGATCGCTTCTCTGGCTCCAAGTCTGGCAACACGGCCTCCCTGACCATCTCTGGGCTC
61  S D R F S G S K S G N T A S L T I S G L

-----Fr3-----|.....CDR3.....
30 241 CAGGCTGAGGACGACGGTGATTATTACTGCAGTTCCTACGCAAGTAGTTCCTATACAAGC
81  Q A E D D G D Y Y C S S Y A S S S Y T S

....CDR3...|----Fr4-----
301 AGTACCACTTGGGTGTTTCGGCGGGG (SEQ ID NO:111)
35 101 S T T W V F G G (SEQ ID NO:112)

```

40 Translation of DNAMAN12(1-425)
 Universal code
 Total amino acid number: 141, MW=14855
 Max ORF: 1-423, 141 AA, MW=14855

45

T-E3-Tie1-heavy

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-----Fr1-----
50 1  GAAGTTCAATTGTTAGAGTCTGGTGGCGGTCTTGTTTCAGCCTGGTGGTTCCTTTACGTCTT
1  E V Q L L E S G G G L V Q P G G S L R L

-----Fr1-----|.....CDR1.....|----Fr2-----
55 61 TCTTGCGCTGCTTCCGATTCACTTTCTCTATGTACGGTATGGTTTGGGTTCCGCCAAGCT
21  S C A A S G F T F S M Y G M V W V R Q A

-----Fr2-----|.....CDR2.....
121 CCTGGTAAAGGTTTGGAGTGGGTTTCTGTTATCTCTCCTTCTGGTGGCAATACTGGTTAT

```

```

41      P G K G L E W V S V I S P S G G N T G Y
      .....CDR2.....|-----Fr3-----
5  181  GCTGACTCCGTTAAAGGTCGCTTCACTATCTCTAGAGACAACTCTAAGAATACTCTCTAC
61      A D S V K G R F T I S R D N S K N T L Y

      -----Fr3-----|.CDR3.
10  241  TTGCAGGTGAACAGCTTAAGGGCTGAGGACACTGCAGTCTACTATTGTGCGAGAGCCCCA
81      L Q V N S L R A E D T A V Y Y C A R A P

      .....CDR3.....|-----Fr4-----
15  301  CGTGGATACAGCTATGGTTACTACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCAAGC
101      R G Y S Y G Y Y Y W G Q G T L V T V S S

361  GCCTCCACCAAGGGCCCATCGGTCTTCCCGCTAGCACCTCCTCCAAGAGCACCTCTGGG
121      A S T K G P S V F P L A P S S K S T S G

421  GGCAC (SEQ ID NO:113)
141  G (SEQ ID NO:114)
20

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Translation of C1-E3_pUCrev(1-322)

Universal code

Total amino acid number: 107, MW=11650

25 Max ORF: 1-321, 107 AA, MW=11650

T-E3-Tie1-kappa-light

DPK4/A20+

jk5

30

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      -----Fr1-----
35  1  GACATCCAGATGACCCAGTCTCCACTCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC
1  D I Q M T Q S P L S L S A S V G D R V T

      --Fr1---|.CDR1.....|-----Fr2-----
61  ATCACTTGCCGGGCGAGTCAGGGCATTGGCCATTATTTAGCCTGGTATCAGCAGAAACCA
10  21  I T C R A S Q G I G H Y L A W Y Q Q K P

      -----Fr2-----|.CDR2.....|-----Fr3-----
121  GGGAAAGTTCCTAAGCTCCTGATCTATACTGCATCCACTTTGCAATCAGGGGTCCCATCT
41  G K V P K L L I Y T A S T L Q S G V P S

15  -----Fr3-----
181  CGGTTTCAGTGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAACAGCCTGCAGCCT
61  R F S G S G S G T D F T L T I N S L Q P

      -----Fr3-----|.CDR3.....|-----Fr4-----
10  241  GAGGATGTTGCAACTTATTACTGTCAACAGTTTAATAGTTACCCTCACACCTTCGGCCAA
81  E D V A T Y Y C Q Q F N S Y P H T F G Q

      -----Fr4-----
301  GGGACACGACTGGATATTAAAC (SEQ ID NO:115)
5  101  G T R L D I K (SEQ ID NO:116)

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Example 4: Alignment

Tables 3 and 4 the list CDR and FR regions of exemplary antibodies, the sequences of which are listed herein.

Table 3: Heavy Chain Sequences

Isolate name	H-FR1	H-CDR1	FR2	H-CDR2	
p-A1	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	IYKMS	WVRQAPGKGLEWVS	SIYPSGGQTKYADSVKG	RFTISRDN SKNTLYI
p-A10	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYKMG	WVRQAPGKGLEWVS	WIYPSGGGTTYADSVKG	RFTISRDN SKNTLYI
p-B1	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	RYPMV	WVRQAPGKGLEWVS	VISPSGGQTFYADSVKG	RFTISRDN SKNTLYI
p-B3	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	RYGMH	WVRQAPGKGLEWVS	VISPSGGMTYYADSVKG	RFTISRDN SKNTLYI
p-C6	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	HYGMT	WVRQAPGKGLEWVS	VISPSGGQTKYADSVKG	RFTISRDN SKNTLYI
p-D12	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	GYGMH	WVRQAPGKGLEWVS	VISPSGGQTSYADSVKG	RFTISRDN SKNTLYI
p-F3	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	MYGME	WVRQAPGKGLEWVS	VISPSGGQTAYADSVKG	RFTISRDN SKNTLYI
p-F4	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	AYMMS	WVRQAPGKGLEWVS	SIYPSGGYTTYADSVKG	RFTISRDN SKNTLYI
p-G3	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	HYMMV	WVRQAPGKGLEWVS	SIYPSGGWTTYADSVKG	RFTISRDN SKNTLYI
s-A10	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	NYVMV	WVRQAPGKGLEWVS	GIYPSGGHTKYADSVKG	RFTISRDN SKNTLYI
s-H1	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	AYQMV	WVRQAPGKGLEWVS	SIYPSGGWTTYADSVKG	RFTISRDN SKNTLYI
s-A2	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	RYTMM	WVRQAPGKGLEWVS	GIYPSGGVTLYADSVKG	RFTISRDN SKNTLYI
s-B2	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	IYGMA	WVRQAPGKGLEWVS	VISPSGGQTFYADSVKG	RFTISRDN SKNTLYI
s-B9	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYVMM	WVRQAPGKGLEWVS	GIYPSGGWTTYTDSVKG	RFTISRDN SKNTLYI
s-C10	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	AYGMS	WVRQAPGKGLEWVS	VIYPSGGWTTYADSVKG	RFTISRDN SKNTLYI
s-C2	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	RYKMK	WVRQAPGKGLEWVS	VIYPSGGGTGYADSVKG	RFTISRDN SKNTLYI
s-C7	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	RYVMY	WVRQAPGKGLEWVS	VIYPSGGATYYADSVKG	RFTISRDN SKNTLYI
s-D11	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	GYAMW	WVRQAPGKGLEWVS	SISPSGGATAYADSVKG	RFTISRDN SKNTLYI
s-E11	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	GYVMF	WVRQAPGKGLEWVS	GIYPSGGWTVYADSVKG	RFTISRDN SKNTLYI
s-G10	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	RYKMF	WVRQAPGKGLEWVS	VIYPSGGPTMYADSVKG	RFTISRDN SKNTLYI
s-H4	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYKMG	WVRQAPGKGLEWVS	SIYPSGGWTHYADSVKG	RFTISRDN SKNTLYI
G2	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	SYKMG	WVRQAPGRGLEWVS	SIYPSGGWTHYADSVKG	RFTISRDN SKNTLYI
E3	EVQLLESGGGLVQPGGSLRLSCAASGFTFS	MYGMV	WVRQAPGKGLEWVS	VISPSGGNTGYADSVKG	RFTISRDN SKNTLYI

Table 4: Light Chain Sequences

Isolatename	L-FR1	L-CDR1	L-FR2	L-CDR2	L-FR3
p-A1	QDIQMTQSPGTLSSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTIS
p-A10	QDIQMTQSPGTLSSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTIS
p-B1	QDIQMTQSPSSLSASVGDRTITC	RASQNINSYLN	WYQQKPGQAPKLLIY	AASNLET	AVPSRFSGSGSGTDFTLTIS
p-B3	QDIQMTQSPATLSSLSPGERATLSC	RASQSVSTYLA	WYQQKPGQAPRLLIY	DASNRAT	GIPGRFSGSGSGTDFTLTIS
p-C6	QDIQMTQSPATLSSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	DASNRAT	GIPARFSGSGSGTDFTLTIS
p-D12	QDIQMTQSPSSLSASVGDRTITC	RASQSISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTIS
p-F3	QDIQMTQSPSTLSASLGDRVTITC	RASESISRWLA	WYQQKPGKAPKLLMY	EASTLES	GVPSRFTGTGSGTEFTLTIS
p-F4	QDIQMTQSPSTLSAYVGDSTITC	RASQSVRRSLA	WYQQRPGKAPKSLIY	KASTLET	GVPPRFSGSGSGTEFTLTIS
p-G3	QSVLTQPHSVSASPGKTVITC	TRSSGNIASNFVQ	WYQQRPGSVPTTVIY	EDDRRPS	GVPRFSGSGIDSSSNAPLS
s-A10	QDIQMTQSPGTLSSLSPGERATLFC	RASQRTVSNLSA	WYQQRPGQAPRLLIY	DASTRAT	GIPDRFSGSGSGRDTFTLTIS
s-H1	QDIQMTQSPGTLSSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTIS
s-A2	QDIQMTQSPGTLSSLSPGERATLSC	RASRSVIISYVA	WYQQKPGQAPRLLIY	GASTRAT	GIPDRFSGSGSGTDFTLTIS
s-B2	QDIQMTQSPATLSSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	DASNRAT	GIPARFSGSGSETDFTLTIS
s-B9	QDIQMTQSPSSLSASVGDRTITC	RASQSVSSHLS	WFQQRPGKAPNLLIY	HASSLQS	GVPSRFSGSGSGTDFTLTIS
s-C10	QDIQMTQSPGTLSSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTIS
s-C2	QSVLTQPDVSGSPGESITISC	TGSSRDVGGYNYVS	WYQQHPGKAPKLLLY	DVTYRPS	GISGRFSGSKSGDTASLTIS
s-C7	QSVLTQPASVSGSPGQSITISC	TGTSSDIGRYNYAS	WYQQRPGKSPKLLIY	EVSDRPS	GVSNRFSGSKSGNTASLIIS
s-D11	QDIQMTQSPATLSSLSPGERATLSC	RASQSISSSYLA	WYQQKPGQPPRLLIY	DASSRVT	GIPARFSGSGFGTDFTLTIS
s-E11	QDIQMTQSPGTLSSLSPGERATLSC	RASQSVSSSYLA	WYQQKPGQAPRLLIY	GASSRAT	GIPDRFSGSGSGTDFTLTIS
s-G10	QDIQMTQSPSSLSASVGDRTITC	RASQTISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTIS
s-H4	QSVLTQPASVSGSPGQSITISC	TGTSSDVGGYKYVS	WYQQHPGKAPKLIIS	DVNNRPS	GVSDRFSGSKSGNTASLTIS
G2	QSVLTQPASVSGSPGQSITISC	TGTSSDVGGYKYVS	WYQQHPGKAPKLIIS	DVNNRPS	GVSDRFSGSKSGNTASLTIS
E3	DIQMTQSPLSLASVGDRTITC	RASQGIGHYLA	WYQQKPGKVPKLLIY	TASTLQS	GVPSRFSGSGSGTDFTLTIN

Example 5:

Sequence alignment of B2 and D11

B2 and D11 are both antagonists of Tiel since they counteract the agonistic activity of E3 in the BaF3 activation assay. B2 and D11 both have a kappa light chain and are similar in sequence (8 amino acid differences):

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                                <--CDR1-->                                <CDR2>
1 (B2)      DIQMTQSPATLSLSPGERATLSCRASQSVSSYLAWEYQQKPGQAPRLLIYDASNRAT
.0          |||||||:|||||:|||||.|.
1 (D11)     DIQMTQSPATLSLSPGERATLSCRASQSISSYLAWEYQQKPGQPPRLLIYDASSRVT

                                <--CDR3-->
15          GIPARFSGSGSETDFTLTISLSEPEDFAVYYCQQRSKWPRTFGQGTKLEIK SEQ ID NO:157
61          |||||||:|||||:|||||.|.
61          GIPARFSGSGFGTDFTLTISLSEPEDFAVYYCQRSSWPRTFGQGTKLEIK SEQ ID NO:158

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Example 6 : Inhibition of tube formation by HUVECS cells using anti Tiel E3-IgG

To demonstrate the ability of E3 to inhibit angiogenesis *in vitro*, purified E3 was tested for its ability to inhibit tube formation by human umbilical cord endothelial cells (HUVECS). Human Umbilical vein endothelial cells (HUVEC) were obtained by treating fresh human umbilical cord veins with Trypsin-EDTA (1x) (Gibco/Invitrogen) for 20-25 minutes at 37°C. The cells were cultured in a T-25 flask coated with attachment factor (AF), (Cascade Biologics) in RPMI 1640 medium supplemented with 10 % FCS, 0.4 % BBE, 1% l-glutamin, 1% penicillin/streptomycin. Primary cultures were detached with warm Trypsin-EDTA and used when confluent at the second or third passage. The cells were maintained in a proliferative state by culturing them in a split ratio 1:2 at an approximate density of the monolayer of about 60-80%. To dissociate the cells, HUVEC monolayers were treated with trypsin/EDTA (500 l/dish) at 37°C for 3 min. Trypsin activity was stopped by adding 3 volumes of complete RPMI medium. The cells were carefully scraped, separated by repeated pipetting, and finally washed with PBS.

After 2 passages HUVECs were seeded in their culture medium (40.10³/50µl/well of a 96-well plate) on a collagen gel (50µl of collagen I 1.5mg/ml) prepared by mixing 7.5 volumes of 2mg/ml collagen (Collagen R; Serva, Heidelberg, Germany), 1 volume of 10X MEM, 1.5 volume of NaHCO₃ (15.6mg/ml) and ~ 1 volume of NaOH to adjust the pH to

7.4. After 1h30, the culture medium was then discarded and the cells were covered with a new layer of collagen (1.5mg/ml, new preparation, 50µl/well). After polymerization of the gel, culture medium was added to each well in presence or in absence of E3 antibody (1 ng/ml to 10µg/ml). The assay was performed with a streptavidin antibody used as a control (from 1ng/ml to 10µg/ml). The total length of the tube network on the culture surface was quantified at x40 magnification by the METAVUE™ Software (Universal Imaging Corporation). Results from triplicate wells were expressed as mean vessel area per field ± SEM (relative units). Each assay was performed at least three times.

E3 is a potent inhibitor of tube formation by HUVECS even at a concentration of 10 ng/ml. The control anti streptavidin has no effect on the ability of HUVECS to form tubes. This results indicates that E3 can inhibit one aspect of angiogenesis.

Example 7 : Immunohistochemical analysis of E3 binding to matched tumor and normal tissue sections

To evaluate the binding of E3 to Tie1 in primary tumor and normal tissue the antibody was produced as an IgG and biotin labeled. The E3 antibody and two other anti Tie1 antibodies B2 and D11 were reformatted as full length IgG molecules. Nucleic acids encoding these IgGs were transiently transfected into HEK293T cells. Plasmid preparations for transient cell transfections were performed using the HP-GENELUTE™ MIDI prep kit (Sigma, cat. no. NA0200). HEK293T cells (GenHunter Corp. cat. no. Q401) were seeded 24 hours before transfection; 6×10^6 cells were plated per 10-cm culture dish. Transfections were carried out using LIPOFECTAMINE™ 2000 reagent (Invitrogen, cat. no. 11668019) following the manufacturer's instructions. Five micrograms of plasmid DNA was used per 10-cm dish. Cells were cultured in DMEM (Invitrogen, cat. no. 31966021) supplemented with 10 % "ultra-low IgG" fetal calf serum (Invitrogen, cat. no. 16250078), at 37 °C, 5 % CO₂, in a water saturated atmosphere. Conditioned media were harvested 72 hours and 144 hours after transfection, pooled and sterile filtered.

One hundred microliters of Protein A beads (rProtein A Sepharose 4 Fast Flow, Amersham Biosciences, cat. no. 17-1279-01) equilibrated in PBS were added to the cell culture supernatants, and these were rotated overnight at 4°C, e.g., in 50 ml tubes. The beads were collected by centrifugation, transferred to a 96-well filter plate (UNI-FILTER

800 GF/B, Whatman, cat. no. 7700-2803) and washed extensively with PBS using a vacuum manifold (Macherey Nagel, cat. no. 760681). Elution of the antibodies was achieved by resuspending the beads in 400 μ l of 12.5 mM citric acid. After a 30 to 60 second incubation, the bead eluates were collected, using the vacuum manifold, into the wells of a 96-well collection plate (UNIPLATE 750, Whatman, cat. no. 7701-5750). Each well of the collection plate contained 60 μ l of 1 M HEPES pH 7.5 buffer to immediately neutralize the eluted fractions. The elution step was performed twice to maximize antibody recovery. The eluted samples were then dialyzed against PBS using dialysis cassettes (Slide-A-Lyser Dialysis Cassettes, MWCO 10,000, Pierce, cat. no. 66380) and protein concentration was determined from the absorbance at 280 nm assuming that a 1 mg/ml solution has an absorbance of 1.35. The quality of the preparations was analyzed by reducing and non-reducing SDS-PAGE.

