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(54) INTEGRIN ANTAGONISTS WITH ENHANCED ANTIBODY DEPENDENT CELL-MEDIATED CYTOXICITY ACTIVITY

(75) Inventors: **Herren Wu**, Boyds, MD (US); **Changshou Gao**, Potomac, MD (US)

Correspondence Address:
JOHNATHAN KLEIN-EVANS
ONE MEDIMMUNE WAY
GAITHERSBURG, MD 20878 (US)

(73) Assignee: **MEDIMMUNE, INC.**, Gaithersburg, MD

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C12P	*21/06*	(2006.01)
C12N	*5/06*	(2006.01)

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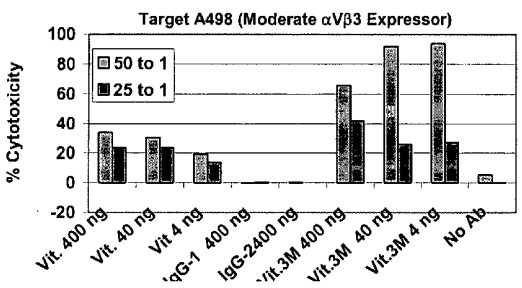
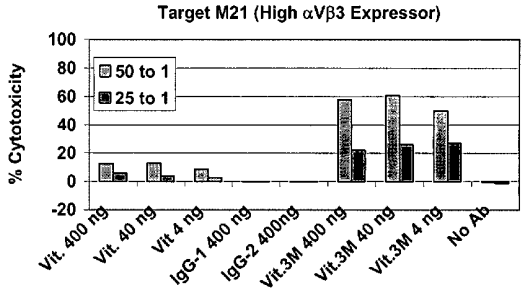
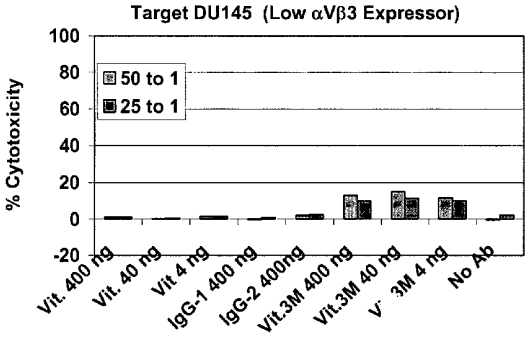
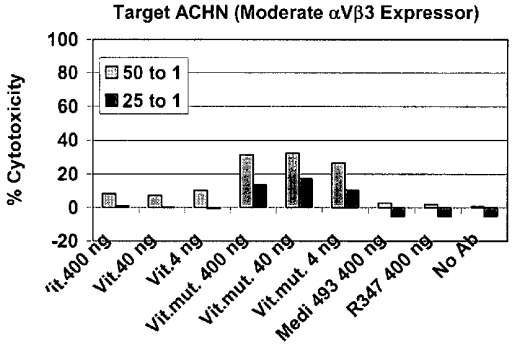
(57) ABSTRACT

The present invention relates to novel Fc variants of antibodies that immunospecifically binds to Integrin $\alpha_v\beta_3$. The Fc variants comprise a variable region that immunospecifically binds to Integrin $\alpha_v\beta_3$ and a Fc region that further comprises at least one novel amino acid residue which may provide for enhanced effector function. More specifically, this invention provides Fc variants that have modified binding affinity to one or more FcγR and/or C1q. Additionally, the Fc variants have altered antibody dependent cell-mediated cytotoxicity (ADCC) and/or complement dependent cytotoxicity (CDC) activity. The invention further provides methods and protocols for the application of said Fc variants of an antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$, particularly for therapeutic purposes.









A

```
CAG GTG CAG CTG GTG GAG TCT GGG GGA GGC GTT GTG CAG CCT GGA AGG      48
Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg      16

TCC CTG AGA CTC TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT AGC TAT      96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr      32

GAC ATG TCT TGG GTT CGC CAG GCT CCG GGC AAG GGT CTG GAG TGG GTC     144
Asp Met Ser Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val      48

GCA AAA GTT AGT AGT GGT GGT GGT AGC ACC TAC TAT TTA GAC ACT GTG     192
Ala Lys Val Ser Ser Gly Gly Gly Ser Thr Tyr Tyr Leu Asp Thr Val      64

CAG GGC CGA TTC ACC ATC TCC AGA GAC AAT AGT AAG AAC ACC CTA TAC     240
Gln Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr      80

CTG CAA ATG AAC TCT CTG AGA GCC GAG GAC ACA GCC GTG TAT TAC TGT     288
Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys      96

GCA AGA CAT CTG CAT GGC AGT TTT GCT TCT TGG GGC CAA GGG ACT ACA     336
Ala Arg His Leu His Gly Ser Phe Ala Ser Trp Gly Gln Gly Thr Thr     112

GTG ACT GTT TCT AGT                                                351
Val Thr Val Ser Ser                                                117
```

```
GAG ATT GTG CTA ACT CAG TCT CCA GCC ACC CTG TCT CTC AGC CCA GGA      48
Glu Ile Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Leu Ser Pro Gly      16

GAA AGG GCG ACT CTT TCC TGC CAG GCC AGC CAA AGT ATT AGC AAC TTC      96
Glu Arg Ala Thr Leu Ser Cys Gln Ala Ser Gln Ser Ile Ser Asn Phe      32

CTA CAC TGG TAT CAA CAA AGG CCT GGT CAA GCC CCA AGG CTT CTC ATC     144
Leu His Trp Tyr Gln Gln Arg Pro Gly Gln Ala Pro Arg Leu Leu Ile      48

CGC TAT CGT TCC CAG TCC ATC TCT GGG ATC CCC GCC AGG TTC AGT GGC     192
Arg Tyr Arg Ser Gln Ser Ile Ser Gly Ile Pro Ala Arg Phe Ser Gly      62

AGT GGA TCA GGG ACA GAT TTC ACC CTC ACT ATC TCC AGT CTG GAG CCT     240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Glu Pro      80

GAA GAT TTT GCA GTC TAT TAC TGT CAA CAG AGT GGC AGC TGG CCT CTG     288
Glu Asp Phe Ala Val Tyr Tyr Cys Gln Gln Ser Gly Ser Trp Pro Leu      96

ACG TTC GGA GGG GGG ACC AAG GTG GAA ATT AAG                        321
Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys                        107
```

FIG. 1

A

```
CAAATGCAGCTGGTGCAGTCTGGGCCTGAGGTGAAGAAGCCTGGGACCTCAGTGAAGGTC  60
 Gln Met Gln Leu Val Gln Ser Gly Pro Glu Val Lys Lys Pro Gly Thr Ser Val Lys Val

TCCTGCAAGGCTTCTGGATTCACCTTTGACGATTACTCCATGAACTGGGTGCGACAGGCT  120
 Ser Cys Lys Ala Ser Gly Phe Thr Phe Asp Asp Tyr Ser Met Asn Trp Val Arg Gln Ala

CGTGGACAACGCCTTGAGTGGATAGGATTTATTAGAAACAAAGCTAATGACTACACAACA  180
 Arg Gly Gln Arg Leu Glu Trp Ile Gly Phe Ile Arg Asn Lys Ala Asn Asp Tyr Thr Thr

GAGTACGCTGACTCTGTGAAGGGTAGAGTCACCATTACCAGGGACATGTCCACGAGCACA  240
 Glu Tyr Ala Asp Ser Val Lys Gly Arg Val Thr Ile Thr Arg Asp Met Ser Thr Ser Thr

GCCTACATGGAGCTGAGCAGCCTGAGATCCGAGGACACGGCCGTGTATTACTGTGCGAGA  300
 Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg

TACCCTAGGCATCATGCTATGGACTCCTGGGGCCAAGGAACCTCGGTCACCGTCTCCTCA  360
 Tyr Pro Arg His His Ala Met Asp Ser Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser
```

```
GACATCCAGATGACCCAGTCTCCATCCTCCCTGTCTGCATCTGTAGGAGACAGAGTCACC  60
 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val Thr

ATCACTTGCAGGGCCAGCCAAAGTATTAGCAACAACCTACACTGGTATCAGCAGAAACCA  120
 Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Asn Asn Leu His Trp Tyr Gln Gln Lys Pro

GGGAAAGCCCCTAAGCTCCTGATCAAGTATGCCTTCCAGTCCATCTCTGGGGTCCCATCA  180
 Gly Lys Ala Pro Lys Leu Leu Ile Lys Tyr Ala Phe Gln Ser Ile Ser Gly Val Pro Ser

AGGTTCAGTGGAAGTGGATCTGGGACAGATTTTACTTTCACCATCAGCAGCCTGCAGCCT  240
 Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro

GAAGATTTTGCAACATATTACTGTCAACAGGCCAACAGCTGGCCGCTCACGTTCGGCGGA  300
 Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ala Asn Ser Trp Pro Leu Thr Phe Gly Gly

GGGACCAAGGTGGAGATCAAA  321
 Gly Thr Lys Val Glu Ile Lys
```

FIG. 2

A

```
GAG GTG CAG CTG GTG GAG TCT GGG GGA GGT GTG GTA CGG CCT GGG GGG     48
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Arg Pro Gly Gly     16

TCC CTG AGA CTC TCC TGT GCA GCC TCT GGG TTC ACC GTC AGT GAT TAC     96
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Val Ser Asp Tyr     32

TCC ATG AAC TGG GTC CGC CAG GCT CCA GGG AAG GGC CTG GAG TGG ATT    144
Ser Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile     48

GGG TTT ATT AGA AAC AAA GCT AAT GCC TAC ACA ACA GAG TAC AGT GCA    192
Gly Phe Ile Arg Asn Lys Ala Asn Ala Tyr Thr Thr Glu Tyr Ser Ala     64

TCT GTG AAG GGT AGA TTC ACC ATC TCA AGA GAT GAT TCA AAA AAC ACG    240
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Asn Thr     80

CTG TAT CTG CAA ATG AAC AGC CTG AAA ACC GAG GAC ACA GCC GTG TAT    288
Leu Tyr Leu Gln Met Asn Ser Leu Lys Thr Glu Asp Thr Ala Val Tyr     96

TAC TGT ACC ACA TAC CCT AGG TAT CAT GCT ATG GAC TCC TGG GGC CAG    336
Tyr Cys Thr Thr Tyr Pro Arg Tyr His Ala Met Asp Ser Trp Gly Gln    112

GGC ACC ATG GTC ACC GTC TCC TCA                                   360
Gly Thr Met Val Thr Val Ser Ser                                   120
```

```
GCC ATC CAG TTG ACT CAG TCT CCA TCC TCC CTG TCT GCA TCT GTA GGA     48
Ala Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly     16

GAC AGA GTC ACC ATC ACT TGC AGG GCC AGC CAA AGT ATT AGC AAC AAC     96
Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Asn Asn     32

CTA CAC TGG TAC CTG CAG AAG CCA GGG CAG TCT CCA CAG CTC CTG ATC    144
Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro Gln Leu Leu Ile     48

TAT TAT GGC TTC CAG TCC ATC TCT GGG GTC CCA TCA AGG TTC AGT GGC    192
Tyr Tyr Gly Phe Gln Ser Ile Ser Gly Val Pro Ser Arg Phe Ser Gly     64

AGT GGA TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AGT CTG CAA CCT    240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro     80

GAA GAT TTT GCA ACT TAC TAC TGT CAA CAG GCC AAC AGC TGG CCG CTC    288
Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ala Asn Ser Trp Pro Leu     96

ACG TTC GGC GGA GGG ACC AAG CTG GAG ATC AAA                       321
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys                       107
```

FIG. 3

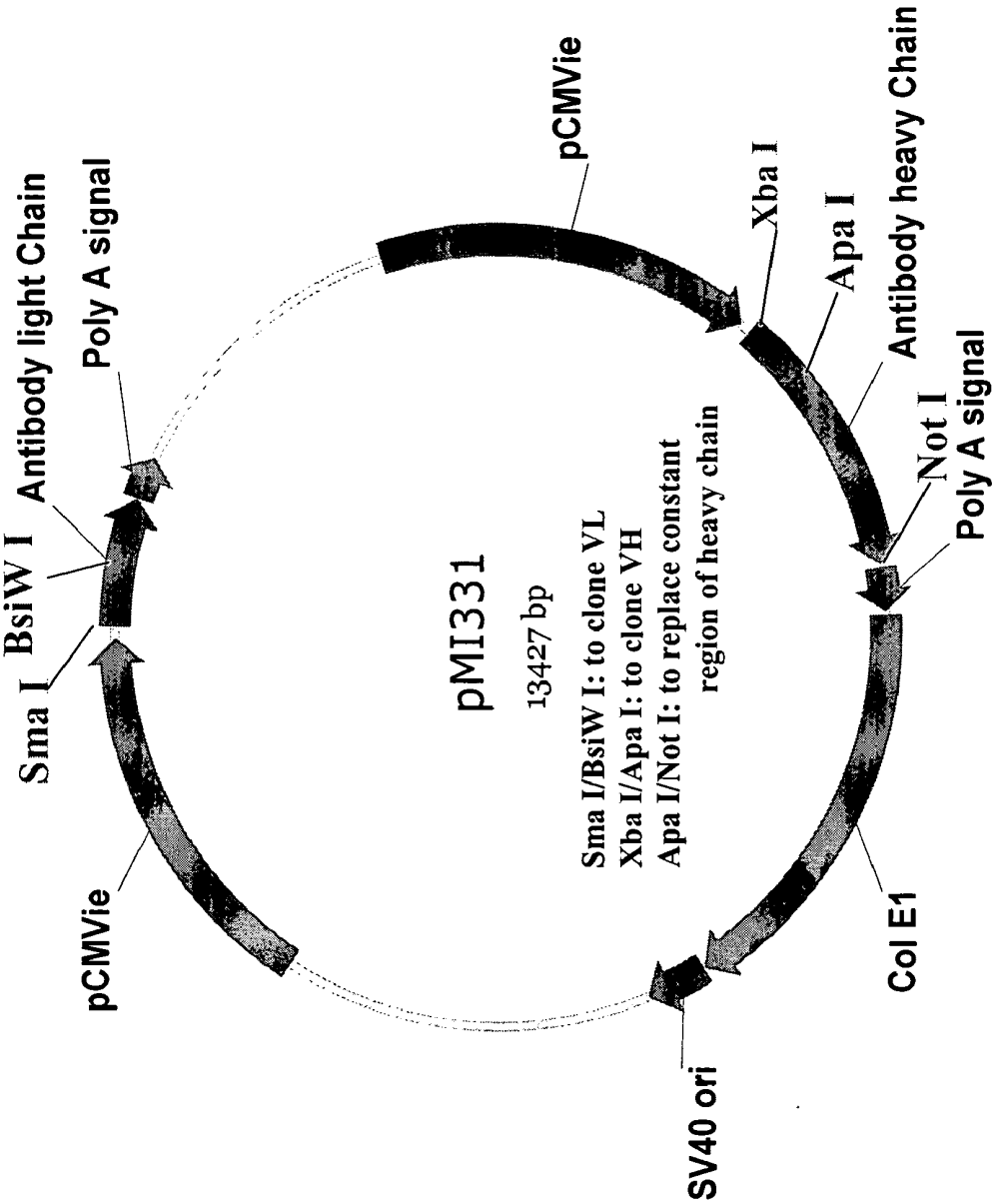
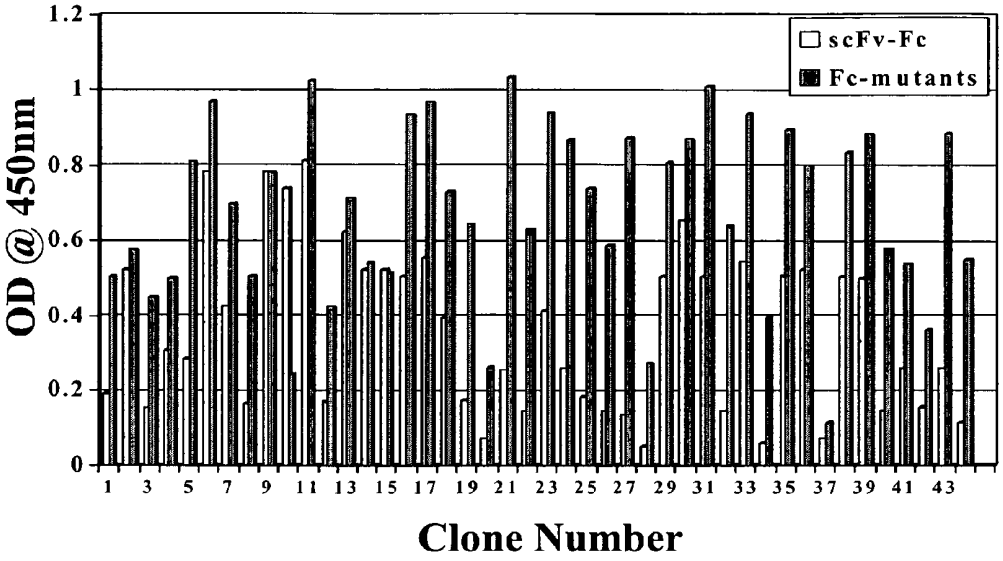

pCMVie
Xba I
Apa I
Antibody heavy Chain
Not I
Poly A signal
Col E1
SV40 ori
pCMVie
Sma I
BsiW I
Antibody light Chain
Poly A signal
pMI331
13427 bp
Sma I/BsiW I: to clone VL
Xba I/Apa I: to clone VH
Apa I/Not I: to replace constant
region of heavy chain

FIG. 4



Clone 1: RI-1 (L234E)
Clone 2: RI-2 (L235R)
Clone 3: RI-13 (L235A)
Clone 4: RI-16 (L235W)
Clone 5: RI-61 (L235P)
Clone 6: RI-63 (L235P)
Clone 7: RI-69 (L235V)
Clone 8: RI-83 (G236E)
Clone 9: RI-102 (L235Y)
Clone 10: RII-III-19 (D265L)
Clone 11: RII-III-81 (S298I)
Clone 12: RII-III-121 (S298T)
Clone 13: RII-III-123 (S298F)
Clone 14: RII-III-145 (E269S)
Clone 15: RII-III-20-4-F5 (E269G)
Clone 16: RIV-2 (P329Q)
Clone 17: RIV-3 (I332E)
Clone 18: RIV-21(L328S)
Clone 19: RIV-22 (A330K)
Clone 20: RIV-23 (I332E)
Clone 21: RIV-43 (A327W)
Clone 22: RIV–47 (I332H)
Clone 23: RIV-49 (I332E)
Clone 24: RIV-50 (A330V)
Clone 25: RIV-59 (A330G)
Clone 26: RIV-65 (A330Y)
Clone 27: RIV-84 (I332S)
Clone 28: RIV-90 (A330G)
Clone 29: RIV-100 (P329H)
Clone 30: RIV-112 (I332W)
Clone 31: RIV-116 (L328V)
Clone 32: RIV-122 (A330T)
Clone 33: RIV-125 (I332F)
Clone 34: RIV-135 (I332Y)
Clone 35: RIV-141(A330L)
Clone 36: RIV-150 (A327N)
Clone 37: RIV-151 (A330I)
Clone 38: RIV-159 (I332Y)
Clone 39: RIV-161 (I332Y)
Clone 40: RIV-165 (A327G)
Clone 41: RIV-168 (L328V)
Clone 42: RIV-173 (L328S)
Clone 43: RIV-189 (A330R)
Clone 44: RIV-203 (A330C)

FIG. 5

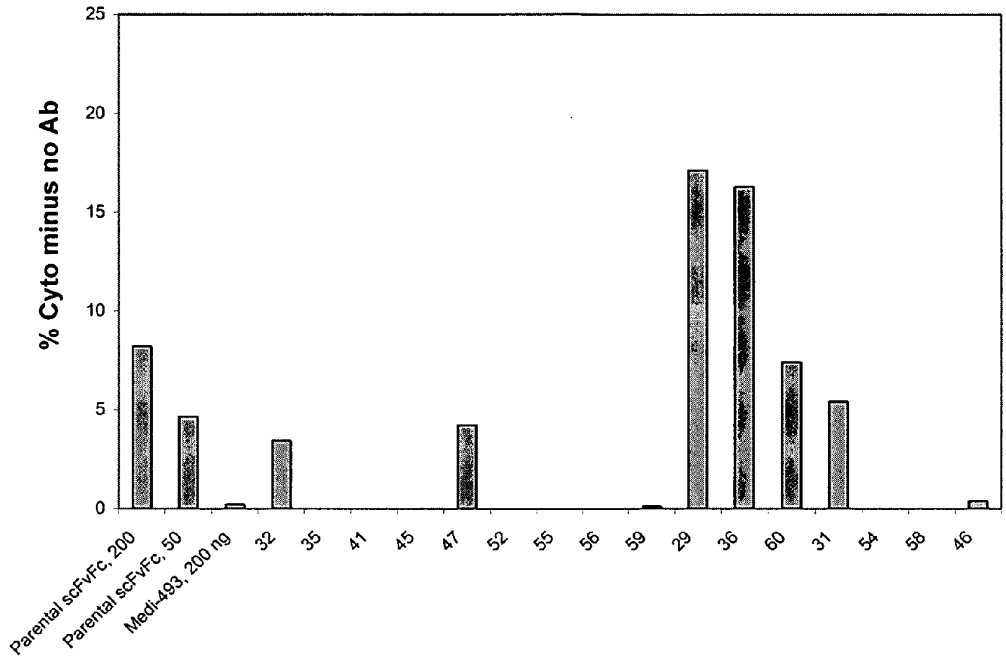

% Cyto minus no Ab
25
20
15
10
5
0
Parental scFvFc, 200
Parental scFvFc, 50
Medi-493, 200 ng
32
35
41
45
47
52
55
56
59
29
36
60
31
54
58
46

FIG. 6

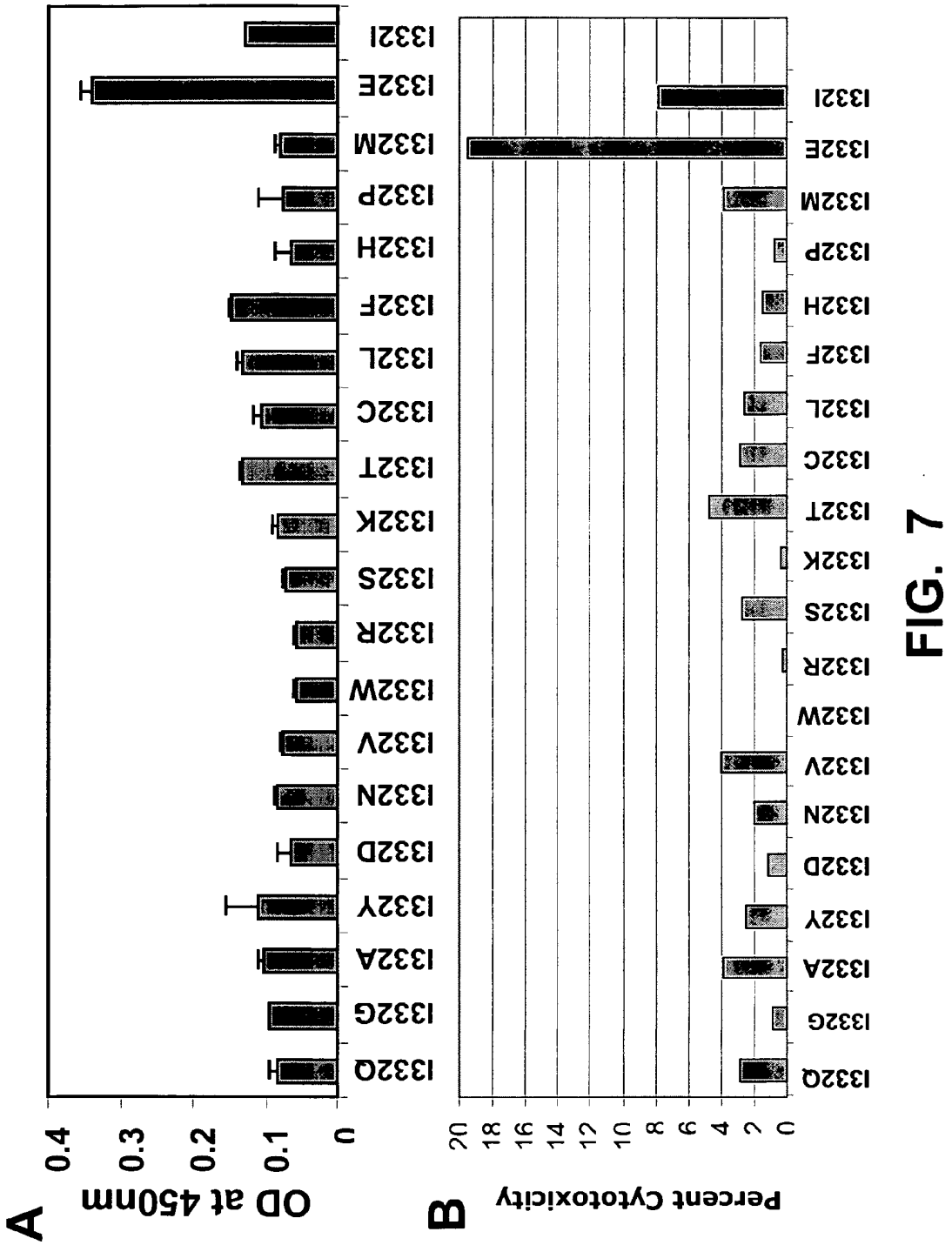
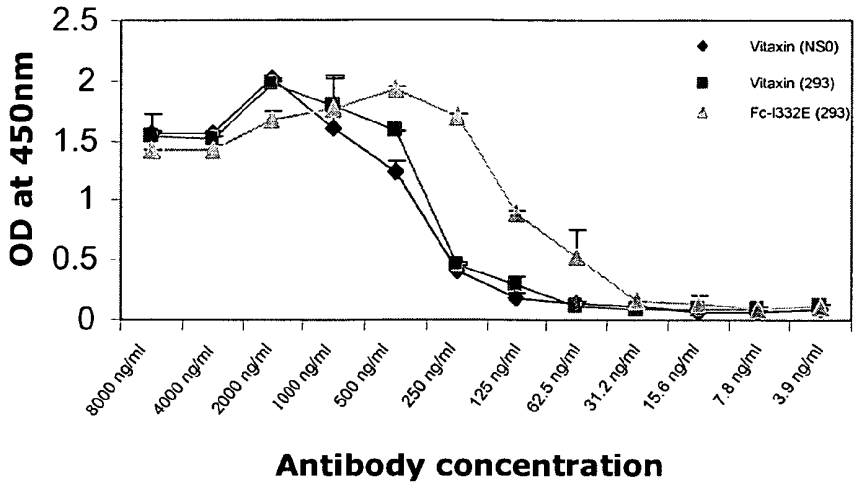

A
OD at 450nm
0.4
0.3
0.2
0.1
0
I332Q
I332G
I332A
I332Y
I332D
I332N
I332V
I332W
I332R
I332S
I332K
I332T
I332C
I332L
I332F
I332H
I332P
I332M
I332E
I332I
B
Percent Cytotoxicity
20
18
16
14
12
10
8
6
4
2
0
I332Q
I332G
I332A
I332Y
I332D
I332N
I332V
I332W
I332R
I332S
I332K
I332T
I332C
I332L
I332F
I332H
I332P
I332M
I332E
I332I

FIG. 7

A


2.5
2
1.5
1
0.5
0
OD at 450nm
Vitaxin (NS0)
Vitaxin (293)
Fc-I332E (293)
8000 ng/ml
4000 ng/ml
2000 ng/ml
1000 ng/ml
500 ng/ml
250 ng/ml
125 ng/ml
62.5 ng/ml
31.2 ng/ml
15.6 ng/ml
7.8 ng/ml
3.9 ng/ml
Antibody concentration

B

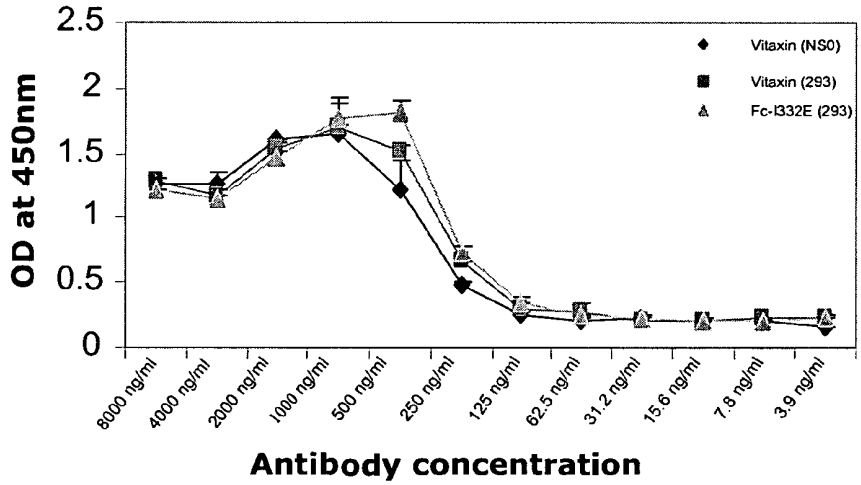

2.5
2
1.5
1
0.5
0
OD at 450nm
Vitaxin (NS0)
Vitaxin (293)
Fc-I332E (293)
8000 ng/ml
4000 ng/ml
2000 ng/ml
1000 ng/ml
500 ng/ml
250 ng/ml
125 ng/ml
62.5 ng/ml
31.2 ng/ml
15.6 ng/ml
7.8 ng/ml
3.9 ng/ml
Antibody concentration

FIG. 8

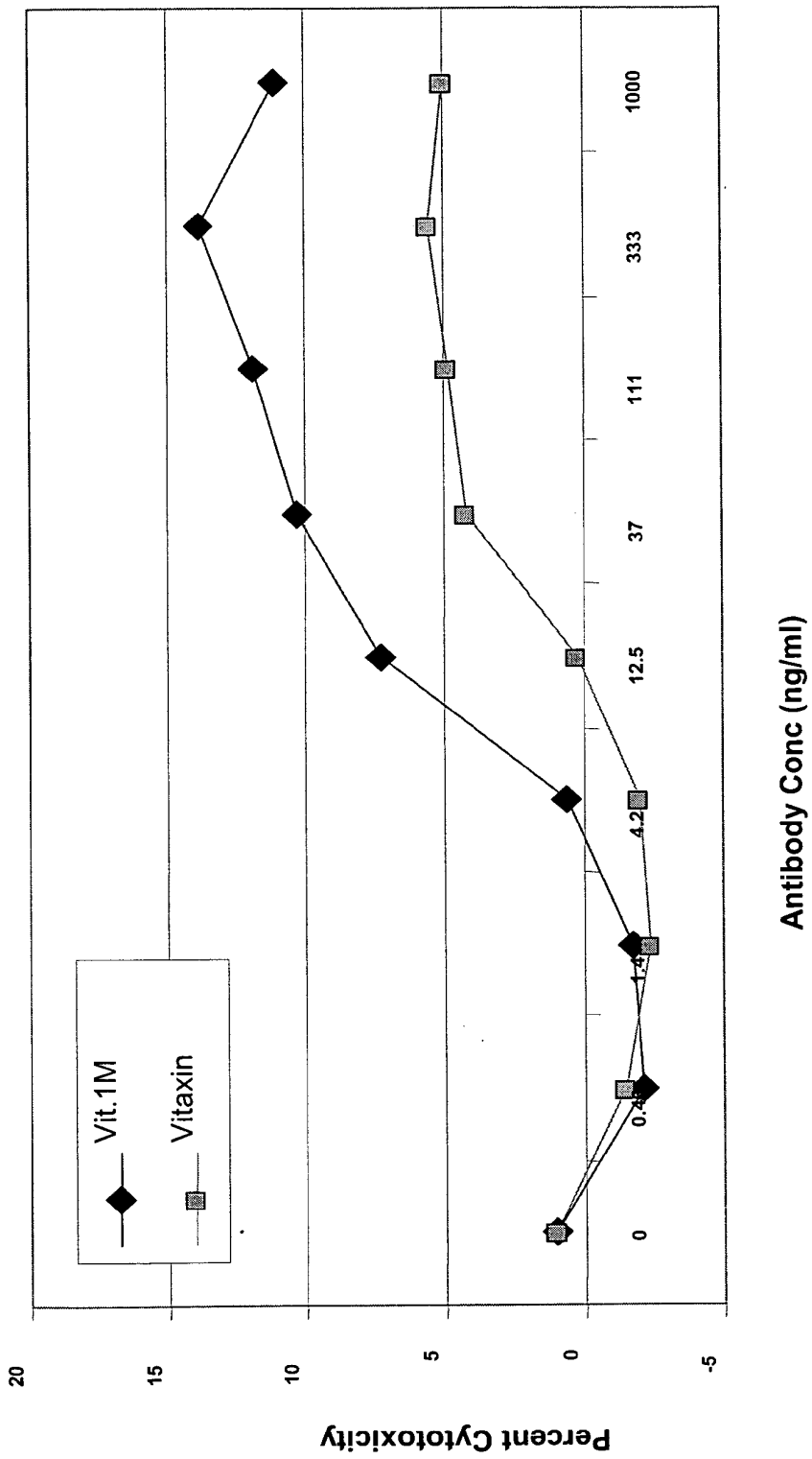

Percent Cytotoxicity
20
15
10
5
0
-5
Vit.1M
Vitaxin
0
0.4
1.4
4.2
12.5
37
111
333
1000
Antibody Conc (ng/ml)

FIG. 9

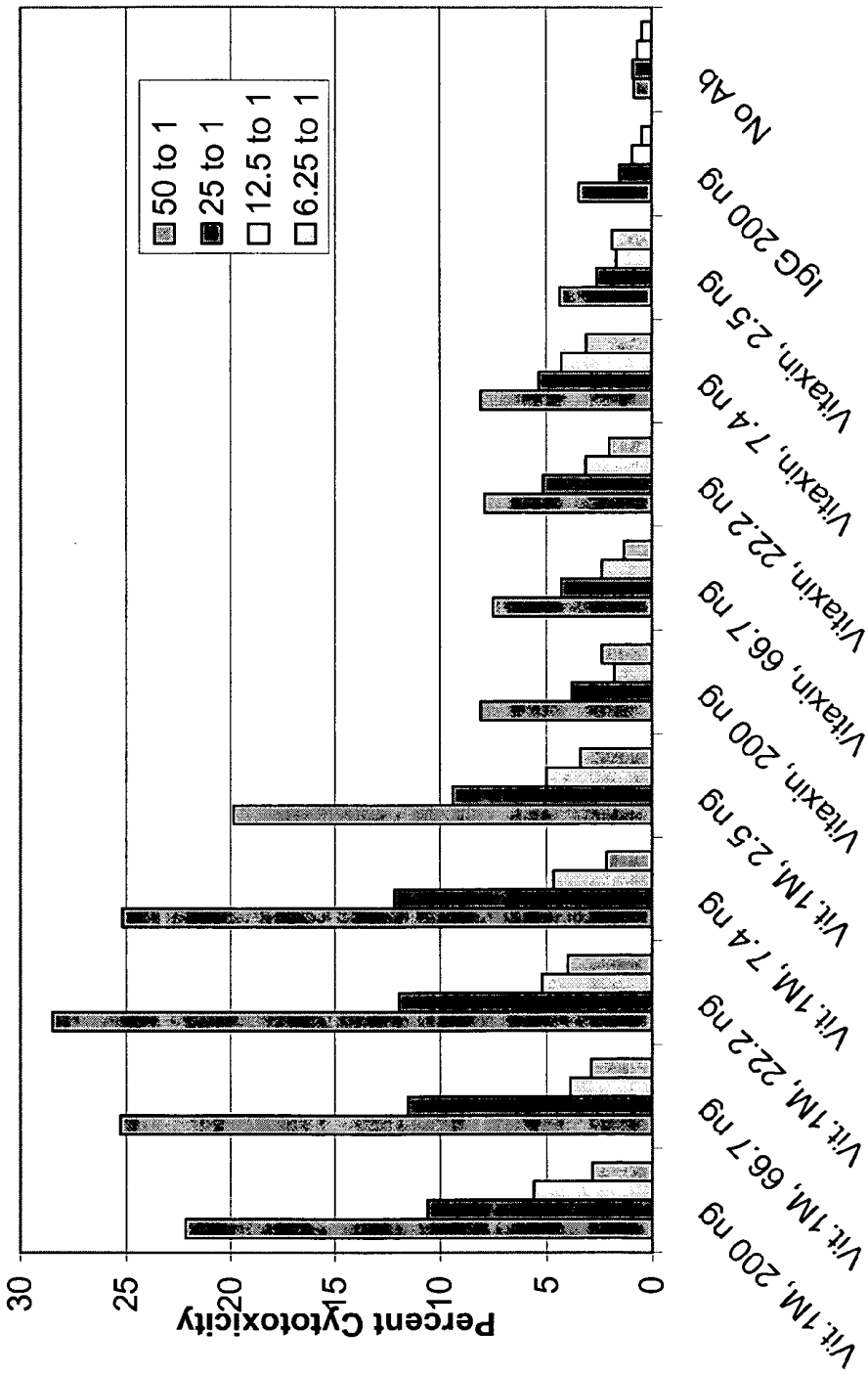

50 to 1
25 to 1
12.5 to 1
6.25 to 1
Percent Cytotoxicity
0
5
10
15
20
25
30
Vit. 1M, 200 ng
Vit. 1M, 66.7 ng
Vit. 1M, 22.2 ng
Vit. 1M, 7.4 ng
Vit. 1M, 2.5 ng
Vitaxin, 200 ng
Vitaxin, 66.7 ng
Vitaxin, 22.2 ng
Vitaxin, 7.4 ng
Vitaxin, 2.5 ng
IgG 200 ng
No Ab

FIG. 10

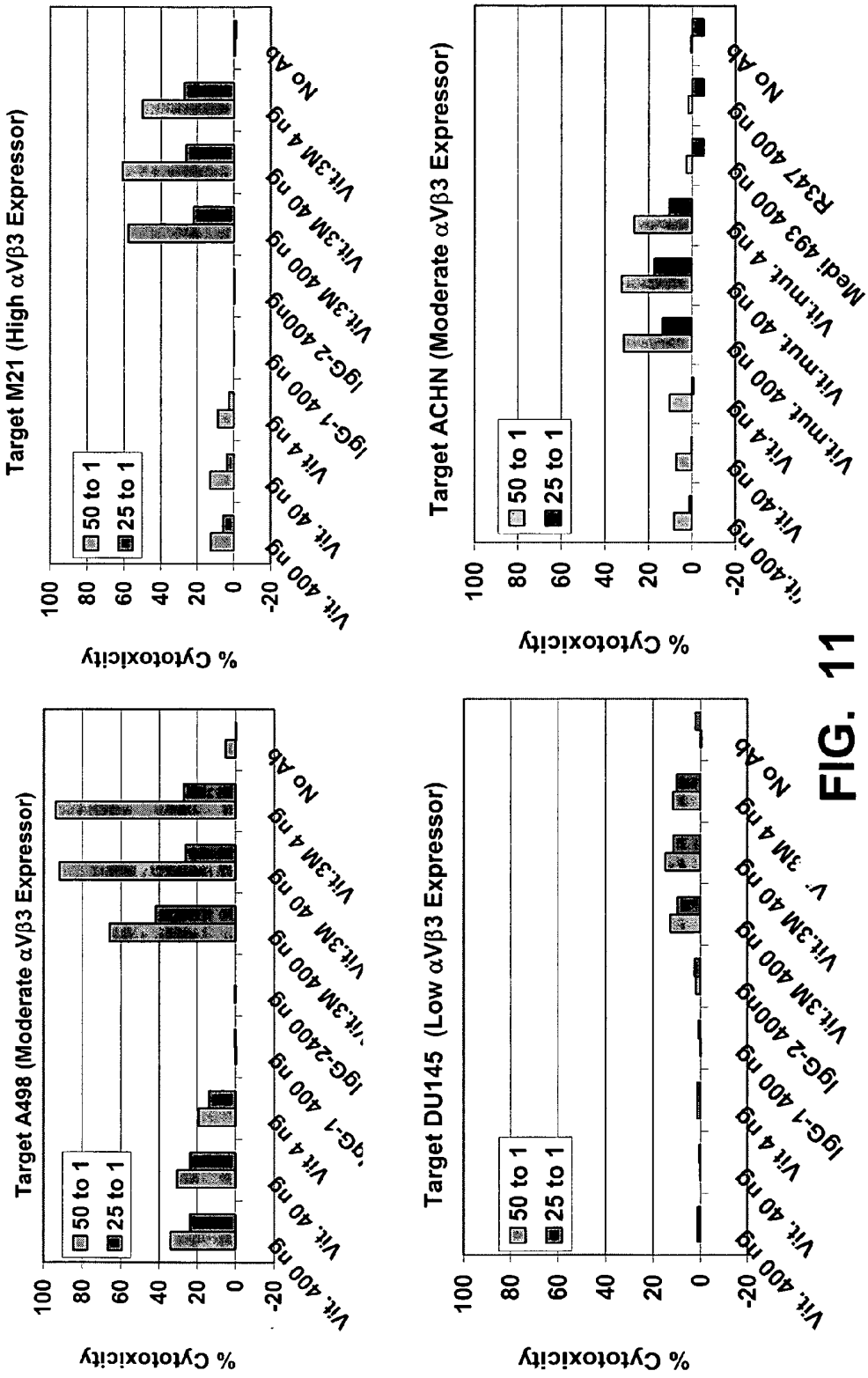

Target M21 (High αVβ3 Expressor)
% Cytotoxicity
50 to 1
25 to 1
Vit. 400 ng
Vit. 40 ng
Vit 4 ng
IgG-1 400 ng
IgG-2 400ng
Vit.3M 400 ng
Vit.3M 40 ng
Vit.3M 4 ng
No Ab
Target ACHN (Moderate αVβ3 Expressor)
% Cytotoxicity
50 to 1
25 to 1
Vit.400 ng
Vit.40 ng
Vit.4 ng
Vit.mut. 400 ng
Vit.mut. 40 ng
Vit.mut. 4 ng
Medi 493 400 ng
R347 400 ng
No Ab
Target A498 (Moderate αVβ3 Expressor)
% Cytotoxicity
50 to 1
25 to 1
Vit. 400 ng
Vit. 40 ng
Vit 4 ng
IgG-1 400 ng
IgG-2400 ng
Vit.3M 400 ng
Vit.3M 40 ng
Vit.3M 4 ng
No Ab
Target DU145 (Low αVβ3 Expressor)
% Cytotoxicity
50 to 1
25 to 1
Vit. 400 ng
Vit. 40 ng
Vit 4 ng
IgG-1 400 ng
IgG-2 400ng
Vit.3M 400 ng
Vit.3M 40 ng
V. 3M 4 ng
No Ab

FIG. 11

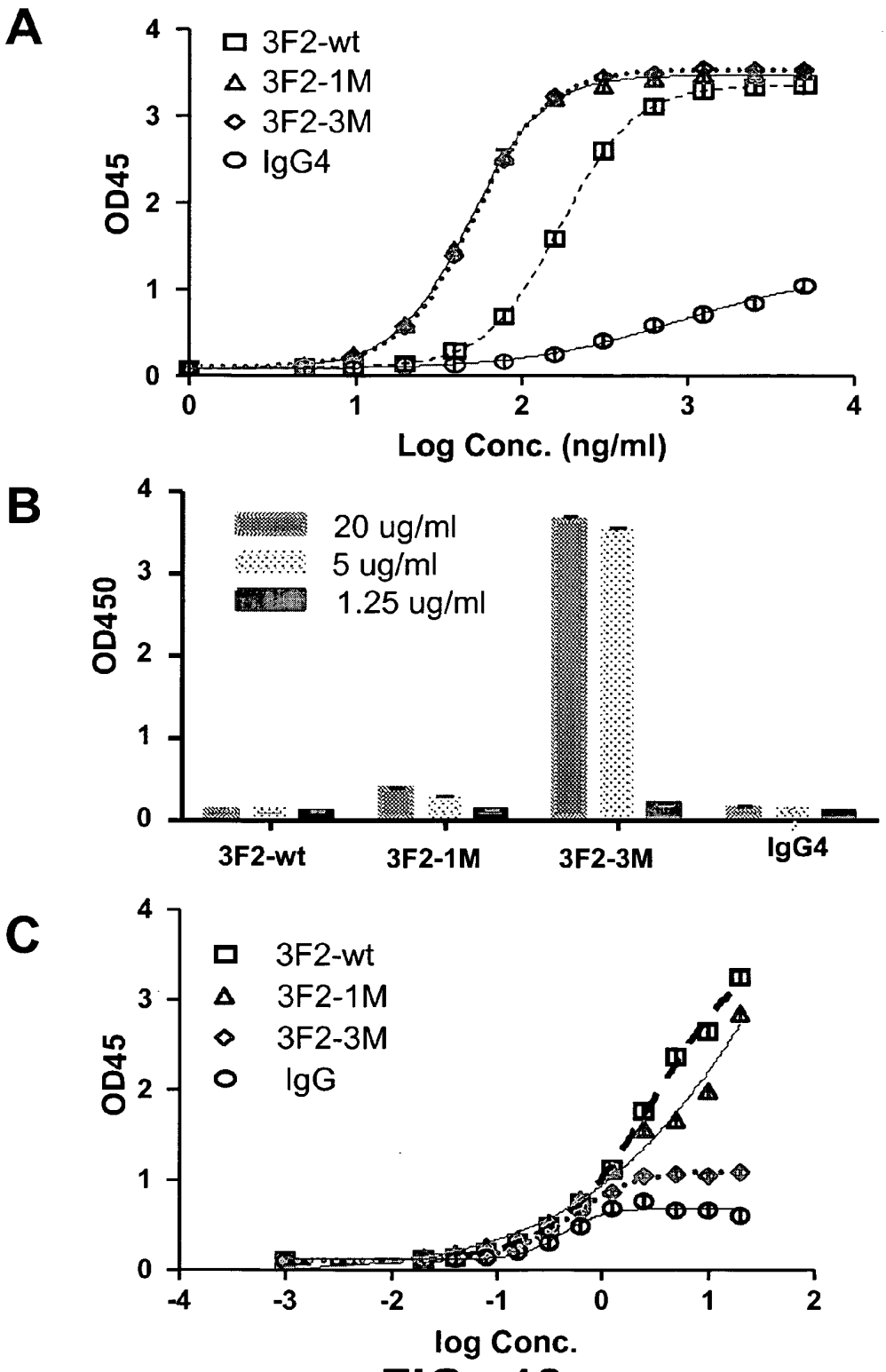

A
3F2-wt
3F2-1M
3F2-3M
IgG4
OD45
Log Conc. (ng/ml)
B
20 ug/ml
5 ug/ml
1.25 ug/ml
OD450
3F2-wt
3F2-1M
3F2-3M
IgG4
C
3F2-wt
3F2-1M
3F2-3M
IgG
OD45
log Conc.

