(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 February 2018 (15.02.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/031258 A1

(51) International Patent Classification:
C07K 14/735 (2006.01) *C07K 16/28* (2006.01)
C07K 16/18 (2006.01)

(21) International Application Number:
PCT/US2017/044393

(22) International Filing Date:
28 July 2017 (28.07.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/374,261 12 August 2016 (12.08.2016) US

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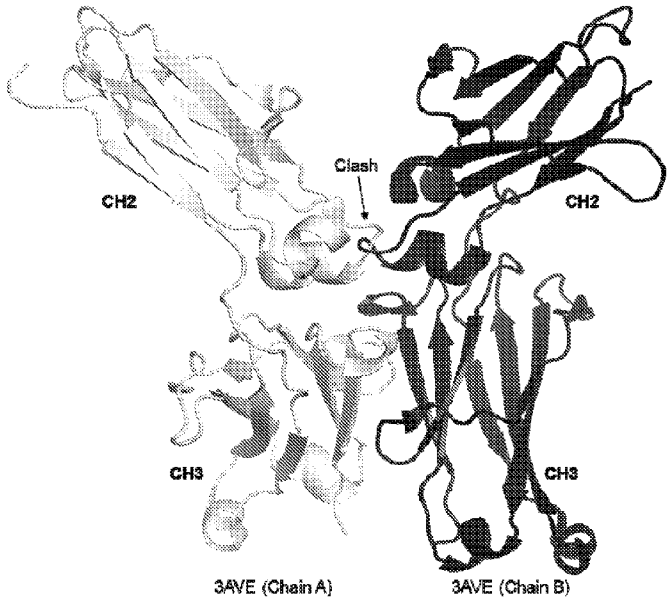
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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: ENGINEERED ANTIBODIES AND OTHER FC-DOMAIN CONTAINING MOLECULES WITH ENHANCED AGONISM AND EFFECTOR FUNCTIONS

Figure 1.



(57) Abstract: The present invention relates to engineered antibodies and other Fc-domain containing molecules with enhanced agonism and effector functions.

[Continued on next page]

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Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

ENGINEERED ANTIBODIES AND OTHER FC-DOMAIN CONTAINING MOLECULES WITH ENHANCED AGONISM AND EFFECTOR FUNCTIONS

SEQUENCE LISTING

This application contains a Sequence Listing submitted via EFS-Web, the entire content of which incorporated herein by reference in its entirety. The ASCII text file, created on 26 June 2017, is named JBI5094WOPCT_ST25.txt and is 205 kilobytes in size.

FIELD OF THE INVENTION

The present invention relates to engineered antibodies and other Fc-domain containing molecules with enhanced agonism and effector functions.

BACKGROUND OF THE INVENTION

Engineering fit-for purpose antibodies and other Fc-domain containing molecules to achieve the desired therapeutic response includes Fc domain engineering approaches to modulate for example antibody effector functions, half-life and stability. In addition, for certain types of molecules, such as antibodies binding tumor necrosis factor (TNFR) superfamily members, engineering approaches have been developed to induce agonism to stimulate their anti-tumor immunity effects.

There is a need for additional engineering approaches to modulate the Fc domain-mediated functions of antibodies and other Fc-domain containing therapeutic constructs.

BRIEF SUMMARY OF THE INVENTION

The invention provides an isolated engineered anti-tumor necrosis factor receptor (TNFR) superfamily member antibody, wherein the antibody comprises a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation when compared to a parental wild-type antibody, residue numbering according to the EU Index.

The invention also provides an isolated engineered anti-TNFR superfamily member antibody comprising the T437R mutation.

The invention also provides an isolated engineered anti-TNFR superfamily member antibody comprising the T437R/K248E mutation.

The invention also provides an isolated engineered anti-TNFR superfamily member antibody comprising the T437R/K338A mutation.

The invention also provides a pharmaceutical composition comprising the antibody of the invention and a pharmaceutically acceptable carrier.

The invention also provides for a method of enhancing agonistic activity of an anti-TNFR superfamily member antibody in a subject, comprising providing the anti-TNFR superfamily member antibody, introducing aT437R mutation, a K248E mutation, a K338A mutation, a T437R/K248E mutation or a T437R/K338A mutation into the antibody to generate an engineered antibody specifically binding the TNFR superfamily member, and administering the engineered antibody to the subject.