The Tie-1 antibodies were biotinylated using the EZ-link Sulfo-NHS-SS-Biotin (Pierce, Cat. 21331). For Tie-1/Fc and Tie-1-His, the reaction was performed for 2 hours on ice in 50 mM sodium carbonate buffer, pH 9.6, in the presence of a 5-fold molar excess of biotinylating agent. For the antibodies, the reaction was performed for 2 hours on ice in PBS, in the presence of a 15-fold molar excess of EZ-link Sulfo-NHS-SS-Biotin. The reaction was stopped by the addition of Tris-HCl, pH 7.5 (50 mM final concentration) followed by a 1-hour incubation on ice. Samples were then dialyzed against PBS.

Various normal and tumor tissue sections were stained with biotinylated antibodies. A mouse monoclonal anti-Tie1 antibody (7e8) (Alitalo laboratory, University of Helsinki) was used as a positive control. Sections without primary antibody served as negative control. All samples were fresh frozen tissues and staining was performed with the TSA-kit (Perkin-Elmer Life Sciences). After acetone fixation (10-20 min, -20°C) the slides were treated with 0.73% H₂O₂ for 10 min to reduce endogenous peroxidase activity followed by blocking for 30 min with TNB buffer. Sections (5-10 mm thick) were incubated with primary antibodies (10 μ g/ml) overnight at 4°C. Sections with the mouse monoclonal anti-tie1 antibody (7e8) were treated with biotinylated anti-mouse antibodies (VectaStain) before the addition of streptavidin-HRP. Signal was amplified by using a TSA kit and the visualized by AEC (235 ml NaAc, 15 ml AEC (stock solution: 1600 mg 3-amino-9-ethyl-carbazole and 480 ml N-dimethylformamide), 250 μ l H₂O₂).

In general, Tie-1 expression was upregulated in tumor tissue when compared with matching normal tissue. However, in the tumor tissues the anti Tie1 antibodies stained other structures in addition to the vessels. Furthermore, some tissue specificity in the expression of certain epitopes was observed. For example, the E3 antibody stained vessels
5 in the lung and kidney but not in the skin while the B2 antibody stained vessels very faintly in other normal tissues than in the breast. Shedding of the ectodomain of Tie-1 into the tumor tissues can explain observed differences.

In skin tissue, the E3, B2, and D11 antibodies stained blood vessels very faintly whereas the murine 7e8 control antibody gave a clear staining in the normal skin. In
10 melanoma tissue, the 7e8 antibody stained vessels only but the E3, B2, and D11 antibodies also stained other surrounding structures. The staining pattern was similar with all three of the E3, B2, and D11 antibodies.

In lung tissue, we observed that the E3 antibody stained especially clearly the large veins in the lung, whereas D11 and 7e8 gave a faint staining. B2 did not stain the same
15 veins. The expression of Tie-1 was dramatically upregulated in lung carcinoma and all the antibodies stained vessels more strongly in samples with lung carcinoma than in samples from normal lung. In the lung tumors, the E3, B2, and D11 antibodies stained structures other than vessels.

In kidney, the E3 and D11 antibodies stained kidney tubules in addition to the
20 vessels. B2 gave only very faint staining of either tubules or vessels while 7e8 stained only vessels. In hypernephroma tissue, only the E3 antibody gave a clear staining.

In breast, E3 gave the brightest staining in the veins and capillaries of the mammary tissue, B2 and 7e8 gave a similar staining while D11 stained those structures rather faintly. In breast carcinoma the Tie-1 expression was substantially upregulated, and
25 the E3, B2, and D11 antibodies stained also other structures in addition to vessels.

Example 8 : Binding to mouse endothelial cell lines of anti Tie1 E3-IgG using flow cytometry

We evaluated if E3 cross reacts with mouse Tie1 *in situ* and thus if we can evaluate
30 E3 activity in mouse tumor xenograft models binding to mouse endothelial cells was tested and compared with human and transfected cell lines.

Specific binding of the Tie-1 antibodies and of control Mabs to mouse endothelial cells was measured by flow cytometry analysis (FACSscan, Becton Dickinson, Oxnard, Epics, Coulter). Mouse endothelial cell lines MS1, Le-2 (Alitalo) Bend3, SVEC (ATCC, Rockville) and Tie-1 transfected Le-2 cells (Alitalo) were stained. Cell staining was
5 modified from existing protocols. About 200,000 cells were used in each experiment: after trypsinization, cells were washed one time in PBS and resuspended PBS, 10% Heat inactivated human serum (incubation buffer). To test specificity, antibodies were incubated at different dilutions for 1 h at room temperature. Cells were spun down by centrifugation for 3 min at 611 g. Between incubations cells were washed twice with PBS.
10 Then relevant biotinylated antibodies (A2 against streptavidin, E3 against Tie-1, were added and incubated for 1 h at room temperature). This was followed by incubation with Streptavidin- R-phycoerythrin (Dako, Glostrup, Denmark) for 1 hour at room temperature in incubation buffer. After the final incubation step bound antibodies were detected by means of flow cytometry on a FACSCan and Epics Altra (Becton Dickinson, Oxnard,
15 Coulter,) and results analyzed.

Intracellular Tie-1 was measured as described above, except for the addition of Saponin to the incubation buffer to a final concentration of 0.1 % during incubations. The anti-Tie-1 antibody E3 binds to mouse endothelial cell lines indicating a cross reactivity of E3 with mouse and human Tie1 *in situ*. The binding pattern in mouse cell lines detected
20 by flow cytometry is different from the binding pattern in HUVEC in that in mouse cells there is a greater cell surface staining than that compared to primary human endothelial cell lines.

Example 9 : Determination of anti Tie1 E3-IgG binding to human platelets using flow cytometry

Binding experiments with a purified polyclonal goat antiserum against Tie-1 (R&D systems) had showed binding to human platelets in a previous study (Tsiamis et al., 2000). The conclusion from this study was that platelets represent a large pool of Tie-1 immunoreactivity which could present a problem for development of Tie1 as a therapeutic target. To determine if the antibody E3 binds to platelets we performed flow cytometric analysis on both activated and inactivated platelets and compared the staining pattern with the purified anti Tie1 polyclonal serum.

To avoid platelet activation, human platelets were isolated from plasma of healthy donors using the platelet GelSep kit (Biocytex, Marseille, France) kit according to the guidelines of the manufacturer. Platelets were activated by the addition of thrombin to a final concentration of 0.8 U/ml. To distinguish activated from non-activated platelets double staining was performed with Tie-1 antibodies/control antibodies and antibody CD42 (total platelets) or CD62 (activated platelets).

After preparation, platelets were resuspended in buffer 2 of the GelSep kit, 10% heat inactivated human serum (incubation buffer) and incubated for 1 hour. To test specificity, biotinylated antibodies human anti-Tie1(E3), human anti-streptavidin (A2), human anti-FITC and goat anti-Tie (R&D systems) were incubated with 500 000 platelets per test for 1 hour at different dilutions (2 µg/ml, 10 µg/ml) for 1 h at room temperature. Platelets were spun down by centrifugation for 10 min at 611 g. Between incubations platelets were washed twice with Buffer 1 of the GelSep kit. Then, Streptavidin- R-phycoerythrin together with anti-CD42-PercP or anti-CD62-PercP were incubated for 30 minutes at room temperature in incubation buffer. After the last incubation and washing detection of bound antibodies was performed by means of flow cytometry on a FACSScan and Epics Altra (Becton Dickinson, Oxnard, Coulter,) and results analyzed. Cells were gated on SSC and anti-CD42-PercP for the total platelets in case non-activated platelets were used and on SSC and anti-CD62-PercP for the activated platelets.

The polyclonal goat anti-Tie-1 antibody indeed binds to platelets under the conditions tested. This binding is lower when platelets are activated. In contrast, the human anti-Tie1 antibody E3 shows no significant binding to total platelets, nor to activated platelets.