FIG. 12

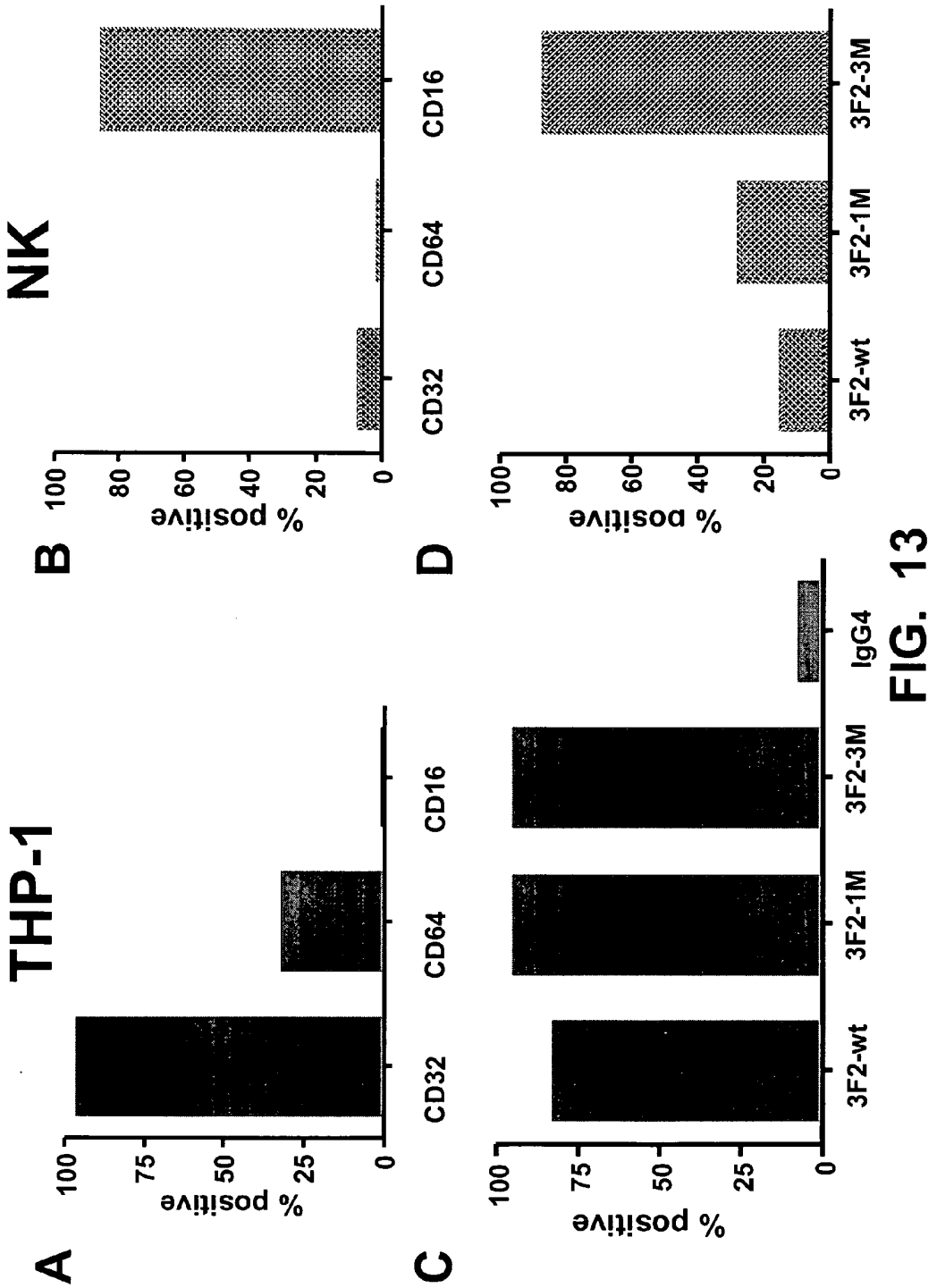
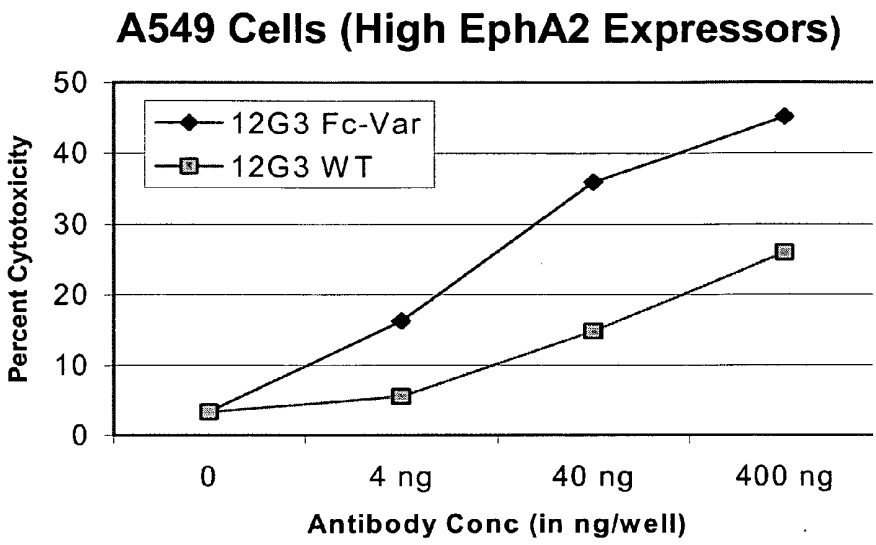

A
THP-1
% positive
100
75
50
25
0
CD32
CD64
CD16
B
NK
% positive
100
80
60
40
20
0
CD32
CD64
CD16
C
% positive
100
75
50
25
0
3F2-wt
3F2-1M
3F2-3M
IgG4
D
% positive
100
80
60
40
20
0
3F2-wt
3F2-1M
3F2-3M

FIG. 13

A


A549 Cells (High EphA2 Expressors)
Percent Cytotoxicity
50
40
30
20
10
0
12G3 Fc-Var
12G3 WT
0
4 ng
40 ng
400 ng
Antibody Conc (in ng/well)

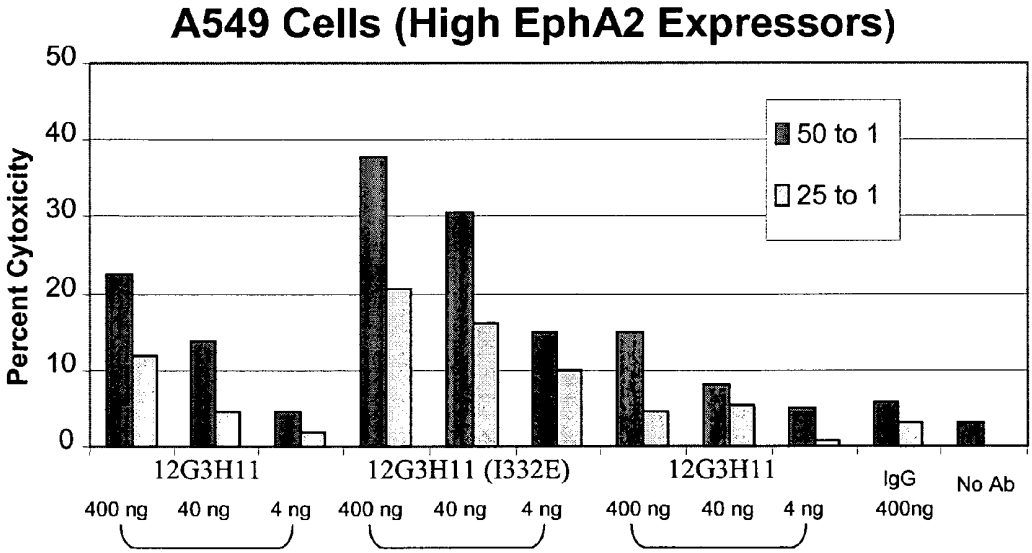
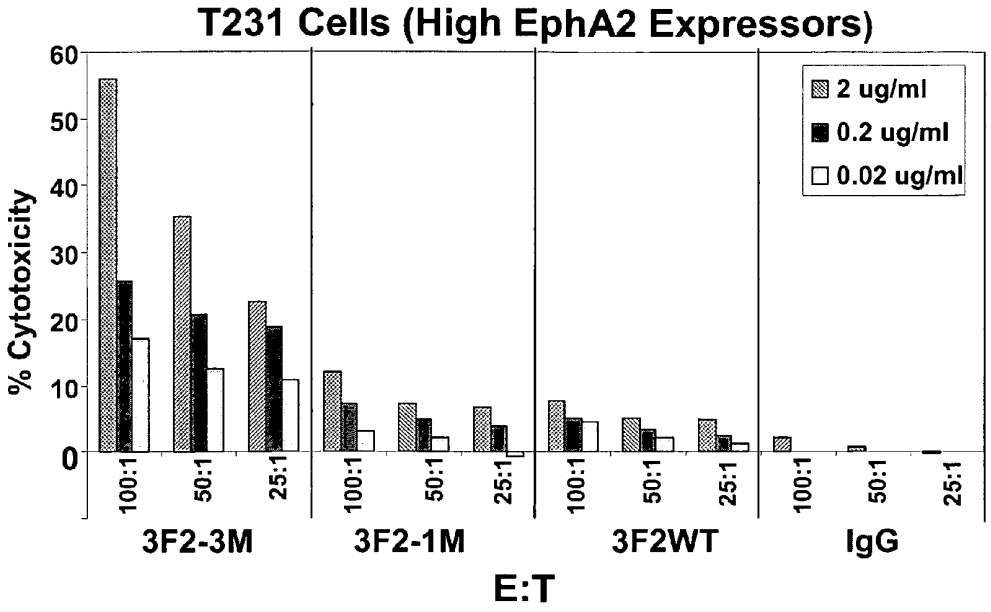

A549 Cells (High EphA2 Expressors)
Percent Cytoxicity
50
40
30
20
10
0
50 to 1
25 to 1
12G3H11
12G3H11 (I332E)
12G3H11
IgG
No Ab
400 ng
40 ng
4 ng
400 ng
40 ng
4 ng
400 ng
40 ng
4 ng
400ng

FIG. 14


T231 Cells (High EphA2 Expressors)
% Cytotoxicity
60
50
40
30
20
10
0
2 ug/ml
0.2 ug/ml
0.02 ug/ml
100:1
50:1
25:1
3F2-3M
3F2-1M
3F2WT
IgG
E:T

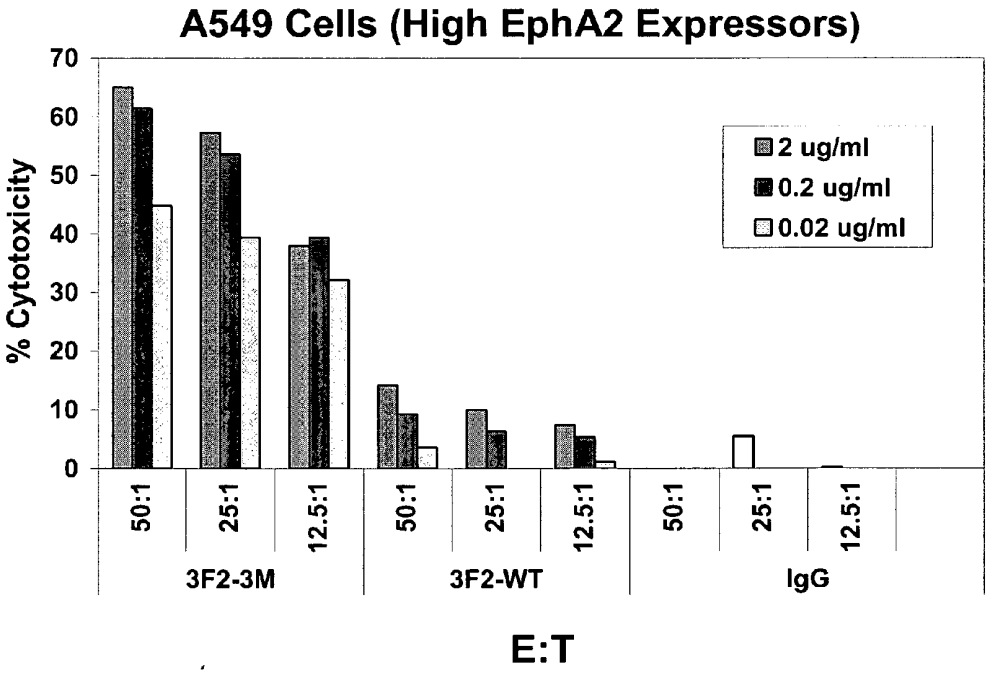
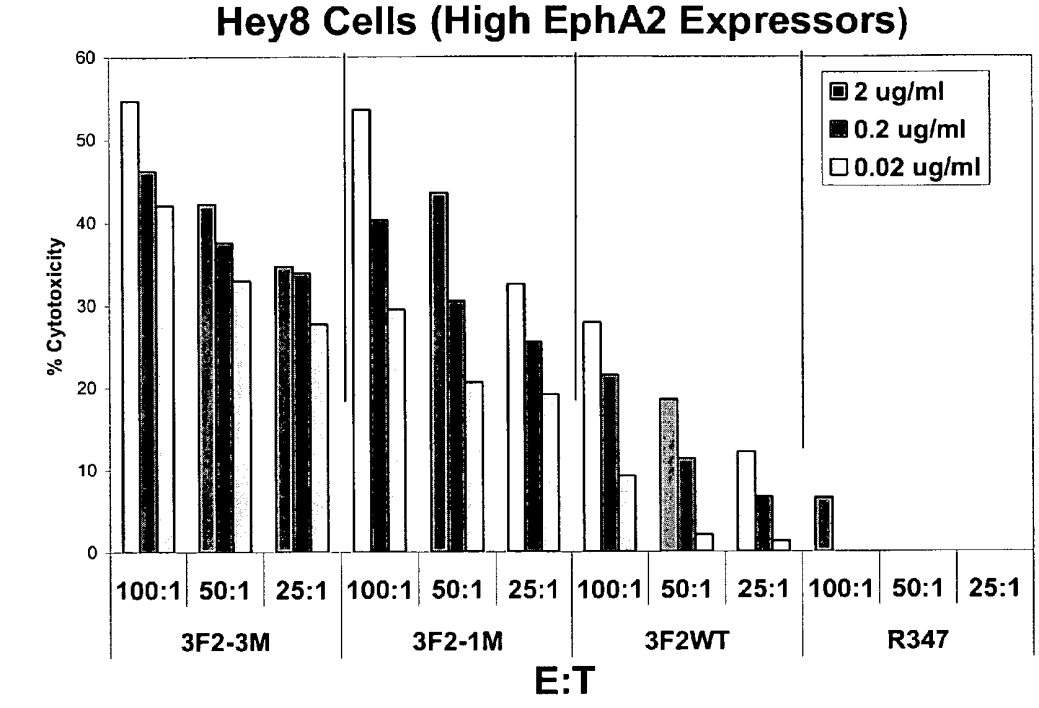

A549 Cells (High EphA2 Expressors)
% Cytotoxicity
70
60
50
40
30
20
10
0
2 ug/ml
0.2 ug/ml
0.02 ug/ml
50:1
25:1
12.5:1
3F2-3M
3F2-WT
IgG
E:T

FIG. 15


Hey8 Cells (High EphA2 Expressors)
% Cytotoxicity
60
50
40
30
20
10
0
2 ug/ml
0.2 ug/ml
0.02 ug/ml
100:1
50:1
25:1
3F2-3M
3F2-1M
3F2WT
R347
E:T

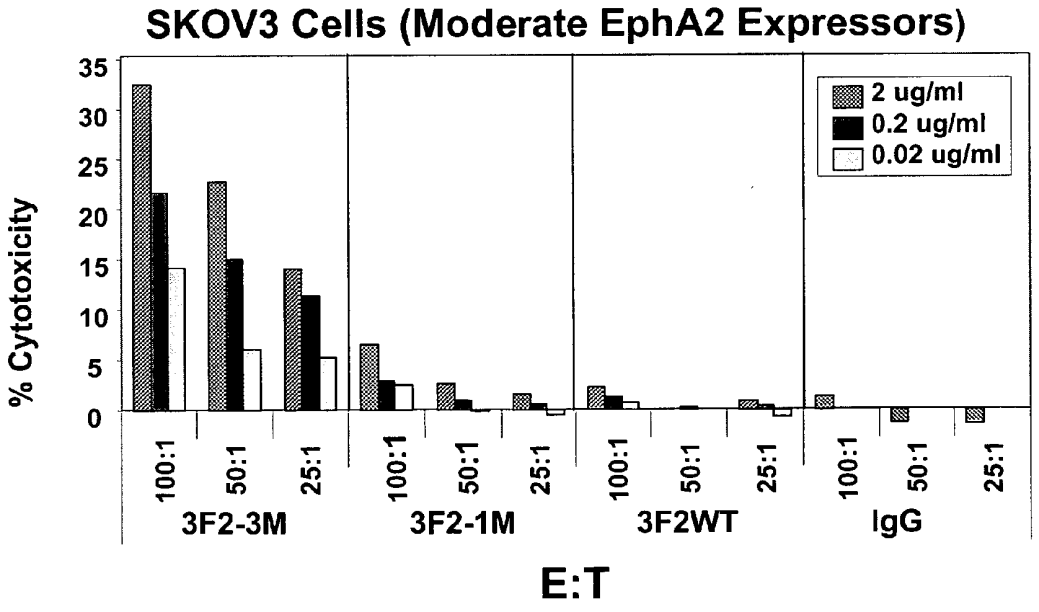
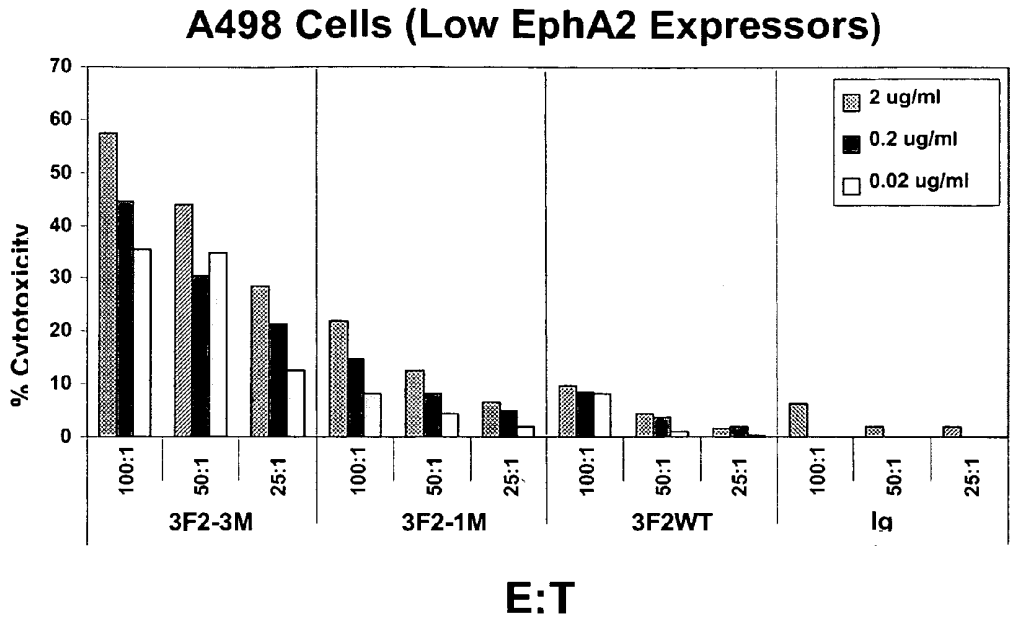

SKOV3 Cells (Moderate EphA2 Expressors)
% Cytotoxicity
35
30
25
20
15
10
5
0
2 ug/ml
0.2 ug/ml
0.02 ug/ml
100:1
50:1
25:1
3F2-3M
3F2-1M
3F2WT
IgG
E:T

FIG. 16


A498 Cells (Low EphA2 Expressors)
% Cytotoxicity
70
60
50
40
30
20
10
0
2 ug/ml
0.2 ug/ml
0.02 ug/ml
100:1
50:1
25:1
3F2-3M
3F2-1M
3F2WT
Ig
E:T

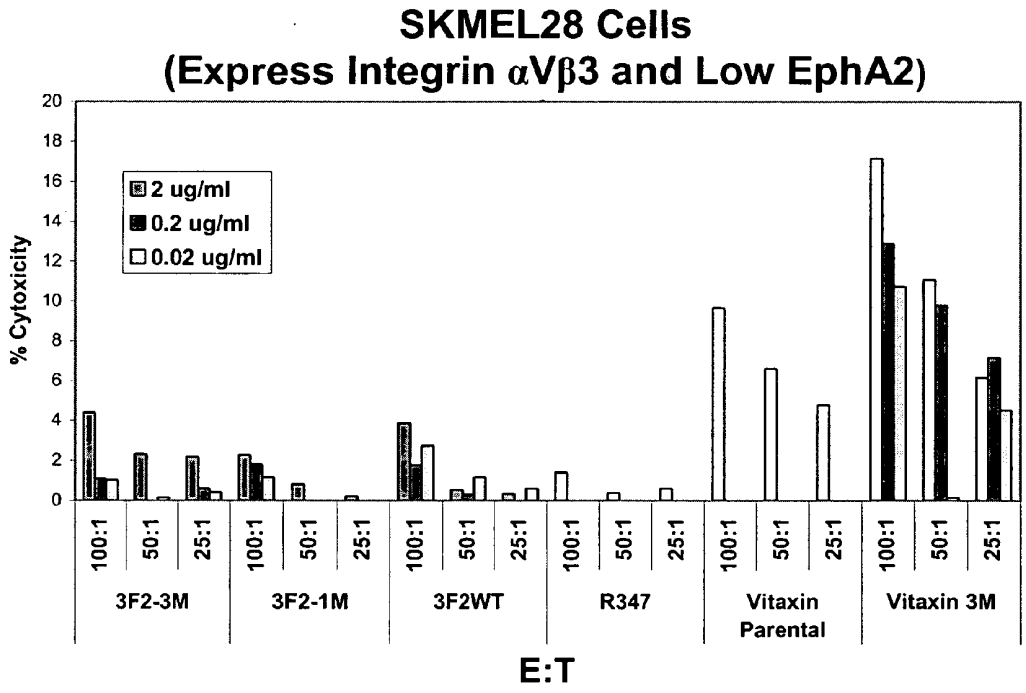

SKMEL28 Cells
(Express Integrin αVβ3 and Low EphA2)
% Cytoxicity
20
18
16
14
12
10
8
6
4
2
0
2 ug/ml
0.2 ug/ml
0.02 ug/ml
100:1
50:1
25:1
3F2-3M
3F2-1M
3F2WT
R347
Vitaxin Parental
Vitaxin 3M
E:T

FIG. 17

INTEGRIN ANTAGONISTS WITH ENHANCED ANTIBODY DEPENDENT CELL-MEDIATED CYTOXICITY ACTIVITY

1. CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit under 35 U.S.C. §119(e) of the following U.S. Provisional Application Nos. 60/601,634, filed, Aug. 16, 2004 and 60/608,852, filed, Sep. 13, 2004. The priority applications are hereby incorporated by reference herein in their entirety for all purposes.

2. FIELD OF THE INVENTION

[0002] The present invention provides novel antibodies comprising immunologically active fragments of immunoglobulin molecules and an Fc region that further comprises at least one novel amino acid residue of the invention. The present invention also relates to novel antibodies comprising a variable region, or fragment thereof, that immunospecifically binds to Integrin $\alpha_v\beta_3$ and a Fc region that further comprises at least one high effector function amino acid residue (e.g., 239D, 330L, 332E). The present invention further relates to novel variants of antibodies that immunospecifically bind to Integrin $\alpha_v\beta_3$ (e.g., VITAXIN® (Wu et al., 1998, *PNAS USA* 95:6037-6042)) which contain one or more substitutions in their Fc regions. Collectively, these two types of novel antibodies are referred to herein as "Fc variants of the invention" or "Fc variants." In one embodiment, the Fc variants of the invention have enhanced effector function. In another embodiment the Fc variants of the invention have altered binding affinity to one or more Fc ligands (e.g., FcγRs, complement protein C1q). In another embodiment, the Fc variants of the invention have enhanced binding to FcγRIIIA and increased ability to mediate antibody dependent cell-mediated cytotoxicity (ADCC). In another embodiment, the Fc variants have reduced binding to FcγRIIIA and decreased ability to mediate ADCC. In still another embodiment, the Fc variants have enhanced binding to the C1q and increased ability to mediate complement dependent cytotoxicity (CDC). In yet another embodiment, the Fc variants have reduced binding to C1q and decreased ability to mediate CDC. In particular, the present invention relates to Fc variants that can act as inhibitors and/or antagonists of Integrin $\alpha_v\beta_3$. In addition the present invention provides methods and protocols for the application or use of Fc variants, particularly for therapeutic purposes. Specifically, the methods and protocols involve the administration of a prophylactically or therapeutically effective amount of one or more Fc variants alone or in combination with the administration of one or more other therapies useful for cancer therapy. The Fc variants utilized for therapeutic purposes may or may not be conjugated or fused to a moiety (e.g., a therapeutic agent or drug). The methods of the invention are particularly useful for the prevention, management, treatment or amelioration numerous forms of cancer including cancers that have the potential to metastasize or have metastasized to other organs or tissues. The invention also provides methods for screening for an antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$ as well as methods to manipulate the Fc region and thereby modulate the ability of said Fc region to mediate ADCC and/or CDC activity and/or the binding affinity for one or more Fc ligands (e.g., FcγRs, C1q). The invention also provides methods for generating Fc variant fusions that immunospecifically binds to Integrin $\alpha_v\beta_3$. Further, the invention provides pharmaceutical formulations and kits for use in preventing, managing, treating or ameliorating cancer or one or more symptoms thereof.

3. BACKGROUND OF THE INVENTION

3.1 Cancer

[0003] A neoplasm, or tumor, is a neoplastic mass resulting from abnormal uncontrolled cell growth, which can be benign or malignant. Benign tumors generally remain localized. Malignant tumors are collectively termed cancers. The term "malignant" generally means that the tumor can invade and destroy neighboring body structures and spread to distant sites to cause death (for review, see Robbins and Angell, 1976, *Basic Pathology,* 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-122). Cancer can arise in many sites of the body and behave differently depending upon its origin. Cancerous cells destroy the part of the body in which they originate and then spread to other part(s) of the body where they start new growth and cause more destruction. The progressive growth and metastasis of tumor cells is dependent on the ability of tumor cells to stimulate the formation of new blood vessels in a process called angiogenesis.

[0004] More than 1.2 million Americans develop cancer each year. Cancer is the second leading case of death in the United States and if current trends continue, cancer is expected to be the leading cause of the death by the year 2010. Lung and prostate cancer are the top cancer killers for men in the United States. Lung and breast cancer are the top cancer killers for women in the United States. One in two men in the United States will be diagnosed with cancer at some time during his lifetime. One in three women in the United States will be diagnosed with cancer at some time during her lifetime.

[0005] A cure for cancer has yet to be found. Current treatment options, such as surgery, chemotherapy and radiation treatment, are oftentimes either ineffective or present serious side effects.

3.2 Integrins

[0006] Integrins are a class of cell adhesion receptors that mediate both cell-cell and cell-extracellular matrix adhesion events. Integrins consist of heterodimeric polypeptides where a single α chain polypeptide noncovalently associates with a single β chain. There are now about 16 distinct α chain polypeptides and at least about 8 different β chain polypeptides that constitute the integrin family of cell adhesion receptors. In general, different binding specificities and tissue distributions are derived from unique combinations of the α and β chain polypeptides or integrin subunits. The family to which a particular integrin is associated with is usually characterized by the β subunit. However, the ligand binding activity of the integrin is largely influenced by the α subunit.

[0007] As cell adhesion receptors, integrins are involved in a variety of physiological processes including, for example, cell attachment, cell migration and cell proliferation. Different integrins play different roles in each of these biological processes and the inappropriate regulation of their function or activity can lead to various pathological conditions. For example, inappropriate endothelial cell prolifera-

tion during angiogenesis, also called neovascularization, of a tumor was found to be mediated by cells expressing vitronectin binding integrins. In this regard, the inhibition of the vitronectin-binding Integrin $\alpha_v\beta_3$ also inhibits this process of tumor angiogenesis. By this same criteria, Integrin $\alpha_v\beta_3$ has also been shown to mediate the abnormal cell proliferation associated with restenosis and granulation tissue development in cutaneous wounds, for example. Additional disease or pathological states mediated or influenced by Integrin $\alpha_v\beta_3$ include, for example, metastasis, osteoporosis, age-related macular degeneration, diabetic retinopathy and inflammatory diseases such as rheumatoid arthritis and psoriasis. There is now considerable evidence that progressive tumor growth is dependent upon angiogenesis (Gastl et al., 1997, Oncol 54:177-184). Thus, agents which can specifically inhibit Integrin $\alpha_v\beta_3$, thus preventing or inhibiting angiogenesis, would be valuable for the therapeutic treatment of diseases including cancer.

3.3 Cancer Therapy

[0008] Currently, cancer therapy may involve surgery, chemotherapy, hormonal therapy and/or radiation treatment to eradicate neoplastic cells in a patient (see, for example, Stockdale, 1998, "Principles of Cancer Patient Management", in Scientific American: Medicine, vol. 3, Rubenstein and Federman, eds., Chapter 12, Section IV). All of these approaches pose significant drawbacks for the patient. Surgery, for example, may be contraindicated due to the health of the patient or may be unacceptable to the patient. Additionally, surgery may not completely remove the neoplastic tissue. Radiation therapy is only effective when the neoplastic tissue exhibits a higher sensitivity to radiation than normal tissue, and radiation therapy can also often elicit serious side effects. Hormonal therapy is rarely given as a single agent and although can be effective, is often used to prevent or delay recurrence of cancer after other treatments have removed the majority of the cancer cells.

[0009] With respect to chemotherapy, there are a variety of chemotherapeutic agents available for treatment of cancer. A significant majority of cancer chemotherapeutics act by inhibiting DNA synthesis (see, for example, Gilman et al., Goodman and Gilman's: The Pharmacological Basis of Therapeutics, Eighth Ed. (Pergamom Press, New York, 1990)). As such chemotherapy agents are inherently nonspecific. In addition almost all chemotherapeutic agents are toxic, and chemotherapy causes significant, and often dangerous, side effects, including severe nausea, bone marrow depression, immunosuppression, etc. (see, for example, Stockdale, 1998, "Principles Of Cancer Patient Management" in Scientific American Medicine, vol. 3, Rubenstein and Federman, eds., ch. 12, sect. 10). Furthermore, even with administration of combinations of chemotherapeutic agents, many tumor cells are resistant or develop resistance to the chemotherapeutic agents.

[0010] Recently, cancer therapy could also involve biological therapy or immunotherapy. Biological therapies/immunotherapies are limited in number and although more specific then chemotherapeutic agents many still target both health and cancerous cells. In addition, such therapies may produce side effects such as rashes or swellings, flu-like symptoms, including fever, chills and fatigue, digestive tract problems or allergic reactions.

[0011] Thus, there is a significant need for alternative cancer treatments, particularly for treatments that more specifically target cancer cells. Integrin $\alpha_v\beta_3$ is one of the best characterized integrins implicated in tumor induced angiogenesis. Integrin $\alpha_v\beta_3$ is highly expressed on some human tumors (e.g., breast tumors), but not readily detected in benign breast tissue. Thus a cancer treatment that would specifically inhibit Integrin $\alpha_v\beta_3$ would be a powerful tool for the treatment and prevention of cancers.

3.4 Antibodies for the Treatment of Cancer

[0012] Antibodies are immunological proteins that bind a specific antigen. In most mammals, including humans and mice, antibodies are constructed from paired heavy and light polypeptide chains. Each chain is made up of two distinct regions, referred to as the variable (Fv) and constant (Fc) regions. The light and heavy chain Fv regions contain the antigen binding determinants of the molecule and are responsible for binding the target antigen. The Fc regions define the class (or isotype) of antibody (IgG for example) and are responsible for binding a number of natural proteins to elicit important biochemical events.

[0013] The Fc region of an antibody interacts with a number of ligands including Fc receptors and other ligands, imparting an array of important functional capabilities referred to as effector functions. An important family of Fc receptors for the IgG class are the Fc gamma receptors (FcγRs). These receptors mediate communication between antibodies and the cellular arm of the immune system (Raghavan et al., 1996, *Annu Rev Cell Dev Biol* 12:181-220; Ravetch et al., 2001, *Annu Rev Immunol* 19:275-290). In humans this protein family includes FcγRI (CID64), including isoforms FcγRIA, FcγRIB, and FcγRIC; FcγRII (CD32), including isoforms FcγRIIA, FcγRIIB, and FcγRIIC; and FcγRIII (CID16), including isoforms FcγRIIIA and FcγRIIB (Jefferis et al., 2002, *Immunol Lett* 82:57-65). These receptors typically have an extracellular domain that mediates binding to Fc, a membrane spanning region, and an intracellular domain that may mediate some signaling event within the cell. These different FcγR subtypes are expressed on different cell types (reviewed in Ravetch et al., 1991, *Annu Rev Immunol* 9:457-492). For example, in humans, FcγRIIIB is found only on neutrophils, whereas FcγRIIIA is found on macrophages, monocytes, natural killer (NK) cells, and a subpopulation of T-cells.

[0014] Formation of the Fc/FcγR complex recruits effector cells to sites of bound antigen, typically resulting in signaling events within the cells and important subsequent immune responses such as release of inflammation mediators, B cell activation, endocytosis, phagocytosis, and cytotoxic attack. The ability to mediate cytotoxic and phagocytic effector functions is a potential mechanism by which antibodies destroy targeted cells. The cell-mediated reaction wherein nonspecific cytotoxic cells that express FcγRs recognize bound antibody on a target cell and subsequently cause lysis of the target cell is referred to as antibody dependent cell-mediated cytotoxicity (ADCC) (Raghavan et al., 1996, *Annu Rev Cell Dev Biol* 12:181-220; Ghetie et al., 2000, *Annu Rev Immunol* 18:739-766; Ravetch et al., 2001, *Annu Rev Immunol* 19:275-290). Notably, the primary cells for mediating ADCC, NK cells, express only FcγRIIIA only, whereas monocytes express FcγRI, FcγRII and FcγRIII (Ravetch et al., 1991, supra).

[0015] Another important Fc ligand is the complement protein C1q. Fc binding to C1q mediates a process called

complement dependent cytotoxicity (CDC) (reviewed in Ward et al., 1995, *Ther Immunol* 2:77-94). C1q is capable of binding six antibodies, although binding to two IgGs is sufficient to activate the complement cascade. C1q forms a complex with the C1r and C1s serine proteases to form the C1 complex of the complement pathway.

[0016] Several key features of antibodies including but not limited to, specificity for target, ability to mediate immune effector mechanisms, and long half-life in serum, make antibodies powerful therapeutics. Numerous monoclonal antibodies are currently in development or are being used therapeutically for the treatment of a variety of conditions including cancer. For example Vitaxin® (MedImmune), a humanized Integrin $\alpha_v\beta_3$ antibody (e.g., PCT publication WO 2003/075957), Herceptin® (Genentech), a humanized anti-Her2/neu antibody approved to treat breast cancer (e.g., U.S. Pat. No. 5,677,171), CNTO 95 (Centocor), a human Integrin α_v antibody (PCT publication WO 02/12501), Rituxan® (IDEC/Genentech/Roche), a chimeric anti-CD20 antibody approved to treat Non-Hodgkin's lymphoma (e.g., U.S. Pat. No. 5,736,137) and Erbitux® (ImClone), a chimeric anti-EGFR antibody (e.g., U.S. Pat. No. 4,943,533).

[0017] There are a number of possible mechanisms by which antibodies destroy tumor cells, including anti-proliferation via blockage of needed growth pathways, intracellular signaling leading to apoptosis, enhanced down regulation and/or turnover of receptors, ADCC, CDC, and promotion of an adaptive immune response (Cragg et al., 1999, *Curr Opin Immunol* 11:541-547; Glennie et al., 2000, *Immunol Today* 21:403-410). However, despite widespread use, antibodies are not optimized for clinic use and many have suboptimal anticancer potency. Thus, there is a significant need to enhance the capacity of antibodies to destroy targeted cancer cells. Methods for enhancing the anti-tumor-potency of antibodies via enhancement of their ability to mediate cytotoxic effector functions such as ADCC and CDC are particularly promising. The importance of FcγR-mediated effector functions for the anti-cancer activity of antibodies has been demonstrated in mice (Clynes et al., 1998, *Proc Natl Acad Sci USA* 95:652-656; Clynes et al., 2000, *Nat Med* 6:443-446), and the affinity of the interaction between Fc and certain FcγRs correlates with targeted cytotoxicity in cell-based assays (Shields et al., 2001, *J Biol Chem* 276:6591-6604; Presta et al., 2002, *Biochem Soc Trans* 30:487-490; Shields et al., 2002, *J Biol Chem* 277:26733-26740). Together these data suggest that manipulating the binding ability of the Fc region of an IgG1 antibody to certain FcγRs may enhance effector functions resulting in more effective destruction of cancer cells in patients. Furthermore, because FcγRs can mediate antigen uptake and processing by antigen presenting cells, enhanced Fc/FcγR affinity may also improve the capacity of antibody therapeutics to elicit an adaptive immune response.

[0018] While enhancing effector function can increase the capacity of antibodies to destroy target cells, for some antibody therapies reduced or eliminated effector function may be more desirable. This is particularly true for those antibodies designed to deliver a drug (e.g., toxins and isotopes) to the target cell where the Fc/FcγR mediated effector functions bring healthy immune cells into the proximity of the deadly payload, resulting in depletion of normal lymphoid tissue along with the target cells (Hutchins et al., 1995, *PNAS USA* 92:11980-11984; White et al., 2001, *Annu Rev Med* 52:125-145). In these cases the use of Fc variants that poorly recruit complement or effector cells would be of tremendous benefit (see for example, Wu et al., 2000, *Cell Immunol* 200:16-26; Shields et al., 2001, *J. Biol Chem* 276:6591-6604; U.S. Pat. No. 6,194,551; U.S. Pat. No. 5,885,573 and PCT publication WO 04/029207).