The invention also provides for a method of treating a cancer in a subject, comprising administering to the subject an antibody specifically binding a TNFR superfamily member comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation when compared to a parental wild-type antibody, residue numbering according to the EU Index, for a time sufficient to treat the cancer.

The invention also provides an isolated Fc domain containing molecule comprising a T437R mutation in the Fc domain.

The invention also provides an isolated Fc domain containing molecule comprising a K338A mutation in the Fc domain.

The invention also provides an isolated Fc domain containing molecule comprising a T437R/K248E mutation in the Fc domain.

The invention also provides an isolated Fc domain containing molecule comprising a T437R/K338A mutation in the Fc domain.

The invention also provides an isolated polynucleotide encoding the Fc domain containing molecule comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation in the Fc domain.

The invention also provides an isolated polynucleotide encoding the Fc domain of SEQ ID NOs: 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73 or 74.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NOs: 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, or 86.

The invention also provides an isolated vector comprising the polynucleotide of the invention.

The invention also provides a host cell comprising the vector of the invention.

The invention also provides for a method of producing the Fc domain containing molecule of the invention, comprising culturing the host cell of the invention in conditions wherein the Fc domain containing molecule is expressed, and isolating the Fc domain containing molecule.

The invention also provides an isolated anti-tumor necrosis factor receptor (TNFR) superfamily member antibody comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation when compared to a parental wild-type antibody, residue numbering according to the EU Index, for use in the treatment of a cancer.

The invention also provides for use of an isolated anti-tumor necrosis factor receptor (TNFR) superfamily member antibody comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation, residue numbering according to the EU Index, in the manufacture of a medicament for the treatment of cancer.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows two half Fc molecules (black and light grey) derived from the crystal structure of human IgG1 Fc (Protein Data Bank entry 3AVE) following alignment to CH3 domains within the multimeric model indicating a clash of respective CH2 domains.

Figure 2A shows the agonistic activity of the singularly mutated antibodies OX4020E5IgG1K248E, OX4020E5IgG1T437R, OX4020E5IgG1K338A in solution, the agonism assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 2B shows the agonistic activity of the singularly mutated antibodies OX4020E5IgG1K248E, OX4020E5IgG1T437R, OX4020E5IgG1K338A when cross-linked with Daudi cells, the agonism assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 2C shows the agonistic activity of double mutated antibodies OX4020E5IgG1T437R/K248E, OX4020E5IgG1T437R/K338A and OX4020E5IgG1K248E/K338A in relation to OX4020E5IgG1T437R and OX4020E5IgG1 in solution, the agonism assessed as percent (%) activity relative to the OX40 ligand (OX40L).

Figure 2D shows that the agonistic activity of OX4020E5IgG1T437R/K248E is enhanced upon cross-linking with Daudi cells, the agonism assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 2E shows that the agonistic activity of OX4020E5IgG1T437R/K338A is enhanced upon cross-linking with Daudi cells, the agonism assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 2F shows that the agonistic activity of OX4020E5IgG1 is enhanced upon cross-linking with Daudi cells, the agonism assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 2G shows that the agonistic activity of OX4020E5IgG1T437R is enhanced upon cross-linking with Daudi cells, the agonism assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 3A shows the agonistic activity of OX40SF2IgG1T437R, OX40SF2IgG1T437R/K248E, OX40SF2IgG1T437R/K338A and OX40SF2IgG1 in solution, the agonism assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 3B shows that the agonistic activity of OX40SF2IgG1T437R is enhanced upon cross-linking with Daudi cells, the agonism assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 3C shows that the agonistic activity of OX40SF2IgG1T437R/K248E is enhanced upon cross-linking with Daudi cells, the agonism assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 4A shows that agonistic activity of OX40SF2IgG1T437R is further enhanced by cross-linking in FcγRIIB dependent manner. The enhancement was blocked by an anti-FcγRIIB antibody 2B6. The agonism was assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 4B shows that agonistic activity of OX40SF2IgG1T437R/K248E is further enhanced by cross-linking in FcγRIIB dependent manner. The enhancement was blocked by an anti-FcγRIIB antibody 2B6. The agonism was assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 5 shows the corrected bioluminescence resonance energy transfer (BRET) ratios obtained from the NanoBRET™ PPI assay for OX40SF2IgG1, OX40SF2IgG1E345R, OX40SF2IgG1T437R and OX40SF2IgG1T437R/K248E, indicative of degree of antibody multimerization on the surface of OX40-expressing cell. OX40SF2IgG1T437R/K248E and OX40SF2IgG1E345R showed elevated corrected NanoBRET ratios across concentrations ranging from 10 to 1000 ng/mL, indicating antibody association at the cell surface. The