Example 10 : Assessment of Tie1 immunoreactivity in human platelets using immunoprecipitation with anti Tie1 E3-IgG (Paula Henderikx, Laetitia Devy)

5 A previous study with a purified polyclonal goat antiserum against Tie-1 (R&D Systems) had showed binding to human platelets (Tsiamis et al., 2000). The conclusion from this study was that platelets represent a large pool of Tie-1 immunoreactivity which could present a problem for development of Tie1 as a therapeutic target . To exclude the possibility that the antibody E3 binds to platelets immunoprecipitation of lysates prepared
10 from platelets and HUVECS were performed. Both, activated and inactivated platelets were tested. Anti-Tie-1 antibodies B2, D11, E3, the goat polyclonal AF619 (R&D) and negative control antibodies anti-FITC and anti-Streptavidin were used. HUVECS were retrieved from culture dishes by trypsinization and platelets were prepared with the platelet GelSep kit (Biocytex, Marseille, France) kit according to the guidelines of the
15 manufacturer. Per immunoprecipitation experiment $3-5 \times 10^6$ and 3×10^8 cells platelets were used for each antibody tested. Platelets and cells were washed with PBS and spun down at 1400 rpm for 4 minutes and supernatant was removed. Then cells were lysed in 1 ml lysis buffer containing 50mM Tris HCL pH 7.5, 150 mM NaCl, 0.5% Deoxycholic acid (DOC) and 0.5% NP-40 for 5 minutes. The lysed cells were spin down for 10 minutes at 14.000
20 rpm and 5 µg/ml antibody was added to the supernatant and incubated at 4°C on a rotator. 100 µl/sample protein A beads (Uppsala, Sweden) were washed 3 times with lysis buffer (centrifugation speed: 15 seconds, 2000 rpm) then cell lysates incubated with antibody were added for 30 minutes 4°C. Then beads were washed three times with washing buffer containing 50mM Tris HCL pH 7.5, 400 mM NaCl, 0.5% DOC, 0.5% NP-40.
25 Finally, beads are spun down and the pellets was resuspended in an equal amount in sample buffer to perform SDS-page and Western blotting. In Western blotting Tie-1 was detected with the polyclonal goat anti-Tie-1 antibody. The conclusions of this study are that E3 is able to immune precipitate Tie-1 in HUVEC but not in platelets.

Example 11 : Distribution of Tie1 in HUVECS cells determined by staining with anti Tie1 E3-IgG

We analyzed the staining pattern of E3 in HUVECS using confocal microscopy. HUVEC were trypsinised, washed with PBS and spotted at a density of 60 000 cells on a gelatine coated microscope slide and incubated for 24 hours in a humidified incubator at 37°C. Cells were air dried and fixed with 4% paraformaldehyde for 20 minutes at room temperature. The slides were washed with PBS. The slides were incubated with 10% Heat inactivated human serum (incubation buffer).

-) For measuring specific binding to Tie-1, biotinylated antibody E3 and biotinylated negative control antibody A2 were used at a concentration of 10 µg/ml and incubated for 1 hour at room temperature. Slides were washed twice with PBS. Then, Streptavidin- R-phycoerythrin (Dako, Glostrup, Denmark) was added and incubated for 1 hour at room temperature. After the last incubation and washing detection of bound antibodies was performed by means of confocal microscopy.

E3 binds specifically to HUVEC as detected by confocal microscopy. The staining is pre-dominantly located inside of the cell which suggests a large intracellular pool of Tie1 relative to a smaller pool of cell surface localized Tie1. The localization of E3 was consistent with co-localization of Tie1 with a cytoskeletal protein.

Example 12 : Conversion of somatic mutations positioned in the framework region of anti Tie1 E3 to germline residues

To reduce potential immunogenicity of E3 in humans, all non germline amino acid residues in the LC framework regions were corrected back to germline. An initial analysis was performed which aligned the LC of E3 with a database containing all kappa and lambda light chain germline genes. The LC of E3 was shown to have closest homology to DPK4 and three substitutions in E3 relative to the germline framework regions were identified.

We constructed a germlined version of E3 in which the LC framework regions were altered to include sequences identical to the DPK4 germline framework regions. The germlined E3 antibody was constructed by engineering a nucleic acid encoding the desired

sequence. Changes to nucleic acids encoding the E3 LC variable domain were made by PCR and other standard molecular biological techniques and verified by nucleic acid sequencing.

The germlined E3 sequence is as follows:

DIQMTQSPSSLSASVGDRVTITCRASQGIGHYLA^WYQQKPGKVPKLLIYTASTLQ
SGVPSRFRSGSGSGTDFTLTIS^SLQPEDVATYYCQQFN^SYPHTFGQGTRLEIK (SEQ ID
NO:159)

The altered positions are underscored.

We produced the germlined version of the E3 antibody as both a soluble Fab and as an IgG. The Fab cassette of the positive sFAB-expressing clone was PCR amplified with oligonucleotides, ligated into a mammalian expression vector containing the human IgG4 Fc region and electroporated into XL1 Blue MRF' cells. The prokaryotic ribosomal binding sequence and gene three leader sequence were replaced with a mammalian internal ribosomal entry and heavy chain leader sequences. Reformatted antibody clones were sequenced to confirm accuracy following the cloning procedure. Endotoxin-free DNA was prepared and used for transient transfection studies.

0 **Example 13 : Production and testing of germlined anti Tiel E3 - Fab for binding to recombinant Tiel-Fc in ELISA**

To evaluate if the conversion of any of the somatic mutations in the framework of E3 back to germline residues had any effect on binding activity the soluble Fabs were produced. The soluble expression vector containing the parental E3 Fab and the germlined E3 Fab construct were grown overnight at 30°C in 2xTY broth containing 100 µg/ml ampicillin and 2 % glucose and use 4 ml of this overnight culture to inoculate 400 ml of 2xTY broth containing 100 µg/ml ampicillin and 0.1% glucose. Cells were grow at 37°C until an OD₆₀₀ of 0.8-1.0, 1mM IPTG was added and the culture was maintained at 30 °C for 4 hours. The cultures were spun down at 4,000 rpm for 15 min at 4 °C. The supernatants were discarded and resuspend the pellets resuspended in 4.8 ml of ice cold TES buffer (0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose, pH 8.0) containing proteases inhibitors (protease inhibitor cocktail tablets [Roche]: dissolve 1 tablet in 1 ml of

water and dilute 50-times in TES buffer). Transfer to 50 ml Falcon tubes and place on ice for 5-10 min. During this incubation, wash the centrifugation bottles with 5.25 ml TES:H₂O (1:3) containing proteases inhibitors and add this to the cells. Incubate for 20 more min on ice. Spin at 3000 g for 15 min at 4°C and transfer the supernatants into new centrifugation tubes. Resuspend the cell pellets in 6 ml TES containing 15 mM MgSO₄ and proteases inhibitors and incubate on ice for 15 min. Centrifuge at 3000 g for 15 min at 4°C. Transfer the supernatants into the centrifugation tubes and spin at 8000 g for 20 min at 4°C. Collect the supernatants and dialyze against PBS. The Fabs were purified by metal chelate chromatography. Incubate the dialyzed periplasmic extracts with 1 ml of TALON™ Metal Affinity Resin (Clontech) and rotate at room temperature for 2 hours. Transfer the beads into empty gravity column (Poly-Prep chromatography columns, Bio-Rad, Cat. 731-1550). Wash the beads with 5 mM imidazole in PBS and elute the Fabs with 150 mM imidazole in PBS. Dialyze against PBS using dialysis cassettes (SLIDE-A-LYSER™ Dialysis Cassettes, MWCO 10,000, Pierce, cat. no. 66380) and determine the protein concentration from the absorbance at 280 nm assuming that a 1 mg/ml solution has an absorbance of 0.86. The quality of the preparations can be analyzed by reducing and non-reducing SDS-PAGE.

Wells of an IMMULON™ 2 HB plate coated overnight with 500 ng or 50 ng of purified recombinant human Tie-1-Fc target antigen per 100 microliters 0.1 M sodium bicarbonate buffer, pH 8.5. Parental E3, E3 germlined (E3g) or a negative control soluble Fab were loaded into wells at either 5 micrograms or 1 microgram per 100 microliters of PBST. Recombinant human Tie-1-Fc target antigen is dissolved in an appropriate amount of acetic acid and subsequently diluted into 0.1 M sodium bicarbonate buffer, pH 9.6 at final concentrations of 500 ng and 50 ng per 100 microliters. After addition of the target antigen to the wells the microtitre plate is incubated overnight at 4°C. The plate is subsequently washed 5 times with PBST and blocked with 1% BSA in PBS at 37°C for 2 hours. The plate is again washed plate times with wash buffer, PBST and 100 microliters per well of purified Fab at 5 or 1 micrograms per 100 microliter PBST was added followed by incubation at room temperature for 1 hour. After washing plate 7 times with PBST 100 microliters of a 1:5000 dilution of anti-sFab-HRP in PBST was added (Pierce Product #31414). After washing the wells seven times 100 microliters TMB-H₂O₂ solution

was added to each well and the plate read at 630 nm in an ELISA. Both E3 and germlined E3 bound to the recombinant human Tie-1-Fc target antigen by this assay.