[0019] All FcγRs bind the same region on the Fc of the IgG subclass, but with different affinities (e.g., FcγRI is a high affinity while FcγRII and FcγRIII are low affinity binders). Other differences between the FcγRs are mechanistic. For example, FcγRI, FcγRIIA/C, and FcγRIIIA are positive regulators of immune complex triggered activation, characterized by having an immunoreceptor tyrosine-based activation motif (ITAM) while FcγRIIB has an immunoreceptor tyrosine-based inhibition motif (ITIM) and is therefore inhibitory. Thus, the balance between activating and inhibiting receptors is an important consideration. For example, enhancing Fc binding to the positive regulators (e.g., FcγRIIIA) while leaving unchanged or even reducing Fc binding to the negative regulator FcγRIIB could result in optimized effector function such as enhanced ADCC mediated destruction of tumor cells. Another critical consideration is that Fc variants should be engineered such that the binding to FcγRs and/or C1q is modulated in the desired manner but so that they maintain their stability, solubility, structural integrity as well as their ability to interact with other important Fc ligands such as FcRn and proteins A and G.

[0020] Numerous mutagenesis studies have been carried out on the Fc domain (See for example, Duncan et al., 1988, *Nature* 332:563-564; Lund et al., 1995, *Faseb J* 9:115-119; Lund et al., 1996, *J Immunol* 157:4963-4969; Armour et al., 1999, *Eur J Immunol* 29:2613-2624; Shields et al., 2001, *J Biol Chem* 276:6591-6604; Jefferis et al., 2002, *Immunol Lett* 82:57-65; Presta et al., 2002, *Biochem Soc Trans* 30:487-490; U.S. Pat. Nos. 5,624,821, 5,885,573 and PCT publication Nos. WO 00/42072, WO 99/58572 and WO 04/029207). While the vast majority of substitutions reduce or ablate Fc binding with FcγRs some have resulted in higher FcγR affinity. However, most of the methods disclosed resulted in only modest improvements in FcRγIIIA binding and ADCC activity. The present invention provides for the first time a modified Fc of antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$ that has increased binding to FcRγIIIA binding, significant enhancement in ADCC and does not show an increase in FcRγIIB binding.

[0021] Citation or discussion of a reference herein shall not be construed as an admission that such is prior art to the present invention.

4. SUMMARY OF THE INVENTION

[0022] The present invention provides novel antibodies comprising immunologically active fragments of immunoglobulin molecules and an Fc region that further comprises at least one novel amino acid residue of the invention (also referred to herein as "high effector function amino acid residue(s))". Said novel antibodies are referred to herein as "Fc variants of the invention" or "Fc variants." Fc binding interactions are essential for a variety of effector functions and downstream signaling events including, but not limited to, antibody dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC). Accord-

ingly, the invention provides Fc variants that exhibit altered binding affinity for at least one or more Fc ligands (e.g., FcγRs, C1q) relative to an antibody having the same amino acid sequence as the molecule of the invention but not comprising the novel amino acids residues of the invention (referred to herein as a "comparable molecule") such as, for example, an antibody comprising an unmodified Fc region containing naturally occurring amino acid residues at the corresponding position in the Fc domain. In addition, the present invention provides novel Fc variants comprising a variable region, or fragment thereof, that immunospecifically bind to Integrin $\alpha_v\beta_3$ and at least one high effector function amino acid residue (e.g., 239D, 330L, 332E).

[0023] The present invention further provides Fc variants of antibodies that immunospecifically bind to Integrin $\alpha_v\beta_3$, said Fc variants comprising an Fc region in which at least one amino acid residue has been substituted. It is specifically contemplated that said Fc variants may be generated by methods well known to one skilled in the art. Briefly, such methods include but are not limited to, combining a variable region with the desired specificity (e.g., a variable region isolated from a phage display or expression library or derived from a human or non-human antibody) with an Fc region containing at least one high effector function amino acid residue. Alternatively, one skilled in the art may generate an Fc variant by substituting at least one amino acid residue in the Fc region of an antibody.

[0024] The present invention also provides Fc variants that have altered binding affinity for one or more Fc ligands (e.g., FcγRs, C1q) relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region). In one embodiment, the Fc variants have higher binding affinity to activating FcγRs (e.g., FcγRIIIA) and/or unchanged or lower binding affinity to inhibitory FcγRs (e.g., FcγRIIB) relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region)). The present invention further provides Fc variants with enhanced ADCC function relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region)). In another embodiment, the Fc variants of the invention have enhanced ability to mediate ADCC ("referred to herein as ADCC activity") in addition to the above changes in FcγR affinities relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region)). In still another embodiment, the Fc variants of the invention are variants of an antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$. Furthermore, the Fc variants of the invention do not have significantly altered antigen binding specificity.

[0025] The present invention also provides Fc variants have lower binding affinity to activating FcγRs (e.g., FcγRIIIA) and/or increased binding affinity to inhibitory FcγRs (e.g., FcγRIIB) relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region). The present invention further provides Fc variants with decreased ADCC activity relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region). In one embodiment, the Fc variants of the invention exhibit decreased ADCC activity in addition to the above changes in FcγR affinities relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region). In another embodiment, the Fc variants of the invention are variants of an antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$. Furthermore, the Fc variants of the invention do not have significantly altered antigen binding specificity.

[0026] The present invention additionally provides Fc variants that have altered binding affinity to the complement protein C1q relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region). In one embodiment, the Fc variants have enhanced binding affinity to C1q and enhanced ability to mediate CDC (referred to herein as "CDC activity"). In another embodiment, the Fc variants have reduced binding affinity to C1q and reduced CDC activity relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region). In still another embodiment, the Fc variants of the invention are variants of an antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$.

[0027] In a specific embodiment, Fc variants of the invention comprise an Fc region comprising at least one high effector function amino acid reside selected from the group consisting of: 234E, 235R, 235A, 235W, 235P, 235V, 235Y, 236E, 239D, 265L, 269S, 269G, 298I, 298T, 298F, 327N, 327G, 327W, 328S, 328V, 329H, 329Q, 330K, 330V, 330G, 330Y, 330T, 330L, 3301, 330R, 330C, 332E, 332H, 332S, 332W, 332F, 332D, and 332Y, wherein the numbering system is that of the EU index as set forth in Kabat et al. (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, Va.).

[0028] In another specific embodiment, Fc variants of the invention comprise an Fc region comprising at least one high effector function amino acid residue selected from the group consisting of: 239D, 330K, 330V, 330G, 330Y, 330T, 330L, 3301, 330R, 330C, 332E, 332H, 332S, 332W, 332F, 332D, and 332Y wherein the numbering system is that of the EU index as set forth in Kabat.

[0029] In still another specific embodiment, Fc variants of the invention comprise an Fc region comprising at least one high effector function amino acid residue selected from the group consisting of: 239D, 330L and 332E. In yet another embodiment, Fc variants of the invention comprise an Fc region comprising at least the high effector function amino acid residue 332E. In a specific embodiment, Fc variants of the invention comprise an Fc region comprising the high effector function amino acid residues 239D, 330L and 332E.

[0030] In one embodiment, the Fc variants comprise at least one amino acid substitution at a position selected from the group consisting of: 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225,226,227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 239, 242, 246, 250, 251, 257, 259, 260, 261, 265, 269, 273, 274, 275, 277, 281, 282, 284, 287, 291, 298, 300, 302, 304, 306, 308, 310, 314, 316, 318, 319, 321, 323, 327, 328, 329, 330, 332 and 336, wherein the numbering of the residues in the Fc region is that of the EU index as set forth in Kabat.

[0031] In a specific embodiment, the Fc variants comprise at least one substitution selected from the group consisting of: L234E, L235R, L235A, L235W, L235P, L235V, L235Y, G236E, S239D, D265L, E269S, E269G, S2981, S298T, S298F, A327N, A327G, A327W, L328S, L328V, P329H, P329Q, A330K, A330V, A330G, A330Y, A330T, A330L, A3301, A330R, A330C, I332E, I332H, I332S, I332W, I332F, I332D, and I332Y, wherein the numbering system is

that of the EU index as set forth in Kabat. In another embodiment, the Fc variants comprise at least one substitution selected from the group consisting of S239D, A330L and I332E. In still another embodiment, the Fc variants comprise at least each of the following substitutions, S239D, A330L and I332E. In yet another embodiment, the Fc variants have at least the amino acid substitution I332E.

[0032] It is an object of the present invention to provide a Fc variants that bind with greater affinity to one or more Fc ligand (e.g., FcγRs, C1q). In one embodiment, said variants have an affinity for one or more Fc ligand (e.g., FcγRs, C1q) that is at least 2 fold greater than that of a comparable molecule (e.g., an antibody prior to Fc modification). In another embodiment, the Fc variants of the invention have affinity for an Fc ligand (e.g., FcγR, C1q) that is between about 2 fold and about 500 fold greater than that of a comparable molecule (e.g., an antibody prior to Fc modification). In still another embodiment, the Fc variants of the invention have affinity for an Fc ligand (e.g., FcγR, C1q) that is between 2 fold and 500 fold greater than that of a comparable molecule (e.g., an antibody prior to Fc modification). In one specific embodiment, an Fc variant of the invention has a greater affinity for FcγRIIIA. In another specific embodiment, an Fc variant of the invention has a greater affinity for FcγRIIB. In yet another specific embodiment, an Fc variant of the invention has a greater affinity for C1q.

[0033] It is a further object of the present invention to provide Fc variants that bind with reduced affinity to one or more Fc ligand (e.g., FcγRs, C1q). In one embodiment, the Fc variants of the invention have an affinity for one or more Fc ligand (e.g., FcγRs, C1q) that is between 2 fold and 500 fold lower than that of a comparable molecule (e.g., an antibody prior to Fc modification). In another embodiment, the Fc variants of the invention have an affinity for one or more Fc ligand (e.g., FcγRs, C1q) that is between about 2 fold and about 500 fold lower than that of a comparable molecule (e.g., an antibody prior to Fc modification). In a specific embodiment, the Fc variants of the invention have an affinity for FcγRIIB that is either unchanged, or reduced. In another specific embodiment, the Fc variants of the invention have an affinity for FcγRIIIA that is reduced. In yet another embodiment, the Fc variants of the invention have an affinity for C1q that is reduced.

[0034] It is a further object of the present invention to provide Fc variants that have enhanced ADCC and/or CDC activity. In one embodiment, Fc variants of the invention have ADCC and/or CDC activity that is at least 2 fold greater then that of a comparable molecule (e.g., an antibody prior to Fc modification). In another embodiment, the Fc variants of the invention have ADCC and/or CDC activity that is between about 2 fold and about 100 fold greater then that of a comparable molecule. In yet another embodiment, the Fc variants of the invention have ADCC and/or CDC activity that is between 2 fold and 100 fold greater then that of a comparable molecule.

[0035] It is a further object of the present invention to provide Fc variants that have reduced ADCC and/or CDC activity. In one embodiment, Fc variants of the invention have ADCC and/or CDC activity that is at least 2 fold lower then that of a comparable molecule (e.g., an antibody prior to Fc modification). In another embodiment, the Fc variants of the invention have ADCC and/or CDC activity that is between about 2 fold and about 100 fold lower then that of a comparable molecule. In another embodiment, the Fc variants of the invention have ADCC and/or CDC activity that is between 2 fold and 100 fold lower then that of a comparable molecule.

[0036] In one specific embodiment, an Fc variant of the invention has an increased affinity for FcγRIIIA and an affinity for FcγRIIB that is unchanged or reduced and enhanced ADCC activity relative to a comparable molecule (e.g., an antibody prior to Fc modification). In another specific embodiment, an Fc variant of the invention has an equilibrium dissociation constant (K_D) that is decreased between about 2 fold and about 10 fold, or between about 5 fold and about 50 fold, or between about 25 fold and about 250 fold, or between about 100 fold and about 500 fold, relative to a comparable molecule. In another specific embodiment, an Fc variant of the invention has an equilibrium dissociation constant (K_D) that is decreased between 2 fold and 10 fold, or between 5 fold and 50 fold, or between 25 fold and 250 fold, or between 100 fold and 500 fold, relative to a comparable molecule. In another specific embodiment, an Fc variant of the invention has a ratio of FcγRIIIA/FcγRIIB equilibrium dissociation constants (K_D) that is decreased and enhanced ADCC activity relative to a comparable molecule.

[0037] In one embodiment, an Fc variant of the invention has an increased affinity for FcγRIIIA and an affinity for FcγRIIB that is unchanged or reduced, an affinity for C1q that is reduced and enhanced ADCC activity relative to a comparable molecule (e.g., an antibody prior to Fc modification).

[0038] In another embodiment, an Fc variant of the invention has a decreased affinity for FcγRIIIA, an affinity for FcγRIIB that is increased and reduced ADCC activity relative to a comparable molecule (e.g., an antibody prior to Fc modification). In still another embodiment, an Fc variant of the invention has a ratio of FcγRIIIA/FcγRIIB equilibrium dissociation constants (K_D) that is increased and reduced ADCC activity relative to a comparable molecule.

[0039] The binding properties of a receptor for its ligand, may be determined by a variety of methods well-known in the art, including but not limited to, equilibrium methods (e.g., enzyme-linked immunoabsorbent assay (ELISA) or radioimmunoassay (RIA)), or kinetics (e.g., BIACORE® analysis), and other methods such as indirect binding assays, competitive inhibition assays, fluorescence resonance energy transfer (FRET), gel electrophoresis and chromatography (e.g., gel filtration). These and other well-known methods may utilize a label on one or more of the components being examined and/or employ a variety of detection methods including but not limited to chromogenic, fluorescent, luminescent, or isotopic labels. A detailed description of binding affinities and kinetics can be found in Paul, W. E., ed., Fundamental Immunology, 4th Ed., Lippincott-Raven, Philadelphia (1999), which focuses on antibody-immunogen interactions.

[0040] The Fc variants of the present invention may be combined with other Fc modifications (e.g., other amino acid substitutions, altered glycosylation, etc.), including but not limited to modifications that alter Fc ligand binding and/or effector function. The invention encompasses com-

bining an Fc variant of the invention with other Fc modifications to provide additive, synergistic, or novel properties in antibodies or Fc fusions. In one embodiment, the other Fc modifications enhance the phenotype of the Fc variants of the present invention (e.g., Fc variant comprising at least one high effector function amino acid) with which they are combined. For example, if an Fc variant (i.e., incorporating a hinge modification of the invention) is combined with a mutant known to bind FcγRIIIA with a higher affinity than a comparable molecule comprising a wild type Fc region; the combination results in a greater fold enhancement in FcγRIIIA affinity.

[0041] The invention encompasses molecules that comprise homodimers or heterodimers of Fc regions wherein at least one Fc region incorporates at least one high effector function amino acid of the invention. Heterodimers comprising Fc regions refer to molecules where the two Fc chains have different sequences. In some embodiments, in the heterodimeric molecules comprising an Fc region incorporating at least one high effector function amino acid and/or other Fc modification, each chain has one or more different modifications from the other chain. In other embodiments, in the heterodimeric molecules comprising an Fc region incorporating a hinge modification, one chain contains the wild-type Fc region and the other chains comprises one or more modifications. Methods of engineering heterodimeric Fc containing molecules are known in the art and encompassed within the invention.

[0042] In one embodiment, an Fc variant of the invention with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity immunospecifically binds to Integrin $\alpha_v\beta_3$. In another embodiment, said Fc variants are antagonists of Integrin $\alpha_v\beta_3$. An antagonist of Integrin $\alpha_v\beta_3$ is any molecule that blocks, inhibits, reduces or neutralizes the function, activity and/or expression of Integrin $\alpha_v\beta_3$. Thus, an antagonist of Integrin can block angiogenesis (also commonly referred to as neovascularization) and/or tumor cell growth resulting in, for example, tumor regression. In another embodiment, an Fc variant of the invention is a variant of an LM609 antibody or an antibody derived therefrom that immunospecifically binds Integrin $\alpha_v\beta_3$, such as chimerized and/or humanized versions of LM609, such as, for example, the antibody Vitaxin®. Such antibodies have been described in PCT Publication Nos. WO 89/05155, WO 98/33919 and WO 00/78815 as well as U.S. Pat. No. 5,753,230, which are incorporated by reference herein in their entireties. In a particular embodiment, said Fc variant is an antibody that competes with LM609 or Vitaxin®, or an antigen-binding fragment thereof for binding to Integrin $\alpha_v\beta_3$.

[0043] In one embodiment, an Fc variant of the invention with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity preferentially binds Integrin $\alpha_v\beta_3$ over other integrins. In another embodiment, said Fc variant of the invention does not immunoreact with an α_v subunit. In another embodiment, said Fc variant of the invention does immunoreact with an α_v subunit. In another embodiment, the Fc variant of the invention does not immunoreact with a β_3 subunit. In yet another embodiment, the Fc variant of the invention does immunoreact with a β_3 subunit. In still another embodiment, the Fc variant of the invention does not immunoreact with integrins other than $\alpha_v\beta_3$. In still another embodiment, the Fc variant of the invention immunoreacts with both Integrin $\alpha_v\beta_3$ and Integrin $\alpha_v\beta_5$ or with more than one Integrin $\alpha\beta$ complex. The Fc variant may have the same immunoreactivity for both Integrin $\alpha_v\beta_3$ and Integrin $\alpha_v\beta_5$ or alternatively, the Fc variant may immunoreact more strongly with Integrin $\alpha_v\beta_3$ than with Integrin $\alpha_v\beta_5$, or more strongly with Integrin $\alpha_v\beta_5$ than with Integrin $\alpha_v\beta_3$. In another embodiment the Fc variant binds an integrin other than Integrin $\alpha_v\beta_3$ (e.g., $\alpha_v\beta_1$, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_D\beta_2$, $\alpha_{IIb}\beta_2$).

[0044] The present invention also encompasses Fc variants with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$ conjugated or fused to a moiety (e.g., therapeutic agent or drug).

[0045] The present invention encompasses the use of Fc variants with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically binds to Integrin $\alpha_v\beta_3$ to inhibit or reduce angiogenesis.

[0046] The invention also encompasses the use of Fc variants with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$ conjugated or fused to a moiety (e.g., therapeutic agent or drug) to inhibit or reduce angiogenesis.

[0047] The present invention also encompasses the use of Fc variants with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$ for the prevention, treatment, management or amelioration of Integrin $\alpha_v\beta_3$-mediated diseases and disorders or one or more symptoms thereof, including but not limited to cancer, inflammatory and autoimmune diseases either alone or in combination with other therapies.

[0048] The invention also encompasses the use of Fc variants with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immuno-specifically bind to Integrin $\alpha_v\beta_3$ conjugated or fused to a moiety (e.g., therapeutic agent or drug) for the prevention, treatment, management or amelioration of Integrin $\alpha_v\beta_3$-mediated diseases and disorders or one or more symptoms thereof, including but not limited to cancer, inflammatory and autoimmune diseases either alone or in combination with other therapies.

[0049] The invention further encompasses treatment protocols that enhance the prophylactic or therapeutic effect of Fc variants with altered binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$.

[0050] The invention also provides methods for screening for antibody antagonists of Integrin $\alpha_v\beta_3$ including but not limited to assays that monitor Integrin $\alpha_v\beta_3$ activity (e.g., cell adhesion, angiogenesis, tumor cell growth and tumor progression) and/or plasma concentration. In addition, the invention provides methods for identifying monoclonal antibodies that bind to the heterodimerized $\alpha_v\beta_3$ but not the α_v or the β_3 chains when not included in a heterodimer. Further, the invention provides for a method to manipulate both the ADCC and or CDC activity as well as the binding affinities for FcγR and/or C1q of antibodies identified using such screening methods. The antibodies identified and manipu-

lated utilizing such methods can be used for the prevention, treatment, management or amelioration of Integrin $\alpha_v\beta_3$-mediated diseases and disorders or one or more symptoms thereof, including but not limited to cancer, inflammatory and autoimmune diseases either alone or in combination with other therapies.

[0051] The present invention provides kits comprising one or more Fc variants with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$ conjugated or fused to a detectable agent, therapeutic agent or drug, in one or more containers, can be used for the prevention, treatment, management or amelioration of Integrin $\alpha_v\beta_3$-mediated diseases and disorders or one or more symptoms thereof, including but not limited to cancer, inflammatory and autoimmune diseases either alone or in combination with other therapies.

5. BRIEF DESCRIPTION OF THE FIGURES

[0052] **FIG. 1**. The nucleotide and deduced amino acid sequence of the variable region of the antibody Vitaxin®, (A) heavy chain variable region (SEQ ID NO: 1 and SEQ ID NO: 3, respectively) (B) light chain variable region (SEQ ID NO: 2 and SEQ ID NO: 4, respectively). The CDRs are underlined.

[0053] **FIG. 2**. The nucleotide and deduced amino acid sequence of the variable region of the antibody 12G3H11 (abbreviated "12G3) (A) heavy chain variable region (SEQ ID NO: 62 and SEQ ID NO: 64, respectively) (B) light chain variable region (SEQ ID NO: 63 and SEQ ID NO: 65, respectively). The CDRs are underlined.

[0054] **FIG. 3**. The nucleotide and deduced amino acid sequence of the variable region of the antibody 3F2 (A) heavy chain variable region (SEQ ID NO: 66 and SEQ ID NO: 68, respectively) (B) light chain variable region (SEQ ID NO: 67 and SEQ ID NO: 69, respectively). The CDRs are underlined.

[0055] **FIG. 4**. Map of the expression plasmid used for the production of full length IgGs. SmaI/BsiWI restriction sites used to clone the light chain variable region, XbaI/ApaI restriction sites used to clone variable region of heavy chain and ApaI/NotI restriction sites were used to replace the constant region of the heavy chain.

[0056] **FIG. 5**. Screening of Vitaxin Fc variant clones by characterizing their relative binding to FcγRIIIA compared to parental scFv-Fc as determined by ELISA. Numerous clones were seen to have improved binding.

[0057] **FIG. 6**. Relative ADCC activity of several Vitaxin Fc variant clones against M21 cells as determined by a cell-based assay. Several Fc variants, including I332E, showed improved ADCC activity relative to the parental scFv-Fc.

[0058] **FIG. 7**. All 20 amino acids were substituted at position 332 of Vitaxin. The relative binding affinities of each position 332 Fc variant to FcγRIIIA was determined by ELISA (panel A). The relative ADCC activity of each position 332 Fc variant was determined by a cell-based assay (panel B). The I322E Fc variant was seen to provide the greatest improvement in both binding and in ADCC activity.

[0059] **FIG. 8**. Binding of Vitaxin® and the I332E (Vitaxin-1M) Fc variant to FcγRIIIA (A) and FcγRIIB (B) as determined by ELISA. The binding of Vitaxin-1M Fc variant to Fc FcγRIIIA is improved while the binding to FcγRIIB appears unchanged.

[0060] **FIG. 9**. Cell-based ADCC assay of Vitaxin® and the I332E (Vitaxin-1M) Fc variant using 50:1 ratio of effector to target cells at a variety of antibody concentrations from 0.4 to 1000 ng/ml. The I332E Fc variant shows higher ADCC activity over a wide range of antibody concentrations.

[0061] **FIG. 10**. Cell-based ADCC assay of Vitaxin® and the I332E (Vitaxin-1M) Fc variant using different ratios of effector to target cells and different amounts of antibody ranging from 2.5 ng to 200 ng per well. The I332E Fc variant shows higher ADCC activity over a wide range of antibody concentrations at all E:T ratios.

[0062] **FIG. 11**. Cell-based ADCC assay of Vitaxin® and the Vitaxin S239D/A330L/I332E (Vitaxin-3M) Fc variant against several target cell lines expressing different levels of Integrin αVβ3, A498 (moderate), DU145 (low), M21 (high) and ACHN (moderate), using two different E:T ratios and antibody amounts ranging from 4 ng to 400 ng per well. In all cases the S239D/A330L/I332E (Vitaxin-3M) Vitaxin Fc variant shows higher ADCC activity.

[0063] **FIG. 12**. ELISA analysis of the wild type anti-EphA2 antibody 3F2 and the 3F2 I332E (3F2-1M) and 3F2 S239D/A330L/I332E (3F2-3M) Fc variants binding to FcγRIIIA tetramer (panel A), FcγRIIIA monomer (panel B) and C1q (panel C). Both the 3F2-1M and 3F2-3M Fc variants bind better to FcγRIIIA monomers and tetramers, although the 3F2-3M Fc variant binds the monomer significantly better then either the wild type antibody or 3F2-1M Fc variant. In contrast both the 3F2-1M and 3F2-3M Fc variants did not bind C1q to the same degree as the wild type antibody with the 3M Fc variant showing the largest decrease in binding.

[0064] **FIG. 13**. FACS analysis of anti-EphA2 antibody 3F2-WT, 3F2-1M and 3F2-3M binding to cells via Fc-domain interactions. THP-1 and NK cells were stained with antibodies to FcγRI, FcγRII and FcγRIII (also commonly referred to CD64, CD32 and CD16, respectively). THP-1 cells have high levels of CD32 on their cell surface, moderate levels of CD64 and very low levels of CD16 (panel A). NK cells however show the opposite profile, high levels of CD16 and low levels of CD32 and CD64 (panel B). All three versions of 3F2 (wt, 1M and 3M) bound to a similar degree to THP-1 cells (panel C). However, the variants were seen to bind to a greater extent to NK cells, with the 3F2-3M Fc variant showing the largest increase in binding (panel D).

[0065] **FIG. 14**. Cell-based ADCC assay of 12G3H11 (anti-EphA2 antibody) and its I332E Fc variant using 50:1 ratio of effector to A549 target cells (panel A) and a similar study using two different E:T ratios from (panel B). In both studies the amount of antibody ranged from 4 ng to 400 ng per well. The I332E Fc variant shows higher ADCC activity over a wide range of antibody concentrations at all E:T ratios.

[0066] **FIG. 15**. Cell-based ADCC assay of anti-EphA2 antibody 3F2 and the 3F2-1M and 3F2-3M Fc variants to target cells expressing high (T231, A549) levels of EphA2.

In each assay the antibody concentration ranged from 0.02 ug/ml to 2 μg/ml. E:T ratios varied from 12.5:1 to 100:1 depending on the assay. The 3F2-3M Fc variant was seen to have the highest ADCC activity against all cell types. Although the 3F2-1M Fc variant showed higher ADCC activity against most cell types than the 3F2 wild type, it was generally not as active as the 3F2-3M Fc variant.

[0067] **FIG. 16**. Cell-based ADCC assay of anti-EphA2 antibody 3F2-WT, 3F2-1M and 3F2-3M Fc variants to target cells expressing high (Hey8) and moderate (SKOV3) levels of EphA2. The antibody concentration and E:T ratios are the same as for **FIG. 15**. The 3F2-3M Fc variant was seen to have the highest ADCC activity against all cell types. Although the 3F2-1M Fc variant showed higher ADCC activity against most cell types than the 3F-WT, it was generally not as active as the 3F2-3M Fc variant.

[0068] **FIG. 17**. Cell-based ADCC assay of anti-EphA2 antibody 3F2, 3F2-1M and 3F2-3M Fc variants to target cells expressing low (A498, SKMEL28) levels of EphA2. The SKMEL28 cells express Integrin αVβ5 as were also used as target cells for the Vitaxin and Vitaxin-3M antibodies. The antibody concentration and E:T ratios are the same as for **FIG. 15**. None of the 3F2 antibodies were seen to have activity against SKMEL28 cells although both Vitaxin and the Vitaxin-3M antibodies had activity the Vitaxin-3M Fc variant was significantly more active.

6. DETAILED DESCRIPTION OF THE INVENTION

[0069] The present invention provides certain amino acid residues in the Fc region of an IgG antibody that correlate with high effector function. Further, the invention provides high effector function residues in the Fc region of an antibody which exhibit high binding affinity for the Fc receptor, FcγRIIIA. In further embodiments, the invention encompasses the introduction of at least one of the high effector amino acid residues of the invention that does not result in a concomitant increase in binding the FcγRIIB receptor. In another embodiment, the invention encompasses the introduction of at least one of the high effector amino acid residues of the invention that results in a concomitant decrease in binding the FcγRIIB receptor and/or C1q. In still another embodiment, the introduction of at least one of the high effector amino acid residues of the invention that results in a concomitant increase in binding to both the FcγRIIIA and FcγRIIB receptors. In yet another embodiment, the ratio of FcγRIIIA/FcγRIIB equilibrium dissociation constants (K_D), is decreased. Furthermore, the presence of at least one of the high effector amino acid residue of the invention results in antibodies with an enhanced antibody dependent cell-mediated cytotoxicity (ADCC) activity. Accordingly, the invention provides Fc variants that exhibit altered effector function (e.g., ADCC, CDC, etc.) and/or altered binding affinity for at least one Fc ligand (e.g., FcγRIIIA, FcγRIIB, C1q, etc.) relative to an antibody (or other Fc-domain containing polypeptide) having the same amino acid sequence as the molecule of the invention but not comprising the novel amino acids residues of the invention (referred to herein as a "comparable molecule") such as an antibody comprising an unmodified Fc region containing naturally occurring amino acid residues at the corresponding position in the Fc domain. In particular, the present invention provides Fc variants comprising a variable region, or fragment thereof, that immunospecifically binds to Integrin $\alpha_v\beta_3$ and a Fc region that further comprises at least one high effector function amino acid residue (e.g., 239D, 330L, 332E).

[0070] The present invention further provides Fc variants of antibodies that immunospecifically bind to Integrin $\alpha_v\beta_3$, said Fc variants comprising an Fc region in which at least one amino acid residue has been substituted. The present invention also relates to Fc variants with altered binding affinity to their FcγRs compared to that of a comparable molecule (e.g., an antibody having an original unmodified Fc region). In one embodiment, the Fc variants have higher binding affinity to activating FcγRs (e.g., FcγRIIIA). In a specific embodiment, the Fc variants of the invention have equilibrium dissociation constants (K_D) that are decreased relative to a comparable molecule. In another embodiment the Fc variants have higher binding affinity to activating FcγRs and unchanged or lower binding affinity to inhibitory FcγRs (e.g., FcγRIIB). Also contemplated, are Fc variants which have a ratio of FcγRIIIA/FcγRIIB equilibrium dissociation constants (K_D) that are decreased relative to a comparable molecule. In one embodiment, the Fc variants of the invention also exhibit increased ADCC activity when compared to a comparable molecule (e.g., an antibody having an original unmodified Fc region) in addition to the above changes in FcγR affinities. In another embodiment, the Fc variants of the invention are variants of an antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$. In a specific embodiment, the Fc variants of the invention immunospecifically bind Integrin $\alpha_v\beta_3$ and are Integrin $\alpha_v\beta_3$ antagonists.

[0071] The antibodies of the present invention may be produced "de novo" by combining a variable domain, or fragment thereof, that immunospecifically binds Integrin $\alpha_v\beta_3$ with an Fc domain comprising one or more of the high effector function residues disclosed herein, or may be produced by modifying an Fc domain-containing antibody that binds $\alpha_v\beta_3$ Integrin by introducing one or more high effector function residues into the Fc domain.

[0072] The present invention also relates to novel Fc variants with a higher binding affinity to inhibitory FcγRs and a lower binding affinity to activating FcγRs (e.g., FcγRIIIA) when reative to a comparable molecule (e.g., an antibody having an original unmodified Fc region). It is contemplated that said Fc variants will also exhibit a reduced ability to mediate ADCC activity relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region). In one embodiment, the Fc variants of the invention are variants of an antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$. In a specific embodiment, the Fc variants of the invention with a higher binding affinity to inhibitory FcγRs and a lower binding affinity to activating FcγRs immunospecifically bind Integrin $\alpha_v\beta_3$ and are Integrin $\alpha_v\beta_3$ antagonists.

[0073] In addition, the present invention further provides novel Fc variants with altered binding to C1q relative to a comparable molecule (e.g., an antibody having an original unmodified Fc region). Specifically, the Fc variants of the invention may exhibit a higher binding affinity for C1q and increased CDC activity. Alternatively, the Fc variants of the invention may exhibit a lower binding affinity for C1q and reduced CDC activity. In other situations, the Fc variants of the invention with altered binding to C1q exhibit CDC

activity that is unchanged relative to a comparable molecule. It is specifically contemplated that Fc variants with alterations in C1q binding and CDC activity may also exhibit alterations in binding to one or more FcγRs and/or ADCC activity. In one embodiment, the Fc variants of the invention are variants of an antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$. In a In another embodiment embodiment, the Fc variants of the invention altered binding to C1q immunospecifically bind Integrin $\alpha_v\beta_3$ and are Integrin $\alpha_v\beta_3$ antagonists.

[**0074**] Also encompassed by the invention are Fc variants that inhibit the functional activity of Integrin $\alpha_v\beta_3$ or inhibit Integrin $\alpha_v\beta_3$-mediated pathologies, such molecules are also referred to herein as Integrin $\alpha_v\beta_3$ antagonists. Accordingly, the invention provides antibodies useful for the inhibition of angiogenesis or the inhibition of other functions mediated or influenced by Integrin $\alpha_v\beta_3$, including but not limited to cell proliferation, cell attachment, cell migration, granulation tissue development, tumor growth, tumor cell invasion and/or inflammation. Such antibodies have been described in International Publication Nos. WO 89/05155, WO 98/33919 and WO 00/78815 as well as U.S. Pat. No. 5,753,230, which are incorporated by reference herein in their entireties.

[**0075**] As used herein, the terms "antibody" and "antibodies" refer to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies, camelised antibodies, chimeric antibodies, single-chain Fvs (scFv), disulfide-linked Fvs (sdFv), Fab fragments, F (ab') fragments, and anti-idiotypic (anti-Id) antibodies (including, e.g., anti-Id antibodies to antibodies of the invention), and epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, i.e., molecules that contain an antigen binding site, these fragments may or may not be fused to another immunoglobulin domain including but not limited to, an Fc region or fragment thereof. As outlined herein, the terms "antibody" and "antibodies" specifically include the Fc variants described herein, full length antibodies and variant Fc-Fusions comprising Fc regions, or fragments thereof, comprising at least one novel amino acid residue described herein fused to an immunologically active fragment of an immunoglobulin or to other proteins as described herein. Such variant Fc fusions include but are not limited to, scFv-Fc fusions, variable region (e.g., VL and VH)—Fc fusions, scFv-scFv-Fc fusions. Immunoglobulin molecules can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass.

[**0076**] As used herein, the term "immunospecifically binds to Integrin $\alpha_v\beta_3$" and analogous terms refer to peptides, polypeptides, proteins, fusion proteins and antibodies or fragments thereof that specifically bind to Integrin $\alpha_v\beta_3$ or a fragment thereof. A peptide, polypeptide, protein, or antibody that immunospecifically binds to an Integrin $\alpha_v\beta_3$ or a fragment thereof may bind to other peptides, polypeptides, or proteins with lower affinity as determined by, e.g., immunoassays, BIAcore, or other assays known in the art. Antibodies or fragments that immunospecifically bind to Integrin $\alpha_v\beta_3$ or a fragment thereof may be cross-reactive with related antigens. It is contemplated that antibodies or fragments that immuno-specifically bind to Integrin $\alpha_v\beta_3$ or a fragment thereof preferentially bind Integrin $\alpha_v\beta_3$ over other antigens. However, the present invention specifically encompasses antibodies with multiple specificities (e.g., an antibody with specificity for two or more discrete antigens (reviewed in Cao et al., 2003, *Adv Drug Deliv Rev* 55:171-197; Hudson et al., 2003, *Nat Med* 1: 129-134)) in the definition of an antibody that "immunospecifically binds to Integrin $\alpha_v\beta_3$." For example, bispecific antibodies contain two different binding specificities fused together. In the simplest case a bispecific antibody would bind to two adjacent epitopes on a single target antigen, such an antibody would not cross-react with other antigens (as described supra). Alternatively, bispecific antibodies can bind to two different antigens, such an antibody immunospecifically binds to two different molecules but not to other unrelated molecules. In addition, an antibody that immunospecifically binds Integrin $\alpha_v\beta_3$ may cross-react with related integrins. Another class of multispecific antibodies may recognize a shared subunit of multi-subunit complexes in the context of one or more specific complexes. For example CNTO 95 (Trikha et al., 2004, *Int J Cancer* 110:326-335) recognizes Integrin α_v in the context of both Integrin $\alpha_v\beta_3$ and Integrin $\alpha_v\beta_5$. Thus, a multispecific antibody may immunospecifically bind to both Integrin $\alpha_v\beta_3$ and one or more additional molecules such as Integrin $\alpha_v\beta_5$.

[**0077**] Antibodies or fragments that immunospecifically bind to Integrin $\alpha_v\beta_3$ or a fragment thereof can be identified, for example, by immunoassays, BIAcore, or other techniques known to those of skill in the art. An antibody or fragment thereof binds specifically to Integrin $\alpha_v\beta_3$ or a fragment thereof when it binds to Integrin $\alpha_v\beta_3$ or a fragment thereof with higher affinity than to any cross-reactive antigen as determined using experimental techniques, such as radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISAs). See, e.g., Paul, ed., 1989, Fundamental Immunology Second Edition, Raven Press, New York at pages 332-336 for a discussion regarding antibody specificity.

[**0078**] Without wishing to be bound by any particular theory, the amino acid substitutions of the invention alter the affinity of an antibody for its FcγRs and/or the complement protein C1q by modulating one or more of the factors that regulate protein-protein interactions (e.g., receptor-ligand and antibody-antigen interactions). Such factors include but are not limited to, factors affecting protein folding or three dimensional configuration such as hydrogen bonds, hydrophobic interactions, ionic interactions, Von der Waals forces and/or disulfide bonds as well as factors affecting allosteric interactions, solubility and covalent modifications.

[**0079**] Without wishing to be bound by any particular theory, the amino acid substitutions of the invention modulate the ADCC and/or CDC activity of an antibody by altering one more of the factors that influence downstream effector function including but not limited to, the affinity of the antibody for its FcγRs and/or to C1q, ability to mediate cytotoxic effector and/or complement cascade functions, protein stability, antibody half life and recruitment of effector cells and/or molecules.

[**0080**] It will be understood that Fc region (also referred to herein as "Fc" and "Fc polypeptide") as used herein includes the polypeptides comprising the constant region of an antibody excluding the first constant region immunoglobulin domain. Thus Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgG, and the last three constant region immunoglobulin domains of IgE and IgM, and the flexible hinge N-terminal to these domains. For IgA and IgM Fc may include the J chain. For IgG, Fc comprises immunoglobulin domains Cgamma2 and Cgamma3 (Cγ2 and Cγ3) and the hinge between Cgamma1

(Cγ1) and Cgamma2 (Cγ2). Although the boundaries of the Fc region may vary, the human IgG heavy chain Fc region is usually defined to comprise residues C226 or P230 to its carboxyl-terminus, wherein the numbering is according to the EU index as in Kabat et al. (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, Va.). The "EU index as set forth in Kabat" refers to the residue numbering of the human IgG1 EU antibody as described in Kabat et al. supra. Fc may refer to this region in isolation, or this region in the context of an antibody, antibody fragment, or Fc fusion protein. Note: Polymorphisms have been observed at a number of Fc positions, including but not limited to Kabat 270, 272, 312, 315, 356, and 358, and thus slight differences between the presented sequence and sequences in the prior art may exist.

[0081] It will be understood that the complementarity determining regions (CDRs) residue numbers referred to herein are those of Kabat et al. (1991, NIH Publication 91-3242, National Technical Information Service, Springfield, Va.). Specifically, residues 24-34 (CDR1), 50-56 (CDR2) and 89-97 (CDR3) in the light chain variable domain and 31-35 (CDR1), 50-65 (CDR2) and 95-102 (CDR3) in the heavy chain variable domain. Note that CDRs vary considerably from antibody to antibody (and by definition will not exhibit homology with the Kabat consensus sequences). Maximal alignment of framework residues frequently requires the insertion of "spacer" residues in the numbering system, to be used for the Fv region. It will be understood that the CDRs referred to herein are those of Kabat et al. supra. In addition, the identity of certain individual residues at any given Kabat site number may vary from antibody chain to antibody chain due to interspecies or allelic divergence.

[0082] In one embodiment, Fc variants of the invention will have at least one amino acid substitution of the Fc region wherein said antibody variant has a modified binding affinity for its FcγRs and/or for C1q relative to a comparable molecule (e.g., the original antibody without said substitution).

[0083] In a specific embodiment, Fc variants comprise an Fc region comprising at least one high effector function amino acid reside selected from the group consisting of: 234E, 235R, 235A, 235W, 235P, 235V, 235Y, 236E, 239D, 265L, 269S, 269G, 2981, 298T, 298F, 327N, 327G, 327W, 328S, 328V, 329H, 329Q, 330K, 330V, 330G, 330Y, 330T, 330L, 3301, 330R, 330C, 332E, 332H, 332S, 332W, 332F, 332D, and 332Y, wherein the numbering system is that of the EU index as set forth in Kabat. Specific high effector function amino acid residues of the invention are also set forth in Table 1.

[0084] In another embodiment, the Fc variants comprise an Fc region comprising at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 20, or at least 30, or at least 40, or at least 50, or at least 60, or at least 70, or at least 80, or at least 90, or at least 100, or at least 200 high effector function amino acid residues.

[0085] In another specific embodiment, Fc variants of the invention comprise an Fc region comprising at least one high effector function amino acid residue selected from the group consisting of: 239D, 330K, 330V, 330G, 330Y, 330T, 330L, 3301, 330R, 330C, 332E, 332H, 332S, 332W, 332F, 332D, and 332Y, wherein the numbering system is that of the EU index as set forth in Kabat.

[0086] In still another specific embodiment, Fc variants of the invention comprise an Fc region comprising at least one high effector function amino acid residue selected from the group consisting of: 239D, 330L and 332E. In another embodiment, Fc variants of the invention comprise an Fc region comprising at least the high effector function amino acid residue 332E. In a specific embodiment, Fc variants of the invention comprise an Fc region comprising the high effector function amino acid residues 239D, 330L and 332E.