corrected NanoBRET ratio for OX40SF2IgG1 and OX40SF2IgG1T437R was at background level. n = antibody conjugated to Nanoluc. h = antibody conjugated to Halotag.

Figure 6 shows that the T437R/K248E mutation rescues agonism on Fc silent antibodies OX40SF2IgG2sigma and OX40SF2IgG4PAA. The agonism was assessed as percent (%) activity relative to 1 μg/mL OX40 ligand (OX40L).

Figure 7 shows that OX40SF2IgG1T437R and OX40SF2IgG1T437R/K248E mediate ADCC with increased potency when compared to OX40SF2IgG1. Y-axis indicates fold of activation of ADCC activity in relation to a sample without the antibody.

Figure 8 shows that OX40SF2IgG1T437R and OX40SF2IgG1T437R/K248E mediate ADCP at comparable levels when compared to OX40SF2IgG1. Y-axis indicates percentage (%) of cells killed.

Figure 9A shows that OX40SF2IgG1T437R and OX40SF2IgG1T437R/K248E have enhanced CDC when compared to OX40SF2IgG1. Y-axis indicates percentage (%) cytotoxicity.

Figure 9B shows that OX40SF2IgG2sigmaT437R/K248E mediates ADCP with increased potency when compared to OX40SF2IgG2sigma. Y-axis indicates percentage (%) of cells killed.

Figure 10 shows the PK profiles of Tg32 hemizygous mice dosed with indicated antibodies. Data was normalized to the first time of the linear (beta) phase of the curve, and expressed as % maximum concentration for the doses following a 2 mg/kg body weight bolus dose. Serum concentrations at day 14 and day 21 for 3 groups are not shown because levels were below the detectable range. Each data point represents the mean $\pm$ standard error of 5 animals per group.

Figure 11 shows the PK profiles of Tg32 homozygous SCID mice dosed with indicated antibodies. Half-life values, $t_{1/2}$, were estimated as follows: OX40SF2IgG1T437R, $t_{1/2}$ = 9.5 $\pm$ 0.7 d; OX40SF2IgG1T437R/K248E, $t_{1/2}$ = 8.3 $\pm$ 0.5 d; OX40SF2IgG1, $t_{1/2}$ = 9.2 $\pm$ 0.6 d.

DETAILED DESCRIPTION OF THE INVENTION

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

Although any methods and materials similar or equivalent to those described herein may be used in the practice for testing of the present invention, exemplary materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used.

As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a cell" includes a combination of two or more cells, and the like.

"Anti-tumor necrosis factor receptor (TNFR) superfamily member antibody" or anti-TNFR superfamily member antibody refers to an antibody that specifically binds a TNFR superfamily member.

"TNFR superfamily member" includes receptors that belong to the TNFR superfamily, including the receptors shown in **Table 1,** including naturally occurring variants of the TNFRs**.** The TNFRs are typically expressed as type I transmembrane proteins and contain one to six cysteine-rich domains in their extracellular domain. Signaling occurs as a TNFR trimer. An amino acid sequence for one isoform for each TNFR is shown in **Table 1**. The ligand(s) of the particular TNFR is also indicated in **Table 1**.

Table 1.