Example 14 : Production and testing of germlined anti Tie1 - E3 –Fab for binding to recombinant human Tie1 in BIAcore

Recombinant purified human Tie1-Fc antigen (Stock 2.45 mg/ml) was biotinylated using the EZ-link Sulfo-NHS-SS-Biotin (Pierce, Cat. 21331). The reaction was performed for 2 hours on ice in 50 mM sodium carbonate buffer, pH 9.6, in the presence of a 5-fold molar excess of biotinylating agent and was stopped by the addition of Tris-HCl, pH 7.5 (50 mM final concentration) followed by a 1-hour incubation on ice. Samples were then dialyzed against PBS. The antigen was then diluted 1/100 fold in HBS and was then captured onto a streptavidin chip. This was coated to a density of 830RU (resonance units). All analysis was performed in HBS buffer. The parental Fab E3 and germlined E3 Fab were prepared as described above. A stock solution of 0.587 mg/ml (11740nM) was diluted 1/587 in HBS + BSA to obtain a stock of 20nM and the germlined Fab E3 0.025 mg/ml (500 nM) was diluted 1/25 in HBS + BSA to obtain a stock of 20nM. Serial dilutions were made of each Fab preparation to obtain 10nM, 5nM, 2.5nM, and 1.25nM solutions. For the association phase samples were injected at 30µl/min for 4 minutes using kinject program. This was followed by a 10 minutes dissociation phase, any remaining sample was stripped from the Tie-1 Fc surface at a flow of 50 µl/min with a single injection of 5 mM NaOH + 1M NaCl for 18 seconds. All samples were run and analyzed in duplicate.

Sensorgrams were analyzed using the simultaneous k_a/k_d fitting program with 1:1 model in the BIAEVALUATION™ software 3.1. From the analysis we can see that the germlining of the E3 antibody has had minimal effect on the binding activity of the antibody.

Table 5: Comparison of the binding affinity of parental and germlined E3 Fab

E3 Fab	Tie-1 Fc	k_a (1/Ms)	k_d (1/s)	$KD(1)$ nM
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parental	Human	3.00E+05	6.10E-04	2.0
germlined	Human	3.00E+05	1.02E-03	3.4

5 **Example 15 : Comparison of affinity of germlined anti Tie1 E3 –IgG to parental anti Tie1 E3 for binding to recombinant human Tie1 using BIAcore**

In order to evaluate if the binding behavior had been affected in any way by the conversion of the somatic mutations back to germline residues, the germlined antibody
 0 was produced and tested as an IgG. The germlined E3-IgG construct used to transiently transfect HEK293T cells and purified.

The germlined E3 IgG1 stock solution 0.63 mg/ml was diluted 1/50 in a buffer of pH4.5 and the parental E3 IgG1 stock solution 0.56 mg/ml (2143-001) was diluted 1/50 in a buffer of pH 4.5. The IgG were directly coated onto a CM5 chip. The surface of the
 15 chips was activated with a 7 minute pulse of 0.05M NHS/0.2M EDC and the IgG was flowed over until 780RU germlined E3-IgG and 728 non germlined E3 IgG was coated onto the surface. All flow cells were subsequently deactivated with a 7 minute pulse of 1M ethanolamine hydrochloride pH 8.5. All analysis was performed in HBS buffer. Purified recombinant human Tie-1 Fc was diluted 1/28.7 in HBS to obtain a 400 nM stock solution.
 20 Serial dilutions were made to obtain 200nM, 100nM, 50nM and 25 nM Tie-1 Fc stocks. For analysis of the association phase samples were injected at 30 μ l/min for 8.3 minutes using kinject program. This was followed by a 40 minutes dissociation phase. Any antigen remaining associated to the surface was stripped from the IgG surface at a flow of 50 μ l/min with two injections of 10mM glycine pH 1.5 for 30secods. All samples were run
 25 and analyzed in duplicate

Sensorgrams were analyzed using the simultaneous k_a/k_d fitting program with 1:1 model in the BIAEVALUATION™ software 3.1. Germlining had minimal impact on the binding activity of the E3 IgG with respect to human Tie1 Fc.

Table 6: Comparison of the binding affinity of parental and germlined E3 IgG

E3 IgG	Tie-1 Fc	ka (1/Ms)	kd (1/s)	KD(1) nM
parental	Human	6.19E+03	3.61E-05	5.83
germlined	Human	7.09E+03	3.67E-05	5.17

Example 16 : Production and testing of germlined anti Tie1 - E3 –Fab for binding to recombinant mouse Tie1 in BIAcore

Mouse Tie 1-Fc antigen (0.5 mg/ml stock) was biotinylated using established procedures and after dilution 1/100 fold in HBS this was then used for capturing to a streptavidin chip. This was coated to a resonance value of 740RU. All analysis was performed in HBS buffer. The parental Fab E3 0.587 mg/ml (11740 nM) was diluted 1/587 in HBS + BSA to obtain a stock of 20 nM and the germlined Fab E3 0.025 mg/ml (500 nM) was diluted 1/25 in HBS + BSA to obtain a stock of 20 nM. Serial dilutions were made of each Fab preparation to obtain 10 nM, 5 nM, 2.5nM, and 1.25nM. For the association phase samples were injected at 30µl/min for 4 minutes using kinject program. This was followed by a 10 minutes dissociation phase, any remaining sample was stripped from the Tie-1 Fc surface at a flow of 50 µl/min with a single injection of 50 mM NaOH + 1 M NaCl for 18 seconds. All samples were run and analyzed in duplicate.

Sensorgrams were analyzed using the simultaneous ka/kd fitting program with 1:1 model in the BIAEVALUATION™ software 3.1. The germlining of the E3 antibody has had minimal effect on the binding activity of the antibody.

Table 7: Comparison of the binding affinity of parental and germlined E3 Fab

E3 Fab	Tie-1 Fc	ka (1/Ms)	kd (1/s)	KD(1) nM
parental	Mouse	2,46 E+05	9,50E-04	3,9
germlined	Mouse	3,40E+05	1,04E-03	3,1

Example 17 : Comparison of affinity of germlined anti Tie1 E3 –IgG to parental anti Tie1 E3 for binding to recombinant mouse Tie1 using BIAcore

In order to evaluate if the binding behavior had been affected in any way by the conversion of the somatic mutations back to germline, the germlined antibody was produced and tested as a IgG. The germlined E3 was reformatted to an IgG as described. This was then used to transiently transfect HEK293T cells using established procedures. The IgG was purified from the culture supernatant using protein A column chromatography using established procedures and the subsequent IgG was then tested for binding activity using surface plasmon resonance (BIAcore). The germlined E3 IgG1 stock solution 0,63 mg/ml (2146-002) was diluted 1/50 in a buffer of pH 4.5 and the parental E3 IgG1 stock solution 0,56 mg/ml (2143-001) was diluted 1/50 in a buffer of pH 4.5. The IgG were directly coated via onto a CM5 chip. The surface of the chips was activated with a 7 minute pulse of 0.05M NHS/0.2M EDC and the IgG was flowed over until 780RU germlined E3-IgG and 728 non germlined E3 IgG was coated onto the surface. All flow cells were subsequently deactivated with a 7 minute pulse of 1M ethanolamine hydrochloride pH8,5. All analysis was performed in HBS buffer. Purified recombinant mouse Tie-1 Fc was diluted 1/6,5 in HBS to obtain a 400nM stock solution. Serial dilutions were made to obtain 200nM, 100nM, 50nM and 25 nM Tie-1 Fc stocks. For analysis of the association phase samples were injected at 30µl/min for 8,3 minutes using kinject program. This was followed by a 40 minutes dissociation phase. Any antigen remaining associated to the surface was stripped from the IgG surface at a flow of 50 µl/min with two injections of 10mM glycine pH1,5 for 30seconds. All samples were run and analyzed in duplicate

Sensorgrams were analyzed using the simultaneous k_a/k_d fitting program with 1:1 model in the BIAEVALUATION™ software 3.1. The germlining process had minimal impact on the binding activity of the E3 IgG with respect to mouse Tie1-Fc.