[0087] In a specific embodiment, Fc variants will have one or more amino acid substitutions at positions selected from the group consisting of: 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223,224, 225,226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 239, 242, 246, 250, 251, 257, 259, 260, 261, 265, 269, 273, 274, 275, 277, 281, 282, 284, 287, 291, 298, 300, 302, 304, 306, 308, 310, 314, 316, 318, 319, 321, 323, 327, 328, 329, 330, 332 and 336, of the Fc region wherein the numbering of the residues in the Fc region is that of the EU index as set forth in Kabat.

[0088] In another specific embodiment, the Fc variants comprise at least one substitution selected from the group consisting of: L234E, L235R, L235A, L235W, L235P, L235V, L235Y, G236E, S239D, D265L, E269S, E269G, S298I, S298T, S298F, A327N, A327G, A327W, L328S, L328V, P329H, P329Q, A330K, A330V, A330G, A330Y, A330T, A330L, A330I, A330R, A330C, I332E, I332H, I332S, I332W, I332F, I332D, and I332Y, wherein the numbering system is that of the EU index as set forth in Kabat. Specific amino acid substitutions of the invention are also set forth in Table 1.

[0089] In another embodiment, the Fc variants comprise at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, or at least 20, or at least 30, or at least 40, or at least 50, or at least 60, or at least 70, or at least 80, or at least 90, or at least 100, or at least 200 amino acid substitutions of the Fc region.

TABLE 1

Specific Amino Acid Residues with High Effector Function (HEF)

Position[a]	Amino Acid[b]	HEF Residue(s)[c]
234	L	E
235	L	R, A, W, P, V, Y
236	G	E
239	S	D
265	D	L
269	E	S, G
298	S	I, T, F
327	A	N, G, W
328	L	S, V
329	P	H, Q
330	A	K, V, G, Y, T, L, I, R, C
332	I	E, H, S, W, F, Y, D

[a]heavy chain position number and amino acid residue
[b]amino acid residue present in naturally occurring antibody
[c]residues that can be engineered into corresponding position to generate an Fc region with high effector function.

[0090] In one embodiment, the Fc variants comprise at least one substitution selected from the group consisting of S239D, A330L and I332E. In another preferred embodiment, the Fc variants comprise at least each of the following substitutions, S239D, A330L and I332E. In another embodiment, the Fc variants of the invention have at least the amino acid substitution I332E.

[0091] It is specifically contemplated that conservative amino acid substitutions may be made for said amino acid

substitutions in the Fc of the antibody of interest, described supra (see Table 1). It is well known in the art that "conservative amino acid substitution" refers to amino acid substitutions that substitute functionally-equivalent amino acids. Conservative amino acid changes result in silent changes in the amino acid sequence of the resulting peptide. For example, one or more amino acids of a similar polarity act as functional equivalents and result in a silent alteration within the amino acid sequence of the peptide. Substitutions that are charge neutral and which replace a residue with a smaller residue may also be considered "conservative substitutions" even if the residues are in different groups (e.g., replacement of phenylalanine with the smaller isoleucine). Families of amino acid residues having similar side chains have been defined in the art. Several families of conservative amino acid substitutions are shown in Table 2.

TABLE 2

Families of Conservative Amino Acid Substitutions

Family	Amino Acids
non-polar	Trp, Phe, Met, Leu, Ile, Val, Ala, Pro
uncharged polar	Gly, Ser, Thr, Asn, Gln, Tyr, Cys
acidic/negatively charged	Asp, Glu
basic/positively charged	Arg, Lys, His
Beta-branched	Thr, Val, Ile
residues that influence chain orientation	Gly, Pro
aromatic	Trp, Tyr, Phe, His

[0092] The term "conservative amino acid substitution" also refers to the use of amino acid analogs or variants. Guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie et al., "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," (1990, Science 247:1306-1310).

[0093] In another embodiment, the Fc variants have at least the amino acid substitution I332D.

[0094] One skilled in the art will understand that that the Fc variants of the invention may have altered FcγR and/or C1 q binding properties (examples of binding properties include but are not limited to, binding specificity, equilibrium dissociation constant (K_D), dissociation and association rates (K_{off} and K_{on} respectively), binding affinity and/or avidity) and that certain alterations are more or less desirable. It is well known in the art that the equilibrium dissociation constant (K_D) is defined as k_{off}/k_{on}. It is generally understood that a binding molecule (e.g., and antibody) with a low K_D is preferable to a binding molecule (e.g., and antibody) with a high K_D. However, in some instances the value of the k_{on} or k_{off} may be more relevant than the value of the K_D. One skilled in the art can determine which kinetic parameter is most important for a given antibody application. For example a modification that enhances Fc binding to one or more positive regulators (e.g., FcγRIIIA) while leaving unchanged or even reducing Fc binding to the negative regulator FcγRIIB would be more preferable for enhancing ADCC activity. Alternatively, a modification that reduced binding to one or more positive regulator and/or enhanced binding to FcγRIIB would be preferable for reducing ADCC activity. Accordingly, the ratio of binding affinities (e.g., equilibrium dissociation constants (K_D)) can indicate if the ADCC activity of an Fc variant is enhanced or decreased. For example a decrease in the ratio of FcγRIILA/FcγRIIB equilibrium dissociation constants (K_D), will correlate with improved ADCC activity, while an increase in the ratio will correlate with a decrease in ADCC activity. Additionally, modifications that enhanced binding to C1q would be preferable for enhancing CDC activity while modification that reduced binding to C1q would be preferable for reducing or eliminating CDC activity.

[0095] The affinities and binding properties of an Fc domain for its ligand, may be determined by a variety of in vitro assay methods (biochemical or immunological based assays) known in the art for determining Fc-FcγR interactions, i.e., specific binding of an Fc region to an FcγR including but not limited to, equilibrium methods (e.g., enzyme-linked immunoabsorbent assay (ELISA); see Example 3, or radioimmunoassay (RIA)), or kinetics (e.g., BIACORE® analysis), and other methods such as indirect binding assays, competitive inhibition assays, fluorescence resonance energy transfer (FRET), gel electrophoresis and chromatography (e.g., gel filtration). These and other methods may utilize a label on one or more of the components being examined and/or employ a variety of detection methods including but not limited to chromogenic, fluorescent, luminescent, or isotopic labels. A detailed description of binding affinities and kinetics can be found in Paul, W. E., ed., Fundamental Immunology, 4th Ed., Lippincott-Raven, Philadelphia (1999), which focuses on antibody-immunogen interactions.

[0096] In a one embodiment, the Fc variants of the invention bind FcγRIIIA with increased affinity relative to a comparable molecule. In another embodiment, the Fc variants of the invention bind FcγRIIIA with increased affinity and bind FcγRIIB with a binding affinity that is unchanged relative to a comparable molecule. In still another embodiment, the Fc variants of the invention bind FcγRIIIA with increased affinity and bind FcγRIIB with a decreased affinity relative to a comparable molecule. In yet another embodiment, the Fc variants of the invention have a ratio of FcγRIIIA/FcγRIIB equilibrium dissociation constants (K_D) that is decreased relative to a comparable molecule.

[0097] In one embodiment, the Fc variants of the invention bind FcγRIIIA with increased affinity and bind FcγRIIB with a decreased affinity when relative to a comparable molecule and immunospecifically bind Integrin αVβ3.

[0098] In one embodiment, said Fc variants bind with increased affinity to FcγRIIIA. In one embodiment, said Fc variants have affinity for FcγRIIIA that is at least 2 fold, or at least 3 fold, or at least 5 fold, or at least 7 fold, or a least 10 fold, or at least 20 fold, or at least 30 fold, or at least 40 fold, or at least 50 fold, or at least 60 fold, or at least 70 fold, or at least 80 fold, or at least 90 fold, or at least 100 fold, or at least 200 fold greater than that of a comparable molecule.

[0099] In another embodiment, an Fc variant of the invention has an equilibrium dissociation constant (K_D) that is decreased between about 2 fold and about 10 fold, or between about 5 fold and about 50 fold, or between about 25 fold and about 250 fold, or between about 100 fold and about 500 fold, or between about 250 fold and about 1000 fold relative to a comparable molecule. In another embodiment, an Fc variant of the invention has an equilibrium dissocia-

tion constant (K_D) that is decreased between 2 fold and 10 fold, or between 5 fold and 50 fold, or between 25 fold and 250 fold, or between 100 fold and 500 fold, or between 250 fold and 1000 fold relative to a comparable molecule. In a specific embodiment, said Fc variants have an equilibrium dissociation constants (K_D) for FcγRIIIA that is reduced by at least 2 fold, or at least 3 fold, or at least 5 fold, or at least 7 fold, or a least 10 fold, or at least 20 fold, or at least 30 fold, or at least 40 fold, or at least 50 fold, or at least 60 fold, or at least 70 fold, or at least 80 fold, or at least 90 fold, or at least 100 fold, or at least 200 fold, or at least 400 fold, or at least 600 fold, relative to a comparable molecule.

[0100] In one embodiment, said Fc variant binds to FcγRIIB with an affinity that is unchanged or reduced. In another embodiment said Fc variants have affinity for FcγRIIB that is unchanged or reduced by at least 1 fold, or by at least 3 fold, or by at least 5 fold or by at least 10 or by at least 20 fold, or by at least 50 fold relative to a comparable molecule.

[0101] In another embodiment, said Fc variants have an equilibrium dissociation constants (K_D) for FcγRIIB that is unchanged or increased by at least at least 2 fold, or at least 3 fold, or at least 5 fold, or at least 7 fold, or a least 10 fold, or at least 20 fold, or at least 30 fold, or at least 40 fold, or at least 50 fold, or at least 60 fold, or at least 70 fold, or at least 80 fold, or at least 90 fold, or at least 100 fold, or at least 200 fold relative to a comparable molecule.

[0102] In another embodiment, the Fc variants of the invention bind FcγRIIIA with decreased affinity and bind FcγRIIB with increased affinity when compared to the original antibodies without the substituted Fc. In still another embodiment said Fc variants have affinity for FcγRIIIA that is reduced by at least 1 fold, or by at least 3 fold, or by at least 5 fold or by at least 10 or by at least 20 fold, or by at least 50 fold when compared to that of the original antibody without the substituted Fc. In yet another embodiment said Fc variants have affinity for FcγRIIB that is at least 2 fold, or at least 3 fold, or at least 5 fold, or at least 7 fold, or a least 10 fold, or at least 20 fold, or at least 50 fold or at least 100 fold, greater than that of a comparable molecule.

[0103] In still another embodiment, the Fc variants have an equilibrium dissociation constants (K_D) for FcγRIIIA that are increased by at least 1 fold, or by at least 3 fold, or by at least 5 fold or by at least 10 or by at least 20 fold, or by at least 50 fold when compared to that of the original antibody without the substituted Fc. In yet another embodiment said Fc variants have equilibrium dissociation constants (K_D) for FcγRIIB that are decreased at least 2 fold, or at least 3 fold, or at least 5 fold, or at least 7 fold, or a least 10 fold, or at least 20 fold, or at least 50 fold or at least 100 fold, relative to a comparable molecule.

[0104] "Antibody-dependent cell-mediated cytotoxicity" or "ADCC" refers to a form of cytotoxicity in which secreted Ig bound onto Fc receptors (FcRs) present on certain cytotoxic cells (e.g., Natural Killer (NK) cells, neutrophils, and macrophages) enables these cytotoxic effector cells to bind specifically to an antigen-bearing target cell and subsequently kill the target cell with cytotoxins. Specific high-affinity IgG antibodies directed to the surface of target cells "arm" the cytotoxic cells and are absolutely required for such killing. Lysis of the target cell is extracellular, requires direct cell-to-cell contact, and does not involve complement.

[0105] The ability of any particular antibody to mediate lysis of the target cell by ADCC can be assayed. To assess ADCC activity an antibody of interest is added to target cells in combination with immune effector cells, which may be activated by the antigen antibody complexes resulting in cytolysis of the target cell. Cytolysis is generally detected by the release of label (e.g. radioactive substrates, fluorescent dyes or natural intracellular proteins) from the lysed cells. Useful effector cells for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer (NK) cells. Specific examples of in vitro ADCC assays are described in Wisecarver et al., 1985 79:277-282; Bruggemann et al., 1987, *J Exp Med* 166:1351-1361; Wilkinson et al., 2001, *J Immunol Methods* 258:183-191; Patel et al., 1995 *J Immunol Methods* 184:29-38 and herein (see Example 3). Alternatively, or additionally, ADCC activity of the antibody of interest may be assessed in vivo, e.g., in a animal model such as that disclosed in Clynes et al., 1998, PNAS USA 95:652-656.

[0106] It is contemplated that the Fc variants of the invention are also characterized by in vitro functional assays for determining one or more FcγR mediator effector cell functions (See Example 3). In certain embodiments, the molecules of the invention have similar binding properties and effector cell functions in in vivo models (such as those described and disclosed herein) as those in in vitro based assays However, the present invention does not exclude molecules of the invention that do not exhibit the desired phenotype in in vitro based assays but do exhibit the desired phenotype in vivo.

[0107] The present invention further provides Fc variants with enhanced ADCC function. In one embodiment, the Fc variants of the invention have increased ADCC activity. In another embodiment said Fc variants have ADCC activity that is at least 2 fold, or at least 3 fold, or at least 5 fold or at least 10 fold or at least 50 fold or at least 100 fold greater than that of a comparable molecule. In a specific embodiment, Fc variants of the invention bind FcγRIIIA with increased affinity, bind FcγRIIB with decreased affinity and have enhanced ADCC activity relative to a comparable molecule.

[0108] In one embodiment, the Fc variants of the invention have enhanced ADCC activity and immunospecifically bind to Integrin $\alpha_v\beta_3$. In one embodiment the Fc variants of the invention have enhanced ADCC activity and have a ratio of FcγRIIIA/FcγRIIB equilibrium dissociation constants (K_D) that is decreased relative to a comparable molecule and immunospecifically bind to Integrin $\alpha_v\beta_3$ In another embodiment, the Fc variants of the invention have enhanced ADCC activity, bind activating FcγRs (e.g., FcγRIIIA) with higher affinity and bind inhibitory FcγRs (e.g., FcγRIIB) with unchanged or lower affinity and immunospecifically bind to Integrin $\alpha_v\beta_3$.

[0109] The present invention also provides Fc variants with reduced ADCC function. In one embodiment, the Fc variants of the invention have reduced ADCC activity. In one embodiment said Fc variants have ADCC activity that is at least 2 fold, or at least 3 fold, or at least 5 fold or at least 10 fold or at least 50 fold or at least 100 fold less than that

of a comparable molecule. In a specific embodiment, Fc variants of the invention bind FcγRIIIA with decreased affinity, bind FcγRIIB with increased affinity and have reduced ADCC activity.

[0110] In one embodiment, the Fc variants of the invention have reduced ADCC activity and immunospecifically bind to Integrin $\alpha_v\beta_3$. In another embodiment, the antibody variants of the invention have reduced ADCC activity, bind activating FcγRs (e.g., FcγRIIIA) with lower affinity, bind inhibitory FcγRs (e.g., FcγRIIB) with higher affinity and immunospecifically bind to Integrin $\alpha_v\beta_3$.

[0111] "Complement dependent cytotoxicity" and "CDC" refer to the lysing of a target cell in the presence of complement. The complement activation pathway is initiated by the binding of the first component of the complement system (C1q) to a molecule, an antibody for example, complexed with a cognate antigen. To assess complement activation, a CDC assay, e.g. as described in Gazzano-Santoro et al., 1996, *J. Immunol. Methods,* 202:163, may be performed.

[0112] The present invention further provides Fc variants with enhanced CDC function. In one embodiment, the Fc variants of the invention have increased CDC activity. In another embodiment said Fc variants have CDC activity that is at least 2 fold, or at least 3 fold, or at least 5 fold or at least 10 fold or at least 50 fold or at least 100 fold greater than that of a comparable molecule. In another embodiment, an Fc variant of the invention binds C1q with an affinity that is at least 2 fold, or at least 3 fold, or at least 5 fold, or at least 7 fold, or a least 10 fold, or at least 20 fold, or at least 50 fold or at least 100 fold, greater than that of a comparable molecule. In a specific embodiment, Fc variants of the invention bind C1q with increased affinity; have enhanced CDC activity and immunospecifically bind to Integrin $\alpha_v\beta_3$.

[0113] The present invention also provides Fc variants with reduced CDC function. In one embodiment, the Fc variants of the invention have reduced CDC activity. In another embodiment said Fc variants have CDC activity that is at least 2 fold, or at least 3 fold, or at least 5 fold or at least 10 fold or at least 50 fold or at least 100 fold less than that of relative to a comparable molecule. In another embodiment, an Fc variant of the invention binds C1q with an affinity that is reduced by at least 1 fold, or by at least 3 fold, or by at least 5 fold or by at least 10 or by at least 20 fold, or by at least 50 fold relative to a comparable molecule. In a specific embodiment, Fc variants of the invention bind to Integrin $\alpha_v\beta_3$, bind C1q with decreased affinity have reduced CDC activity and immunospecifically bind to Integrin $\alpha_v\beta_3$.

[0114] It is also specifically contemplated that the Fc variants of the invention may contain inter alia one or more additional amino acid residue substitutions, mutations and/or modifications which result in an antibody with desired characteristics including but not limited to: increased serum half life, increase binding affinity, reduced immunogenicity, increased production, altered Fc ligand binding, enhanced or reduced ADCC or CDC activity, altered glycosylation and/or disulfide bonds and modified binding specificity (for examples see infra). The invention encompasses combining an Fc variant of the invention with other Fc modifications to provide additive, synergistic, or novel properties in antibodies or Fc fusions. In one embodiment, the other Fc modifications enhance the phenotype of the Fc variant with which they are combined. For example, if an Fc variant of the invention is combined with a mutant known to bind FcγRIIIA with a higher affinity than a comparable molecule comprising a wild type Fc region; the combination with a mutant of the invention results in a greater fold enhancement in FcγRIIIA affinity.

[0115] In one embodiment, the Fc variants of the present invention may be combined with other known Fc variants such as those disclosed in Ghetie et al., 1997, *Nat Biotech.* 15:637-40; Duncan et al, 1988, *Nature* 332:563-564; Lund et al., 1991, *J. Immunol* 147:2657-2662; Lund et al, 1992, Mol Immunol 29:53-59; Alegre et al, 1994, *Transplantation* 57:1537-1543; Hutchins et al., 1995, *Proc Natl. Acad Sci USA* 92:11980-11984; Jefferis et al, 1995, *Immunol Lett.* 44:111-117; Lund et al., 1995, *Faseb J* 9:115-119; Jefferis et al, 1996, *Immunol Lett* 54:101-104; Lund et al, 1996, *J Immunol* 157:4963-4969; Armour et al., 1999, *Eur J Immunol* 29:2613-2624; Idusogie et al, 2000, *J Immunol* 164:4178-4184; Reddy et al, 2000, *J Immunol* 164:1925-1933; Xu et al., 2000, *Cell Immunol* 200:16-26; Idusogie et al, 2001, *J Immunol* 166:2571-2575; Shields et al., 2001, *J Biol Chem* 276:6591-6604; Jefferis et al, 2002, *Immunol Lett* 82:57-65; Presta et al., 2002, *Biochem Soc Trans* 30:487-490); U.S. Pat. Nos. 5,624,821; 5,885,573; 5,677,425; 6,165,745; 6,277,375; 5,869,046; 6,121,022; 5,624,821; 5,648,260; 6,194,551; 6,737,056; 6,821,505; 6,277,375; U.S. patent application Ser. Nos. 10/370,749 and PCT Publications WO 94/2935; WO 99/58572; WO 00/42072; WO 02/060919, WO 04/029207, each of which is incorporated herein by reference in its entirety.

[0116] In some embodiments, the Fc variants of the present invention comprises one or more engineered glycoforms, i.e., a carbohydrate composition that is covalently attached to a molecule comprising an Fc region. Engineered glycoforms may be useful for a variety of purposes, including but not limited to enhancing or reducing effector function. Engineered glycoforms may be generated by any method known to one skilled in the art, for example by using engineered or variant expression strains, by co-expression with one or more enzymes, for example DI N-acetylglucosaminyltransferase III (GnTI11), by expressing a molecule comprising an Fc region in various organisms or cell lines from various organisms, or by modifying carbohydrate(s) after the molecule comprising Fc region has been expressed. Methods for generating engineered glycoforms are known in the art, and include but are not limited to those described in Umana et al, 1999, *Nat. Biotechnol* 17:176-180; Davies et al., 20017 *Biotechnol Bioeng* 74:288-294; Shields et al, 2002, *J Biol Chem* 277:26733-26740; Shinkawa et al., 2003, *J Biol Chem* 278:3466-3473) U.S. Pat. No. 6,602,684; U.S. Ser. No. 10/277,370; U.S. Ser. No. 10/113,929; PCT WO 00/61739A1; PCT WO 01/292246A1; PCT WO 02/311140A1; PCT WO 02/30954A1; Potillegent™ technology (Biowa, Inc. Princeton, N.J.); GlycoMAb™ glycosylation engineering technology (GLYCART biotechnology AG, Zurich, Switzerland); each of which is incorporated herein by reference in its entirety. See, e.g., WO 00061739; EA01229125; U.S. 20030115614; Okazaki et al., 2004, *JMB,* 336: 1239-49 each of which is incorporated herein by reference in its entirety. Additional methods are described in section 6.2 entitled "Antibodies of the Invention," infra.

[0117] In another embodiment, the Fc variants of the invention are variants of Vitaxin®, its derivatives, analogs, and epitope-binding fragments thereof (such as but not limited to, those disclosed in U.S. Pat. Nos. 6,531,580; 6,590,079; 6,596,850; PCT Publications WO 89/05155, WO 98/33919, and WO 00/78815) each of which is incorporated herein by reference in its entirety.

[0118] Integrins are receptor proteins which are of crucial importance. They are the main way that cells both bind to and respond to the extracellular matrix and are involved in a variety of cellular functions such as wound healing, cell differentiation, homing of tumor cells and apoptosis. They are part of a large family of cell adhesion receptors which are involved in cell-extracellular matrix and cell-cell interactions. Integrin-ligand interactions are accompanied by clustering and activation of the integrins on the cell surface, which is also accompanied by the transduction of signals into intracellular signal transduction pathways that mediate a number of intracellular events. Molecules known to be involved in the downstream signaling events include Focal adhesion kinase (FAK), mitogen activated protein kinase (MAPK) and phospholipase C-gamma (PLC-gamma) among others. As cell surface molecules the Integrins are readily accessible target molecules for antibody directed therapies. In another embodiment, the Fc variants of the invention are variants of an antibody that immunospecifically binds to an Integrin other than Integrin $\alpha_v\beta_3$. Integrins to which an Fc variant of the invention immunospecifically binds to include but are not limited to, Integrin $\alpha_v\beta_1$, Integrin $\alpha_1\beta_1$, Integrin $\alpha_2\beta_1$, Integrin $\alpha_3\beta_1$, Integrin $\alpha_4\beta_1$, Integrin $\alpha_4\beta_7$ Integrin $\alpha_5\beta_1$, Integrin $\alpha_6\beta_1$, Integrin $\alpha_6\beta_4$, Integrin $\alpha_7\beta_1$, Integrin $\alpha_8\beta_1$, Integrin $\alpha_9\beta_1$, Integrin $\alpha_D\beta_2$, Integrin $\alpha_L\beta_2$, Integrin $\alpha_M\beta_2$, Integrin $\alpha_v\beta_1$, Integrin $\alpha_v\beta_5$, Integrin $\alpha_v\beta_6$, Integrin $\alpha_v\beta_8$, Integrin $\alpha_X\beta_2$, Integrin $\alpha_v\beta_1$, Integrin $\alpha_{IIb}\beta_5$, Integrin $\alpha_{IIb}\beta_3$, Integrin $\alpha_{IELb}\beta_7$.

[0119] In a particular embodiment, the Fc variants of the invention are antibodies or fragments thereof that compete with Vitaxin® or an antigen-binding fragment thereof for binding to Integrin $\alpha_v\beta_3$.

[0120] The present invention further encompasses the use of Fc variants of the invention that have a high binding affinity for integrin $\alpha_v\beta_3$. In a specific embodiment, an Fc variant of the invention that immunospecifically binds to integrin $\alpha_v\beta_3$ has an association rate constant or k_{on} rate (Fc variant (Ab)+antigen $(Ag)^k{}_{on}\leftrightarrows$Ab-Ag) of at least $10^5M^{-1}s^{-1}$, at least $5\times10^5M^{-1}s^{-1}$, at least $10^6M^{-1}s^{-1}$, at least $5\times10^6M^{-1}s^{-1}$, at least $10^7M^{-1}s^{-1}$, at least $5\times10^7M^{-1}s^{-1}$, or at least $10^8M^{-1}s^{-1}$. In another embodiment, an Fc variant that immunospecifically binds to integrin $\alpha_v\beta_3$ has a k_{on} of at least $2\times10^5M^{-1}s^{-1}$, at least $5\times10^5M^{-1}s^{-1}$, at least $10^6M^{-1}s^{-1}$, at least $5\times10^6M^{-1}s^{-1}$, at least $10^7M^{-1}s^{-1}$, at least $5\times10^7M^{-1}s^{-1}$, or at least $10^8M^{-1}s^{-1}$.

[0121] In another embodiment, an Fc variant of the invention that immunospecifically binds to integrin $\alpha_{v\beta3}$ has a k_{off} rate (Fc variant (Ab)+antigen $(Ag)^k{}_{off}\leftrightarrows$Ab−Ag) of less than $10^{-1}s^{-1}$, less than $5\times10^{-1}s^{-1}$, less than $10^{-2}s^{-1}$, less than $5\times10^{-2}s^{-1}$, less than $10^{-3}s^{-1}$, less than $5\times10^{-3}s^{-1}$, less than $10^{-4}s^{-1}$, less than $5\times10^{-4}s^{-1}$, less than $10^{-5}s^{-1}$, less than $5\times10^{-5}s^{-1}$, less than $10^{-6}s^{-1}$, less than $5\times10^{-6}s^{-1}$, less than $10^{-7}s^{-1}$, less than $5\times10^{-7}s^{-1}$, less than $10^{-8}s^{-1}$, less than $5\times10^{-8}s^{-1}$, less than $10^{-9}s^{-1}$, less than $5\times10^{-9}s^{-1}$, or less than $10^{-10-1}s^{-1}$. In another embodiment, an Fc variant that immunospecifically binds to integrin $\alpha_v\beta_3$ has a k_{off}, of less than $5\times10^{-4}s^{-1}$, less than $10^{-5}s^{-1}$, less than $5\times10^{-5}s^{-1}$, less than $10^{-6}s^{-1}$, less than $5\times10^{-6}s^{-1}$, less than $10^{-7}s^{-1}$, less than $5\times10^{-7}s^{-1}$, less than $10^{-8}s^{-1}$, less than $5\times10^{-8}s^{-1}$, less than $10^{-9}s^{-1}$, less than $5\times10^{-9}s^{-1}$, or less than $10^{-10}s^{-1}$.

[0122] In another embodiment, an Fc variant of the invention that immunospecifically binds to integrin $\alpha_v\beta_3$ has an affinity constant or K_a (k_{on}/k_{off}) of at least 10^2M^{-1}, at least $5\times10^2M^{-1}$, at least 10^3M^{-1}, at least $5\times10^3M^{-1}$, at least 10^4M^{-1}, at least $5\times10^4M^{-1}$, at least 10^5M^{-1}, at least $5\times10^5M^{-1}$, at least 10^6M^{-1}, at least $5\times10^6M^{-1}$, at least 10^7M^{-1}, at least $5\times10^7M^{-1}$, at least 10^8M^{-1}, at least $5\times10^8M^{-1}$, at least 10^9M^{-1}, at least $5\times10^9M^{-1}$, at least $10^{10}M^{-1}$, at least $5\times10^1M^{-1}$, at least $10^{11}M^{-1}$, at least $5\times10^{11}M^{-1}$, at least $10^{12}M^{-1}$, at least $5\times10^{12}M$, at least $10^{13}M^{-1}$, at least $5\times10^{13}M^{-1}$, at least $10^{14}M^{-1}$, at least $5\times10^{14}M^{-1}$, at least $10^{15}M^{-1}$, or at least $5\times10^{15}M^{-1}$.

[0123] In yet another embodiment, an Fc variant that immunospecifically binds to integrin $\alpha_v\beta_3$ has a dissociation constant or K_d (k_{off}/k_{on}) of less than $10^{-2}M$, less than $5\times10^{-2}M$, less than $10^{-3}M$, less than $5\times10^{-3}M$, less than $10^{-4}M$, less than $5\times10^{-4}M$, less than $10^{-5}M$, less than $5\times10^{-5}M$, less than $10^{-6}M$, less than $5\times10^{-6}M$, less than $10^{-7}M$, less than $5\times10^{-7}M$, less than $10^{-8}M$, less than $5\times10^{-8}M$, less than $10^{-9}M$, less than $5\times10^{-9}M$, less than $10^{-10}M$, less than $5\times10^{-10}M$, less than $10^{-11}M$, less than $5\times10^{-11}M$, less than $10^{-12}M$, less than $5\times10^{-12}M$, less than $10^{-13}M$, less than $5\times10^{13}M$, less than $10^{-14}M$, less than $5\times10^{-14}M$, less than $10^{-15}M$, or less than $5\times10^{-15}M$.

6.1 Fc Variants that Immunospecifically Bind to Integrin $\alpha_v\beta_3$

[0124] As discussed above, the invention encompasses Fc comprising a variable region that immunospecifically binds to Integrin $\alpha_v\beta_3$ and a Fc region that further comprises at least one high effector function amino acid residue (e.g., 239D, 330L, 332E wherein the numbering of the residues is that of the EU index as set forth in Kabat). The invention further encompasses Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$, have altered ADCC and/or CDC activity and modified binding affinities for one or more Fc ligand (e.g., FcγRs, C1q) relative to a comparable molecule. The invention encompasses Fc variants of anti-Integrin $\alpha_v\beta_3$ antibodies including, but not limited to, LM609 (Scripps), the murine monoclonal LM609 (PCT Publication WO 89/015155 and U.S. Pat. No. 5,753,230, each of which is incorporated herein by reference in its entirety); the humanized monoclonal antibody MEDI-522 (a.k.a. VITAXIN®, MedImmune, Inc., Gaithersburg, Md.; Wu et al., 1998, PNAS USA 95(11): 6037-6042; PCT Publications WO 90/33919 and WO 00/78815, each of which is incorporated herein by reference in its entirety); D12 (PCT Publication WO 98/40488); anti-Integrin $\alpha_v\beta_3$ antibody PDE 117-706 (ATCC access No. HB-12224), P112-4C1 (ATCC access No. HB-12225), P113-12A6 (ATCC access No. HB-12226), P112-11D2 (ATCC access No. HB-12227), P112-IOD4 (ATCC access No. HB-12228) and P113-IF3 (ATCC access No. HB-12229). (G.D, Searle & Co., PCT Publication WO 98/46264); 17661-37E and 17661-37E 1-5 (USBiological), MON 2032 and 2033 (CalTag), ab7166 (BV3) and ab 7167 (BV4) (Abcam), WOW-1 (Kiosses et al., 2001, *Nature Cell Biology,* 3:316-320), CNTO 95 (Centocor, PCT publication WO 02/12501 which is incorporated herein by reference in its entirety) and analogs, derivatives, or fragments thereof.

[0125] In one embodiment embodiment, the Fc variant is an Fc variant of Vitaxin®, a humanized blocking monoclonal antibody that binds Integrin $\alpha_v\beta_3$. The amino acid sequence of Vitaxin® is disclosed, e.g., in PCT Publications WO 98/33919; WO 00/78815; and WO 02/070007; U.S. application Ser. No. 09/339,922, each of which is incorporated herein by reference in its entirety. The amino acid sequences for the heavy chain variable region and light chain variable region are provided herein as SEQ ID NO: 3 and SEQ ID NO: 4, respectively **(FIGS. 1A and 1B)**. The nucleotide sequence for the heavy chain variable and light chain variable region are provided herein as SEQ ID NO: 1 and SEQ ID NO: 2, respectively **(FIGS. 1A and 1B)**. In another embodiment, Fc variant of the present invention binds to the same epitope as Vitaxin® or competes with Vitaxin® for binding to Integrin $\alpha_v\beta_3$. In an alternative embodiment, the Fc variant of the invention that immunospecifically binds to Integrin $\alpha_v\beta_3$ is not an Fc variant of Vitaxin®.

[0126] In a specific embodiment, an Fc of the invention is generated by combining a antigen binding domain (e.g., variable region) or fragment thereof of an antibody or fragment thereof that immunospecifically binds Integrin $\alpha_v\beta_3$ (examples supra) with an Fc region comprising at least one high effector function amino acid residue. Methods for generating such a recombinant antibody are well know to one skilled in the art and are further described infra.

[0127] In one embodiment, the Fc variant of the invention preferentially binds Integrin $\alpha_v\beta_3$ over other integrins. In another embodiment, the Fc variant of the invention does not immunoreact with an α_v subunit. In another embodiment, said Fc variant of the invention does immunoreact with an α_v subunit. In still another embodiment, the Fc variant of the invention does not immunoreact with an P3 subunit. In yet another embodiment, the Fc variant of the invention does not immunoreact with integrins other than $\alpha_v\beta_3$. In yet another embodiment, the Fc variant of the invention does immunoreact with a β_3 subunit. In still another embodiment, the Fc variant of the invention immunoreacts with both Integrin $\alpha_v\beta_3$ and Integrin $\alpha_v\beta_5$ or with more then one Integrin $\alpha\beta$ complex. The variant may have the same immunoreactivity for both Integrin $\alpha_v\beta_3$ and Integrin $\alpha_v\beta_5$ or alternatively, the Fc variant may immunoreact more strongly with Integrin $\alpha_v\beta_3$ than with Integrin $\alpha_v\beta_5$, or more strongly with Integrin $\alpha_v\beta_5$ than with Integrin $\alpha_v\beta_3$. In another embodiment the Fc variant binds an integrin other then Integrin $\alpha_v\beta_3$ (e.g., $\alpha_v\beta_1$, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_D\beta_2$, $\alpha_{IIb}\beta_3$).

[0128] The present invention encompasses Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$, said antibodies comprising a variable heavy ("VH") domain having an amino acid sequence of the VH domain for LM609 or VITAXIN®. The present invention also encompasses Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$, said antibodies comprising a variable light ("VL") domain having an amino acid sequence of the VL domain for LM609 or VITAXIN®. The invention further encompasses Fc variants that immuno-specifically bind to Integrin $\alpha_v\beta_3$, said antibodies comprising a VH domain disclosed herein combined with a VL domain disclosed herein, or other VL domain. The present invention further encompasses Fc variants Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$, said Fc variants comprising a VL domain disclosed herein combined with a VH domain disclosed herein, or other VH domain.

[0129] The present invention encompasses Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$, said antibodies comprising a VH CDR having an amino acid sequence of any one of the VH CDRs listed in Table 3 infra. The present invention also encompasses Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$ said antibodies comprising a VL CDR having an amino acid sequence of any one of the VL CDRs listed in Table 3 infra. The present invention also encompasses Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$, said Fc variants comprising one or more VH CDRs and one or more VL CDRs listed in Table 3. The present invention further encompasses Fc variants that immunospecifically binds to Integrin $\alpha_v\beta_3$ and Fc variants comprising any combination of some or all of the VH CDRs and VL CDRs listed in Table 3 infra.

TABLE 3

CDR Sequences Of LM609 and Vitaxin ®

CDR	Sequence	SEQ ID NO:
LM609 VH1	SYDMS	5
LM609 VH2	KVSSGGGSTYYLDTVQG	6
LM609 VH3	HNYGSFAY	7
LM609 VL1	QASQSISNHLH	8
LM609 VL2	YRSQSIS	9
LM609 VL3	QQSGSWPHT	10
Vitaxin ® VH1	SYDMS	70
Vitaxin ® VH2	KVSSGGGSTYYLDTVQG	71
Vitaxin ® VH3	HLHGSFAS	72
Vitaxin ® VL1	QASQSISNFLH	73
Vitaxin ® VL2	TRSQSIS	74
Vitaxin ® VL3	QQSGSYPLT	75

[0130] The present invention also encompasses Fc variants that compete with Vitaxin®, LM609 or CNTO 95 or an antigen-binding fragment thereof for binding to Integrin $\alpha_v\beta_3$. Competition assays, which can be used to identify such antibodies, are well known to one skilled in the art. In a particular embodiment, 1 μg/ml of an antibody of the invention prevents 75%, 80%, 85% or 90% of ORIGEN TAG labeled LM609, Vitaxin® or CNTO 95 from binding to biotin-labeled Integrin $\alpha_v\beta_3$ as measured by well-known ORIGEN analysis. In another embodiment, the invention encompasses Fc variants of antibodies other than those disclosed in WO 98/40488 that compete with Vitaxin®, LM609 or an antigen-binding fragment thereof for binding to Integrin $\alpha_v\beta_3$.

[0131] The present invention also provides Fc variants that comprise a framework region known to those of skill in the art. In one embodiment, the fragment region of an antibody of the invention or fragment thereof is human or humanized. In a specific embodiment, an Fc variant of the invention comprises the framework region of Vitaxin® and/or one or more CDRs from Vitaxin® (Table 3 supra).

[0132] The present invention encompasses Fc variants comprising the amino acid sequence of Vitaxin® with mutations (e.g., one or more amino acid substitutions) in the framework or variable regions in addition to any other substitutions or changes (e.g., Fc substitution(s) as described supra). In one embodiment, mutations in these antibodies maintain or enhance the avidity and/or affinity of the antibodies for the Integrin $\alpha_v\beta_3$ to which they immunospecifically bind. Standard techniques known to those skilled in the

art (e.g., immunoassays) can be used to assay the affinity of an antibody for a particular antigen.

[0133] The present invention encompasses the use of a nucleic acid molecule(s), generally isolated, encoding an Fc variant that immunospecifically binds to Integrin $\alpha_v\beta_3$. In a specific embodiment, an isolated nucleic acid molecule encodes an Fc variant that immunospecifically binds to Integrin $\alpha_v\beta_3$, said Fc variant having the amino acid sequence of LM609 or Vitaxin® containing one or more Fc substitution (e.g. supra). In another embodiment, an isolated nucleic acid molecule encodes an Fc variant that immunospecifically binds to Integrin $\alpha_v\beta_3$ said Fc variant comprising a VH domain having the amino acid sequence of the VH domain of LM609 or Vitaxin®. In another embodiment, an isolated nucleic acid molecule encodes an Fc variant that immunospecifically binds to Integrin $\alpha_v\beta_3$, said antibody comprising a VL domain having the amino acid sequence of the VL domain of LM609 or Vitaxin®.

[0134] The invention encompasses the use of an isolated nucleic acid molecule encoding an Fc variant that immunospecifically binds to Integrin $\alpha_v\beta_3$, said Fc variant comprising a VH CDR having the amino acid sequence of any of the VH CDRs listed in Table 3, supra. In particular, the invention encompasses the use of an isolated nucleic acid molecule encoding an Fc variant that immunospecifically binds to Integrin $\alpha_v\beta_3$ said antibody comprising one, two, or more VH CDRs having the amino acid sequence of any of the VH CDRs listed in Table 3, supra.

[0135] The present invention encompasses the use of an isolated nucleic acid molecule encoding an Fc variant that immunospecifically binds to Integrin $\alpha_v\beta_3$, said Fc variant comprising a VL CDR having an amino acid sequence of any of the VL CDRs listed in Table 3, supra. In particular, the invention encompasses the use of an isolated nucleic acid molecule encoding an Fc variant that immunospecifically binds to Integrin $\alpha_v\beta_3$, said antibody comprising one, two or more VL CDRs having the amino acid sequence of any of the VL CDRs listed in Table 3, supra.

[0136] The present invention encompasses the use of Fc variants that immuno-specifically bind to Integrin $\alpha_v\beta_3$, Fc variants comprising derivatives of the VH domains, VH CDRs, VL domains, or VL CDRs described herein that immunospecifically bind to Integrin $\alpha_v\beta_3$. Standard techniques known to those of skill in the art can be used to introduce mutations (e.g., additions, deletions, and/or substitutions) in the nucleotide sequence encoding an antibody of the invention, including, for example, site-directed mutagenesis and PCR-mediated mutagenesis are routinely used to generate amino acid substitutions. In one embodiment, the VH and/or VL CDRs derivatives include less than 25 amino acid substitutions, less than 20 amino acid substitutions, less than 15 amino acid substitutions, less than 10 amino acid substitutions, less than 5 amino acid substitutions, less than 4 amino acid substitutions, less than 3 amino acid substitutions, or less than 2 amino acid substitutions in the relative to the original VH and/or VL CDRs. In another embodiment, the VH and/or VL CDRs derivatives have conservative amino acid substitutions (e.g. supra) are made at one or more predicted non-essential amino acid residues (i.e., amino acid residues which are not critical for the antibody to immunospecifically bind to Integrin $\alpha_v\beta_3$). Alternatively, mutations can be introduced randomly along all or part of the VH and/or VL CDR coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis, the encoded antibody can be expressed and the activity of the antibody can be determined.

[0137] The present invention encompasses Fc variants of LM609 or Vitaxin® with one or more additional amino acid residue substitutions in the variable light (VL) domain and/or variable heavy (VH) domain. The present invention also encompasses Fc variants of LM609 or Vitaxin® with one or more additional amino acid residue substitutions in one or more VL CDRs and/or one or more VH CDRs. The antibody generated by introducing substitutions in the VH domain, VH CDRs, VL domain and/or VL CDRs of an Fc variant of LM609 or Vitaxin® can be tested in vitro and in vivo, for example, for its ability to bind to Integrin $\alpha_v\beta_3$ and/or FcγRs (by, e.g., immunoassays including, but not limited to ELISAs and BIAcore), or for its ability to mediate ADCC, prevent, treat, manage or ameliorate cancer or one or more symptoms thereof.

[0138] The present invention also encompasses the use of Fc variants that immuno-specifically bind to Integrin $\alpha_v\beta_3$ or a fragment thereof, said Fc variants comprising an amino acid sequence of a variable heavy chain and/or variable light chain that is at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of the variable heavy chain and/or light chain of Vitaxin® (i.e., SEQ ID NO:3 and/or SEQ ID NO:4). The present invention further encompasses the use of Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$ or a fragment thereof, said antibodies or antibody fragments comprising an amino acid sequence of one or more CDRs that is at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of one or more CDRs of Vitaxin®. The determination of percent identity of two amino acid sequences can be determined by any method known to one skilled in the art, including BLAST protein searches.