TNFR superfamily member		Ligand(s) of the TNFR superfamily member	
Name	SEQ ID NO:	Name	SEQ ID NO:
Tumor necrosis factor receptor 1 (CD120a)	1	TNF-alpha (cachectin)	28
Tumor necrosis factor receptor 2 (CD120b)	2	TNF-alpha (cachectin)	28

Lymphotoxin beta receptor (CD18)	3	Lymphotoxin beta (TNF-C)	29
OX40 (CD134)	4	OX40L	30
CD40	5	CD154	31
Fas receptor (CD95)	6	FasL	32
Decoy receptor 3 (TR6)	7	FasL, LIGHT, TL1A	32 (FASL), 33(LIGHT), 34(TL1A)
CD27	8	CD70, Siva1	35 (CD70), 36 (Siva1)
CD30	9	CD153	37
4-1BB (CD137)	10	4-1BB ligand	38
Death receptor 4 (TRAILR1)	11	TRAIL	39
Death receptor 5 (TRAILR2)	12	TRAIL	39
Decoy receptor 1 (TRAILR3)	13	TRAIL	39
Decoy receptor 2 (TRAILR4)	14	TRAIL	39
RANK (CD265)	15	RANKL	40
Osteoprotegerin	16	RANKL	40
TWEAK receptor	17	TWEAK	41
TACI (CD267)	18	APRIL, BAFF, CAMLG	42 (APRIL, 43 (BAFF), 44 (CAMLG)
BAFF receptor (CD268)	19	BAFF	43
Herpesvirus entry mediator (CD270)	20	LIGHT	33

Nerve growth factor receptor (CD271)	21	NGF, BDNF, NT-3, NT-4	45 (NGF), 46 (BDNF), 47 (NT-3), 48 (NT-4)
B-cell maturation antigen (CD269)	22	BAFF	43
Glucocorticoid-induced TNFR-related (CD357)	23	GITR ligand	49
TROY (TRADE)	24	unknown	
Death receptor 6 (CD358)	25	unknown	
Death receptor 3 (Apo-3)	26	TL1A	34
Ectodysplasin A2 receptor (XEDAR)	27	EDA-A2	50

SEQ ID NO: 1

MGLSTVPDLLLPLVLLELLVGIYPSGVIGLVPHLGDREKRDSVCPQGKYIHPQNNSI
CCTKCHKGTYLYNDCPGPGQDTDCRECESGSFTASENHLRHCLSCSKCRKEMGQ
VEISSCTVDRDTVCGCRKNQYRHYWSENLFQCFNCSLCLNGTVHLSCQEKQNTV
CTCHAGFFLRENECVSCSNCKKSLECTKLCLPQIENVKGTEDSGTTVLLPLVIFFGL
CLLSLLFIGLMYRYQRWKSKLYSIVCGKSTPEKEGELEGTTTKPLAPNPSFSPTPGF
TPTLGFSPVPSSTFTSSSTYTPGDCPNFAAPRREVAPPYQGADPILATALASDPIPNP
LQKWEDSAHKPQSLDTDDPATLYAVVENVPPLRWKEFVRRLGLSDHEIDRLELQ
NGRCLREAQYSMLATWRRRTPRREATLELLGRVLRDMDLLGCLEDIEEALCGPA
ALPPAPSLLR

SEQ ID NO: 2

MAPVAVWAALAVGLELWAAAHALPAQVAFTPYAPEPGSTCRLREYYDQTAQMC
CSKCSPGQHAKVFCTKTSDTVCDSCEDSTYTQLWNWVPECLSCGSRCSSDQVETQ
ACTREQNRICTCRPGWYCALSKQEGCRLCAPLRKCRPGFGVARPGTETSDVVCKP
CAPGTFSNTTSSTDICRPHQICNVVAIPGNASMDAVCTSTSPTRSMAPGAVHLPQP

VSTRSQHTQPTPEPSTAPSTSFLLPMGPSPPAEGSTGDFALPVGLIVGVTALGLLIIG
VVNCVIMTQVKKKPLCLQREAKVPHLPADKARGTQGPEQQHLLITAPSSSSSSLES
SASALDRRAPTRNQPQAPGVEASGAGEARASTGSSDSSPGGHGTQVNVTCIVNVC
SSSDHSSQCSSQASSTMGDTDSSPSESPKDEQVPFSKEECAFRSQLETPETLLGSTEE
KPLPLGVPDAGMKPS