Table 8: Comparison of the binding affinity of parental and germlined E3 IgG

E3 IgG	Tie-1 Fc	k_a (1/Ms)	k_d (1/s)	KD(1) nM
parental	Mouse	6.17E+03	9.20E-05	14.9
germlined	Mouse	6.00E+03	8.99E-05	15

Example 18: Comparison of IC₅₀ of germlined anti Tie1 - E3 and parental anti Tie1 - E3 in tube formation assays using HUVECS

Germlined E3 and its parental antibody were evaluated in the tube formation assay in a collagen type-I matrix. Human Umbilical vein endothelial cells (HUVEC) (freshly isolated) were obtained by treating human umbilical cord veins with Trypsin-EDTA (1x) (Gibco/Invitrogen) for 20-25 minutes at 37°C. The cells were then cultured in a T-25 flask coated with attachment factor (AF), (Cascade Biologics) in RPMI 1640 medium supplemented with 10 % FCS, 0.4 % BBE, 1% l-glutamin, 1% penicillin/streptomycin.

- 1) Primary cultures were detached with warm Trypsin-EDTA and used when confluent at the second or third passage. During culturing, the cells were kept in a proliferative state by culturing them in a split ratio 1:2 at an approximate density of the monolayer of about 60-80%. HUVEC monolayers were treated with trypsin/EDTA (500µl/dish) at 37°C for 3 min. Trypsin activity was stopped by adding 3 volumes of complete RPMI medium. The cells were carefully scraped, separated by repeated pipetting, and finally washed with PBS. HUVECs (passage 2) were seeded in their culture medium (40.10³/50µl/well of a 96-well plate) on a collagen gel (50µl of coll I 1.5mg/ml) prepared by mixing 7.5 volumes of 2mg/ml collagen (Collagen R; Serva, Heidelberg, Germany), 1 volume of 10X MEM, 1.5 volume of NaHCO₃ (15.6 mg/ml) and ~ 1 volume of NaOH to adjust the pH to 7.4. After 1h30, the culture medium was then discarded and the cells were covered with a new layer of collagen (1.5mg/ml, new preparation, 50µl/well). After polymerization of the gel, culture medium was added to each well in presence or in absence of E3 antibody or germlined E3 antibody (0.1 ng/ml to 100ng/ml). The total length of the tube network on the culture surface was quantified at 40 × magnification by the Metavue Software (Universal Imaging Corporation). Results from triplicate wells were expressed as mean vessel area per field ±SEM (relative units). Each assay was performed at least three times (Figure 16, Figure17). The conclusions are that conversion of the 3 somatic mutations to germline amino acids in E3 has had little effect on the potency of E3. Both parental E3 and germlined E3 inhibit tube formation *in vitro* with an essentially identical IC₅₀ = 10 ng/ml, i.e. 66 pM)

Example 19 : Analysis of germlined anti Tie1 - E3 in tube formation assays with mouse endothelial cells

In order to assess mouse Tie-1 cross-reactivity and biological activity on mouse Tie1, both E3 and germlined E3 were evaluated for their ability to inhibit tube formation *in vitro* using mouse endothelial cell line (LEII).

LEII lung mouse endothelial cell line (ATCC) was cultured in a T-25 flask in MEM medium with GLUTAMAX™ (Life Technologies Ltd., Paisley, Scotland) supplemented with 10 % FCS, and 1% penicillin/streptomycin. During culturing, the cells were kept in a proliferative state by culturing them in a split ratio 1:5 at an approximate density of the monolayer of about 80%. LEII monolayers were treated with trypsin/EDTA (500 µl/dish) at 37°C for 3 min. Trypsin activity was stopped by adding 3 volumes of complete MEM medium. The cells were carefully scraped, separated by repeated pipetting, and finally washed with PBS. LEII cells were seeded in their culture medium (20-40 × 10³/50µl/well of a 96-well plate) on a basement membrane (BIOCOAT™ Angiogenesis System; Becton Dickinson). After polymerization of the MATRIGEL™ (30 min at 37°C, 5% CO₂ environment) the endothelial cell suspension resuspended in complete culture medium in the presence of the desired molecules (4.10⁵ cells/ml; 50µl/well) was added to each well. The angiogenesis assay plate was then incubated for 16 to 18 hours at 37°C, 5% CO₂ atmosphere. The total length of the tube network was then quantified at 40× magnification by the METAVUE™ Software (Universal Imaging Corporation). Results from triplicate wells were expressed as mean vessel area per field ± SEM (relative units). Each assay was performed at least two times. Germlined E3 is a potent inhibitor of tube formation in mouse endothelial cells.

Example 20 : Immunohistochemical analysis of mouse tumor tissue sections using anti Tie1 - E3

We determined if antibody E3 binds to mouse endothelial cells in mouse xenographs. Immunohistochemistry was performed with biotinylated antibody E3 and control antibodies anti-CD31 (endothelial cell specific marker) and anti-PCNA (proliferating cell nuclear antigen). Formalin-fixed tumor tissues from a mouse-xenograph containing SW480 cells (ATCC) were tested for the binding pattern of the

human anti-Tie1 antibody E3. 5 μ m sections of paraffin embedded tissues were deparaffinized, rehydrated and pretreated with warm the citrate buffer (0.01 M sodium citrate, pH6 at 95°C) for 45 min. The slides were cooled down in fresh citrate buffer for 20 min and rinsed with distilled water. The slides were hydrogen peroxide treated, (0.3 % H_2O_2 in PBS), and preincubated with PBS, 5 % FCS, 5% heat inactivated human serum (HS) for 1 hour. Between antibody incubations slides were washed 3 times 5 minutes in PBS. Biotinylated antibodies E3 and A2 were diluted to a concentration of 10 μ g/ml in PBS, 10 % HS and incubated for 1 hour at RT. Slides were then incubated with an avidin-HRP (Dako) for 30 minutes at room temperature. Staining was detected by AEC (Vector Laboratories, Burlingame) and H_2O_2 . The peroxidase reaction was stopped with water and slides were counter-stained with haematoxylin. The tissues were evaluated for their binding reactivity. The staining pattern was consistent with staining of mouse endothelial cell Tie-1 and also with Tie1 expressed by the E3 binds to Tie-1 expressed by SW480 tumor cells in a mouse xenograft.

WHAT IS CLAIMED:

1. An isolated protein comprising a heavy chain immunoglobulin variable domain sequence and a light chain immunoglobulin variable domain sequence, wherein the protein binds to Tie1 ectodomain and the heavy chain immunoglobulin variable domain sequence comprises one or more of the following properties:

i) a HC CDR1 that includes an amino acid sequence as follows:

(AGSR)-Y-(GVK)-M-(GSVF), (SEQ ID NO:117)

(AGSIMRH)-Y-(GVMK)-M-(GSVMFH) (SEQ ID NO:118), or

(AGSIMRNH)-Y-(AGTVMKPQ)-M-(AGSTVMYWFKH) (SEQ ID NO:119);

ii) a HC CDR2 that includes an amino acid sequence as follows:

X-I-Y-P-S-G-G-X-T-X-Y-A-D-S-V-K-G (SEQ ID NO:120), wherein X is any amino acid,

(GSV)-I-(SY)-P-S-G-G-(WQ)-T-(GY) (SEQ ID NO:121),

(GSV)-I-(SY)-P-S-G-G-(WNQ)-T-(GY) (SEQ ID NO:160),

(GSV)-I-(SY)-P-S-G-G-(WQ)-T-(GY)-Y-A-D-S-V-K-G (SEQ ID NO:122), or

(GSVW)-I-(SY)-P-S-G-G-(AGVMYWPQH)-T-(AGSTLVMYFKH)

(SEQ ID NO:123);

iii) a HC CDR3 that includes an amino acid sequence as follows:

V-(four or five residues)-F-D-(I/Y) (SEQ ID NO:124),

G-Y-G-P-I-A-P-G-L-D-Y (SEQ ID NO:125),

(GV)-N-Y-Y-(GYD)-S-(SD)-G-Y-G-P-I-A-P-G-L-D-Y (SEQ ID NO:126),

(GVD)-(AGLN)-(LYR)-(GSTLYH)-(GYD)-(AGSYFP)-(SFD)-(AGYD)-(IY)-

(GFD)-(YDP)-(IP)-A-P-G-L-D-Y (SEQ ID NO:127), or

VNYYDSSGYGPIAPGLDY (SEQ ID NO:128).