[0139] The present invention also encompasses the use of Fc variants that immuno-specifically bind to Integrin $\alpha_v\beta_3$ or fragments thereof, where said Fc variants are encoded by a nucleotide sequence that hybridizes to the nucleotide sequence of Vitaxin® (i.e., SEQ ID NO: 1 and/or SEQ ID NO: 2) under stringent conditions. In another embodiment, the invention encompasses Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$ or a fragment thereof, said Fc variants comprising one or more CDRs encoded by a nucleotide sequence that hybridizes under stringent conditions to the nucleotide sequence of one or more CDRs of Vitaxin®. Stringent hybridization conditions include, but are not limited to, hybridization to filter-bound DNA in 6× sodium chloride/sodium citrate (SSC) at about 45° C. followed by one or more washes in 0.2×SSC/0.1% SDS at about 50-65° C., highly stringent conditions such as hybridization to filter-bound DNA in 6×SSC at about 45° C. followed by one or more washes in 0.1×SSC/0.2% SDS at about 60° C., or any other stringent hybridization conditions known to those skilled in the art (see, for example, Ausubel, F. M. et al., eds. 1989 Current Protocols in Molecular

Biology, vol. 1, Green Publishing Associates, Inc. and John Wiley and Sons, Inc., NY at pages 6.3.1 to 6.3.6 and 2.10.3).

[0140] Set forth below, is a more detailed description of the antibodies encompassed within the various aspects of the invention.

6.2 Antibodies of the Invention

[0141] Fc variants of the invention may include, but are not limited to, synthetic antibodies, monoclonal antibodies, oligoclonal antibodies, recombinantly produced antibodies, intrabodies, multispecific antibodies, bispecific antibodies, human antibodies, humanized antibodies, chimeric antibodies, synthetic antibodies, single-chain FvFcs (scFvFc), single-chain Fvs (scFv), and anti-idiotypic (anti-Id) antibodies. In particular, antibodies used in the methods of the present invention include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules. The antibodies of the invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA, and IgA_2) or subclass of immunoglobulin molecule.

[0142] Fc variants of the invention may be from any animal origin including birds and mammals (e.g., human, murine, donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken). In one embodiment, the antibodies are human or humanized monoclonal antibodies. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from mice that express antibodies from human genes.

[0143] Antibodies like all polypeptides have an Isoelectric Point (pI), which is generally defined as the pH at which a polypeptide carries no net charge. It is known in the art that protein solubility is typically lowest when the pH of the solution is equal to the isoelectric point (pI) of the protein. It is possible to optimize solubility by altering the number and location of ionizable residues in the antibody to adjust the pI. For example the pI of a polypeptide can be manipulated by making the appropriate amino acid substitutions (e.g., by substituting a charged amino acid such as a lysine, for an uncharged residue such as alanine). Without wishing to be bound by any particular theory, amino acid substitutions of an antibody that result in changes of the pI of said antibody may improve solubility and/or the stability of the antibody. One skilled in the art would understand which amino acid substitutions would be most appropriate for a particular antibody to achieve a desired pI. The pI of a protein may be determined by a variety of methods including but not limited to, isoelectric focusing and various computer algorithms (see for example Bjellqvist et al., 1993, *Electrophoresis* 14:1023-1031). In one embodiment, the pI of the Fc variants of the invention is higher then about 6.5, about 7.0, about 7.5, about 8.0, about 8.5, or about 9.0. In one embodiment, substitutions resulting in alterations in the pI of the Fc variant of the invention will not significantly diminish its binding affinity for Integrin $\alpha_v\beta_3$. In another embodiment, the pI of the Fc variants of the invention is higher then 6.5, 7.0, 7.5, 8.0, 8.5, or 9.0. It is specifically contemplated that the substitution(s) of the Fc region that result in altered binding to FcγR (described supra) may also result in a change in the pI. In another embodiment, substitution(s) of the Fc region are specifically chosen to effect both the desired alteration in FcγR binding and any desired change in pI. As used herein the pI value is defined as the pI of the predominant charge form. The pI of a protein may be determined by a variety of methods including but not limited to, isoelectric focusing and various computer algorithms (see, e.g., Bjellqvist et al., 1993, *Electrophoresis* 14:1023).

[0144] The Tm of the Fab domain of an antibody, can be a good indicator of the thermal stability of an antibody and may further provide an indication of the shelf-life. A lower Tm indicates more aggregation/less stability, whereas a higher Tm indicates less aggregation/more stability. Thus, antibodies having higher Tm are preferable. In one embodiment, the Fab domain of an Fc variant has a Tm value higher than at least 50° C., 55° C., 60° C., 65° C., 70° C., 75° C., 80° C., 85° C., 90° C., 95° C., 100° C., 105° C., 1101C, 115° C. or 120° C. Thermal melting temperatures (Tm) of a protein domain (e.g., a Fab domain) can be measured using any standard method known in the art, for example, by differential scanning calorimetry (see, e.g., Vermeer et al., 2000, *Biophys. J.* 78:394-404; Vermeer et al., 2000, *Biophys. J.* 79: 2150-2154).

[0145] Fc variants of the invention may be monospecific, bispecific, trispecific or have greater multispecificity. Multispecific antibodies may immunospecifically bind to different epitopes of desired target molecule or may immunospecifically bind to both the target molecule as well as a heterologous epitope, such as a heterologous polypeptide or solid support material. See, e.g., International Publication Nos. WO 94/04690; WO 93/17715; WO 92/08802; WO 91/00360; and WO 92/05793; Tutt, et al., 1991, *J. Immunol.* 147:60-69; U.S. Pat. Nos. 4,474,893, 4,714,681, 4,925,648, 5,573,920, and 5,601,819; and Kostelny et al., 1992, *J. Immunol.* 148:1547-1553). In the present case, one of the binding specificities is for Integrin $\alpha_v\beta_3$, the other one is for any other antigen (i.e., another integrin, a signaling or effector molecule).

[0146] Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by the instant invention. Examples of BsAbs include without limitation those with one arm directed against a Integrin $\alpha_v\beta_3$ and the other arm directed against any other antigen. Methods for making bispecific antibodies are known in the art. Traditional production of full-length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., 1983, *Nature,* 305:537-539 which is incorporated herein by reference in its entirety). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., 1991, *EMBO J.,* 10:3655-3659. A more directed approach is the generation of a Di-diabody a tetravalent bispecific antbodiy. Methods for producing a Di-diabody are known in the art (see e.g., Lu et al., 2003, J Immunol Methods 279:219-32; Marvin et al., 2005, Acta Pharmacolical Sinica 26:649).

[0147] According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when, the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

[0148] In one embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm (e.g., Integrin $\alpha_v\beta_3$), and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 (incorporated herein by reference in its entirety). For further details of generating bispecific antibodies see, for example, Suresh et al., 1986, *Methods in Enzymology,* 121:210 (incorporated herein by reference in its entirety). According to another approach described in WO96/27011 (incorporated herein by reference in its entirety), a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the CH3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

[0149] Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089) The above referencecs are each incorporated herein by reference in their entireties. Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques. Each of the above references is incorporated herein by reference in its entirety.

[0150] Antibodies with more than two valencies incorporating at least one hinge modification of the invention are contemplated. For example, trispecific antibodies can be prepared. See, e.g., Tutt et al. J. Immunol. 147: 60 (1991), which is incorporated herein by reference.

[0151] The Fc variants of the invention encompass single domain antibodies, including camelized single domain antibodies (see e.g., Muyldermans et al., 2001, *Trends Biochem. Sci.* 26:230; Nuttall et al., 2000, *Cur. Pharm. Biotech.* 1:253; Reichmann and Muyldermans, 1999, *J. Immunol. Meth.* 231:25; International Publication Nos. WO 94/04678 and WO 94/25591; U.S. Pat. No. 6,005,079; which are incorporated herein by reference in their entireties).

[0152] Other antibodies specifically contemplated are "oligoclonal" antibodies. As used herein, the term "oligoclonal" antibodies" refers to a predetermined mixture of distinct monoclonal antibodies. See, e.g., PCT publication WO 95/20401; U.S. Pat. Nos. 5,789,208 and 6,335,163 which are incorporated by reference herein. In one embodiment, oligoclonal antibodies consist of a predetermined mixture of antibodies against one or more epitopes are generated in a single cell. In another embodiment, oligoclonal antibodies comprise a plurality of heavy chains capable of pairing with a common light chain to generate antibodies with multiple specificities (e.g., PCT publication WO 04/009618 which is incorporated by reference herein). Oligoclonal antibodies are particularly useful when it is desired to target multiple epitopes on a single target molecule (e.g., Integrin $\alpha_v\beta_3$). Those skilled in the art will know or can determine what type of antibody or mixture of antibodies is applicable for an intended purpose and desired need.

[0153] Antibodies of the present invention also encompass Fc variants that have half-lives (e.g., serum half-lives) in a mammal, (e.g., a human), of greater than 5 days, greater than 10 days, greater than 15 days, greater than 20 days, greater than 25 days, greater than 30 days, greater than 35 days, greater than 40 days, greater than 45 days, greater than 2 months, greater than 3 months, greater than 4 months, or greater than 5 months. The increased half-lives of the antibodies of the present invention in a mammal, (e.g., a human), results in a higher serum titer of said antibodies or antibody fragments in the mammal, and thus, reduces the frequency of the administration of said antibodies or antibody fragments and/or reduces the concentration of said antibodies or antibody fragments to be administered. Antibodies having increased in vivo half-lives can be generated by techniques known to those of skill in the art. For example, antibodies with increased in vivo half-lives can be generated by modifying (e.g., substituting, deleting or adding) amino acid residues identified as involved in the interaction between the Fc domain and the FcRn receptor (see, e.g., International Publication Nos. WO 97/34631; WO 04/029207; U.S. Pat. No. 6,737056 and U.S. Patent Publication No. 2003/0190311, each of which are incorporated herein by reference in their entireties).

[0154] In one embodiment, the Fc variants of the invention may be chemically modified (e.g., one or more chemical moieties can be attached to the antibody) or be modified to alter its glycosylation, again to alter one or more functional properties of the antibody.

[0155] In still another embodiment, the glycosylation of the Fc variants of the invention is modified. For example, an aglycoslated antibody can be made (i.e., the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for a target antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in further detail in U.S. Pat. Nos. 5,714,350 and 6,350,861, each of which is incorporated herein by reference in its entirety.

[0156] Additionally or alternatively, an Fc variant can be made that has an altered type of glycosylation, such as a hypofucosylated antibody having reduced amounts of fucosyl residues or an antibody having increased bisecting GlcNAc structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an antibody with altered glycosylation. See, for example, Shields, R. L. et al. (2002) *J. Biol. Chem.* 277:26733-26740; Umana et al. (1999) *Nat. Biotech.* 17:176-1, as well as, European Patent No: EP 1,176,195; PCT Publications WO 03/035835; WO 99/54342, each of which is incorporated herein by reference in its entirety.

[0157] In still another embodiment, the glycosylation of an Fc variant of the invention is modified. For example, an aglycoslated antibody can be made (i.e., the antibody lacks glycosylation). Glycosylation can be altered to, for example, increase the affinity of the antibody for a target antigen. Such carbohydrate modifications can be accomplished by, for example, altering one or more sites of glycosylation within the antibody sequence. For example, one or more amino acid substitutions can be made that result in elimination of one or more variable region framework glycosylation sites to thereby eliminate glycosylation at that site. Such aglycosylation may increase the affinity of the antibody for antigen. Such an approach is described in further detail in U.S. Pat. Nos. 5,714,350 and 6,350,861, each of which is incorporated herein by reference in its entirety.

[0158] Additionally or alternatively, an Fc variant can be made that has an altered type of glycosylation, such as a hypofucosylated Fc variant having reduced amounts of fucosyl residues or an Fc variant having increased bisecting GlcNAc structures. Such altered glycosylation patterns have been demonstrated to increase the ADCC ability of antibodies. Such carbohydrate modifications can be accomplished by, for example, expressing the antibody in a host cell with altered glycosylation machinery. Cells with altered glycosylation machinery have been described in the art and can be used as host cells in which to express recombinant antibodies of the invention to thereby produce an antibody with altered glycosylation. See, for example, Shields, R. L. et al. (2002) *J. Biol. Chem.* 277:26733-26740; Umana et al. (1999) *Nat. Biotech.* 17:176-1, as well as, European Patent No: EP 1,176,195; PCT Publications WO 03/035835; WO 99/54342, each of which is incorporated herein by reference in its entirety.

6.3 Antibody Conjugates and Derivatives

[0159] Fc variants of the invention include derivatives that are modified (i.e., by the covalent attachment of any type of molecule to the antibody such that covalent attachment). For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Additionally, the derivative may contain one or more non-classical amino acids.

[0160] Antibodies or fragments thereof with increased in vivo half-lives can be generated by attaching to said antibodies or antibody fragments polymer molecules such as high molecular weight polyethyleneglycol (PEG). PEG can be attached to said antibodies or antibody fragments with or without a multifunctional linker either through site-specific conjugation of the PEG to the N- or C-terminus of said antibodies or antibody fragments or via epsilon-amino groups present on lysine residues. Linear or branched polymer derivatization that results in minimal loss of biological activity will be used. The degree of conjugation will be closely monitored by SDS-PAGE and mass spectrometry to ensure proper conjugation of PEG molecules to the antibodies. Unreacted PEG can be separated from antibody-PEG conjugates by, e.g., size exclusion or ion-exchange chromatography.

[0161] Further, antibodies can be conjugated to albumin in order to make the antibody or antibody fragment more stable in vivo or have a longer half life in vivo. The techniques are well known in the art, see e.g., International Publication Nos. WO 93/15199, WO 93/15200, and WO 01/77137; and European Patent No. EP 413,622, all of which are incorporated herein by reference. The present invention encompasses the use of antibodies or fragments thereof conjugated or fused to one or more moieties, including but not limited to, peptides, polypeptides, proteins, fusion proteins, nucleic acid molecules, small molecules, mimetic agents, synthetic drugs, inorganic molecules, and organic molecules.

[0162] The present invention encompasses the use of antibodies or fragments thereof recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugations) to a heterologous protein or polypeptide (or fragment thereof, preferably to a polypeptide of at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 amino acids) to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences. For example, antibodies may be used to target heterologous polypeptides to particular cell types, either in vitro or in vivo, by fusing or conjugating the antibodies to antibodies specific for particular cell surface receptors. Antibodies fused or conjugated to heterologous polypeptides may also be used in in vitro immunoassays and purification methods using methods known in the art. See e.g., Interna-

tional publication No. WO 93/21232; European Patent No. EP 439,095; Naramura et al., 1994, Immunol. Lett. 39:91-99; U.S. Pat. No. 5,474,981; Gillies et al., 1992, PNAS 89:1428-1432; and Fell et al., 1991, J. Immunol. 146:2446-2452, which are incorporated by reference in their entireties.

[0163] The present invention further includes formulations comprising heterologous proteins, peptides or polypeptides fused or conjugated to antibody fragments. For example, the heterologous polypeptides may be fused or conjugated to a Fab fragment, Fd fragment, Fv fragment, $F(ab)_2$ fragment, a VH domain, a VL domain, a VH CDR, a VL CDR, or fragment thereof. Methods for fusing or conjugating polypeptides to antibody portions are well known in the art. See, e.g., U.S. Pat. Nos. 5,336,603, 5,622,929, 5,359,046, 5,349,053, 5,447,851, and 5,112,946; European Patent Nos. EP 307,434 and EP 367,166; International publication Nos. WO 96/04388 and WO 91/06570; Ashkenazi et al., 1991, Proc. Natl. Acad. Sci. USA 88: 10535-10539; Zheng et al., 1995, J. Immunol. 154:5590-5600; and Vil et al., 1992, Proc. Natl. Acad. Sci. USA 89:11337-11341 (said references incorporated by reference in their entireties).

[0164] Additional fusion proteins, e.g., of Vitaxin® or other anti-integrin $\alpha_v\beta_3$ antibodies, may be generated through the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling"). DNA shuffiing may be employed to alter the activities of antibodies of the invention or fragments thereof (e.g., antibodies or fragments thereof with higher affinities and lower dissociation rates). See, generally, U.S. Pat. Nos. 5,605,793; 5,811,238; 5,830,721; 5,834,252; and 5,837,458, and Patten et al., 1997, Curr. Opinion Biotechnol. 8:724-33; Harayama, 1998, Trends Biotechnol. 16(2): 76-82; Hansson, et al., 1999, J. Mol. Biol. 287:265-76; and Lorenzo and Blasco, 1998, Biotechniques 24(2): 308-313 (each of these patents and publications are hereby incorporated by reference in its entirety). Antibodies or fragments thereof, or the encoded antibodies or fragments thereof, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. One or more portions of a polynucleotide encoding an antibody or antibody fragment, which portions immunospecifically bind to Integrin $\alpha_v\beta_3$ may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules.

[0165] Moreover, the antibodies or fragments thereof can be fused to marker sequences, such as a peptide to facilitate purification. In specific embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, Calif., 91311), among others, many of which are commercially available. As described in Gentz et al., 1989, Proc. Natl. Acad. Sci. USA 86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984, Cell 37:767) and the "flag" tag.

[0166] In other embodiments, Fc variants of the present invention or analogs or derivatives thereof are conjugated to a diagnostic or detectable agent. Such antibodies can be useful for monitoring or prognosing the development or progression of a cancer as part of a clinical testing procedure, such as determining the efficacy of a particular therapy. Such diagnosis and detection can be accomplished by coupling the antibody to detectable substances including, but not limited to various enzymes, such as but not limited to horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such as but not limited to streptavidin/biotin and avidin/biotin; fluorescent materials, such as but not limited to, umbelliferone, fluorescein, fluorescein isothiocynate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; luminescent materials, such as but not limited to, luminol; bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin; radioactive materials, such as but not limited to iodine (^{131}I, ^{125}I, ^{123}I, ^{121}I,), carbon (^{14}C), sulfur (^{35}S), tritium (^{3}H), indium (^{115}In, ^{113}In, ^{112}In, ^{111}In,), and technetium (^{99}Tc), thallium (^{201}Ti), gallium (^{68}Ga, ^{67}Ga), palladium (^{103}Pd), molybdenum (^{99}Mo), xenon (^{133}Xe), fluorine (^{18}F), ^{153}Sm, ^{177}Lu, ^{159}Gd, ^{149}Pm, ^{140}La, ^{175}Yb, ^{166}Ho, ^{90}Y, ^{47}Sc, ^{186}Re, ^{188}Re, ^{142}Pr, ^{105}Rh, ^{97}Ru, ^{68}Ge, ^{57}Co, ^{65}Zn, ^{85}Sr, ^{32}P, ^{153}Gd, ^{169}Yb, ^{51}Cr, ^{54}Mn, ^{75}Se, ^{113}Sn, and ^{117}Tin; positron emitting metals using various positron emission tomographies, noradioactive paramagnetic metal ions, and molecules that are radiolabelled or conjugated to specific radioisotopes.

[0167] The present invention further encompasses uses of Fc variants of the invention or fragments thereof conjugated to a therapeutic agent.

[0168] In other embodiments, Fc variants of the invention may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytocidal agent, a therapeutic agent or a radioactive metal ion, e.g., alpha-emitters. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include paclitaxel, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, epirubicin, and cyclophosphamide and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BCNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cisdichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), anti-mitotic agents (e.g., vincristine and vinblastine), and auristatin E compounds (e.g. monomethyl auristatin E; see for example U.S. Pat. No. 6,884,869). A more extensive list of therapeutic moieties can be found in PCT publications WO 03/075957;

[0169] In other embodiments, Fc variants of the invention may be conjugated to a therapeutic agent or drug moiety that modifies a given biological response. Therapeutic agents or drug moieties are not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety

may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, Onconase (or another cytotoxic RNase), pseudomonas exotoxin, cholera toxin, or diphtheria toxin; a protein such as tumor necrosis factor, α-interferon, β-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator, an apoptotic agent, e.g., TNF-α, TNF-β, AIM I (see, International Publication No. WO 97/33899), AIM II (see, International Publication No. WO 97/34911), Fas Ligand (Takahashi et al., 1994, *J. Immunol.*, 6:1567), and VEGI (see, International Publication No. WO 99/23105), a thrombotic agent or an anti-angiogenic agent, e.g., angiostatin or endostatin; or, a biological response modifier such as, for example, a lymphokine (e.g., interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), and granulocyte colony stimulating factor ("G-CSF")), or a growth factor (e.g., growth hormone ("GH")).

[0170] In other embodiments, Fc variants of the invention can be conjugated to therapeutic moieties such as a radioactive materials or macrocyclic chelators useful for conjugating radiometal ions (see above for examples of radioactive materials). In certain embodiments, the macrocyclic chelator is 1,4,7,10-tetraazacyclododecane-N,N',N",N"-tetraacetic acid (DOTA) which can be attached to the antibody via a linker molecule. Such linker molecules are commonly known in the art and described in Denardo et al., 1998, *Clin Cancer Res.* 4:2483-90; Peterson et al., 1999, *Bioconjug. Chem.* 10:553; and Zimmerman et al., 1999, *Nucl. Med. Biol.* 26:943-50 each incorporated by reference in their entireties.

[0171] Techniques for conjugating therapeutic moieties to antibodies are well known. Moieties can be conjugated to antibodies by any method known in the art, including, but not limited to aldehyde/Schiff linkage, sulphydryl linkage, acid-labile linkage, cis-aconityl linkage, hydrazone linkage, enzymatically degradable linkage (see generally Garnett, 2002, *Adv Drug Deliv Rev* 53:171-216). Techniques for conjugating therapeutic moieties to antibodies are well known, see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd Ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc. 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., 1982, *Immunol. Rev.* 62:119-58.

[0172] Methods for fusing or conjugating antibodies to polypeptide moieties are known in the art. See, e.g., U.S. Pat. Nos. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851, and 5,112,946; EP 307,434; EP 367,166; PCT Publications WO 96/04388 and WO 91/06570; Ashkenazi et al., 1991, *PNAS USA* 88:10535-10539; Zheng et al., 1995, *J Immunol* 154:5590-5600; and Vil et al., 1992, PNAS USA 89:11337-11341. The fusion of an antibody to a moiety does not necessarily need to be direct, but may occur through linker sequences. Such linker molecules are commonly known in the art and described in Denardo et al., 1998, Clin Cancer Res 4:2483-90; Peterson et al., 1999, *Bioconjug Chem* 10:553; Zimmerman et al., 1999, *Nucl Med Biol* 26:943-50; Garnett, 2002, *Adv Drug Deliv Rev* 53:171-216, each of which is incorporated herein by reference in its entirety.

[0173] Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Pat. No. 4,676,980, which is incorporated herein by reference in its entirety.

[0174] Antibodies may also be attached to solid supports, which are particularly useful for immunoassays or purification of the target antigen. Such solid supports include, but are not limited to, glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

[0175] The therapeutic moiety or drug conjugated to an antibody or fragment thereof that immunospecifically binds to Integrin $\alpha_v\beta_3$ should be chosen to achieve the desired prophylactic or therapeutic effect(s) for a particular disorder in a subject. A clinician or other medical personnel should consider the following when deciding on which therapeutic moiety or drug to conjugate to an antibody or fragment thereof that immunospecifically binds to Integrin $\alpha_v\beta_3$: the nature of the disease, the severity of the disease, and the condition of the subject.

6.4 Methods Of Generating Antibodies

[0176] The Fc variants of the invention can be produced by any method known in the art for the synthesis of antibodies, in particular, by chemical synthesis or by recombinant expression techniques.

[0177] Polyclonal antibodies to Integrin $\alpha_v\beta_3$ can be produced by various procedures well known in the art. For example, Integrin $\alpha_v\beta_3$ or immunogenic fragments thereof can be administered to various host animals including, but not limited to, rabbits, mice, rats, etc. to induce the production of sera containing polyclonal antibodies specific for Integrin $\alpha_v\beta_3$. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *corynebacterium parvum*. Such adjuvants are also well known in the art.

[0178] Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The

term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

[0179] Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. Briefly, mice can be immunized with Integrin $\alpha_v\beta_3$ or a domain thereof (e.g., the extracellular domain) and once an immune response is detected, e.g., antibodies specific for Integrin $\alpha_v\beta_3$ are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Additionally, a RIMMS (repetitive immunization, multiple sites) technique can be used to immunize an animal (Kilpatrick et al., 1997, *Hybridoma* 16:381-9, incorporated herein by reference in its entirety). Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding a polypeptide of the invention. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing mice with positive hybridoma clones.

[0180] Accordingly, monoclonal antibodies can be generated by culturing a hybridoma cell secreting an antibody wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with Integrin $\alpha_v\beta_3$ or immunogenic fragments thereof, with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind Integrin $\alpha_v\beta_3$.

[0181] The Fc variants of the invention contain novel amino acid residues in their Fc regions. Fc variants can be generated by numerous methods well known to one skilled in the art. Non-limiting examples include, isolating antibody coding regions (e.g., from hybridoma) and making one or more desired substitutions in the Fc region of the isolated antibody coding region. Alternatively, the variable regions may be subcloned into a vector encoding an Fc region comprising one or more high effector function amino acid residues. Additional methods and details are provided below.

[0182] Antibody fragments that recognize specific Integrin $\alpha_v\beta_3$ epitopes may be generated by any technique known to those of skill in the art. For example, Fab and F(ab')2 fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). F(ab')2 fragments contain the variable region, the light chain constant region and the CH1 domain of the heavy chain. Further, the antibodies of the present invention can also be generated using various phage display methods known in the art.

[0183] In phage display methods, functional antibody domains are displayed on the surface of phage particles that carry the polynucleotide sequences encoding them. In particular, DNA sequences encoding VH and VL domains are amplified from animal cDNA libraries (e.g., human or murine cDNA libraries of lymphoid tissues). The DNA encoding the VH and VL domains are recombined together with an scFv linker by PCR and cloned into a phagemid vector (e.g., p CANTAB 6 or pComb 3 HSS). The vector is electroporated in *E. coli* and the *E. coli* is infected with helper phage. Phage used in these methods are typically filamentous phage including fd and M13 and the VH and VL domains are usually recombinantly fused to either the phage gene III or gene VIII. Phage expressing an antigen binding domain that binds to the Integrin $\alpha_v\beta_3$ epitope of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman et al., 1995, J. Immunol. Methods 182:41-50; Ames et al., 1995, J. Immunol. Methods 184:177-186; Kettleborough et al., 1994, Eur. J. Immunol. 24:952-958; Persic et al., 1997, Gene 187:9-18; Burton et al., 1994, Advances in Immunology 57:191-280; PCT Publication Nos. WO 90/02809, WO 91/10737, WO 92/01047, WO 92/18619, WO 93/11236, WO 95/15982, WO 95/20401, and WO97/13844; and U.S. Pat. Nos. 5,698,426, 5,223,409, 5,403,484, 5,580,717, 5,427,908, 5,750,753, 5,821,047, 5,571,698, 5,427,908, 5,516,637, 5,780,225, 5,658,727, 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

[0184] As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described below. Techniques to recombinantly produce Fab, Fab' and F(ab')2 fragments can also be employed using methods known in the art such as those disclosed in International Publication No. WO 92/22324; Mullinax et al., 1992, BioTechniques 12(6): 864-869; Sawai et al., 1995, AJRI 34:26-34; and Better et al., 1988, Science 240:1041-1043 (said references incorporated by reference in their entireties).

[0185] To generate whole antibodies, PCR primers including VH or VL nucleotide sequences, a restriction site, and a flanking sequence to protect the restriction site can be used to amplify the VH or VL sequences in scFv clones. Utilizing cloning techniques known to those of skill in the art, the PCR amplified VH domains can be cloned into vectors expressing a VH constant region, e.g., the human gamma constant, and the PCR amplified VL domains can be cloned into vectors expressing a VL constant region, e.g., human kappa or lamba constant regions. In one embodiment, the constant region is an Fc region containing at least one high effector function amino acid. In a specific embodiment, the vectors for expressing the VH or VL domains comprise a promoter, a secretion signal, a cloning site for both the variable and constant domains, as well as a selection marker such as neomycin. The VH and VL domains may also be cloned into one vector expressing the desired constant regions. The heavy chain conversion vectors and light chain conversion vectors are then co-transfected into cell lines to generate stable or transient cell lines that express fill-length antibodies, e.g., IgG, using techniques known to those of skill in the art.

[0186] A chimeric antibody is a molecule in which different portions of the antibody are derived from different immunoglobulin molecules. Methods for producing chimeric antibodies are known in the art. See e.g., Morrison, 1985, Science 229:1202; Oi et al., 1986, BioTechniques

4:214; Gillies et al., 1989, J. Immunol. Methods 125:191-202; and U.S. Pat. Nos. 5,807,715, 4,816,567, 4,8 16397, and 6,311,415, which are incorporated herein by reference in their entirety.

[0187] For some uses, including in vivo use of antibodies in humans and in vitro detection assays, it may be preferable to use human or chimeric antibodies. Completely human antibodies are particularly desirable for therapeutic treatment of human subjects. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also U.S. Pat. Nos. 4,444,887 and 4,716,111; and PCT Publication Nos. WO 98/46645, WO 98/50433, WO 98/24893, WO98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

[0188] A humanized antibody is an antibody or its variant or fragment thereof which is capable of binding to a predetermined antigen and which comprises a framework region having substantially the amino acid sequence of a human immunoglobulin and a CDR having substantially the amino acid sequence of a non-human immunoglobulin. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, Fabc, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (i.e., donor antibody) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. In one embodiment, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. Ordinarily, the antibody will contain both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain. The humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including IgG1, IgG2, IgG3 and IgG4. Usually the constant domain is a complement fixing constant domain where it is desired that the humanized antibody exhibit cytotoxic activity, and the class is typically IgG.sub. 1. Where such cytotoxic activity is not desirable, the constant domain may be of the IgG.sub.2 class. The humanized antibody may comprise sequences from more than one class or isotype, and selecting particular constant domains to optimize desired effector functions is within the ordinary skill in the art. The framework and CDR regions of a humanized antibody need not correspond precisely to the parental sequences, e.g., the donor CDR or the consensus framework may be mutagenized by substitution, insertion or deletion of at least one residue so that the CDR or framework residue at that site does not correspond to either the consensus or the import antibody. Such mutations, however, will not be extensive. Usually, at least 75% of the humanized antibody residues will correspond to those of the parental framework region (FR) and CDR sequences, more often 90%, and most preferably greater than 95%. Humanized antibody can be produced using variety of techniques known in the art, including but not limited to, CDR-grafting (European Patent No. EP 239,400; International Publication No. WO 91/09967; and U.S. Pat. Nos. 5,225,539, 5,530,101, and 5,585,089), veneering or resurfacing (European Patent Nos. EP 592,106 and EP 519,596; Padlan, 1991, Molecular Immunology 28(4/5): 489-498; Studnicka et al., 1994, Protein Engineering 7(6): 805-814; and Roguska et al., 1994, PNAS 91:969-973), chain shuffling (U.S. Pat. No. 5,565, 332), and techniques disclosed in, e.g., U.S. Pat. No. 6,407, 213, U.S. Pat. No. 5,766,886, WO 9317105, Tan et al., J. Immunol. 169:1119-25 (2002), Caldas et al., Protein Eng. 13(5): 353-60 (2000), Morea et al., Methods 20(3): 267-79 (2000), Baca et al., J. Biol. Chem. 272(16): 10678-84 (1997), Roguska et al., Protein Eng. 9(10): 895-904 (1996), Couto et al., Cancer Res. 55 (23 Supp): 5973s-5977s (1995), Couto et al., Cancer Res. 55(8): 1717-22 (1995), Sandhu J S, Gene 150(2): 409-10 (1994), and Pedersen et al., J. Mol. Biol. 235(3): 959-73 (1994). Often, framework residues in the framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Pat. No. 5,585,089; and Riechmann et al., 1988, Nature 332:323, which are incorporated herein by reference in their entireties.)

[0189] Human antibodies can also be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring that express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, e.g., Integrin $\alpha_v\beta_3$ or immunogenic fragments thereof. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar (1995, *Int. Rev. Immunol.* 13:65-93). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, e.g., International Publication Nos. WO 98/24893, WO 96/34096, and WO 96/33735; and U.S. Pat. Nos. 5,413,923, 5,625,126, 5,633, 425, 5,569,825, 5,661,016, 5,545,806, 5,814,318, and 5,939, 598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, Calif.), Genpharm (San Jose, Calif.) and Medarex

(Princeton, N.J.) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

[0190] Further, the antibodies of the invention can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" Integrin $\alpha_v\beta_3$ using techniques well known to those skilled in the art. (See, e.g., Greenspan & Bona, 1989, FASEB J. 7(5): 437-444; and Nissinoff, 1991, J. Immunol. 147(8): 2429-2438). For example, antibodies of the invention which bind to and competitively inhibit the binding of Integrin $\alpha_v\beta_3$ (as determined by assays well known in the art and disclosed infra) to its ligands can be used to generate anti-idiotypes that "mimic" Integrin $\alpha_v\beta_3$ binding domains and, as a consequence, bind to and neutralize Integrin $\alpha_v\beta_3$ and/or its ligands. Such neutralizing anti-idiotypes or Fab fragments of such anti-idiotypes can be used in therapeutic regimens to neutralize Integrin $\alpha_v\beta_3$. The invention provides methods employing the use of polynucleotides comprising a nucleotide sequence encoding an antibody of the invention or a fragment thereof.

[0191] In one embodiment, the nucleotide sequence encoding an antibody that immunospecifically binds Integrin $\alpha_v\beta_3$ is obtained and used to generate the Fc variants of the invention. The nucleotide sequence can be obtained from sequencing hybridoma clone DNA. If a clone containing a nucleic acid encoding a particular antibody or an epitope-binding fragment thereof is not available, but the sequence of the antibody molecule or epitope-binding fragment thereof is known, a nucleic acid encoding the immunoglobulin may be chemically synthesized or obtained from a suitable source (e.g., an antibody cDNA library, or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from any tissue or cells expressing the antibody, such as hybridoma cells selected to express an antibody) by PCR amplification using synthetic primers that hybridize to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, e.g., a cDNA clone from a cDNA library that encodes the antibody. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

[0192] Once the nucleotide sequence of the antibody is determined, the nucleotide sequence of the antibody may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, e.g., recombinant DNA techniques, site directed mutagenesis, PCR, etc. (see, Or example, the techniques described in *Current Protocols in Molecular Biology*, F. M. Ausubel et al., ed., John Wiley & Sons (Chichester, England, 1998); *Molecular Cloning: A Laboratory Manual,* 3nd Edition, J. Sambrook et al., ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y., 2001); *Antibodies: A Laboratory Manual*, E. Harlow and D. Lane, ed., Cold Spring Harbor Laboratory Press (Cold Spring Harbor, N.Y., 1988); and *Using Antibodies: A Laboratory Manual*, E. Harlow and D. Lane, ed., Cold Spring Harbor Laboratory (Cold Spring. Harbor, N.Y., 1999) which are incorporated by reference herein in their entireties), to generate antibodies having a different amino acid sequence by, for example, introducing deletions, and/or insertions into desired regions of the antibodies.

[0193] In one embodiment, one or more substitutions are made within the Fc region (e.g. supra) of an antibody able to immunospecifically bind Integrin $\alpha_v\beta_3$. In another embodiment, the amino acid substitutions modify binding to one or more Fc ligand (e.g., FcγRs, C1q) and alter ADCC and/or CDC activity.

[0194] In a specific embodiment, one or more of the CDRs is inserted within framework regions using routine recombinant DNA techniques. The framework regions may be naturally occurring or consensus framework regions, specifically contemplated are human framework regions (see, e.g., Chothia et al., 1998, J. Mol. Biol. 278: 457-479 for a listing of human framework regions). In one embodiment, the polynucleotide generated by the combination of the framework regions and CDRs encodes an antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$. In another embodiment, as discussed supra, one or more amino acid substitutions may be made within the framework regions, it is contemplated that the amino acid substitutions improve binding of the antibody to its antigen. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the polynucleotide are encompassed by the present invention and within the skill of the art.

6.5 Polypeptides and Fusion Proteins That Bind to Integrin $\alpha_v\beta_3$

[0195] The present invention encompasses polypeptides and fusion proteins that immunospecifically bind to Integrin $\alpha_v\beta_3$.

[0196] In a specific embodiment, a polypeptide or a fusion protein that immunospecifically binds to Integrin $\alpha_v\beta_3$ inhibits or reduces the interaction between Integrin $\alpha_v\beta_3$ and its ligands by about 25%, about 30%, about 35%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 95%, or about 98% in an in vivo or in vitro assay described herein or well-known to one of skill in the art. In this context "about" means plus or minus 0.1% to 2.5%. In another specific embodiment, a polypeptide or a fusion protein that immunospecifically binds to Integrin $\alpha_v\beta_3$ inhibits or reduces the interaction between Integrin $\alpha_v\beta_3$ and its ligands by 25%, 30%, 35%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 98% in an in vivo or in vitro assay described herein or well-known to one of skill in the art. In alternative embodiment, a polypeptide or a fusion protein that immunospecifically binds to Integrin $\alpha_v\beta_3$ does not significantly inhibit the interaction between Integrin $\alpha_v\beta_3$ and its ligands in an in vivo or in vitro assay described herein or well-known to one of skill in the art.

[0197] In a one embodiment, a polypeptide or a fusion protein that immuno-specifically binds to Integrin $\alpha_v\beta_3$ comprises an Integrin $\alpha_v\beta_3$ ligand or a fragment thereof which immunospecifically binds to an Integrin $\alpha_v\beta_3$ fused to an Fc domain. It is specifically contemplated that the Fc domain of said fusion protein comprises at least one high effector function amino acid and/or substitution as described supra. In another embodiment, said Fc domain is that of an Fc variant of the present invention, the Fc domain of an Fc variant is hereafter referred to as a variant Fc domain. Examples of Integrin $\alpha_v\beta_3$ ligands include, but are not limited to, vitronectin, osteopontin, bone sialoprotein,

echistatin, RGD-containing peptides, and RGD mimetics. (See e.g., Dresner-Pollak et al., J. Cell Biochem. 56(3): 323-30; Duong et al., Front. Biosci. 1(3): d757-68).

[0198] In another embodiment, a polypeptide or a fusion protein that immunospecifically binds to Integrin $\alpha_v\beta_3$ comprises a bioactive molecule fused to a variant Fc domain of the present invention. In accordance with these embodiments, the bioactive molecule immunospecifically binds to Integrin $\alpha_v\beta_3$. Bioactive molecules that immunospecifically bind to Integrin $\alpha_v\beta_3$ include, but are not limited to, peptides, polypeptides, proteins, small molecules, mimetic agents, synthetic drugs, inorganic molecules, and organic molecules. In still another embodiment, a bioactive molecule that immunospecifically binds to Integrin $\alpha_v\beta_3$ is a polypeptide comprising at least 5, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90 or at least 100 contiguous amino acid residues, and is heterologous to the amino acid sequence of the variant Fc domain of the invention.

[0199] In another embodiment, a peptide, a polypeptide or a fusion protein that immunospecifically binds to Integrin $\alpha_v\beta_3$ comprises a polypeptide having an amino acid sequence that is at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% identical to the amino acid sequence of an Integrin $\alpha_v\beta_3$ ligand or a fragment thereof fused to a variant Fc domain of the present invention

[0200] The present invention provides polypeptides or fusion proteins that immunospecifically bind to Integrin $\alpha_v\beta_3$ comprising a variant Fc domain of the present invention fused to a polypeptide encoded by a nucleic acid molecule that hybridizes to the nucleotide sequence encoding an Integrin $\alpha_v\beta_3$ ligand or a fragment thereof.

[0201] In a specific embodiment, a polypeptide or a fusion protein that immunospecifically binds to Integrin $\alpha_v\beta_3$ comprises a variant Fc domain of the present invention fused to a polypeptide encoded by a nucleic acid molecule that hybridizes to the nucleotide sequence encoding an Integrin $\alpha_v\beta_3$ ligand or a fragment thereof under stringent conditions, e.g., hybridization to filter-bound DNA in 6× sodium chloride/sodium citrate (SSC) at about 45° C. followed by one or more washes in 0.2×SSC/0.1% SDS at about 50-65° C., under highly stringent conditions, e.g., hybridization to filter-bound nucleic acid in 6×SSC at about 45° C. followed by one or more washes in 0.1×SSC/0.2% SDS at about 68° C., or under other stringent hybridization conditions which are known to those of skill in the art (see, for example, Ausubel, F. M. et al., eds., 1989, *Current Protocols in Molecular Biology, Vol.* 1, Green Publishing Associates, Inc. and John Wiley & Sons, Inc., New York at pages 6.3.1-6.3.6 and 2.10.3).

[0202] The present invention also encompasses polypeptides and fusion proteins that immunospecifically bind to Integrin $\alpha_v\beta_3$ comprising of a variant Fc domain, fused to marker sequences, such as but not limited to, a peptide, to facilitate purification. In other embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, Calif., 91311), among others, many of which are commercially available. Other peptide tags useful for purification include, but are not limited to, the hemagglutinin "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson et al., 1984, Cell 37:767) and the "flag" tag.

[0203] The present invention further encompasses polypeptides and fusion proteins that immunospecifically bind to Integrin $\alpha_v\beta_3$ fused to a variant Fc further conjugated to a therapeutic moiety. A polypeptide or a fusion protein that immunospecifically binds to Integrin $\alpha_v\beta_3$ may be conjugated to a therapeutic moiety such as a cytotoxin, e.g., a cytostatic or cytocidal agent, an agent which has a potential therapeutic benefit, or a radioactive metal ion, e.g., alpha-emitters. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples of a therapeutic moieties and cytotoxin or cytotoxic agents are listed supra (see section 6.3 entitled "Antibody Conjugates And Derivatives," infra).

[0204] Polypeptides, proteins and fusion proteins can be produced by standard recombinant DNA techniques or by protein synthetic techniques, e.g., by use of a peptide synthesizer. For example, a nucleic acid molecule encoding a peptide, polypeptide, protein or a fusion protein can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, e.g., *Current Protocols in Molecular Biology*, Ausubel et al., eds., John Wiley & Sons, 1992). Moreover, a nucleic acid encoding a bioactive molecule can be cloned into an expression vector containing the variant Fc domain or a fragment thereof such that the bioactive molecule is linked in-frame to the variant Fc domain or variant Fc domain fragment.

[0205] Methods for fusing or conjugating polypeptides to the constant regions of antibodies are known in the art. See, e.g., U.S. Pat. Nos. 5,336,603, 5,622,929, 5,359,046, 5,349,053, 5,447,851, 5,723,125, 5,783,181, 5,908,626, 5,844,095, and 5,112,946; EP 307,434; EP 367,166; EP 394,827; International Publication Nos. WO 91/06570, WO 96/04388, WO 96/22024, WO 97/34631, and WO 99/04813; Ashkenazi et al., 1991, Proc. Natl. Acad. Sci. USA 88: 10535-10539; Traunecker et al., 1988, Nature, 331:84-86; Zheng et al., 1995, J. Immunol. 154:5590-5600; and Vil et al., 1992, Proc. Natl. Acad. Sci. USA 89:11337-11341, which are incorporated herein by reference in their entireties.

[0206] The nucleotide sequences encoding a bioactive molecule and an Fc domain or fragment thereof may be obtained from any information available to those of skill in the art (i.e., from Genbank, the literature, or by routine cloning). The nucleotide sequences encoding Integrin ligands may be obtained from any available information, e.g., from Genbank, the literature or by routine cloning. See, e.g., Xiong et al., Science, 12; 294(5541): 339-45 (2001). The nucleotide sequence coding for a polypeptide a fusion protein can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. A variety of host-vector systems may be utilized in the present invention to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, aden-

ovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

6.6 Recombinant Expression Of Antibodies and Fusion Proteins

[0207] Recombinant expression of an Fc variant or fusion protein comprising a variant Fc domain (referred to herein as an "variant Fc fusion protein", or "variant Fc fusion"), derivative, analog or fragment thereof, (e.g., a heavy or light chain of an antibody of the invention or a portion thereof or a single chain antibody of the invention), requires construction of an expression vector containing a polynucleotide that encodes the antibody, or fusion protein. Once a polynucleotide encoding an antibody molecule or a heavy or light chain of an antibody, or fusion protein of the invention has been obtained, the vector for the production of the antibody or fusion protein molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a polynucleotide containing an antibody or fusion protein encoding nucleotide sequence are described herein. Methods that are well known to those skilled in the art can be used to construct expression vectors containing antibody or fusion protein coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques, and in vivo genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding an Fc variant or variant Fc fusion of the invention, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (see, e.g., International Publication No. WO 86/05807; International Publication No. WO 89/01036; and U.S. Pat. No. 5,122,464) and the variable domain of the antibody, or a polypeptide for generating an variant Fc fusion may be cloned into such a vector for expression of the full length antibody chain (e.g. heavy or light chain), or complete variant Fc fusion protein.

[0208] The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce an Fc variant or variant Fc fusion protein of the invention. Thus, the invention includes host cells containing a polynucleotide encoding an Fc variant or variant Fc fusion protein of the invention or fragments thereof, or a heavy or light chain thereof, or portion thereof, or a single chain antibody of the invention, operably linked to a heterologous promoter. In other embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

[0209] A variety of host-expression vector systems may be utilized to express the antibody or fusion protein molecules of the invention (see, e.g., U.S. Pat. No. 5,807,715). Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express an antibody or fusion protein molecule of the invention in situ. These include but are not limited to microorganisms such as bacteria (e.g., *E. coli* and *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody or fusion protein coding sequences; yeast (e.g., *Saccharomyces Pichia*) transformed with recombinant yeast expression vectors containing antibody or fusion protein coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing antibody or fusion protein coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing antibody or fusion protein coding sequences; or mammalian cell systems (e.g., COS, CHO, BHK, 293, NS0, and 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody or fusion protein molecules, are used for the expression of a recombinant antibody or fusion protein molecules. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for antibodies (Foecking et al., 1986, Gene 45:101; and Cockett et al., 1990, Bio/Technology 8:2). In a specific embodiment, the expression of nucleotide sequences encoding antibodies or fusion protein that bind to Integrin $\alpha_v\beta_3$ is regulated by a constitutive promoter, inducible promoter or tissue specific promoter.

[0210] In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the antibody or fusion protein molecule being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of an antibody or fusion protein molecule, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO 12:1791), in which the antibody or fusion protein coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a lac Z-fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione 5-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

[0211] In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody or fusion protein coding

sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

[0212] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the antibody or fusion protein coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the antibody or fusion protein molecule in infected hosts (e.g., see Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 8 1:355-359). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see, e.g., Bittner et al., 1987, Methods in Enzymol. 153:516-544).

[0213] The expression of an antibody or a fusion protein may be controlled by any promoter or enhancer element known in the art. Promoters which may be used to control the expression of the gene encoding an antibody or fusion protein include, but are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), the tetracycline (Tet) promoter (Gossen et al., 1995, Proc. Nat. Acad. Sci. USA 89:5547-5551); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroffet al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25; see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94); plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286); neuronal-specific enolase (NSE) which is active in neuronal cells (Morelli et al., 1999, Gen. Virol. 80:571-83); brain-derived neurotrophic factor (BDNF) gene control region which is active in neuronal cells (Tabuchi et al., 1998, Biochem. Biophysic. Res. Com. 253:818-823); glial fibrillary acidic protein (GFAP) promoter which is active in astrocytes (Gomes et al., 1999, Braz J Med Biol Res 32(5): 619-631; Morelli et al., 1999, Gen. Virol. 80:571-83) and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

[0214] Expression vectors containing inserts of a gene encoding an antibody or fusion protein can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a gene encoding a peptide, polypeptide, protein or a fusion protein in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted gene encoding the peptide, polypeptide, protein or the fusion protein, respectively. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of a nucleotide sequence encoding an antibody or fusion protein in the vector. For example, if the nucleotide sequence encoding the antibody or fusion protein is inserted within the marker gene sequence of the vector, recombinants containing the gene encoding the antibody or fusion protein insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the gene product (e.g., antibody or fusion protein) expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the fusion protein in in vitro assay systems, e.g., binding with anti-bioactive molecule antibody.

[0215] In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered fusion protein may be con-

trolled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, phosphorylation of proteins). Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system will produce an unglycosylated product and expression in yeast will produce a glycosylated product. Eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript (e.g., glycosylation, and phosphorylation) of the gene product may be used. Such mammalian host cells include, but are not limited to, CHO, VERY, BHK, Hela, COS, MDCK, 293, 3T3, W138, NS0, and in particular, neuronal cell lines such as, for example, SK-N-AS, SK-N-FI, SK-N-DZ human neuroblastomas (Sugimoto et al., 1984, J. Natl. Cancer Inst. 73: 51-57), SK-N-SH human neuroblastoma (Biochim. Biophys. Acta, 1982, 704: 450-460), Daoy human cerebellar medulloblastoma (He et al., 1992, Cancer Res. 52: 1144-1148) DBTRG-05MG glioblastoma cells (Kruse et al., 1992, In Vitro Cell. Dev. Biol. 28A: 609-614), IMR-32 human neuroblastoma (Cancer Res., 1970, 30: 2110-2118), 1321N1 human astrocytoma (Proc. Natl. Acad. Sci. USA, 1977, 74: 4816), MOG-G-CCM human astrocytoma (Br. J. Cancer, 1984, 49: 269), U87MG human glioblastoma-astrocytoma (Acta Pathol. Microbiol. Scand., 1968, 74: 465-486), A172 human glioblastoma (Olopade et al., 1992, Cancer Res. 52: 2523-2529), C6 rat glioma cells (Benda et al., 1968, Science 161: 370-371), Neuro-2a mouse neuroblastoma (Proc. Natl. Acad. Sci. USA, 1970, 65: 129-136), NB41A3 mouse neuroblastoma (Proc. Natl. Acad. Sci. USA, 1962, 48: 1184-1190), SCP sheep choroid plexus (Bolin et al., 1994, J. Virol. Methods 48: 211-221), G355-5, PG-4 Cat normal astrocyte (Haapala et al., 1985, J. Virol. 53: 827-833), Mpf ferret brain (Trowbridge et al., 1982, In Vitro 18: 952-960), and normal cell lines such as, for example, CTX TNA2 rat normal cortex brain (Radany et al., 1992, Proc. Natl. Acad. Sci. USA 89: 6467-6471) such as, for example, CRL7030 and Hs578Bst. Furthermore, different vector/host expression systems may effect processing reactions to different extents.

[0216] For long-term, high-yield production of recombinant proteins, stable expression is often preferred. For example, cell lines which stably express an antibody or fusion protein may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched medium, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci that in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express an Fc variant or variant Fc fusion protein that specifically binds to Integrin $\alpha v\beta_3$. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the activity of a polypeptide or a fusion protein that immunospecifically binds to Integrin $\alpha v\beta_3$.

[0217] A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy et al., 1980, Cell 22:817) genes can be employed in tk-, hgprt- or aprt-cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., 1981, J. Mol. Biol. 150:1); and hygro, which confers resistance to hygromycin (Santerre et al., 1984, Gene 30:147) genes.

[0218] Once a peptide, polypeptide, protein or a fusion protein of the invention has been produced by recombinant expression, it may be purified by any method known in the art for purification of a protein, for example, by chromatography (e.g., ion exchange, affinity, particularly by affinity for the specific antigen after Protein A, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

[0219] The expression levels of an antibody or fusion protein molecule can be increased by vector amplification (for a review, see Bebbington and Hentschel, The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells in DNA cloning, Vol. 3. (Academic Press, New York, 1987)). When a marker in the vector system expressing an antibody or fusion protein is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the antibody or fusion protein will also increase (Crouse et al., 1983, Mol. Cell. Biol. 3:257).

[0220] The host cell may be co-transfected with two expression vectors of the invention. For example, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers, which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used which encodes, and is capable of expressing, a fusion protein or both heavy and light chain polypeptides. The coding sequences for the fusion protein or heavy and light chains may comprise cDNA or genomic DNA.

6.7 Antagonists of Integrin $\alpha_v\beta_3$

[0221] The invention specifically encompasses Fc variants, or variant Fc fusions, of the invention that are Integrin $\alpha_v\beta_3$ antagonists. As used herein, the terms "antagonist" and "antagonists" refer to any protein, polypeptide, peptide, peptidomimetic, glycoprotein, antibody, antibody fragment, carbohydrate, nucleic acid, organic molecule, inorganic molecule, large molecule, or small molecule that blocks, inhibits, reduces or neutralizes the function, activity and/or expression of another molecule. In various embodiments, an antagonist reduces the function, activity and/or expression of another molecule by at least 10%, at least 15%, at least

20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% relative to a control such as phosphate buffered saline (PBS). More specifically, an Integrin $\alpha_v\beta_3$ antagonist inhibits, reduces or neutralizes the function, activity and/or expression of Integrin $\alpha_v\beta_3$ or inhibits or reduces Integrin $\alpha_v\beta_3$-mediated pathologies.

[0222] In one embodiment, integrin $\alpha_v\beta_3$ antagonists inhibit or reduce angiogenesis. In particular embodiments, integrin $\alpha_v\beta_3$ antagonists inhibit or reduce angiogenesis in a subject by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% relative to a control such as PBS, as measured by, for example, changes in regional blood volume using dynamic susceptibility contrast-enhanced MRI.

[0223] The invention also provides methods for screening for antagonists for Integrin $\alpha_v\beta_3$. Said screening methods include but are not limited to assays that monitor Integrin $\alpha_v\beta_3$ activity (e.g., cell adhesion, angiogenesis, tumor cell growth and tumor progression) and/or plasma concentration. These and additional methods are further described infra (see section 6.8 entitled "Biological Assays," infra) and in PCT publications WO 02/12501, WO 03/075957, WO 04/066956 and U.S. patent applications 2003/0157098 among others.

[0224] In addition, the invention provides methods for identifying monoclonal antibodies that bind to the heterodimerized $\alpha_v\beta_3$ but not the α_v or the β_3 chains when not included in a heterodimer. Further, the invention provides for a method to manipulate both the ADCC activity and the binding affinities for FcγR of antibodies identified using such screening methods.

[0225] Integrin $\alpha_v\beta_3$ and/or amino acid substituted subunits of Integrin $\alpha_v\beta_3$ (see for example PCT publication WO 03/075957) can be used for screening antibodies with specific affinity for particular epitopes by identifying monoclonal antibodies that bind to wild type Integrin $\alpha_v\beta_3$ but not the altered form, or that bind mouse $\alpha_v\beta_3$ integrins with a region substituted with the corresponding region from the human $\alpha_v\beta_3$ but do not bind to wild type mouse Integrin $\alpha_v\beta_3$.

[0226] In addition, the invention provides methods for identifying monoclonal antibodies and other molecules (e.g., Integrin $\alpha_v\beta_3$ ligands and variants thereof) that bind to the heterodimerized $\alpha_v\beta_3$ but not the α_v or β_3 chains when not included in a heterodimer. Such screening can be accomplished by any routine method for assaying antibody specificity and/or protein interactions known in the art, for example, using cell lines that do not express wild type Integrin $\alpha_v\beta_3$ to recombinantly express the mutant Integrin $\alpha_v\beta_3$ or individual α_v or β_3 chains. In one embodiment, new identified antibodies that immunospecifically bind Integrin $\alpha_v\beta_3$ are antagonists of Integrin $\alpha_v\beta_3$. Assays to measure the antagonist activity of a molecule include but are not limited to those described infra.

[0227] The Fc of antibodies identified from such screening methods can be substituted as described supra to alter ADCC and/or CDC activity and to modify binding affinities for one or more Fc ligand (e.g., FcγRs, C1q). Other antagonistic binding molecules (e.g., Integrin $\alpha_v\beta_3$ ligands and variants thereof) identified from such screening methods can be fused to a variant Fc domain of the invention. It is further contemplated that the Fc variants of the newly identified Integrin $\alpha_v\beta_3$ antagonistic antibodies and variant Fc fusions of the newly identified Integrin $\alpha_v\beta_3$ antagonists are useful for the prevention, management and treatment of Integrin $\alpha_v\beta_3$-mediated diseases and disorders, including but not limited to inflammatory diseases, autoimmune diseases, bone metabolism related disorders, angiogenic related disorders, disorders related to aberrant expression and/or activity of $\alpha_v\beta_3$, and cancer. Such Fc variants and/or variant Fc fusions can be used in the methods and formulations of the present invention.

6.8 Biological Assays

[0228] The antagonistic effect of one or more Fc variant, or variant Fc fusion of the invention on Integrin $\alpha_v\beta_3$ activity can be determined by any method known in the art. Methods include but are not limited to those described infra and in PCT publications WO 02/12501, WO 03/075957, WO 03/075741, WO 04/066956 and U.S. patent applications 2003/0157098 among others, each of which is are incorporated herein by reference in its entirety. For example, the blockage of Integrin $\alpha_v\beta_3$ activity and/or the plasma concentration of Integrin $\alpha_v\beta_3$ can be assayed by any technique known in the art that measures the activity and/or expression of Integrin $\alpha_v\beta_3$, including but not limited to, Western blot, Northern blot, RNase protection assays, enzymatic activity assays, in situ hybridization, immunohistochemistry, and immunocytochemistry.

[0229] The binding specificity, affinity and functional activity of an Fc variant, or variant Fc fusion protein of the invention can be characterized in various in vitro binding and cell adhesion assays known in the art, including but limited to, ELISA Western Blot analysis, cell surface staining, inhibition of ligand-receptor interactions, flow cytometric analysis and those disclosed in International Publication Nos. WO 04/014292, WO 03/094859, WO 04/069264, WO 04/028551, WO 03/004057, WO 03/040304, WO 00/78815, WO 02/070007 and WO 03/075957, U.S. Pat. Nos. 5,795,734, 6,248,326 and 6,472,403, Pecheur et al., 2002, FASEB J. 16(10): 1266-1268; Almed et al., The Journal of Histochemistry & Cytochemistry 50:1371-1379 (2002), all of which are incorporated herein by reference. For example, the binding affinity, specificity and the off-rate of an Fc variant and/or variant Fc fusion protein can be determined by a competitive binding assay with the parental anti-Integrin $\alpha_v\beta_3$ antibody, by measuring the inhibitory activity of an Fc variant, or variant Fc fusion protein of the invention on binding to Integrin $\alpha_v\beta_3$. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled Integrin $\alpha_v\beta_3$ (e.g., 3H or 125I) with the Fc variant of interest in the presence of increasing amounts of unlabeled Integrin $\alpha_v\beta_3$, and the detection of the monoclonal antibody bound to the labeled Integrin $\alpha_v\beta_3$. The affinity of an Fc variant for an Integrin $\alpha_v\beta_3$ and the binding off-rates can be determined from the data by scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, an Integrin $\alpha_v\beta_3$ is incubated with an Fc variant conjugated to a labeled com-

pound (e.g., 3H or 125I) in the presence of increasing amounts of a second unlabeled monoclonal antibody.

[0230] The kinetic parameters of an Fc variant, or variant Fc fusion protein may also be determined using any surface plasmon resonance (SPR) based assays known in the art. For a review of SPR-based technology see Mullet et al., 2000, Methods 22: 77-91; Dong et al., 2002, *Review in Mol. Biotech.,* 82: 303-23; Fivash et al., 1998, *Current Opinion in Biotechnology* 9: 97-101; Rich et al., 2000, *Current Opinion in Biotechnology* 11: 54-61; all of which are incorporated herein by reference in their entirety. Additionally, any of the SPR instruments and SPR based methods for measuring protein-protein interactions described in U.S. Pat. Nos. 6,373,577; 6,289,286; 5,322,798; 5,341,215; 6,268,125 are contemplated in the methods of the invention, all of which are incorporated herein by reference in their entirety.

[0231] The binding specificity of an Fc variant, or variant Fc fusion protein of the invention to Integrin $\alpha_v\beta_3$ can be assessed by any method known in the art including but not limited to, measuring binding to Integrin $\alpha_v\beta_3$ and its crossreactivity to other α_v- or β_3-containing integrins, inhibition of Integrin $\alpha_v\beta_3$ binding in cell adhesion assays. In addition, binding affinity and specificity can be determined by a competitive binding assay with the parental anti-Integrin $\alpha_v\beta_3$ antibody against Integrin $\alpha_v\beta_3$ or by measuring the inhibitory activity of an Fc variant, or variant Fc fusion protein of the invention on Integrin $\alpha_v\beta_3$ binding to fibrinogen.

[0232] The inhibitory and/or antagonistic activity of an Fc variant, or variant Fc fusion of the invention can be tested in cell proliferation assays, cell adhesion assays (Lawrenson et al., 2002, J Cell Sci 115:1059 and Davy et al., 2000, EMBO 19:5396) and in endothelial cell migration assays such as the transwell cell migration assay (Choi et al., 1994, *J Vascular Sur* 19:125-134 and Leavesly et al., 1993, *J Cell Biol* 121:163-170).

[0233] Additional examples of in vitro assays, e.g., Western blotting analysis, flow cytometric analysis, cell adhesion assay to cortical bone and extracellular matrix proteins, cell migration assay, cell invasion assay, and cell proliferation assay, can be found in Pecheur et al., 2002, FASEB J. 16(10): 1266-1268, of which the entire text is incorporated herein by reference.

[0234] The anti-cancer activity of an Fc variant, or variant Fc fusion of the invention can be determined by using various experimental animal models for the study of cancer such as the corneal micro pocket assay (see, e.g., Fournier et al., (1981) Invest Opthalmol & Visual Sci. 21:351-54); scid mouse model or transgenic mice where a mouse Integrin $\alpha_v\beta_3$ is replaced with the human Integrin $\alpha_v\beta_3$, nude mice with human xenografts, animal models wherein an antagonist of Integrin $\alpha_v\beta_3$ recognizes the same target as Vitaxin®, such as hamsters, rabbits, etc. known in the art and described in *Relevance of Tumor Models for Anticancer Drug Development* (1999, eds. Fiebig and Burger); *Contributions to Oncology* (1999, Karger); *The Nude Mouse in Oncology Research* (1991, eds. Boven and Winograd); and *Anticancer Drug Development Guide* (1997 ed. Teicher), herein incorporated by reference in their entireties.

[0235] Various animal models known in the art that are relevant to a targeted disease or disorder, e.g., inflammatory diseases, autoimmune diseases, diseases or disorders associated with aberrant bone metabolism and/or aberrant angiogenesis, cancers, disorders associated with aberrant integrin $\alpha_v\beta_3$ expression and/or activity can be used, including but not limited to, those that are disclosed in International Publication No. WO 00/78815, U.S. Pat. No. 6,248,326, U.S. Pat. No. 6,472,403, Pecheur et al., 2002, FASEB J. 16(10): 1266-1268; Almed et al., The Journal of Histochemistry & Cytochemistry 50:1371-1379 (2002), all of which are incorporated herein by reference. Models that can be used include but are not limited to, growth factor or tumor-induced angiogenesis in the chick chorioallantoic membrane (CAM) (see, e.g., Ausprunk et al. (1980) *Am. J. Pathol.,* 79:597-618; Ossonski et al. (1975) *Cancer Res.,* 40:2300-2309; Brooks et al. (1994) *Science,* 264:569-571 and Brooks et al., (1994), *Cell,* 79:1157-1164), Vx2 carcinoma cells in rabbits (see, e.g., Voelkel et al., (1975) *Metabolism* 24:973-86), tumors induced in BALB/c nu/nu mice and SCID mice with subcutaneously implanted human bone fragments (SCID-human-bone model). Additional examples of tumor models can be found in Teicher et al., Tumor Models in Cancer Research, (Humana Press, Totowa, N.J., 2001).

[0236] It is contemplated that the protocols and formulations of the invention are tested in vitro, and then in vivo, for the desired therapeutic or prophylactic activity, prior to use in humans. For example, assays which can be used to determine whether administration of a specific therapeutic protocol, formulation or combination therapy of the invention is indicated, include in vitro cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise contacted with a formulation of the invention, and the effect of such a formulation upon the tissue sample is observed. The tissue sample can be obtained by biopsy from the patient. This test allows the identification of the therapeutically most effective prophylactic or therapeutic agent(s) for each individual patient. In various specific embodiments, in vitro assays can be carried out with representative cells of cell types involved in an autoimmune disorder, an inflammatory disorder, a disorder associated with aberrant expression and/or activity of Integrin $\alpha V\beta 3$, to determine if a formulation of the invention has a desired effect upon such cell types. A lower level of proliferation or survival of the contacted cells indicates that the formulation is effective to treat the condition in the patient. Alternatively, instead of culturing cells from a patient, a formulation of the invention may be screened using cells of a tumor or malignant cell line, osteoclasts, endothelial cells or an endothelial cell line. Many assays standard in the art can be used to assess such survival and/or growth; for example, cell proliferation can be assayed by measuring ^{3}H-thymidine incorporation, by direct cell count, by detecting changes in transcriptional activity of known genes such as proto-oncogenes (e.g., fos, myc) or cell cycle markers; cell viability can be assessed by trypan blue staining, differentiation can be assessed visually based on changes in morphology, etc.

[0237] Prophylactic or therapeutic agents can be tested in suitable animal model systems prior to testing in humans, including but not limited to in rats, mice, chicken, cows, monkeys, rabbits, hamsters, etc. The principle animal models for known in the art and widely used are known and described in the art as described above.

[0238] Further, any assays known to those skilled in the art can be used to evaluate the prophylactic and/or therapeutic

utility of the combinatorial therapies disclosed herein for treatment or prevention of cancer.

6.9 Prophylactic and Therapeutic Uses

[0239] As discussed above, agents that immunospecifically bind Integrin $\alpha_v\beta_3$, can be utilized for the inhibition of angiogenesis or the inhibition of other functions mediated or influenced by Integrin $\alpha_v\beta_3$, including but not limited to cell proliferation, cell attachment, cell migration, granulation tissue development, and/or inflammation. Accordingly, the present invention relates to the use of agents that immunospecifically bind and in particular embodiments, inhibit Integrin $\alpha_v\beta_3$ for the prevention, management, treatment or amelioration of cancer or one or more symptoms thereof and/or the inhibition of angiogenesis.

[0240] Angiogenesis, also called neovascularization, is the process where new blood vessels form from pre-existing vessels within a tissue. As described above, this process is mediated by endothelial cells expressing Integrin $\alpha_v\beta_3$ and inhibition of at least this integrin, inhibits new vessel growth. There are a variety of pathological conditions that require new blood vessel formation or tissue angiogenesis and inhibition of this process inhibits the pathological condition. As such, pathological conditions that require angiogenesis for growth or maintenance are considered to be Integrin $\alpha_v\beta_3$-mediated diseases. The extent of treatment, or reduction in severity, of these diseases will therefore depend on the extent of inhibition of angiogenesis. These Integrin $\alpha_v\beta_3$-mediated diseases include, for example, inflammatory disorders such as immune and non-immune inflammation, thrombosis, acute ischemic stroke, chronic articular rheumatism, psoriasis, disorders associated with inappropriate or inopportune invasion of vessels such as diabetic retinopathy, neovascular glaucoma and capillary proliferation in atherosclerotic plaques as well as cancer disorders.

[0241] Such cancer disorders can include, for example, solid tumors, tumor metastasis, angiofibromas, angiosarcomas, retrolental, fibroplasia, hemangiomas, Kaposi's sarcoma, carcinomas, carcinosarcomas, and other cancers which require neovascularization to support tumor growth. Additional diseases which are considered angiogenic include psoriasis and rheumatoid arthritis as well as retinal diseases such as macular degeneration.

[0242] Diseases other than those requiring new blood vessels which are Integrin $\alpha_v\beta_3$-mediated diseases include, for example, restenosis and osteoporosis.

[0243] Accordingly, the present invention relates to the use of agents that immunospecifically bind and in particular embodiments, inhibit Integrin $\alpha_v\beta_3$ for the prevention, management, treatment or amelioration of cancer, solid tumor metastasis, restenosis, thrombosis, acute ischemic stroke, granulation tissue development in cutaneous wounds, osteoporosis, age-related macular degeneration, diabetic retinopathy, as well as, inflammatory diseases such as rheumatoid arthritis and psoriasis or one or more symptoms thereof and/or the inhibition of angiogenesis or conditions associated therewith.

[0244] In one embodiment, the methods and formulations of the invention are used for inhibiting angiogenesis. In a specific embodiment, the methods and formulations of the invention are used for inhibiting angiogenesis in a solid tumor. In another embodiment, the methods and formulations of the invention are used for inhibiting angiogenesis in an inflamed, angiogenic tissue including but not limited to retinal tissues and joint tissues.

[0245] Further, the present invention provides Fc variants that immunospecifically bind and in particular embodiments, inhibit Integrin $\alpha_v\beta_3$ which are useful for therapeutic purposes, more specifically, for the treatment, prevention, management or amelioration of cancer. Specific examples of cancers that can be prevented, managed, treated or ameliorated in accordance with the invention include, but are not limited to, leukemias, such as but not limited to, acute leukemia, acute lymphocytic leukemia, acute myelocytic leukemias, such as, myeloblastic, promyelocytic, myelomonocytic, monocytic, and erythroleukemia leukemias and myelodysplastic syndrome; chronic leukemias, such as but not limited to, chronic myelocytic (granulocytic) leukemia, chronic lymphocytic leukemia, hairy cell leukemia; polycythemia vera; lymphomas such as but not limited to Hodgkin's disease, non-Hodgkin's disease; multiple myelomas such as but not limited to smoldering multiple myeloma, nonsecretory myeloma, osteosclerotic myeloma, plasma cell leukemia, solitary plasmacytoma and extramedullary plasmacytoma; Waldenstrom's macroglobulinemia; monoclonal gammopathy of undetermined significance; benign monoclonal gammopathy; heavy chain disease; bone and connective tissue sarcomas such as but not limited to bone sarcoma, osteosarcoma, chondrosarcoma, Ewing's sarcoma, malignant giant cell tumor, fibrosarcoma of bone, chordoma, periosteal sarcoma, soft-tissue sarcomas, angiosarcoma (hemangiosarcoma), fibrosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, neurilemmoma, rhabdomyosarcoma, synovial sarcoma; brain tumors such as but not limited to, glioma, astrocytoma, brain stem glioma, ependymoma, oligodendroglioma, nonglial tumor, acoustic neurinoma, craniopharyngioma, medulloblastoma, meningioma, pineocytoma, pineoblastoma, primary brain lymphoma; breast cancer including but not limited to adenocarcinoma, lobular (small cell) carcinoma, intraductal carcinoma, medullary breast cancer, mucinous breast cancer, tubular breast cancer, papillary breast cancer, Paget's disease, and inflammatory breast cancer; adrenal cancer such as but not limited to pheochromocytom and adrenocortical carcinoma; thyroid cancer such as but not limited to papillary or follicular thyroid cancer, medullary thyroid cancer and anaplastic thyroid cancer; pancreatic cancer such as but not limited to, insulinoma, gastrinoma, glucagonoma, vipoma, somatostatin-secreting tumor, and carcinoid or islet cell tumor; pituitary cancers such as but limited to Cushing's disease, prolactin-secreting tumor, acromegaly, and diabetes insipius; eye cancers such as but not limited to ocular melanoma such as iris melanoma, choroidal melanoma, and cilliary body melanoma, and retinoblastoma; vaginal cancers such as squamous cell carcinoma, adenocarcinoma, and melanoma; vulvar cancer such as squamous cell carcinoma, melanoma, adenocarcinoma, basal cell carcinoma, sarcoma, and Paget's disease; cervical cancers such as but not limited to, squamous cell carcinoma, and adenocarcinoma; uterine cancers such as but not limited to endometrial carcinoma and uterine sarcoma; ovarian cancers such as but not limited to, ovarian epithelial carcinoma, borderline tumor, germ cell tumor, and stromal tumor; esophageal cancers such as but not limited to, squamous cancer, adenocarcinoma, adenoid cyctic carcinoma, mucoepidermoid carcinoma, adenosqua-

mous carcinoma, sarcoma, melanoma, plasmacytoma, verrucous carcinoma, and oat cell (small cell) carcinoma; stomach cancers such as but not limited to, adenocarcinoma, fungating (polypoid), ulcerating, superficial spreading, diffusely spreading, malignant lymphoma, liposarcoma, fibrosarcoma, and carcinosarcoma; colon cancers; rectal cancers; liver cancers such as but not limited to hepatocellular carcinoma and hepatoblastoma; gallbladder cancers such as adenocarcinoma; cholangiocarcinomas such as but not limited to pappillary, nodular, and diffuse; lung cancers such as non-small cell lung cancer, squamous cell carcinoma (epidermoid carcinoma), adenocarcinoma, large-cell carcinoma and small-cell lung cancer; testicular cancers such as but not limited to germinal tumor, seminoma, anaplastic, classic (typical), spermatocytic, nonseminoma, embryonal carcinoma, teratoma carcinoma, choriocarcinoma (yolk-sac tumor), prostate cancers such as but not limited to, adenocarcinoma, leiomyosarcoma, and rhabdomyosarcoma; penal cancers; oral cancers such as but not limited to squamous cell carcinoma; basal cancers; salivary gland cancers such as but not limited to adenocarcinoma, mucoepidermoid carcinoma, and adenoidcystic carcinoma; pharynx cancers such as but not limited to squamous cell cancer, and verrucous; skin cancers such as but not limited to, basal cell carcinoma, squamous cell carcinoma and melanoma, superficial spreading melanoma, nodular melanoma, lentigo malignant melanoma, acral lentiginous melanoma; kidney cancers such as but not limited to renal cell carcinoma, adenocarcinoma, hypernephroma, fibrosarcoma, transitional cell cancer (renal pelvis and/or uterer); Wilms' tumor; bladder cancers such as but not limited to transitional cell carcinoma, squamous cell cancer, adenocarcinoma, carcinosarcoma. In addition, cancers include myxosarcoma, osteogenic sarcoma, endotheliosarcoma, lymphangioendotheliosarcoma, mesothelioma, synovioma, hemangioblastoma, epithelial carcinoma, cystadenocarcinoma, bronchogenic carcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma and papillary adenocarcinomas (for a review of such disorders, see Fishman et al., 1985, Medicine, 2d Ed., J.B. Lippincott Co., Philadelphia and Murphy et al., 1997, Informed Decisions: The Complete Book of Cancer Diagnosis, Treatment, and Recovery, Viking Penguin, Penguin Books U.S.A., Inc., United States of America).

[0246] In a specific embodiment, the methods and formulations of the invention are used for the prevention, management, treatment or amelioration of a primary or secondary cancer that expresses Integrin $\alpha_v\beta_3$. In another embodiment, the methods and formulations of the invention are used for the prevention, management, treatment or amelioration of a primary or secondary cancer that does not express Integrin $\alpha_v\beta_3$. In another embodiment, the methods and formulations are used for the prevention, management, treatment or amelioration of a cancer that has the potential to metastasize or has metastasized to other tissues or organs (e.g., bone). In another embodiment, the methods and formulations of the invention are used for the prevention, management, treatment or amelioration of lung cancer, prostate cancer, ovarian cancer, melanoma, bone cancer or breast cancer. Methods using agents that immunospecifically inhibit Integrin $\alpha_v\beta_3$ include but are not limited to those disclosed in PCT publications WO 00/078815, WO 02/070007, WO 03/075957, WO 03/075741 and WO 04/066956, each of which is herein incorporated by reference in its entirety.

[0247] The invention provides methods for screening for antibody and other antagonists of Integrin $\alpha_v\beta_3$. Further, the invention provides for a method to manipulate the ADCC and/or CDC activity and the binding affinities for one or more Fc ligand (e.g., FcγR, C1q) of the antibodies and/or other antagonists identified using such screening methods. The Integrin $\alpha_v\beta_3$ antagonists identified and manipulated utilizing such methods can be used for the prevention, treatment, management or amelioration of Integrin $\alpha_v\beta_3$-mediated diseases and disorders or one or more symptoms thereof, including but not limited to cancer, inflammatory and autoimmune diseases either alone or in combination with other therapies.

[0248] The invention also provides variant Fc fusion proteins that immunospecifically bind to Integrin $\alpha_v\beta_3$. Said variant Fc fusion proteins can be used for the prevention, treatment, management or amelioration of Integrin $\alpha_v\beta_3$-mediated diseases and disorders or one or more symptoms thereof, including but not limited to cancer, inflammatory and autoimmune diseases either alone or in combination with other therapies.

[0249] In a specific embodiment, Fc variants and/or Fc variant fusion proteins of the invention that immunospecifically bind to Integrin $\alpha_v\beta_3$ are used for the prevention, management, treatment or amelioration of cancer or one or more symptoms thereof. In another embodiment, Fc variant antibodies and/or Fc variant fusion proteins of the invention used for the prevention, management, treatment or amelioration of cancer or one or more symptoms thereof are antagonists of Integrin $\alpha_v\beta_3$.

[0250] The invention also encompasses the use of Fc variants and/or variant Fc fusions with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$ conjugated or fused to a moiety (e.g., therapeutic agent or drug) for prevention, treatment, management or amelioration of Integrin $\alpha_v\beta_3$-mediated diseases and disorders or one or more symptoms thereof, including but not limited to cancer, inflammatory and autoimmune diseases. The invention further encompasses treatment protocols that enhance the prophylactic or therapeutic effect of said Fc variants and/or variant Fc fusions.

[0251] The invention provides methods for preventing, managing, treating or ameliorating cancer that has the potential to metastasize or has metastasized to an organ or tissue (e.g., bone) or one or more symptoms thereof, said methods comprising administering to a subject in need thereof one or more doses of a prophylactically or therapeutically amount of one or more Fc variants and/or variant Fc fusion protein of the invention.

[0252] The invention provides methods for preventing, managing, treating or ameliorating cancer or one or more symptoms thereof, said methods comprising administering to a subject in need thereof one or more doses of a prophylactically or therapeutically effective amount of one or more Fc variants and/or variant Fc fusion with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity fused or conjugated to a moiety (e.g., a therapeutic agent or drug). Examples of a moiety that an Fc variant can be fused or conjugated to include, but are not limited to those disclosed in PCT publication WO 2003/075957 which is herein incorporated

by reference in its entirety. Examples of Fc variants and variant Fc fusions with modified binding affinity to their one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity include, but are not limited to, those variants disclosed supra.

[0253] The present invention encompasses protocols for the prevention, management, treatment or amelioration of Integrin $\alpha_v\beta_3$-mediated diseases and disorders or one or more symptoms thereof, including but not limited to cancer, inflammatory and autoimmune diseases or one or more symptoms thereof in which one or more Fc variants and/or variant Fc fusion with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically binds to Integrin $\alpha_v\beta_3$ is used in combination with the administration of a dosage of a prophylactically or therapeutically effective amount of one or more other therapies other than an Fc variant and/or variant fusion protein. The invention is based, in part, on the recognition that the Fc variants and/or variant fusion proteins of the invention potentiate and synergize with, enhance the effectiveness of, improve the tolerance of, and/or reduce the side effects caused by, other therapies, including current standard and experimental chemotherapies. The combination therapies of the invention have additive potency, an additive therapeutic effect or a synergistic effect. The combination therapies of the invention enable lower dosages of the therapy (e.g., prophylactic or therapeutic agents) utilized in conjunction with Fc variants and/or variant Fc fusions for the prevention, management, treatment or amelioration of Integrin $\alpha_v\beta_3$-mediated diseases and disorders or one or more symptoms thereof, including but not limited to, cancer, inflammatory and autoimmune diseases and/or less frequent administration of such prophylactic or therapeutic agents to a subject with an Integrin $\alpha_v\beta_3$-mediated disease (e.g., cancer) to improve the quality of life of said subject and/or to achieve a prophylactic or therapeutic effect. Further, the combination therapies of the invention reduce or avoid unwanted or adverse side effects associated with the administration of current single agent therapies and/or existing combination therapies for diseases, such as cancer, which in turn improves patient compliance with the treatment protocol.

[0254] In one embodiment, the invention provides methods for preventing, managing, treating or ameliorating an Integrin $\alpha_v\beta_3$-mediated disease (e.g., cancer) or one or more symptoms thereof, said methods comprising administering to a subject in need thereof a dose of a prophylactically or therapeutically effective amount of an Fc variant and/or variant Fc fusion in combination with the administration of an Integrin antagonist, a standard or experimental chemotherapy, a hormonal therapy, a biological therapy/immunotherapy and/or a radiation therapy. In another embodiment, the invention provides methods for preventing, managing, treating or ameliorating an Integrin $\alpha_v\beta_3$-mediated disease (e.g., cancer) or one or more symptoms thereof, said methods comprising administering to a subject in need thereof a dose of a prophylactically or therapeutically effective amount of an Fc variant and/or variant Fc fusion in combination with surgery, alone or in further combination with the administration of an Integrin antagonist, a standard or experimental chemotherapy, a hormonal therapy, a biological therapy/immunotherapy and/or a radiation therapy. In accordance with these embodiments, the Fc variant and/or variant Fc fusion utilized to prevent, manage, treat or ameliorate an Integrin $\alpha_v\beta_3$-mediated disease (e.g., cancer) or one or more symptoms thereof may or may not be conjugated or fused to a moiety (e.g., therapeutic agent or drug) and said Fc variants and/or variant Fc fusions are in particular embodiments, antagonists that immunospecifically bind to Integrin $\alpha_v\beta_3$.

[0255] Therapeutic or prophylactic agents include, but are not limited to, small molecules, synthetic drugs, peptides, polypeptides, proteins, nucleic acids (e.g., DNA and RNA nucleotides including, but not limited to, antisense nucleotide sequences, triple helices and nucleotide sequences encoding biologically active proteins, polypeptides or peptides), antibodies, synthetic or natural inorganic molecules, mimetic agents, and synthetic or natural organic molecules. Any agent which is known to be useful, or which has been used or is currently being used for the prevention, treatment or amelioration of Integrin $\alpha_v\beta_3$-mediated disease or disorder including but not limited to cancer, inflammatory and autoimmune diseases or symptom associated therewith can be used in combination with an Fc variant and/or variant Fc fusion in accordance with the invention described herein.

[0256] Exemplary agents to be used in the combination therapies described supra include but are not limited to Integrin antagonists (e.g., RGD peptides and disintegrins), standard or experimental chemotherapy agents (e.g., doxorubicin, epirubicin, cyclophosphamide, 5-fluorouracil, taxanes such as docetaxel and paclitaxel, leucovorin, levamisole, irinotecan, estramustine, etoposide, vinblastine, dacarbazine, nitrosoureas such as carmustine and lomustine, vinca alkaloids, platinum compounds, cisplatin, mitomycin, vinorelbine, gemcitabine, carboplatin, hexamethylmelamine and/or topotecan), immunomodulatory agents (e.g., cytokines, antibodies, interleukins and hemapoietic factors), biological therapies/immunotherapies (e.g., tamoxifen, LHRH agonists, non-steroidal antiandrogens, steroidal antiandrogens, estrogens, aminoglutethimide, hydrocortisone, flutamide withdrawal, progesterone, ketoconazole, prednisone, interferon-alpha, interferon-beta, interferon-gamma, interleukin-2, tumor necrosis factor-alpha, and melphalan), anti-inflammatory agents (e.g., non-steroidal anti-inflammatory drugs (NSAIDs), steroidal anti-inflammatory drugs, beta-agonists, anticholingeric agents, and methyl xanthines), analgesics (e.g., NSAIDs, salicylates, acetominophen, narcotics, and non-narcotic and anxiolytic combinations). Also contemplated is the use of the Fc variants of the invention in combination with other anti-cancer antibody agents including but not limited to, Avastin™ (Genetech), Herceptin™ (Genentech), Rituxin™ (Genentech/Biogen) and Zevalin (Biogen). Additional agents and therapies and their dosages, routes of administration and recommended usage are known in the art and have been described in such literature as the *Physician's Desk Reference* (57th ed., 2003). Other combination therapies are described in PCT applications WO 02/070007; WO 03/075741; WO 03/075957 and WO 04/066956. Each of the above references and patent publications each of which are incorporated herein in their entireties.