2. The protein of claim 1 wherein the light chain immunoglobulin variable domain sequence comprises one or more of the following properties

i) a LC CDR1 that includes an amino acid sequence as follows:

R-A-S-Q-S-(IV)-S-(SR)-X1-Y-L-(AN) (SEQ ID NO:129),

R-A-S-Q-S-V-S-S-X-L (SEQ ID NO:130),

R-A-S-Q-S-(IV)-S-(SR)-(SY)-(LY)-(ALN) (SEQ ID NO:131), or
 R-A-S-(REQ)-(GSTRN)-(IV)-(GSTIRN)-(STIRH)-X1-(SYWNH)-(LV)-(ASN)
 (SEQ ID NO:132), wherein X1 can be serine or absent;

ii) a LC CDR2 that includes an amino acid sequence as follows:

X-A-S-X-R-A-T (SEQ ID NO:133), wherein X can be any amino acid,
 (AGD)-A-S-(STN)-R-A-T (SEQ ID NO:134),
 (AGD)-A-S-(STN)-(LR)-(AEQ)-(ST) (SEQ ID NO:135), or
 (AGTKDEH)-A-S-(STN)-(LR)-(AVEQ)-(ST) (SEQ ID NO:136); and

iii) a LC CDR3 that includes an amino acid sequence as follows:

Q-Q-(SYFR)-(GSYN)-S-(STYW)-(RP)-(LWR)-(TIY)-T (SEQ ID NO:137),
 Q-Q-(SYFR)-(GSYN)-S-(STYW)-(RP)-(LWRH)-(TIY) (SEQ ID NO:161),
 (LQ)-Q-(SYFR)-(GSYN)-(SKN)-(STYW)-(RP)-(LWR)-(TIY)-T (SEQ ID

NO:138),

Q-Q-(YR)-(GS)-S-(SW)-P-R-X1-T (SEQ ID NO:139), wherein X1 is any amino

acid or absent,

(LQ)-(LQ)-(SYFRD)-(GSYN)-(STRKN)-(STYWF)-(RP)-(ILMWRH)-(TIY)-(TI)
 (SEQ ID NO:140), and

(LQ)-(LRQ)-(SYFRD)-(GSYN)-(ASTRKN)-(STYWF)-(SVRP)-(STILMWRH)-
 (TIY)-(STI) (SEQ ID NO:141).

3. The protein of claim 1 wherein the light chain immunoglobulin variable domain sequence comprises one or more of the following properties

i) a LC CDR1 that includes an amino acid sequence as follows:

S-X-(ND)-(IV)-(AG)-X1-X2-X3 (SEQ ID NO:142), or

T-(GR)-(ST)-S-X5-(ND)-(IV)-(AG)-X1-X2-X3-Y-X4-S (SEQ ID NO:143),

wherein X1 is any amino acid (e.g., G or R), X2 is any amino acid (e.g., Y or N), X3 is any amino acid (e.g., F, N, or K), X4 is any amino acid (e.g., aliphatic, e.g., V or A);

ii) a LC CDR2 that includes an amino acid sequence as follows:

(DE)-V-N-N-R-P-S (SEQ ID NO:144), or

(DE)-(VD)-(STDN)-(YRDN)-R-P-S (SEQ ID NO:145); and

iii) a LC CDR3 that includes an amino acid sequence as follows:

(SQ)-S-(SY)-(ASID)-(GSR)-(ST)-(STRN)-(STYR)-(ATLY)-(SVWQ) (SEQ ID NO:146).

4. The protein of claim 1 wherein the HC variable domain sequence comprises
5 SEQ ID NO:118 and SEQ ID NO:160.

5. The protein of claim 1 wherein the LC variable domain sequence comprises
SEQ ID NO:132, SEQ ID NO:136, and SEQ ID NO:161.

0 6. The protein of claim 1 wherein the amino acid sequence of the HC variable
domain sequence is at least 85% identical to the amino acid sequence of the HC variable
domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10,
s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

5 7. The protein of claim 1 wherein the amino acid sequence of the LC variable
domain sequence is at least 85% identical to the amino acid sequence of the LC variable
domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10,
s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4.

20 8. The protein of claim 1 wherein the amino acid sequences of the HC variable
domain sequence comprises CDR1, CDR2, and CDR3 sequences from the E3 clone, and
the LC variable domain sequence comprises CDR1, CDR2, and CDR3 sequences from the
E3 clone.

25 9. The protein of claim 8 wherein the LC variable domain sequence comprises
SEQ ID NO:159.

10. The protein of claim 8 wherein the HC variable domain sequence comprises
SEQ ID NO:114.

30

11. The protein of claim 1 wherein the HC and LC framework regions are human.

12. The protein of claim 1 further comprising an Fc domain.

13. The protein of claim 12 that comprises the constant domains of a human IgG1, IgG2, IgG3, or IgG4.

5

14. An isolated protein comprising a heavy chain immunoglobulin variable domain sequence and a light chain immunoglobulin variable domain sequence, wherein the protein binds to a Tie1 ectodomain and to endothelial cells, but does not substantially bind to platelets.

0

15. The protein of claim 14 that binds to Tie1 with a Kd of less than 5 nM.

16. The isolated protein of claim 14 that comprises the HC and LC immunoglobulin variable domains of the E3 antibody or domains that are at least 85% identical to the HC and LC immunoglobulin variable domains of the E3 antibody, respectively.

5

17. The protein of claim 14 that inhibits tube formation by HUVEC cells in vitro.

18. The protein of claim 14 that recognizes melanoma-associated structures in a histological section.

10

19. An isolated protein comprising a heavy chain immunoglobulin variable domain sequence and a light chain immunoglobulin variable domain sequence, wherein the protein binds to a Tie1 ectodomain and competes with E3 for binding to Tie1 or binds to an epitope that overlaps an epitope that is recognized by E3 or that has at least one, two or three residues in common with an epitope that is recognized by E3.

25

20. An isolated nucleic acid comprising a coding sequence that encodes a polypeptide comprising an immunoglobulin HC variable domain sequence, wherein the coding sequence is at least 85% identical to a reference sequence that encodes the HC variable domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4, or the coding sequence hybridizes to the reference sequence or a complement thereof.

21. The nucleic acid of claim 20 that further comprises a second coding sequence that encodes a polypeptide comprising an immunoglobulin LC variable domain.

22. An isolated nucleic acid comprising a coding sequence that encodes a polypeptide comprising an immunoglobulin LC variable domain sequence, wherein the coding sequence is at least 85% identical to a reference sequence that encodes the LC variable domain of clone E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, or s-H4, or the coding sequence hybridizes to the reference sequence or a complement thereof.

23. The nucleic acid of claim 22 that further comprises a second coding sequence that encodes a polypeptide comprising an immunoglobulin HC variable domain.

24. A host cell that contains a first nucleic acid sequence encoding a polypeptide comprising a HC variable domain of an antibody and a second nucleic acid sequence encoding a polypeptide comprising a LC variable domain of the antibody, wherein the antibody is a protein according to claim 1 or 14.

25. A host cell that contains a first nucleic acid encoding a polypeptide comprising a HC variable region and a second nucleic acid encoding a polypeptide comprising a LC variable region, wherein the HC and the LC variable regions each comprise a sequence at least 85% identical to respective amino acid sequences of the HC and LC variable domains of a clone selected from the group consisting of E3, G2, p-A1, p-A10, p-B1, p-B3, p-C6, p-D12, p-F3, p-F4, p-G3, s-A10, s-H1, s-A2, s-B2, s-B9, s-C10, s-C2, s-C7, s-D11, s-E11, s-G10, and s-H4.

26. A pharmaceutical composition comprising a protein of any of claims 1-19 and a pharmaceutically acceptable carrier.

5 27. A method of modulating angiogenesis, the method comprising:
administering a composition that comprises the protein of claim 1 or 14 to a subject in an amount effective to modulate angiogenesis in the subject.

28. The method of claim 27 wherein the protein antagonizes Tie1 activity.

0 29. The method of claim 27 wherein the subject has an angiogenesis-related disorder.

30. The method of claim 27 wherein the subject has a neoplastic disorder.

5 31. The method of claim 27 wherein the subject has a metastatic cancer.

32. The method of claim 27 wherein the subject has an angiogenesis-dependent cancer or tumor.

20 33. The method of claim 27 wherein the subject has an inflammatory disorder.

34. The method of claim 33 wherein the subject has rheumatoid arthritis.

25 35. The method of claim 33 wherein the subject has psoriasis.

36. The method of claim 27 wherein the protein is delivered systemically.

30 37. The method of claim 27 wherein the protein is administered in an amount effective to reduce one or more of the following activities: sprouting, splitting and remodeling of blood vessels.

38. The method of claim 27 wherein the protein is administered in an amount effective to reduce vasculogenesis or tubule formation.

39. A method of modulating endothelial cell activity in the subject, the method
5 comprising:

administering a composition that comprises the protein of claim 14 to a subject in an amount effective to modulate endothelial cell activity in the subject.

40. A method for detecting the presence of a Tie1 protein, in a sample, in vitro, the
10 method comprising:

(i) contacting the sample with an Tie1-binding protein according to claim 1, under conditions that allow interaction of the Tie1-binding protein and the Tie1 protein to occur; and

(ii) detecting formation of a complex between the Tie1-binding protein and
15 the sample.

41. A method for detecting the presence of Tie1 in vivo, the method comprising:

(i) administering to a human subject an Tie1-binding protein, under conditions that allow interaction of the Tie1-binding ligand and the Tie1 protein to occur;
20 and

(ii) detecting formation of a complex between the Tie1-binding protein and a Tie1 molecule of the subject or detecting distribution of Tie1-binding protein or at least one location of the Tie1-binding protein in the subject.

25

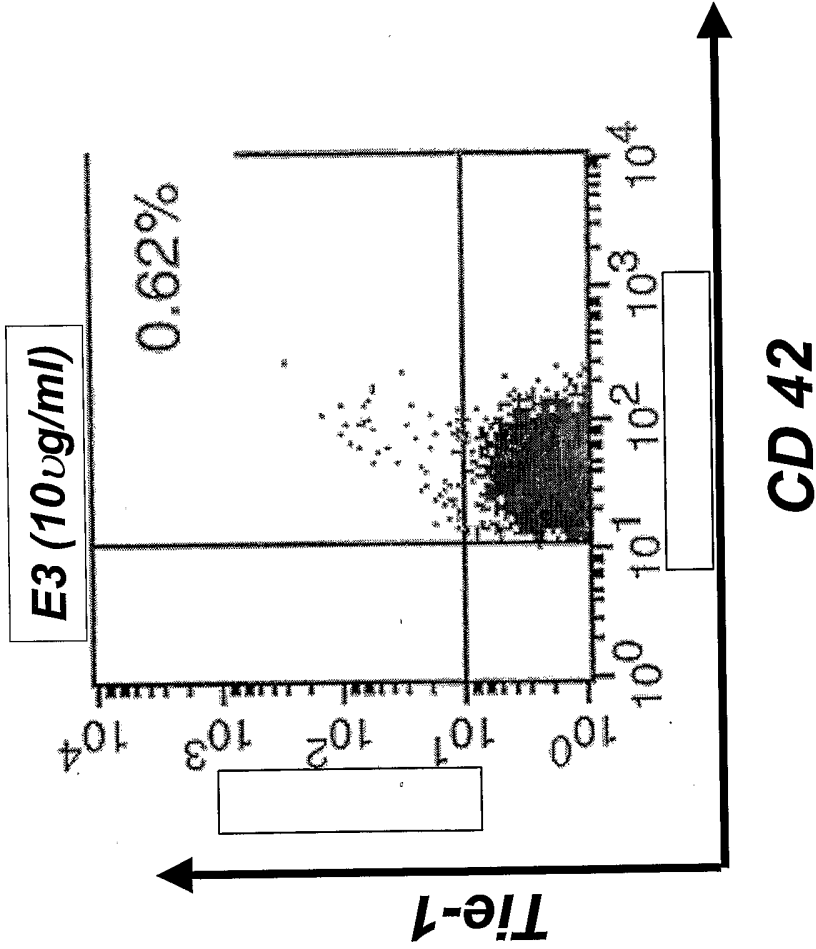


FIG. 1

E3: IC50<1ng/ml (<6.6pM)

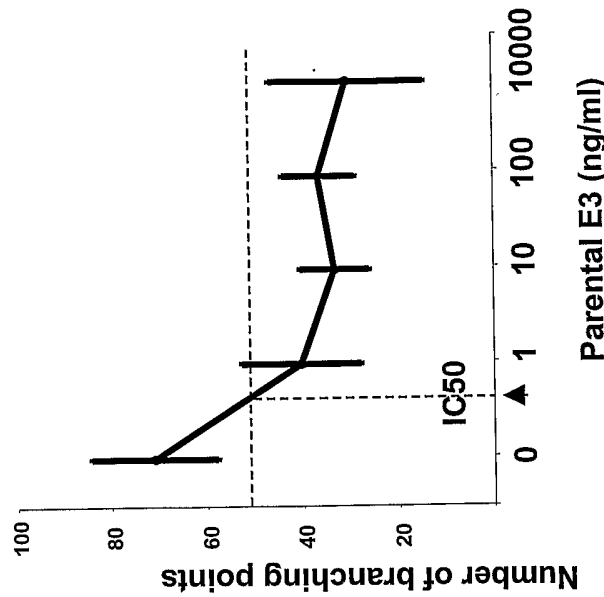


FIG. 2A

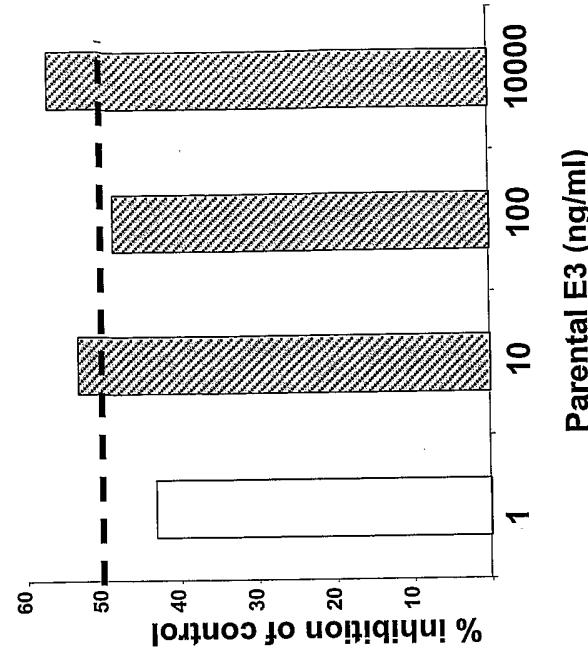


FIG. 2B

gE3: IC50<10ng/ml (<66pM)

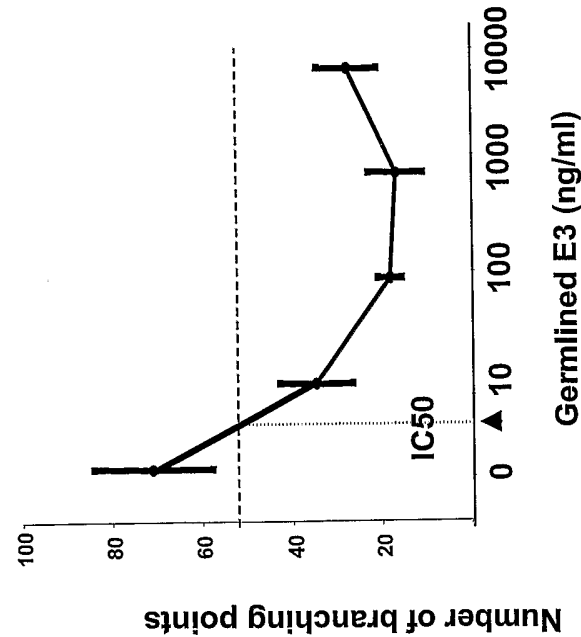


FIG. 3A

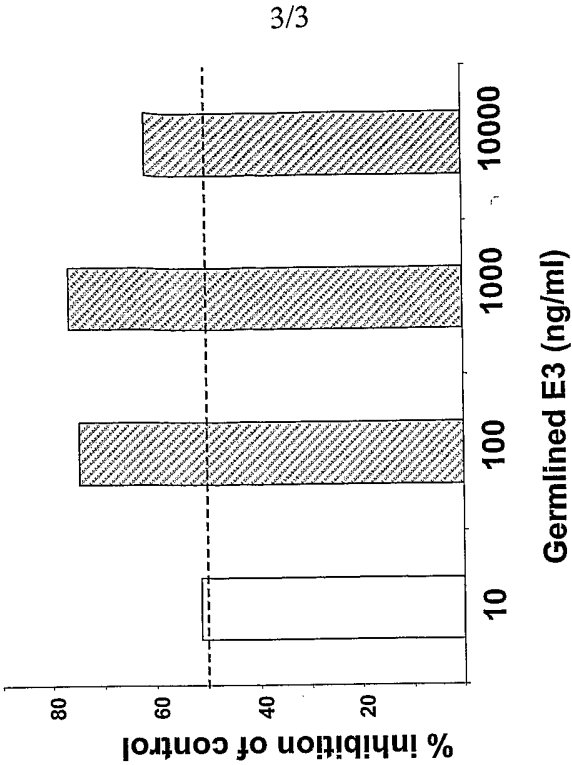


FIG. 3B