[0257] Examples of anti-cancer agents that can be used in combination with the Fc variants and other embodiments of the invention, including pharmaceutical compositions and dosage forms and kits of the invention, include, but are not limited to: acivicin, aclarubicin, acodazole hydrochloride, acronine, adozelesin, aldesleukin, altretamine, ambomycin,

ametantrone acetate, aminoglutethimide, amsacrine, anastrozole, anthramycin, asparaginase, asperlin, azacitidine, azetepa, azotomycin, batimastat, benzodepa, bicalutamide, bisantrene hydrochloride, bisnafide dimesylate, bizelesin, bleomycin sulfate, brequinar sodium, bropirimine, busulfan, cactinomycin, calusterone, caracemide, carbetimer, carboplatin, carmustine, carubicin hydrochloride, carzelesin, cedefingol, chlorambucil, cirolemycin, cisplatin, cladribine, crisnatol mesylate, cyclophosphamide, cytarabine, dacarbazine, dactinomycin, daunorubicin hydrochloride, decarbazine, decitabine, dexormaplatin, dezaguanine, dezaguanine mesylate, diaziquone, docetaxel, doxorubicin, doxorubicin hydrochloride, droloxifene, droloxifene citrate, dromostanolone propionate, duazomycin, edatrexate, eflomithine hydrochloride, elsamitrucin, enloplatin, enpromate, epipropidine, epirubicin hydrochloride, erbulozole, esorubicin hydrochloride, estramustine, estramustine phosphate sodium, etanidazole, etoposide, etoposide phosphate, etoprine, fadrozole hydrochloride, fazarabine, fenretinide, floxuridine, fludarabine phosphate, fluorouracil, flurocitabine, fosquidone, fostriecin sodium, gemcitabine, gemcitabine hydrochloride, hydroxyurea, idarubicin hydrochloride, ifosfamide, ilmofosine, interleukin 2 (including recombinant interleukin 2, or rIL2), interferon alpha 2a, interferon alpha 2b, interferon alpha n1, interferon alpha n3, interferon beta I a, interferon gamma I b, iproplatin, irinotecan hydrochloride, lanreotide acetate, letrozole, leuprolide acetate, liarozole hydrochloride, lometrexol sodium, lomustine, losoxantrone hydrochloride, masoprocol, maytansine, mechlorethamine hydrochloride, megestrol acetate, melengestrol acetate, melphalan, menogaril, mercaptopurine, methotrexate, methotrexate sodium, metoprine, meturedepa, mitindomide, mitocarcin, mitocromin, mitogillin, mitomalcin, mitomycin, mitosper, mitotane, mitoxantrone hydrochloride, mycophenolic acid, nitrosoureas, nocodazole, nogalamycin, ormaplatin, oxisuran, paclitaxel, pegaspargase, peliomycin, pentamustine, peplomycin sulfate, perfosfamide, pipobroman, piposulfan, piroxantrone hydrochloride, plicamycin, plomestane, porfimer sodium, porfiromycin, prednimustine, procarbazine hydrochloride, puromycin, puromycin hydrochloride, pyrazofurin, riboprine, rogletimide, safingol, safingol hydrochloride, semustine, simtrazene, sparfosate sodium, sparsomycin, spirogermanium hydrochloride, spiromustine, spiroplatin, streptonigrin, streptozocin, sulofenur, talisomycin, tecogalan sodium, tegafur, teloxantrone hydrochloride, temoporfin, teniposide, teroxirone, testolactone, thiamiprine, thioguanine, thiotepa, tiazofurin, tirapazamine, toremifene citrate, trestolone acetate, triciribine phosphate, trimetrexate, trimetrexate glucuronate, triptorelin, tubulozole hydrochloride, uracil mustard, uredepa, vapreotide, verteporfin, vinblastine sulfate, vincristine sulfate, vindesine, vindesine sulfate, vinepidine sulfate, vinglycinate sulfate, vinleurosine sulfate, vinorelbine tartrate, vinrosidine sulfate, vinzolidine sulfate, vorozole, zeniplatin, zinostatin, zorubicin hydrochloride. Other anti cancer drugs include, but are not limited to: 20 epi 1,25 dihydroxyvitamin D3, 5 ethynyluracil, abiraterone, aclarubicin, acylfulvene, adecypenol, adozelesin, aldesleukin, ALL TK antagonists, altretamine, ambamustine, amidox, amifostine, aminolevulinic acid, amrubicin, amsacrine, anagrelide, anastrozole, andrographolide, angiogenesis inhibitors, antagonist D, antagonist G, antarelix, anti dorsalizing morphogenetic protein 1, antiandrogens, antiestrogens, antineoplaston, aphidicolin glycinate, apoptosis gene modulators, apoptosis regulators, apurinic acid, ara CDP DL PTBA, arginine deaminase, asulacrine, atamestane, atrimustine, axinastatin 1, axinastatin 2, axinastatin 3, azasetron, azatoxin, azatyrosine, baccatin III derivatives, balanol, batimastat, BCR/ABL antagonists, benzochlorins, benzoylstaurosporine, beta lactam derivatives, beta alethine, betaclamycin B, betulinic acid, bFGF inhibitor, bicalutamide, bisantrene, bisaziridinylspermine, bisnafide, bistratene A, bizelesin, breflate, bropirimine, budotitane, buthionine sulfoximine, calcipotriol, calphostin C, camptothecin derivatives, canarypox IL 2, capecitabine, carboxamide amino triazole, carboxyamidotriazole, CaRest M3, CARN 700, cartilage derived inhibitor, carzelesin, casein kinase inhibitors (ICOS), castanospermine, cecropin B, cetrorelix, chloroquinoxaline sulfonamide, cicaprost, cis porphyrin, cladribine, clomifene analogues, clotrimazole, collismycin A, collismycin B, combretastatin A4, combretastatin analogue, conagenin, crambescidin 816, crisnatol, cryptophycin 8, cryptophycin A derivatives, curacin A, cyclopentanthraquinones, cycloplatam, cypemycin, cytarabine ocfosfate, cytolytic factor, cytostatin, dacliximab, decitabine, dehydrodidemnin B, deslorelin, dexamethasone, dexifosfamide, dexrazoxane, dexverapamil, diaziquone, didemnin B, didox, diethylnorspermine, dihydro 5 azacytidine, dihydrotaxol, dioxamycin, diphenyl spiromustine, docetaxel, docosanol, dolasetron, doxifluridine, droloxifene, dronabinol, duocarmycin SA, ebselen, ecomustine, edelfosine, edrecolomab, eflomithine, elemene, emitefur, epirubicin, epristeride, estramustine analogue, estrogen agonists, estrogen antagonists, etanidazole, etoposide phosphate, exemestane, fadrozole, fazarabine, fenretinide, filgrastim, finasteride, flavopiridol, flezelastine, fluasterone, fludarabine, fluorodaunorunicin hydrochloride, forfenimex, formestane, fostriecin, fotemustine, gadolinium texaphyrin, gallium nitrate, galocitabine, ganirelix, gelatinase inhibitors, gemcitabine, glutathione inhibitors, hepsulfam, heregulin, hexamethylene bisacetamide, hypericin, ibandronic acid, idarubicin, idoxifene, idramantone, ilmofosine, ilomastat, imidazoacridones, imiquimod, immunostimulant peptides, insulin like growth factor 1 receptor inhibitor, interferon agonists, interferons, interleukins, iobenguane, iododoxorubicin, ipomeanol, iroplact, irsogladine, isobengazole, isohomohalicondrin B, itasetron, jasplakinolide, kahalalide F, lamellarin N triacetate, lanreotide, leinamycin, lenograstim, lentinan sulfate, leptolstatin, letrozole, leukemia inhibiting factor, leukocyte alpha interferon, leuprolide+estrogen+progesterone, leuprorelin, levamisole, liarozole, linear polyamine analogue, lipophilic disaccharide peptide, lipophilic platinum compounds, lissoclinamide 7, lobaplatin, lombricine, lometrexol, lonidamine, losoxantrone, lovastatin, loxoribine, lurtotecan, lutetium texaphyrin, lysofylline, lytic peptides, maitansine, mannostatin A, marimastat, masoprocol, maspin, matrilysin inhibitors, matrix metalloproteinase inhibitors, menogaril, merbarone, meterelin, methioninase, metoclopramide, MIF inhibitor, mifepristone, miltefosine, mirimostim, mismatched double stranded RNA, mitoguazone, mitolactol, mitomycin analogues, mitonafide, mitotoxin fibroblast growth factor saporin, mitoxantrone, mofarotene, molgramostim, monoclonal antibody, human chorionic gonadotrophin, monophosphoryl lipid A+myobacterium cell wall sk, mopidamol, multiple drug resistance gene inhibitor, multiple tumor suppressor 1 based therapy, mustard anticancer agent, mycaperoxide B, mycobacterial cell wall extract, myriaporone, N

acetyldinaline, N substituted benzamides, nafarelin, nagrestip, naloxone+pentazocine, napavin, naphterpin, nartograstim, nedaplatin, nemorubicin, neridronic acid, neutral endopeptidase, nilutamide, nisamycin, nitric oxide modulators, nitroxide antioxidant, nitrullyn, O6 benzylguanine, octreotide, okicenone, oligonucleotides, onapristone, ondansetron, ondansetron, oracin, oral cytokine inducer, ormaplatin, osaterone, oxaliplatin, oxaunomycin, paclitaxel, paclitaxel analogues, paclitaxel derivatives, palauamine, palmitoylrhizoxin, pamidronic acid, panaxytriol, panomifene, parabactin, pazelliptine, pegaspargase, peldesine, pentosan polysulfate sodium, pentostatin, pentrozole, perflubron, perfosfamide, perillyl alcohol, phenazinomycin, phenylacetate, phosphatase inhibitors, picibanil, pilocarpine hydrochloride, pirarubicin, piritrexim, placetin A, placetin B, plasminogen activator inhibitor, platinum complex, platinum compounds, platinum triamine complex, porfimer sodium, porfiromycin, prednisone, propyl bis acridone, prostaglandin J2, proteasome inhibitors, protein A based immune modulator, protein kinase C inhibitor, protein kinase C inhibitors, microalgal, protein tyrosine phosphatase inhibitors, purine nucleoside phosphorylase inhibitors, purpurins, pyrazoloacridine, pyridoxylated hemoglobin polyoxyethylene conjugate, raf antagonists, raltitrexed, ramosetron, ras farnesyl protein transferase inhibitors, ras inhibitors, ras GAP inhibitor, retelliptine demethylated, rhenium Re 186 etidronate, rhizoxin, ribozymes, RII retinamide, rogletimide, rohitukine, romurtide, roquinimex, rubiginone B1, ruboxyl, safingol, saintopin, SarCNU, sarcophytol A, sargramostim, Sdi 1 mimetics, semustine, senescence derived inhibitor 1, sense oligonucleotides, signal transduction inhibitors, signal transduction modulators, single chain antigen binding protein, sizofiran, sobuzoxane, sodium borocaptate, sodium phenylacetate, solverol, somatomedin binding protein, sonermin, sparfosic acid, spicamycin D, spiromustine, splenopentin, spongistatin 1, squalamine, stem cell inhibitor, stem cell division inhibitors, stipiamide, stromelysin inhibitors, sulfinosine, superactive vasoactive intestinal peptide antagonist, suradista, suramin, swainsonine, synthetic glycosaminoglycans, tallimustine, tamoxifen methiodide, tauromustine, taxol, tazarotene, tecogalan sodium, tegafur, tellurapyrylium, telomerase inhibitors, temoporfin, temozolomide, teniposide, tetrachlorodecaoxide, tetrazomine, thaliblastine, thalidomide, thiocoraline, thioguanine, thrombopoietin, thrombopoietin mimetic, thymalfasin, thymopoietin receptor agonist, thymotrinan, thyroid stimulating hormone, tin ethyl etiopurpurin, tirapazamine, titanocene bichloride, topsentin, toremifene, totipotent stem cell factor, translation inhibitors, tretinoin, triacetyluridine, triciribine, trimetrexate, triptorelin, tropisetron, turosteride, tyrosine kinase inhibitors, tyrphostins, UBC inhibitors, ubenimex, urogenital sinus derived growth inhibitory factor, urokinase receptor antagonists, vapreotide, variolin B, vector system, erythrocyte gene therapy, velaresol, veramine, verdins, verteporfin, vinorelbine, vinxaltine, vitaxin, vorozole, zanoterone, zeniplatin, zilascorb, and zinostatin stimalamer. Additional anti-cancer drugs are 5-fluorouracil and leucovorin.

[0258] The methods and formulations of the invention are particularly useful in preventing, managing, treating or ameliorating cancers, including, but not limited to, cancer of the head, neck, eye, mouth, throat, esophagus, chest, bone, lung, colon, rectum, colorectal, or other gastrointestinal tract organs, stomach, spleen, renal, skeletal muscle, subcutaneous tissue, metastatic melanoma, endometrial, prostate, breast, ovaries, testicles or other reproductive organs, skin, thyroid, blood, lymph nodes, kidney, liver, pancreas, and brain or central nervous system. Additional specific cancers are described supra. In a specific embodiment, the methods and formulations of the invention are used for the prevention, management, treatment or amelioration of a primary or secondary cancer that expresses Integrin $\alpha_v\beta_3$. In another embodiment, the methods and formulations of the invention are used for the prevention, management, treatment or amelioration of a primary or secondary cancer that does not express Integrin $\alpha_v\beta_3$.

[0259] The methods and formulations of the invention are useful not only in untreated cancer patients but are also useful in the management or treatment of cancer patients partially or completely refractory to current standard and experimental cancer therapies, including, but not limited to, chemotherapies, hormonal therapies, biological therapies, radiation therapies, and/or surgery.

6.10 Formulations and Administration

[0260] As described above, the present invention relates to the use of agents that immunospecifically bind and in particular embodiments, inhibit Integrin $\alpha_v\beta_3$ for the prevention, management, treatment or amelioration of an Integrin $\alpha_v\beta_3$-mediated disease (e.g., cancer) or one or more symptoms thereof and/or the inhibition of angiogenesis. Accordingly, the present invention provides formulations (e.g., a pharmaceutical composition) comprising one or more Fc variants and/or Fc variant fusions with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$ (also referred to herein as "formulation(s) of the invention" or simply "formulation(s)"). In a specific embodiment, said Fc variants and/or Fc variant fusions are antagonists of Integrin $\alpha_v\beta_3$.

[0261] In one embodiment, formulations (e.g., a pharmaceutical composition) comprising one or more Fc variants and/or Fc variant fusions are liquid formulations (referred to herein as "liquid formulation(s)" which are specifically encompassed by the more generic terms "formulation(s) of the invention" and "formulation(s)"). In a specific embodiment, the liquid formulations are substantially free of surfactant and/or inorganic salts. In another specific embodiment, the liquid formulations have a pH ranging from about 5.0 to about 7.0, about 5.5 to about 6.5, or about 5.8 to about 6.2, or about 6.0. In another specific embodiment, the liquid formulations have a pH ranging from 5.0 to 7.0, 5.5 to 6.5, or 5.8 to 6.2, or 6.0. In yet another specific embodiment, the liquid formulations comprise histidine at a concentration ranging from about 1 mM to about 100 mM, or from about 5 mM to about 50 mM, or about 10 mM to about 25 mM. In still another specific embodiment, the liquid formulations comprise histidine at a concentration ranging from 1 mM to 100 mM, or from 5 mM to 50 mM, or 10 mM to 25 mM

[0262] In another embodiment, the liquid formulations have a concentration of one or more Fc variants and/or Fc variant fusions is about 50 mg/ml, about 75 mg/ml, about 100 mg/ml, about 125 mg/ml, about 150 mg/ml, about 175 mg/ml, about 200 mg/ml, about 225 mg/ml, about 250 mg/ml, about 275 mg/ml, or about 300 mg/ml. In another embodiment, the liquid formulations have a concentration of one or more Fc variants and/or Fc variant fusions is 50

mg/ml, 75 mg/ml, 100 mg/ml, 125 mg/ml, 150 mg/ml, 175 mg/ml, 200 mg/ml, 225 mg/ml, 250 mg/ml, 275 mg/ml, or 300 mg/ml. In still another embodiment, the liquid formulations should exhibit one, or more of the following characteristics, stability, low to undetectable levels of antibody fragmentation and/or aggregation, very little to no loss of the biological activities of the antibodies or antibody fragments during manufacture, preparation, transportation, and storage. In certain embodiments the liquid formulations lose less than 50%, or less than 30%, or less than 20%, or less than 10% or even less than 5% or 1% of the antibody activity within 1 year storage under suitable conditions at about 4° C. The activity of an antibody can be determined by a suitable antigen-binding or effector function assay for the respective antibody. In yet another embodiment, the liquid formulations are of low visocisty and turbidity. In a particular embodiment, the liquid formulations have a viscosity of less than 10.00 cP at any temperature in the range of 1 to 26° C. Viscosity can be determined by numerous method well known in the art. For example, the viscosity of a polypeptide solution can be measured using a ViscoLab 4000 Viscometer System (Cambridge Applied Systems) equipped with a ViscoLab Piston (SN:7497, 0.3055", 1-20 cP) and S6S Reference Standard (Koehler Instrument Company, Inc.) and connected to a water bath to regulate the temperature of the samples being analyzed. The sample is loaded into the chamber at a desired starting temperature (e.g., 2° C.) and the piston lowered into the sample. After sample was equilibrated to the temperature of the chamber, measurement is initiated. The temperature is increased at a desired rate to the desired final temperature (e.g., >25° C.). And the viscosity over time is recorded.

[0263] It is contemplated that the liquid formulations may further comprise one or more excipients such as a saccharide, an amino acid (e.g. arginine, lysine, and methionine) and a polyol. Additional descriptions and methods of preparing and analyzed liquid formulations can be found, for example, in PCT publications WO 03/106644; WO 04/066957; WO 04/091658 each of which is herein incorporated by reference in its entirety.

[0264] In one embodiment the formulations (e.g., liquid formulations) of the invention are pyrogen-free formulations which are substantially free of endotoxins and/or related pyrogenic substances. Endotoxins include toxins that are confined inside a microorganism and are released when the microorganisms are broken down or die. Pyrogenic substances also include fever-inducing, thermostable substances (glycoproteins) from the outer membrane of bacteria and other microorganisms. Both of these substances can cause fever, hypotension and shock if administered to humans. Due to the potential harmful effects, it is advantageous to remove even low amounts of endotoxins from intravenously administered pharmaceutical drug solutions. The Food & Drug Administration ("FDA") has set an upper limit of 5 endotoxin units (EU) per dose per kilogram body weight in a single one hour period for intravenous drug applications (The United States Pharmacopeial Convention, Pharmacopeial Forum 26 (1):223 (2000)). When therapeutic proteins are administered in amounts of several hundred or thousand milligrams per kilogram body weight, as can be the case with monoclonal antibodies, it is advantageous to remove even trace amounts of endotoxin. In one embodiment, endotoxin and pyrogen levels in the composition are less then 10 EU/mg, or less then 5 EU/mg, or less then 1 EU/mg, or less then 0.1 EU/mg, or less then 0.01 EU/mg, or less then 0.001 EU/mg.

[0265] It will be apparent to one skilled in the art that a formulation comprising one or more Fc variants and/or Fc variant fusions to be administered to a subject (e.g., a human) in need thereof should be formulated in a pharmaceutically-acceptable excipient. Examples of formulations, pharmaceutical compositions in particular, of the invention include but are not limited to those disclosed in PCT publications WO 02/070007, WO 03/075957 and WO 04/066957 each of which is herein incorporated by reference in its entirety. Briefly, the excipient that is included with the Fc variants and/or variant Fc fusion of the present invention in these formulations (e.g., liquid formulations) can be selected based on the expected route of administration of the formulations in therapeutic applications. The route of administration of the formulations depends on the condition to be treated. For example, intravenous injection may be preferred for treatment of a systemic disorder such as a lymphatic cancer or a tumor which has metastasized. The dosage of the formulations to be administered can be determined by the skilled artisan without undue experimentation in conjunction with standard dose-response studies. Relevant circumstances to be considered in making those determinations include the condition or conditions to be treated, the choice of formulations to be administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms. For example, the actual patient body weight may be used to calculate the dose of the Fc variants and/or variant Fc fusion of the present invention in these formulations in milliliters (mL) to be administered. There may be no downward adjustment to "ideal" weight. In such a situation, an appropriate dose may be calculated by the following formula:

Dose (mL)=[patient weight (kg)×dose level (mg/kg)/
drug concentration (mg/mL)]

[0266] Depending on the condition, the formulations can be administered orally, parenterally, intramuscularly, intranasally, vaginally, rectally, lingually, sublingually, buccally, intrabuccally, intravenously, cutaneously, subcutaneously and/or transdermally to the patient.

[0267] Accordingly, formulations designed for oral, parenteral, intramuscular, intranasal, vaginal, rectal, lingual, sublingual, buccal, intrabuccal, intravenous, cutaneous, subcutaneous and/or transdermal administration can be made without undue experimentation by means well known in the art, for example, with an inert diluent or with an edible carrier. The formulations may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the formulations of the present invention may be incorporated with excipients and used in the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums, and the like.

[0268] Tablets, pills, capsules, troches and the like may also contain binders, recipients, disintegrating agent, lubricants, sweetening agents, and/or flavoring agents. Some examples of binders include microcrystalline cellulose, gum tragacanth and gelatin. Examples of excipients include starch and lactose. Some examples of disintegrating agents include alginic acid, cornstarch, and the like. Examples of lubricants include magnesium stearate and potassium stear-

ate. An example of a glidant is colloidal silicon dioxide. Some examples of sweetening agents include sucrose, saccharin, and the like. Examples of flavoring agents include peppermint, methyl salicylate, orange flavoring, and the like. Materials used in preparing these various formulations should be pharmaceutically pure and non-toxic in the amounts used.

[0269] The formulations of the present invention can be administered parenterally, such as, for example, by intravenous, intramuscular, intrathecal and/or subcutaneous injection. Parenteral administration can be accomplished by incorporating the formulations of the present invention into a solution or suspension. Such solutions or suspensions may also include sterile diluents, such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol and/or other synthetic solvents. Parenteral formulations may also include antibacterial agents, such as, for example, benzyl alcohol and/or methyl parabens, antioxidants, such as, for example, ascorbic acid and/or sodium bisulfite, and chelating agents, such as EDTA. Buffers, such as acetates, citrates and phosphates, and agents for the adjustment of tonicity, such as sodium chloride and dextrose, may also be added. The parenteral preparation can be enclosed in ampules, disposable syringes and/or multiple dose vials made of glass or plastic. Rectal administration includes administering the formulation into the rectum and/or large intestine. This can be accomplished using suppositories and/or enemas. Suppository formulations can be made by methods known in the art. Transdermal administration includes percutaneous absorption of the formulation through the skin. Transdermal formulations include patches, ointments, creams, gels, salves, and the like. The formulations of the present invention can be administered nasally to a patient. As used herein, nasally administering or nasal administration includes administering the formulations to the mucous membranes of the nasal passage and/or nasal cavity of the patient.

[0270] In certain embodiments, the formulations (e.g., liquid formulations) are administered to the mammal by subcutaneous (i.e., beneath the skin) administration. For such purposes, the formulations may be injected using a syringe. However, other devices for administration of the formulations are available such as injection devices (e.g. the Inject-ease_and Genject_devices), injector pens (such as the GenPen™); auto-injector devices, needleless devices (e.g., MediJector and BioJector); and subcutaneous patch delivery systems.

[0271] In another aspect of the invention there is provided a slow release formulations. In a specific embodiment, a slow release formulation comprises a liquid formulation. Slow release formulations may be formulated from a number of agents including, but not limited to, polymeric nano or microparticles and gels (e.g., a hyaluronic acid gel). Besides convenience, slow release formulations offer other advantages for delivery of protein drugs including protecting the protein (e.g., Fc variant and/or variant Fc fusion) over an extended period from degradation or elimination, and the ability to deliver the protein locally to a particular site or body compartment thereby lowering overall systemic exposure.

[0272] The present invention, for example, also contemplates injectable depot formulations in which the protein (e.g., Fc variant and/or variant Fc fusion) is embedded in a biodegradable polymeric matrix. Polymers that may be used include, but are not limited to, the homo- and co-polymers of lactic and glycolic acid (PLGA). PLGA degrades by hydrolysis to ultimately give the acid monomers and is chemically unreactive under the conditions used to prepare, for example, microspheres and thus does not modify the protein. After subcutaneous or intramuscular injection, the protein is released by a combination of diffusion and polymer degradation. By using polymers of different composition and molecular weight, the hydrolysis rate can be varied thereby allowing release to last from days to months. In a further aspect the present invention provides a nasal spray formulation. In a specific embodiment, a nasal spray formulation comprises the liquid formulation of the present invention.

[0273] The formulations of the invention may be used in accordance with the methods of the invention for the prevention, management, treatment or amelioration of cancer, inflammatory and autoimmune diseases (in particular an Integrin $\alpha_v\beta_3$-mediated disease) or one or more symptoms thereof. In one embodiment, the formulations of the invention are sterile and in suitable form for a particular method of administration to a subject with cancer, inflammatory and autoimmune diseases, in particular an Integrin $\alpha_v\beta_3$-mediated disease.

[0274] The invention provides methods for preventing, managing, treating or ameliorating cancer, inflammatory and autoimmune diseases (in particular an Integrin $\alpha_v\beta_3$-mediated disease) or one or more symptoms thereof, said method comprising: (a) administering to a subject in need thereof a dose of a prophylactically or therapeutically effective amount of a formulation comprising one or more Fc variants and/or variant Fc fusions, that immunospecifically bind to Integrin $\alpha_v\beta_3$ and (b) administering one or more subsequent doses of said formulation, to maintain a plasma concentration of the antagonist at a desirable level (e.g., about 0.1 to about 100 μg/ml), which continuously blocks the Integrin $\alpha_v\beta_3$ activity. In a specific embodiment, the plasma concentration of the Fc variants and/or variant Fc fusions is maintained at 10 μg/ml, 15 μg/ml, 20 μg/ml, 25 μg/ml, 30 μg/ml, 35 μg/ml, 40 μg/ml, 45 μg/ml or 50 μg/ml. In a specific embodiment, said effective amount of Fc variant and/or variant Fc fusion to be administered is between at least 1 mg/kg and 100 mg/kg per dose. In another specific embodiment, said effective amount of Fc variant and/or variant Fc fusion to be administered is between at least 1 mg/kg and 20 mg/kg per dose. In another specific embodiment, said effective amount of Fc variant and/or variant Fc fusion to be administered is between at least 4 mg/kg and 10 mg/kg per dose. In yet another specific embodiment, said effective amount of Fc variant and/or variant Fc fusion to be administered is between 50 mg and 250 mg per dose. In still another specific embodiment, said effective amount of Fc variant and/or variant Fc fusion to be administered is between 100 mg and 200 mg per dose.

[0275] The present invention provides kits comprising one or more Fc variants and/or variant Fc fusions with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$ conjugated or fused to a detectable agent, therapeutic agent or drug, in one or more containers, for use in the prevention, treatment, manage-

ment, amelioration, detection, monitoring or diagnosis of cancer, inflammatory and autoimmune diseases, in particular an Integrin $\alpha_v\beta_3$-mediated disease.

[0276] The invention also provides kits comprising one or more Fc variants and/or variant Fc fusions with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$ in a first vial and one or more prophylactic or therapeutic agents, other than Fc variants that immunospecifically bind to Integrin $\alpha_v\beta_3$, in a second vial for use in the prevention, treatment, management, amelioration, detection, monitoring or diagnosis of cancer, inflammatory and autoimmune diseases, in particular an Integrin $\alpha_v\beta_3$-mediated disease. The invention also provides kits comprising one or more Fc variants and/or variant Fc fusions with modified binding affinity to one or more Fc ligand (e.g., FcγRs, C1q) and altered ADCC and/or CDC activity that immunospecifically bind to Integrin $\alpha_v\beta_3$ conjugated or fused to a therapeutic agent or drug in a first vial and one or more prophylactic or therapeutic agents, other than antagonists of Integrin $\alpha_v\beta_3$, in a second vial for use in the prevention, treatment, management, amelioration, detection, monitoring or diagnosis of cancer, inflammatory and autoimmune diseases, in particular an Integrin $\alpha_v\beta_3$-mediated disease. The kits may further comprise packaging materials and/or instructions.

7. EXAMPLES

[0277] The invention is now described with reference to the following examples. These examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teachings provided herein.

7.1 Example 1

Construction and Expression of Novel Fc Variants of Antibodies

[0278] Based on the structural information available for the Fc-FcγRIIIB complex, each of the putative FcγR contact residues of the IgG1 Fc portion was randomly mutated by using degenerated oligonucleotides incorporating all possible single mutations. The contact residues were divided into four regions (RI: Leu^{234}, Leu^{235}, Gly^{236}, Gly^{237}, Pro^{238}, Ser239; RII: Asp^{265}, Ser^{267}, Glu^{269}; RIII: Ser^{298}; and RIV: Ala^{327}, Leu^{328}, Pro^{329}, Ala^{330}, and Ile^{332}). Primers used for the amplification and library construction are listed in table 4. The IgG1 of antibody Vitaxin™, converted into scFv-Fc format, was used as the model for this study. The DNA and corresponding amino acid sequences of the variable regions of the Vitaxin® heavy and light chains used to generate the scFv-Fc are shown in **FIG. 1** (panels A and B, respectively). The scFv-Fc was then harnessed as the template to build three Fc mutant libraries containing single mutations in the Fc region. Library I contains all single mutations in the RI region; library II covers the RII and RIII regions; and library III covers the RIV region. Overlapping PCR approach was used to synthesize entire Fc region containing mutations.

TABLE 4

Primers

Primer	Sequence	Notes	SEQ ID
MDAD-1	CCG TGC CCA GCA CCT GAA NNK CTG GGG GGA CCG TCA GTC	contact Region I	11
MDAD-2	CCG TGC CCA GCA CCT GAA CTC NNK GGG GGA CCG TCA GTC TTC	contact Region I	12
MDAD-3	CCG TGC CCA GCA CCT GAA CTC CTG NNK GGA CCG TCA GTC TTC CTC	contact Region I	13
MDAD-4	CCG TGC CCA GCA CCT GAA CTC CTG GGG NNK CCG TCA GTC TTC CTC TTC	contact Region I	14
MDAD-5	CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA NNK TCA GTC TTC CTC TTC CCC	contact Region I	15
MDAD-6	CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG NNK CT TT CT T CC CC NNK GTC TTC CTC TTC CCC CCA	contact Region I	16
MDAD-7	GTC ACA TGC GTG GTG GTG NNK GTG AGC CAC GAA GAC CCT	contact Region II	17
MDAD-8	GTC ACA TGC GTG GTG GTG GAG GTC NNK CAC GAA GAC CCT GAG GTC	contact Region II	18
MDAD-9	GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC NNK GAC CCT GAG GTC AAG TTC	contact Region II	19
MDAD-10	CGG GAG GAG CAG TAC AAC NNK ACG TAC CGT GTG GTC AGC	contact Region III	20

TABLE 4-continued

Primers

Primer	Sequence	Notes	SEQ ID
MDAD-11	TGC AAG GTC TCC AAC AAA NNK CTC CCA GCC CCC ATC GAG	contact Region IV	21
MDAD-12	TGC AAG GTC TCC AAC AAA GCC NNK CCA GCC CCC ATC GAG AAA	contact Region IV	22
MDAD-13	TGC AAG GTC TCC AAC AAA GCC CTC NNK GCC CCC ATC GAG AAA ACC	contact Region IV	23
MDAD-14	TGC AAG GTC TCC AAC AAA GC CTC CCA NNK CCC ATC GAG AAA ACC ATC	contact Region IV	24
MDAD-15	TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC NNK GAG AAA ACC ATC TCC AAA	contact Region IV	25
MDAD-16	ACT CAC ACA TGT CCA CCG TGC CCA GCA CCT GAA	Fc N-terminus	26
MDAD-17	CAC CAC CAC GCA TGT GAC	RII primer	27
MDAD-18	GTT GTA CTG CTC CTC CCG	RIII primer	28
MDAD-19	TTT GTT GGA GAC CTT GCA	RIV primer	29
MDAD-20	AAC CTC TAC AAA TGT GGT ATG GCT	Fc C- terminus	30
A1	AAG CTT CGG TCC GCC ACC ATG GCA ACT GAA GAT CTC CCA AAG	FcγRIIIA primer	31
A2	GTC TGC CGA ACC GCT GCC TGC CAA ACC TTG AGT GAT GGT	FcγRIIIA primer	32
B1	AGC TTC GGT CCG CCA CCA TGG CTG TGC TAT TCC TGG CAG CTC CCC CAA	FcγRIIB primer	33
B2	GTC TGC CGA ACC GCT GCC CCC CAT CGG TGA AGA GCT GGG AGC	FcγRIIB primer	34
SA1	GGC AGC GGT TCG GCA GAC CCC TCC AAG GAC	Streptavidin primer	35
SA2	CAG GGG CTA GCT TAC TGC TGA ACG GCG TCG AGC GG	Streptavidin primer	36
EA1	TCC ACA GGT GTC CAC TCC CGG ACT GAA GAT CTC CCA AAG	FcγRIIIA primer	37
EA2	GGG AGA ATT CCG CGG CCG CTT ATT TGT CAT CGT CAT CTT TGT AGT CAT GGT GAT GGT GAT GGT GTG CGC CTG CCA AAC CTT GAG TGA TGG T	FcγRIIIA primer	38
EB1	TCC ACA GGT GTG CAC TCC GCT GTG CTA TTC CTG GCA GCT CCC CCA AAG	FcγRIIB primer	39
EB2	GGG AGA ATT CCG CGG CCG CTT ATT TGT CAT CGT CAT CTT TGT AGT CAT GGT GAT GGT GAT GGT GTG CGC CCC CCA TCG GTG AAG AGC TGG GAG C	FcγRLIB primer	40
Oligo 1	GCC CTC CCA GCC CCC gag GAG AAA ACC ATC TCC	I332E	41
Oligo 2	GCC CTC CCA GCC CCC cag GAG AAA ACC ATC TCC	I332Q	42
Oligo 3	GCC CTC CCA GCC CCC ggc GAG AAA ACC ATC TCC	I332G	43
Oligo 4	GCC CTC CCA GCC CCC gcc GAG AAA ACC ATC TCC	I332A	44
Oligo 5	GCC CTC CCA GCC CCC tac GAG AAA ACC ATC TCC	I332Y	45
Oligo 6	GCC CTC CCA GCC CCC gac GAG AAA ACC ATC TCC	I332D	46
Oligo 7	GCC CTC CCA GCC CCC aac GAG AAA ACC ATC TCC	I332N	47
Oligo 8	GCC CTC CCA GCC CCC gtg GAG AAA ACC ATC TCC	I332V	48

TABLE 4-continued

Primers

Primer	Sequence	Notes	SEQ ID
Oligo 9	GCC CTG CCA GCC CCC tgg GAG AAA ACC ATC TCC	I332W	49
Oligo 10	GCC CTC CCA GCC CCC cgc GAG AAA ACC ATC TCC	I332R	50
Oligo 11	GCC CTC CCA GCC CCC agc GAG AAA ACC ATC TCC	I332S	51
Oligo 12	GCC CTC CCA GCC CCC aag GAG AAA ACC ATC TCC	I332K	52
Oligo 13	GCC CTC CCA GCC CCC atg GAG AAA ACC ATC TCC	I332M	53
Oligo 14	GCC CTC CCA GCC CCC acc GAG AAA ACC ATC TCC	I332T	54
Oligo 15	GCC CTC CCA GCC CCC tgc GAG AAA ACC ATC TCC	I332C	55
Oligo 16	GCC CTC CCA GCC CCC ctg GAG AAA ACC ATC TCC	I332L	56
Oligo 17	GCC CTC CCA GCC CCC ttc GAG AAA ACC ATC TCC	I332F	57
Oligo 18	GCC CTC CCA GCC CCC cac GAG AAA ACC ATC TCC	I332H	58
Oligo 19	GCC CTC CCA GCC CCC cct GAG AAA ACC ATC TCC	I332P	59
Oligo 20	CTGGGGGGACCG gac GTCTTCCTCTTC	S239D	60
Oligo 21	AAAGCCCTCCCA ctg CCCgagGAGAAA	A330L/I332E	61

7.1.1 Materials and Methods

[0279] Construction of Fc Libraries: For constructing Fc library I, primers MDAD-16, equimolar mixture of MAD-2 to -6, and MDAD-20 were used in the PCR reaction. The PCR products were gel purified and digested by restriction enzymes Not I/Pci I, and ligated into the expression vector pMI under the control of the CMV promoter. For constructing Fc library II, two PCR products incorporating RII and RIII mutations were mixed at 3:1 molar ratio for cloning into pMI vector. Primers MDAD-16, MDAD-17, equimolar mixture of MDAD-7 to -9, and MDAD-20 were used to amplify Fc region to incorporate RII mutations, and primers MDAD-16, -18, -10, and -20 were used to amplify Fc region to incorporate RIII mutations. For Fc library III, primers MDAD-16, MDAD-19, equimolar mixture of MDAD-11 to -15, and MDAD-20 were used in the PCR reaction.

[0280] Transfection: The plasmids of three Fc libraries (I, II, and III) were linearized by Sal I, ethanol precipitated and resuspended in H_2O. 50 μg of each linearized library DNA was individually transfected into 10^7 NS0 cells by electroporation. After electroporation, the cells were transferred to a tube containing 30 ml of growth medium (Glutamine-free IMDM, 1×GS supplement and 2 mM L-glutamine) and seeded in 96-well plates (50 μl/well) at variable dilutions. The cells were cultured at 37° C. in humid air containing 5% CO_2.

[0281] Selection of Stable Transfectants: The selection of stably transfected NS0 cells expressing scFv-Fc mutants was started 18-24 hours after transfection by converting to selection medium (same as growth medium but without glutamine). The medium was changed twice a week at one half of the total volume. After 2-3 weeks of incubation, the culture supernatants were collected for screening of antibody expression.

[0282] Purification of scFv-Fc Variants: The culture supernatants containing scFv-Fc mutants were purified by using a Protein A spin chromatography kit following manufacturer's protocol (Pierce). The bound scFv-Fc mutants were eluted with 0.1 M citrate buffer and then dialyzed in PBS. All proteins were analyzed by SDS-polyacrylamide gel electrophoresis and were applied to quantitative ELISA using anti-human IgG assay plates (Becton Dickson) or BCA kits (PIERCE) to determine scFv-Fc concentrations.

[0283] Antibody Ouantitation by ELISA: To determine the expression level of the Fc variants, anti-human IgG-coated microtiter plates (Becton Dickson) were used. The culture supernatants were added to the wells at dilutions of 1:10 and 1:100. After a 1 hour incubation at room temperature, the plates were washed with PBST (PBS+0.1% Tween 20) and incubated at room temperature for an additional hour with anti-human IgG (Pierce) at a 1:60000 dilution. The signals were detected by TME substrate (Pierce) and read by an ELISA reader at 450 nm. Purified parental Vitaxin™ scFv-Fc expressed in a pMI vector was employed as a standard (at serial dilutions of 0.003 μg-10 μg/ml).

7.2 Example 2

Construction and Expression of the Extracellular Domains of FcγRIIIA and FcγRIIB

[0284] To facilitate the binding studies of the Fc variants to FcγRs the extracellular domains of FcγRIIIA and FcγRIIB were subcloned for expression as strepavidin fusion proteins in *E. coli* and for expression in mammalian cells. The

FcγRIIIA prepared for analysis is the low affinity (F158) allotype. Two forms of FcγRIIIA and FcγRIIB were prepared, a "tetramer" form, generated as as Strepavidin fusion, and a "monomer" form generated as a Flag-tagged.

7.2.1 Materials and Methods

[0285] Construction and Bacterial Expression of the Extracellular Domains of FcγRIIIA- and FcγRIIB-Strepavidin Fusion Proteins (Tetramer): Primer pairs SA1/SA2, A1/A2, and B1/B2 (see primer list, Table 4) were used to PCR amplify streptavidin and the extracellular domains of FcγR IIIA and FcγR IIB, respectively. The cDNA library of human bone marrow (Clontech) was used as a template for FcγR IIIA and FcγR IIB amplification, and the genomic DNA of *Streptomyces avidinii* was used as the template for the amplification of Streptavidin. Overlapping PCR was used to assemble fusion genes of FcγR IIIA-streptavidin and FcγR IIB-strepavidin. The fusion genes were digested by the restriction enzymes Nco I/Nhe I and cloned into the expression vector pET-28a. The fusion proteins were expressed as inclusion bodies and refolded by dialysis to slowly remove urea as described by C. Gao, et al. (1997, *PNAS USA* 94:11777-82). The refolded fusion proteins were then purified by an immunobiotin column (PIERCE) according to manufacturer's instructions.

[0286] Construction and Mammalian Expression of the Extracellular Domains of FcγRIIIA and FcγRIIB (Monomer): The extracellular domains of FcγR IIIA and FcγR IIB were PCR amplified from the cDNA library of human bone marrow (Clontech) with primers EA1/EA2 and EB1/EB2, respectively (see primer list, Table 4). The PCR products were digested by Xba I/Not I and cloned into the mammalian cell expression vector pMI226 under the control of the CMV promoter to generate proteins in which the extracelluar domains of FcγR IIIA and FcγR IIB are tagged with His6-tag followed by FLAG tag at the C-terminal end. The plasmid DNA was transiently transfected into 293H cells by Lipofectamine 2000 Transfection Reagent (Invitrogen). After three collections within 9 days, the proteins were purified by passing the culture supernatant through anti-FLAG M2 agarose columns (Sigma). The FLAG-tagged FcγRIIIA/IIB proteins were eluted from the column and dialyzed against PBS.

7.3 Example 3

Characterization of the Fc Variants

[0287] After mutagenesis of the Fc domain (see example 1 supra) Fc variants, in the scFV-Fc fusion format, were screened for enhanced binding to FcγRIIIA tetramer by ELISA as detailed below. The results for several clones are shown in **FIG. 5**. In addition, the ADCC activity of these clones was determined against M21 cells. The results for several clones are shown in **FIG. 6**. Based on these studies three substitutions were chosen for further study, S239D, A330L and I332E. These substitutions were introduced into the Fc region of the intact Vitaxin® IgG1 heavy chain and coexpressed with Vitaxin® light chain to produce full length Vitaxin® Fc variant IgG1 molecules. The Vitaxin® Fc variant having the I332E substitution was designated Vitaxin®-1M, the Vitaxin® Fc variant having the S239D, A330L, I332L triple substitution was designated Vitaxin®-3M.

[0288] A panel of Vitaxin® Fc variants, in IgG format, was generated in which each of the standard 20 amino acids was substituted at position 332. These variants were characterized. **FIG. 7A** shows the relative binding to FcγRIIIA of these Fc variants, as determined by ELISA. It can be seen that under these conditions several substitutions showed enhanced binding including I332T, I332L, I332F and most dramatically, I332E. However, as shown in **FIG. 7B**, only the I332E substitution showed a similar increase in ADCC activity.

[0289] Representative binding curves for Vitaxin® and one Fc variant of Vitaxin® (I332E; Vitaxin-IM) to FcγRIIIA and FcγRIIB are shown in **FIGS. 8A and 8B** respectively. Vitaxin® was prepared from two cell sources, NSO and HEK293 cells, no difference in binding was observed between these two sources of Vitaxin. The Vitaxin® Fc variant was then prepared from HEK293 cells. The Vitaxin® Fc variant showed approximately a 2.5 fold increase in binding affinity to FcγRIIIA (**FIG. 8A**) with no corresponding change in binding to FcγRIIB as determined by ELISA (**FIG. 8B**).

[0290] The binding of Vitaxin® and the Vitaxin® Fc variants to FcγRIIIA was further analyzed by BIAcore analysis. The binding of Vitaxin® and the Vitaxin® Fc variants were analyzed with the receptor soluble and the antibody immobile (see methods below). The Vitaxin-1M Fc variant was shown to have a roughly 7 fold increase in binding affinity to FcγIIIA as compared to that of the parental wild type Vitaxin antibody. The interaction of the Vitaxin-3M Fc variant to FcγRIIIA was also analyzed by BIAcore and found to have a binding affinity of ~114 nM, nearly 80 time better than that of the parental wild type Vitaxin antibody. The results are summarized in Table 5.

TABLE 5

Binding Constants (K_D) of wild type antibodies and Fc variants to FcγRIIIA

Antibody	Run #	RUs Immobilized	K_D Isotherm	K_D Scatchard	Fold increase over WT[a]
Vitaxin ®	1	9608	3.47 μM	3.26 μM	
Vitaxin-1M	1	9331	458 nM	458 nM	6.5
Vitaxin ®	2	9434	8.9 μM	7.6 μM	
Vitaxin-1M	2	9383	1.28 μM	1.22 μM	7.0
Vitaxin-3M	2	8284	114 nM	113 nM	78.0
3F2	3	8568	15.6 μM	14.2 μM	
3F2-1M	3	7718	1.77 μM	1.68 μM	8.8
3F2-3M	3	7809	158 nM	162 nM	99

[a]calculated using Isoltherm values

[0291] The Vitaxin-IM Fc variant was further characterized in ADCC assays against M21 cells. First, the ratio of target to effector cells was kept constant at 50:1 and the concentration of the two antibodies was varied from 0.4 to 1000 ng/ml (**FIG. 9**). Next, the concentration of antibody was varied for several different ratios of target to effector cell (6.25:1, 12.5:1, 25:1 and 50:1) (**FIG. 10**). In both assays the ADCC activity of the Vitaxin-IM Fc (I332E) variant was approximately 3 fold higher than that of the parent Vitaxin® antibody.

[0292] The Vitaxin-3M Fc variant was also characterized in ADCC assays against a target cells expressing differing levels of Integrin αVβ3 (**FIG. 11**). The target cell lines used

were M21 (a high expresser), DU145 (a low expressor), A498 and ACHN (moderate expressors). The assays were performed using two different ratios of target to effector cell (50:1 and 25:1) and antibody concentrations ranging from 4 to 400 ng per well. In all cases the ADCC activity of the Vitaxin-3M Fc variant was seen to be higher than wild type Vitaxin®. Vitaxin-3M Fc variant was also shown to have higher ADCC activity compared to the wild type Vitaxin® antibody against SKMEL28 target cells which express Integrin $\alpha_v\beta_3$ **(FIG. 18)**.

7.3.1 Materials and Methods

[0293] ELISA Receptor Binding Assay: Microtiter plates were coated with protein A/G (PIERCE) solution (0.25 μg/ml) and incubated at 4° C. overnight. Any remaining binding sites were blocked with 4% skim milk. Approximately 25 μl per well of mutant antibody solution was added to each well and incubated for 1 h at 37° C. After washing, FcγRIIIA-streptavidin or FcγRIIB-streptavidin fusion protein (in 1% BSA) was added for 1 hour at 37° C., followed by washing and biotin-conjugated HRP for 30 min. Detection was carried out by adding 30 μl of tetramethylbenzidine substrate (Pierce) followed by neutralization with 30 μl of 0.2 M H_2SO_4. The absorbance was read at 450 nm

[0294] Generation of 332 Amino Acid Substitutions: QuikChange® II XL site-directed mutagenesis kit (Stratagene, San Diego) was used to generate all the amino acid substitutions at position 332 of the gene encoding the heavy chain of wild type Vitaxin® in the plasmid pMI331 (see **FIG. 4**). Oligos 1 to 19 (see Table 4) were applied to change the Isoleucine to all other 19 different amino acids at the position 332, using Vitaxin as the template. The mutation was further confirmed by DNA sequencing.

[0295] The plasmid DNA containing antibody genes was transiently transfected into 293H cells by Lipofectamine 2000 Transfection Reagent (Invitrogen). After three collections within 9 days, the culture supernatants containing antibody were affinity purified by using a pre-packed Protein A column (Amersham Biosciences, now belongs to GE healthcare). The bound antibody were eluted with elution buffer (100 mM Glycine, pH3.2), neutralized by 1 M Tris buffer (pH 8.0) and then dialyzed in PBS. All purified antibodies were analyzed by SDS-polyacrylamide gel electrophoresis and were applied to quantitative ELISA using anti-human IgG assay plates (Becton Dickson) or BCA kits (PIERCE) to determine IgG concentrations.

[0296] Generation of Vitaxin®—1M and 3M Fc variants: The I332E substitution was generated by site directed mutagenesis (as described above) of the gene encoding the heavy chain of wild type Vitaxin® in the plasmid pMI331 (see **FIG. 4**). The mutant I332E was designated as Vitaxin 1M. The Vitaxin 3M was further generated by two sequential site directed mutagenesis (as described above), using oligo 20 and 21 (see Table 4) as primers and Vitaxin 1M as template. Expression and purification of the 1M and 3M Vitaxin® Fc variants was the same as described above.

[0297] Kinetic Analysis via BIAcore: for Run 1 the interaction of FcγRIIIA with immobilized Vitaxin® and Vitaxin® Fc variant IgGs were monitored by surface plasmon resonance detection using a BIAcore 3000 instrument (Pharmacia Biosensor, Uppsala, Sweden). Vitaxin® and Vitaxin® Fc variant IgGs were coupled to the dextran matrix of a CM5 sensor chip (Pharmacia Biosensor) using an Amine Coupling Kit, as described (Johnsson et al., 1992, *Anal Biochem* 198:268-277), at a surface density of approximately 9400 RUs (see Table 5). FcγRIIIA was serially diluted in 0.01 M HEPES pH 7.4 containing 0.15 M NaCl, 3 mM EDTA and 0.005% P20, at concentrations ranging from 2 μM down to 7.8 nM. Duplicate injections of each concentration were made. All binding experiments were performed at 25° C., and at a flow rate of 10 μL/min. Binding was monitored for 25 min. Following each injection of FcγRIIIA, the IgG surfaces were regenerated with a 30 sec. pulse of 5 mM HCl. FcγRIIIA was also passed over a blank reference cell which is connected, in series, to the IgG-containing flow cells. The steady-state binding curves were also corrected for injection artifacts by subtraction of buffer injections. This doubly-corrected data was then fit to a steady-state isotherm provided by the instrument manufacturer (Pharmacia Biosensor, Uppsala, Sweden) to derive the respective equilibrium binding constants (K_D). Separately, a Scatchard plot of the Req data from each IgG surface was constructed to confirm the results of the binding isotherms.

[0298] For Run 2 the interaction of The interaction of FcγRIIIA with immobilized Vitaxin® and Vitaxin® Fc variant IgGs were monitored by surface plasmon resonance detection using a BIAcore 3000 instrument (Pharmacia Biosensor, Uppsala, Sweden). Vitaxin® and Vitaxin® Fc variant IgGs were coupled to the dextran matrix of a CM5 sensor chip (Pharmacia Biosensor) using an Amine Coupling Kit, as described (Johnsson et al., 1992, *Anal Biochem* 198:268-277), at a surface density of between approximately 8200 and 9400 RUs. FcγRIIIA was serially diluted in 0.01 M HEPES pH 7.4 containing 0.15 M NaCl, 3 mM EDTA and 0.005% P20, at concentrations ranging from 16 μM down to 7.8 nM. Duplicate injections of each concentration were made. All binding experiments were performed at 25° C., and at a flow rate of 10 μL/min. Binding was monitored for 25 min. Following each injection of FcγRIIIA, the IgG surfaces were regenerated with a 30 sec. pulse of 5 mM HCl. FcγRIIIA was also passed over a blank reference cell which is connected, in series, to the IgG-containing flow cells. The steady-state binding curves were also corrected for injection artifacts by subtraction of buffer injections. This doubly-corrected data was then fit to a steady-state isotherm provided by the instrument manufacturer (Pharmacia Biosensor, Uppsala, Sweden) to derive the respective equilibrium binding constants (K_D). Separately, a Scatchard plot of the Req data from each IgG surface was constructed to confirm the results of the binding isotherms.

[0299] Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay: Antibody-dependent cell cytotoxicity (ADCC) was assayed in a four-hour non-radioactive lactate dehydrogenase (LDH) release assay (Promega Corporation, Madison, Wis.). Briefly, M21, A549, or H358 target cells were distributed into 96-well U-bottomed plates (1×10^4/50 μl) and pre-incubated with serial dilution of antibodies (50 μl) for 20 min at 37° C. Human effector cells (100 μl) were then added at effector to target ratios of 50:1 and 25:1. Human effector cells were peripheral blood mononuclear cells (PBMC) purified from healthy donors using Lymphocyte Separation Medium (MP Biomedicals, Irvine, Calif.). After a 4-h incubation at 37° C., plates were centrifuged, and cell death was analyzed by measuring the release of LDH into the cell supernatant with a 30-minute coupled enzymatic assay. The percentage of specific lysis was calculated

according to the formula: % specific lysis=100×(E_x−E_{spon}−T_{spon})/(T_{max}−T_{spon}) where E_x represents the release from experimental wells, E_{spon} is the spontaneous release of effector cells alone, T_{spon} is spontaneous release of target cells alone, and T_{max} is the maximum release from lysed target cells.

[0300] The cell lines used for the ADCC studies included the following: A498 and ACHN renal cell carcinomas with moderate expression of Integrin αVβ3, M21 a melanoma cell line with high Integrin $\alpha_v\beta_3$ expression, DU145 a prostate cancer cell line with low levels of Integrin αVβ3, SKMEL28 a human melanoma expressing Integrin $\alpha_v\beta_3$ but little or no human EphA2.

7.4 Example 4

Fe Variants of Antibodies Recognizing Other Epitopes

[0301] Given the remarkable improvement in ADCC activity of the Vitaxin® Fc (I332E) variant the (I332E) substitution was made in two other antibodies designated 12G3H11 (abbreviated 12G3) and 3F2, both of which bind the EphA2 tyrosine receptor kinase. The variable regions of 12G3 (**FIG. 2A**) and 3F2 (**FIG. 3A**) heavy chain were fused to the wt and variant Fc domains generated above (see sections 7.1 and 7.3). The variable region of the light chain of Vitaxin® was replaced with the corresponding light chain variable region (i.e., 12G3 or 3F2, see **FIGS. 2B and 3B**, resectively) such that an intact 12G3 or 3F2 antibody was encoded by the plasmid (see **FIG. 4** for a map of the plasmid encoding Vitaxin®). The antibodies containing the single substitutions were designated 12G3-1M and 3F2-1M, respectively. In addition, the S239D, A330L, I332L triple substitution was generated in 3F2, designated 3F2-3M.

[0302] The binding characteristics of the 3F2-wt, 3F2-1M and 3F2-3M Fc variants to several Fc ligands were examined in vitro by ELISA (**FIG. 12**). Representative binding curves for 3F2 and the Fc variants of 3F2 (3F2-1 M and 3F2-3M) to FcγRIIIA tetramers (**FIG. 12**, top panel), FcγRIIIA monomers (**FIG. 12**, middle panel) and C1q (**FIG. 12**, bottom panel). From these data it can be seen that both the 3F2 Fc variants have improved binding to the monomeric and tetrameric forms of FcγRIIIA. In contrast both the 3F2 Fc variants have reduced C1q binding with 3F2-3M having the largest reduction in C1q binding (**FIG. 12**, bottom panel).

[0303] The binding of the 3F2 and the 3F2 Fc variants to FcγRIIIA was further analyzed by BIAcore analysis. The binding of 3F2 and the 3F2 Fc variant was analyzed with the receptor soluble and the antibody immobile (see methods below). The data obtained for 3F2 and the 3F2 Fc variants (Run 3) is similar to that obtained for Vitaxin® and the Vitaxin® Fc variants (Runs 1 & 2) with improvements in binding of about 7 fold and about 80 fold for the Vitaxin® 1M and 3M Fc variants, respectively, and about 9 fold and about 100 fold for the 3F2-1M and 3M Fc variants, respectively. The small differences between these numbers may reflect subtle differences in glycosylation between antibody produced in 293H cells vs NSO cells (Vitaxin antibodies and 3F2 antibodies, respectively) as the variable domain is generally not thought to affect FcγRIIIA binding. The results are summarized in Table 5.

[0304] The binding of 3F2-wt, 3F2-1M and 3F2-3M Fc variants to the surface of cells via Fc ligand interactions was examined. Two cell types were utilized, THP-1 cells and NK cells. To determine which Fc ligands were present on the surface both cell types were stained with antibodies recognizing CD32 (FcγRII); CD64 (FcγRI) or CD16 (FcγIII) and analyzed by FACS. The percent of cell staining positive for each Fc ligand are plotted in **FIG. 13**. As can be seen in **FIG. 14** panel A, THP-1 cells predominantly express CD32 with a small amount of CD64 present on the cell surface. In contrast NK cells express CD16 almost exclusively (**FIG. 13**, panel B). All three versions of 3F2 (wt, 1M and 3M) bound to a similar degree to THP-1 cells (**FIG. 13**, panel C). However, the two Fc variants (3F2-IM and 3F2-3M) were seen to bind to a greater extent to NK cells, with the 3F2-3M Fc variant showing the largest increase in binding (**FIG. 13**, panel D).

[0305] The ADCC activity of all the variants was examined. Shown in **FIGS. 14A and 14B** are ADCC assays performed using the 12G3H11-Fc (I332E) variant and the parental 12G3H11 antibody against A549 target cells using effector cells from two donors. The assays were performed using two different ratios of target to effector cell (50:1 and 25: 1) and antibody concentrations ranging from 4 to 400 ng per well. Remarkably, a 10 fold increase in ADCC activity is seen for the 12G3H11-Fc (I332E) variant compared to the parent antibody.

[0306] **FIGS. 15, 16** and **17** are ADCC assays comparing the activity of 3F2-wt and the 3F2 Fc variants against target cells expressing different levels of EphA2. The target cell lines used were T23,1 A549 and Hey8 (high expressors), SKOV3 (a moderate expressor), A498 and SKMEL28 (low expressors). The assays were performed using three different ratios of target to effector cell (between 12.5:1 and 100:1) and antibody concentrations ranging from 0.02 to 2 μg/ml. In all cases the ADCC activity of the 3F2-3M Fc variant was seen to be higher than wild type 3F2. The activity of the 3F2-1M Fc variant was also higher than the 3F2-wt.

7.4.1 Materials and Methods

[0307] Generation of 12G3 and 3F2 Fc variants: To generate the 12G3 and 3F2 Fc variants, the DNA sequences encoding the variable region of Vitaxin® 1M or 3M heavy chain (VH) was replaced with the variable region of 12G3 or 3F2 heavy chain to create 12G3-1M, 3F2-1M and 3F2-3M Fc variants using Xba I/Apa I restriction sites (see plasmid map, **FIG. 4**). The DNA sequences encoding the variable region of Vitaxin® light chain were also replaced with the variable region of 12G3 or 3F2 light chain using SmaII/BsiWI restriction sites (see plasmid map, **FIG. 4**). The nucleotide sequence of the 12G3 heavy and light chain variable regions are listed as SEQ ID NO.: 62 and 63 respectively. The amino acid sequence of the 12G3 heavy and light chain variable regions are listed as SEQ ID NO.: 64 and 65 respectively. The nucleotide sequence of the 3F2 heavy and light chain variable regions are listed as SEQ ID NO.: 66 and 67 respectively. The amino acid sequence of the 3F2 heavy and light chain variable regions are listed as SEQ ID NO.: 68 and 69 respectively.

[0308] The plasmid DNA containing the 12G3 antibody genes was stably transfected into 293H cells by Lipofectamine 2000 Transfection Reagent (Invitrogen). The plasmid DNA containing the 3F2 antibody genes was stably transfected into NSO by electroporation. Antibodies were purified from cell culture supernatants by using a pre-packed

Protein A column (Amersham Biosciences, now belongs to GE healthcare). The bound antibody were eluted with elution buffer (100 mM Glycine, pH3.2), neutralized by 1M Tris buffer (pH 8.0) and then dialyzed in PBS. All purified antibodies were analyzed by SDS-polyacrylamide gel electrophoresis and were applied to quantitative ELISA using anti-human IgG assay plates (Becton Dickson) or BCA kits (PIERCE) to determine IgG concentrations.

[0309] Kinetic Analysis via BIAcore: for Run 3 the interaction of FcγRIIIA with immobilized Vitaxin® and Vitaxin® Fc variant IgGs were monitored by surface plasmon resonance detection using a BIAcore 3000 instrument (Pharmacia Biosensor, Uppsala, Sweden). Vitaxin® and Vitaxin® Fc variant IgGs were coupled to the dextran matrix of a CM5 sensor chip (Pharmacia Biosensor) using an Amine Coupling Kit, as described (Johnsson et al., 1992, *Anal Biochem* 198:268-277), at a surface density of between approximately 7700 and 9400 RUs (see Table 5). FcγRIIIA was serially diluted in 0.01 M HEPES pH 7.4 containing 0.15 M NaCl, 3 mM EDTA and 0.005% P20, at concentrations ranging from 16 uM down to 7.8 nM. Duplicate injections of each concentration were made. All binding experiments were performed at 25° C., and at a flow rate of 10 uL/min. Binding was monitored for 25 min. Following each injection of FcγRIIIA, the IgG surfaces were regenerated with a 30 sec. pulse of 5 mM HCl. FcγRIIIA was also passed over a blank reference cell which is connected, in series, to the IgG-containing flow cells. The steady-state binding curves were also corrected for injection artifacts by subtraction of buffer injections. This doubly-corrected data was then fit to a steady-state isotherm provided by the instrument manufacturer (Pharmacia Biosensor, Uppsala, Sweden) to derive the respective equilibrium binding constants (K_D). Separately, a Scatchard plot of the Req data from each IgG surface was constructed to confirm the results of the binding isotherms.

[0310] Cell Surface Binding: NK cells were isolated from healthy donor by using NK cell isolation kit from Miltenybiotec (Cat# 130-091-152) THP-1: early passage of THP-1 cells were used. For FACS staining of FcγRs, either THP-1 or human NK cells were resuspended in FACS buffer (1% BSA in PBS, pH 7.2) at 1×10^6 cells/ml and 0.5 ml of the cells were transfered into 96 deep well plate, 10 μl of the anti-CD32-PE (Immunotech), anti-CD16-FITC (Pharmingen) or anti-CD64-FITC (PharMingen) was added to the tubes. The samples were incubated at 40C for 30 min. After incubation, the cells were washed with FACS buffer and the samples were analyzed by using Guava EasyCyte

[0311] For binding of antibody 3F2 to Human NK cell surface (FcγRIIIA), 10 μl of the antibody dilution (10 μg/ml or 1 μg/ml) was added to the cells and incubated at 4° C. for 30 min. The cells were washed with FACS buffer, then stained with goat ant-human IgG (H+L)-FITC (Pierce) for 30 min at 4° C. The cells were washed and analyzed by Guava EasyCyte.

[0312] For binding of antibody 3F2 to THP-1 cell surface (FcγRI and FcγRII), 10 μl of the antibody dilution (10 μg/ml or 1 μg/ml) were added to the cells, incubatee at 4° C. for 30 min. The cells were washed with FACS buffer, then stained with goat ant-human IgG (H+L)-FITC (Pierce) for 30 min at 4° C. The cells were washed and analyzed by Guava EasyCyte.

[0313] ELISA for FcγRIIIA Tetramer Binding: Microtiter plates were coated with protein A/G (PIERCE) solution (0.25 μg/ml) and incubated at 4° C. overnight. The plates were then washed with PB S/0.1% Tween and any remaining binding sites were blocked with 1% BSA. 50 μl of test antibody at 1:1 dilution (from 5000 ng/ml to 4.9 ng/ml), was added to each well and inclubated for 60 min at 37° C. 50 μl of 1:500 dilution of the Fcγtetramer was added to each well and incubated for 60 min at 37° C. followed by washing. 50 μl of 1:1000 dilution of biotin-conjugated HRP (PIERCE) was added to each well and incubated for 30 min at 37° C. Detection was carried out by adding 30 μl of tetramethylbenzidine (TMB) substrate (Pierce) followed by neutralization with 30 μl of 0.2 M H_2SO_4. The absorbance was read at 450 nm.

[0314] ELISA for FcγRIIIA Monomer Binding: Microtiter plates were coated with 50 μl to test antibody at concentration range from 20 μg/ml to 0.0019 μg/ml and incubated at 4° C. overnight. 50 μl of 10 μg/ml FcγRIIIA-flag protein was added to each well and incubated for 60 min at 37° C. 50 μl of 2.5 μg/ml anti-flag-ME-biotin (Sigma) was added to each well and incubated for 30 min at 37° C. 50 μl of 1:1000 diulation of avidin-conjugated HRP (PIERCE) was added to each well and incubated for 30 min at 37° C. Detection was carried out by adding 30 μl of tetramethylbenzidine (TMB) substrate (Pierce) followed by neutralization with 30 μl of 0.2 M H_2SO_4. The absorbance was read at 450 nm.

[0315] ELISA for C1q Binding: Microtiter plates were coated with 50 μl of test antibody at concentration range from 20 μg/ml to 0.0019 g/ml and incubated at 4° C. overnight. The plate was then blocked with 5% nonfat powdered milk for 60 min at 37° C. 50 μl of 5 μg/ml human C1q complement protein (Quidal, SanDiego) was added to each well and inclubated for 60 min at 37° C. 50 μl of 1:1000 dilution of anti-complement C1q antibody (Biodesign) was added to each well and incubated for 60 min at 37° C. 50 μl of 1:1000 dilution of donkey anti-sheep/goat antibody-conjugated HRP (PIERCE) was added to each well and incubated for 60 min at 37° C. Detection was carried out by adding 30 μl of tetramethylbenzidine (TMB) substrate (Pierce) followed by neutralization with 30 μl of 0.2 M H_2SO_4. The absorbance was read at 450 nm.

[0316] Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC) Assay: Antibody-dependent cell cytotoxicity (ADCC) was assayed as described above in section 7.3.1 using different target cells. The target cell lines used for these assays are A549 a human non-small cell lung adenocarcinoma cell line expressing high levels of human EphA2, T231 a more metastatic variant of MDA-MB-231 human breast adenocarcinoma cell line obtained from collaborator Kathy Miller at Indiana University Medical Center expressing high levels of human EphA2, HeyA8 a human ovarian carcinoma expressing high levels of human EphA2, SKOV3 a human ovarian adenocarcinoma derived from ascites expressing moderate levels of human EphA2, A498 a human renal cell carcinoma expressing low levels of human EphA2, SKMEL28 a human melanoma expressing Integrin $\alpha_v\beta_3$ but little or no human EphA2.

[0317] Whereas, particular embodiments of the invention have been described above for purposes of description, it will be appreciated by those skilled in the art that numerous variations of the details may be made without departing from the invention as described in the appended claims.

[0318] All publications, patents and patent applications mentioned in this specification are herein incorporated by reference into the specification to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, U.S. Provisional Patent Application Nos.: 60/601,634, filed, Aug. 16, 2004 and 60/608,852, filed, Sep. 13, 2004, and U.S. patent application entitled "Eph Receptor Fc Variants With Enhanced Antibody Dependent Cell-Mediated Cytotoxicity Activity," Attorney Docket No.: AE702US, filed Aug. 15, 2005, are incorporated by reference in their entirety

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<213> ORGANISM: Mus musculus

<400> SEQUENCE: 10

Gln Gln Ser Gly Ser Trp Pro His Thr
1 5

<210> SEQ ID NO 11
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(20)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 11

ccgtgcccag cacctgaann kctgggggga ccgtcagtc 39

<210> SEQ ID NO 12
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (22)..(23)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 12

ccgtgcccag cacctgaact cnnkgggga ccgtcagtct tc 42

<210> SEQ ID NO 13
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:

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<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(26)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 13

ccgtgcccag cacctgaact cctgnnkgga ccgtcagtct tcctc 45

<210> SEQ ID NO 14
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (28)..(29)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 14

ccgtgcccag cacctgaact cctggggnnk ccgtcagtct tcctcttc 48

<210> SEQ ID NO 15
<211> LENGTH: 51
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (31)..(32)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 15

ccgtgcccag cacctgaact cctgggggga nnktcagtct tcctcttccc c 51

<210> SEQ ID NO 16
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (34)..(35)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 16

ccgtgcccag cacctgaact cctgggggga ccgnnkgtct tcctcttccc ccca 54

<210> SEQ ID NO 17
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(20)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 17

gtcacatgcg tggtggtgnn kgtgagccac gaagaccct 39

<210> SEQ ID NO 18

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<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(26)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 18

gtcacatgcg tggtggtgga cgtgnnkcac gaagaccctg aggtc 45

<210> SEQ ID NO 19
<211> LENGTH: 51
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (31)..(32)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 19

gtcacatgcg tggtggtgga cgtgagccac nnkgaccctg aggtcaagtt c 51

<210> SEQ ID NO 20
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(20)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 20

cgggaggagc agtacaacnn kacgtaccgt gtggtcagc 39

<210> SEQ ID NO 21
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (19)..(20)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 21

tgcaaggtct ccaacaaann kctcccagcc cccatcgag 39

<210> SEQ ID NO 22
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (22)..(23)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 22

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tgcaaggtct ccaacaaagc cnnkccagcc cccatcgaga aa 42

<210> SEQ ID NO 23
<211> LENGTH: 45
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(26)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 23

tgcaaggtct ccaacaaagc cctcnnkgcc cccatcgaga aaacc 45

<210> SEQ ID NO 24
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (28)..(29)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 24

tgcaaggtct ccaacaaagc cctcccannk cccatcgaga aaaccatc 48

<210> SEQ ID NO 25
<211> LENGTH: 54
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (34)..(35)
<223> OTHER INFORMATION: n is a, c, g, or t

<400> SEQUENCE: 25

tgcaaggtct ccaacaaagc cctcccagcc cccnnkgaga aaaccatctc caaa 54

<210> SEQ ID NO 26
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 26

actcacacat gtccaccgtg cccagcacct gaa 33

<210> SEQ ID NO 27
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 27

caccaccacg catgtgac 18

<210> SEQ ID NO 28

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<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 28

gttgtactgc tcctcccg 18

<210> SEQ ID NO 29
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 29

tttgttggag accttgca 18

<210> SEQ ID NO 30
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 30

aacctctaca aatgtggtat ggct 24

<210> SEQ ID NO 31
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 31

aagcttcggt ccgccaccat ggcaactgaa gatctcccaa ag 42

<210> SEQ ID NO 32
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 32

gtctgccgaa ccgctgcctg ccaaaccttg agtgatggt 39

<210> SEQ ID NO 33
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 33

agcttcggtc cgccaccatg gctgtgctat tcctggcagc tcccccaa 48

<210> SEQ ID NO 34
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:

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<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 34

gtctgccgaa ccgctgcccc ccatcggtga agagctggga gc 42

<210> SEQ ID NO 35
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 35

ggcagcggtt cggcagaccc ctccaaggac 30

<210> SEQ ID NO 36
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 36

caggggctag cttactgctg aacggcgtcg agcgg 35

<210> SEQ ID NO 37
<211> LENGTH: 39
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 37

tccacaggtg tccactcccg gactgaagat ctcccaaag 39

<210> SEQ ID NO 38
<211> LENGTH: 91
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 38

gggagaattc cgcggccgct tatttgtcat cgtcatcttt gtagtcatgg tgatggtgat 60

ggtgtgcgcc tgccaaacct tgagtgatgg t 91

<210> SEQ ID NO 39
<211> LENGTH: 48
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 39

tccacaggtg tccactccgc tgtgctattc ctggcagctc ccccaaag 48

<210> SEQ ID NO 40
<211> LENGTH: 94
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

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<400> SEQUENCE: 40

gggagaattc cgcggccgct tatttgtcat cgtcatcttt gtagtcatgg tgatggtgat 60

ggtgtgcgcc ccccatcggt gaagagctgg gagc 94

<210> SEQ ID NO 41
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 41

gccctcccag cccccgagga gaaaaccatc tcc 33

<210> SEQ ID NO 42
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 42

gccctcccag ccccccagga gaaaaccatc tcc 33

<210> SEQ ID NO 43
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 43

gccctcccag cccccggcga gaaaaccatc tcc 33

<210> SEQ ID NO 44
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 44

gccctcccag cccccgccga gaaaaccatc tcc 33

<210> SEQ ID NO 45
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 45

gccctcccag ccccctacga gaaaaccatc tcc 33

<210> SEQ ID NO 46
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 46

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gccctcccag cccccgacga gaaaaccatc tcc 33

<210> SEQ ID NO 47
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 47

gccctcccag cccccaacga gaaaaccatc tcc 33

<210> SEQ ID NO 48
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 48

gccctcccag cccccgtgga gaaaaccatc tcc 33

<210> SEQ ID NO 49
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 49

gccctcccag ccccctggga gaaaaccatc tcc 33

<210> SEQ ID NO 50
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 50

gccctcccag ccccccgcga gaaaaccatc tcc 33

<210> SEQ ID NO 51
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 51

gccctcccag cccccagcga gaaaaccatc tcc 33

<210> SEQ ID NO 52
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 52

gccctcccag cccccaagga gaaaaccatc tcc 33

<210> SEQ ID NO 53

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<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 53

gccctcccag cccccatgga gaaaaccatc tcc 33

<210> SEQ ID NO 54
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 54

gccctcccag cccccaccga gaaaaccatc tcc 33

<210> SEQ ID NO 55
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 55

gccctcccag ccccctgcga gaaaaccatc tcc 33

<210> SEQ ID NO 56
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 56

gccctcccag cccccctgga gaaaaccatc tcc 33

<210> SEQ ID NO 57
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 57

gccctcccag ccccttcga gaaaaccatc tcc 33

<210> SEQ ID NO 58
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 58

gccctcccag cccccacga gaaaaccatc tcc 33

<210> SEQ ID NO 59
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:

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<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 59

gccctcccag ccccccctga gaaaaccatc tcc 33

<210> SEQ ID NO 60
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 60

ctggggggac cggacgtctt cctcttc 27

<210> SEQ ID NO 61
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: synthetic primer

<400> SEQUENCE: 61

aaagccctcc cactgcccga ggagaaa 27

<210> SEQ ID NO 62
<211> LENGTH: 360
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody variable region

<400> SEQUENCE: 62

caaatgcagc tggtgcagtc tgggcctgag gtgaagaagc ctgggacctc agtgaaggtc 60

tcctgcaagg cttctggatt cacctttgac gattactcca tgaactgggt gcgacaggct 120

cgtggacaac gccttgagtg gataggattt attagaaaca aagctaatga ctacacaaca 180

gagtacgctg actctgtgaa gggtagagtc accattacca gggacatgtc cacgagcaca 240

gcctacatgg agctgagcag cctgagatcc gaggacacgg ccgtgtatta ctgtgcgaga 300

taccctaggc atcatgctat ggactcctgg ggccaaggaa cctcggtcac cgtctcctca 360

<210> SEQ ID NO 63
<211> LENGTH: 321
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody variable region

<400> SEQUENCE: 63

gacatccaga tgacccagtc tccatcctcc ctgtctgcat ctgtaggaga cagagtcacc 60

atcacttgca gggccagcca aagtattagc aacaacctac actggtatca gcagaaacca 120

gggaaagccc ctaagctcct gatcaagtat gccttccagt ccatctctgg ggtcccatca 180

aggttcagtg gaagtggatc tgggacagat tttactttca ccatcagcag cctgcagcct 240

gaagattttg caacatatta ctgtcaacag gccaacagct ggccgctcac gttcggcgga 300

gggaccaagg tggagatcaa a 321

<210> SEQ ID NO 64

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<211> LENGTH: 120
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody variable region

<400> SEQUENCE: 64

Gln Met Gln Leu Val Gln Ser Gly Pro Glu Val Lys Lys Pro Gly Thr
1 5 10 15

Ser Val Lys Val Ser Cys Lys Ala Ser Gly Phe Thr Phe Asp Asp Tyr
20 25 30

Ser Met Asn Trp Val Arg Gln Ala Arg Gly Gln Arg Leu Glu Trp Ile
35 40 45

Gly Phe Ile Arg Asn Lys Ala Asn Asp Tyr Thr Thr Glu Tyr Ala Asp
50 55 60

Ser Val Lys Gly Arg Val Thr Ile Thr Arg Asp Met Ser Thr Ser Thr
65 70 75 80

Ala Tyr Met Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
85 90 95

Tyr Cys Ala Arg Tyr Pro Arg His His Ala Met Asp Ser Trp Gly Gln
100 105 110

Gly Thr Ser Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 65
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody variable region

<400> SEQUENCE: 65

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Asn Asn
20 25 30

Leu His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Lys Tyr Ala Phe Gln Ser Ile Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Phe Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ala Asn Ser Trp Pro Leu
85 90 95

Thr Phe Gly Gly Gly Thr Lys Val Glu Ile Lys
100 105

<210> SEQ ID NO 66
<211> LENGTH: 361
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody variable region

<400> SEQUENCE: 66

gaggtgcagc tggtggagtc tgggggaggt gtggtacggc ctgggggtc cctgagactc 60

tcctgtgcag cctctgggtt caccgtcagt gattactcca tgaactgggt ccgccaggct 120

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ccagggaagg gcctggagtg gattgggttt attagaaaca aagctaatgc ctacacaaca 180

gagtacagtg catctgtgaa gggtagattc accatctcaa gagatgattc aaaaaacacg 240

ctgtatctgc aaatgaacag cctgaaaacc gaggacacag ccgtgtatta ctgtaccaca 300

taccctaggt atcatgctat ggactcctgg ggccagggca ccatggtcac cgtctcctca 360

g 361

<210> SEQ ID NO 67
<211> LENGTH: 321
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody variable region

<400> SEQUENCE: 67

gccatccagt tgactcagtc tccatcctcc ctgtctgcat ctgtaggaga cagagtcacc 60

atcacttgca gggccagcca aagtattagc aacaacctac actggtacct gcagaagcca 120

gggcagtctc cacagctcct gatctattat ggcttccagt ccatctctgg ggtcccatca 180

aggttcagtg gcagtggatc tgggacagat ttcactctca ccatcagcag tctgcaacct 240

gaagattttg caacttacta ctgtcaacag gccaacagct ggccgctcac gttcggcgga 300

gggaccaagc tggagatcaa a 321

<210> SEQ ID NO 68
<211> LENGTH: 120
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody variable region

<400> SEQUENCE: 68

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Arg Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Val Ser Asp Tyr
20 25 30

Ser Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Ile
35 40 45

Gly Phe Ile Arg Asn Lys Ala Asn Ala Tyr Thr Thr Glu Tyr Ser Ala
50 55 60

Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asp Ser Lys Asn Thr
65 70 75 80

Leu Tyr Leu Gln Met Asn Ser Leu Lys Thr Glu Asp Thr Ala Val Tyr
85 90 95

Tyr Cys Thr Thr Tyr Pro Arg Tyr His Ala Met Asp Ser Trp Gly Gln
100 105 110

Gly Thr Met Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 69
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody variable region

<400> SEQUENCE: 69

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Ala Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Asn Asn
            20                  25                  30

Leu His Trp Tyr Leu Gln Lys Pro Gly Gln Ser Pro Gln Leu Leu Ile
        35                  40                  45

Tyr Tyr Gly Phe Gln Ser Ile Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Ala Asn Ser Trp Pro Leu
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
            100                 105


<210> SEQ ID NO 70
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody CDR

<400> SEQUENCE: 70

Ser Tyr Asp Met Ser
1               5


<210> SEQ ID NO 71
<211> LENGTH: 17
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody CDR

<400> SEQUENCE: 71

Lys Val Ser Ser Gly Gly Gly Ser Thr Tyr Tyr Leu Asp Thr Val Gln
1               5                   10                  15

Gly


<210> SEQ ID NO 72
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody CDR

<400> SEQUENCE: 72

His Leu His Gly Ser Phe Ala Ser
1               5


<210> SEQ ID NO 73
<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody CDR

<400> SEQUENCE: 73

Gln Ala Ser Gln Ser Ile Ser Asn Phe Leu His
1               5                   10
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<210> SEQ ID NO 74
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody CDR

<400> SEQUENCE: 74

Thr Arg Ser Gln Ser Ile Ser
1 5

<210> SEQ ID NO 75
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: recombinant antibody CDR

<400> SEQUENCE: 75

Gln Gln Ser Gly Ser Tyr Pro Leu Thr
1 5

1. An antibody that immunospecifically binds to Integrin $\alpha_v\beta_3$ comprising an IgG_1 Fc region, wherein the Fc region comprises at least the high effector function amino acid residue 332E, as numbered by the EU index as set forth in Kabat, wherein the antibody comprising at least the high effector function amino acid residue 332E has an altered binding affinity for one or more FcγRs as compared to the same antibody not comprising at least the high effector function amino acid residue 332E.

2. The antibody of claim 1, wherein the Fc region further comprises at least the high effector function amino acid residues 239D and 330L, as numbered by the EU index as set forth in Kabat.

3. The antibody of claim 1, wherein the high effector function amino acid residue is selected from the group consisting of: 234E, 235R, 235A, 235W, 235P, 235V, 235Y, 236E, 239D, 265L, 269S, 269G, 2981, 298T, 298F, 327N, 327G, 327W, 328S, 328V, 329H, 329Q, 330K, 330V, 330G, 330Y, 330T, 330L, 330I, 330R, 330C, 332E, 332H, 332S, 332W, 332F, 332D, and 332Y, wherein the numbering system is that of the EU index as set forth in Kabat.

4. The antibody of claim 1, wherein said altered binding affinity for said one or more FcγRs is increased as compared to the same antibody not comprising at least the high effector function amino acid residue 332E.

5. The antibody of claim 4, wherein said FcγR is FcγRIIIA.

6. The antibody of claim 4, wherein said FcγR is FcγRIIB.

7. The antibody of claim 1, wherein said altered binding affinity for said one or more FcγRs is decreased as compared to the same antibody not comprising at least the high effector function amino acid residue 332E.

8. The antibody of claim 5, wherein the equilibrium dissociation constant (K_D) of binding for FcγRIIIA is decreased at least 2 fold as compared to the same antibody not comprising at least the high effector function amino acid residue 332E.

9. The antibody of claim 8, wherein the equilibrium dissociation constant (K_D) of binding for FcγRIIIA is decreased at least 70 fold as compared to the same antibody not comprising at least the high effector function amino acid residue 332E.

10. The antibody of claim 5, wherein said increased affinity for FcγRIIIA results in an enhanced ADCC activity relative to a comparable molecule not comprising at least the high effector function amino acid residue 332E.

11. The antibody of claim 10, wherein said enhanced ADCC activity is at least 2 fold greater relative to a comparable molecule not comprising at least the high effector function amino acid residue 332E.

12. The antibody of claim 1, wherein said antibody is humanized, fully human, CDR-grafted, or chimeric.

13. The antibody of claim 12, wherein said antibody is Vitaxin®.

14. The antibody of claim 12, wherein said antibody variable sequences comprise SEQ ID Nos. 3 and 4.

15. The antibody of claim 12, wherein said antibody is conjugated to a detectable agent, therapeutic agent or drug.

16. A method of generating the antibody of claim 1, comprising (a) isolating antibody coding regions; and (b) making one or more desired substitutions in said Fc region of said isolated antibody coding region.

17. A method of generating the antibody of claim 1, comprising subcloning variable regions into a vector encoding said Fc region comprising at least one or more high effector function amino acid residues.

18. A formulation comprising a therapeutically effective amount of the antibody of claim 1 in a pharmaceutically-acceptable excipient.

19. A method of ameliorating, treating or preventing cancer by administering the formulation of claim 18 to a patient in need thereof.

20. The method of claim 19, wherein said cancer is of the head, neck, eye, mouth, throat, esophagus, chest, bone, lung, colon, rectum, colorectal, stomach, spleen, renal, skeletal muscle, subcutaneous tissue, metastatic melanoma, endome-

trial, prostate, breast, ovaries, testicles, skin, thyroid, blood, lymph nodes, kidney, liver, pancreas, brain or central nervous system.

21. The method of claim 19, wherein said administration is oral, parenteral, intramuscular, intranasal, vaginal, rectal, lingual, sublingual, buccal, intrabuccal, intravenous, cutaneous, subcutaneous or transdermal.

22. The method of claim 19, further comprising administering said formulation in combination with other therapies, such as chemotherapy, hormonal therapy, biological therapy, immunotherapy or radiation therapy.

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专利名称(译)	整联蛋白拮抗剂具有增强的抗体依赖性细胞介导的细胞毒性活性		
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申请号	US11/203253	申请日	2005-08-15
[标]申请(专利权)人(译)	免疫医疗公司		
申请(专利权)人(译)	MEDIMMUNE INC.		
当前申请(专利权)人(译)	MEDIMMUNE INC.		
[标]发明人	WU HERREN GAO CHANGSHOU		
发明人	WU, HERREN GAO, CHANGSHOU		
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摘要(译)

本发明涉及免疫特异性结合整联蛋白α v β 3 的抗体的新Fc变体。 Fc变体包含免疫特异性结合整联蛋白α v β 3 的可变区和进一步包含至少一个新氨基酸残基的Fc区，其可提供增强的效应子。功能。更具体地，本发明提供了对一种或多种FcγR和/或C1q具有修饰的结合亲和力的Fc变体。另外，Fc变体具有改变的抗体依赖性细胞介导的细胞毒性（ADCC）和/或补体依赖性细胞毒性（CDC）活性。本发明进一步提供了应用免疫特异性结合整联蛋白α v β 3 的抗体Fc变体的方法和方案，特别是用于治疗目的。