SEQ ID NO: 3
MLGLRPPLLALVGLLSLGCVLSQECTKFKVSSCRECIESGPGCTWCQKLNFTGPGD
PDSIRCDTRPQLLMRGCAADDIMDPTSLAETQEDHNGGQKQLSPQKVTLYLRPGQ
AAAFNVTFRRAKGYPIDLYYLMDLSYSMLDDLRNVKKLGGDLLRALNEITESGRI
GFGSFVDKTVLPFVNTHPDKLRNPCPNKEKECQPPFAFRHVLKLTNNSNQFQTEV
GKQLISGNLDAPEGGLDAMMQVAACPEEIGWRNVTRLLVFATDDGFHFAGDGKL
GAILTPNDGRCHLEDNLYKRSNEFDYPSVGQLAHKLAENNIQPIFAVTSRMVKTY
EKLTEIIPKSAVGELSEDSSNVVQLIKNAYNKLSSRVFLDHNALPDTLKVTYDSFCS
NGVTHRNQPRGDCDGVQINVPITFQVKVTATECIQEQSFVIRALGFTDIVTVQVLP
QCECRCRDQSRDRSLCHGKGFLECGICRCDTGYIGKNCECQTQGRSSQELEGSCR
KDNNSIICSGLGDCVCGQCLCHTSDVPGKLIYGQYCECDTINCERYNGQVCGGPG
RGLCFCGKCRCHPGFEGSACQCERTTEGCLNPRRVECSGRGRCRCNVCECHSGYQ
LPLCQECPGCPSPCGKYISCAECLKFEKGPFGKNCSAACPGLQLSNNPVKGRTCKE
RDSEGCWVAYTLEQQDGMDRYLIYVDESRECVAGPNIAAIVGGTVAGIVLIGILLL
VIWKALIHLSDLREYRRFEKEKLKSQWNNDNPLFKSATTTVMNPKFAES

SEQ ID NO: 4
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RCSRSQNTVCRPCGPGFYNDVVSSKPCKPCTWCNLRSGSERKQLCTATQDTVCRC
RAGTQPLDSYKPGVDCAPCPPGHFSPGDNQACKPWTNCTLAGKHTLQPASNSSD
AICEDRDPPATQPQETQGPPARPITVQPTEAWPRTSQGPSTRPVEVPGGRAVAAIL
GLGLVLGLLGPLAILLALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLA
KI

SEQ ID NO: 5

MVRLPLQCVLWGCLLTAVHPEPPTACREKQYLINSQCCSLCQPGQKLVSDCTEFT
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CTSEACESCVLHRSCSPGFGVKQIATGVSDTICEPCPVGFFSNVSSAFEKCHPWTSC
ETKDLVVQQAGTNKTDVVCGPQDRLRALVVIPIIFGILFAILLVLVFIKKVAKKPTN
KAPHPKQEPQEINFPDDLPGSNTAAPVQETLHGCQPVTQEDGKESRISVQERQ

SEQ ID NO: 6
MLGIWTLLPLVLTSVARLSSKSVNAQVTDINSKGLELRKTVTTVETQNLEGLHHD
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GHGLEVEINCTRTQNTKCRCKPNFFCNSTVCEHCDPCTKCEHGIIKECTLTSNTKC
KEEGSRSNLGWLCLLLLPIPLIVWVKRKEVQKTCRKHRKENQGSHESPTLNPETV
AINLSDVDLSKYITTIAGVMTLSQVKGFVRKNGVNEAKIDEIKNDNVQDTAEQKV
QLLRNWHQLHGKKEAYDTLIKDLKKANLCTLAEKIQTIILKDITSDSENSNFRNEIQ
SLV

SEQ ID NO: 7
MRALEGPGLSLLCLVLALPALLPVPAVRGVAETPTYPWRDAETGERLVRLLQALR
VARMPGLERSVRERFLPVH

SEQ ID NO: 8
MARPHPWWLCVLGTLVGLSATPAPKSCPERHYWAQGKLCCQMCEPGTFLVKDC
DQHRKAAQCDPCIPGVSFSPDHHTRPHCESCRHCNSGLLVRNCTITANAECACRN
GWQCRDKECTECDPLPNPSLTARSSQALSPHPQPTHLPYVSEMLEARTAGHMQTL
ADFRQLPARTLSTHWPPQRSLCSSDFIRILVIFSGMFLVFTLAGALFLHQRRKYRSN
KGESPVEPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP

SEQ ID NO: 9
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RPGMFCSTSAVNSCARCFFHSVCPAGMIVKFPGTAQKNTVCEPASPGVSPACASPE
NCKEPSSGTIPQAKPTPVSPATSSASTMPVRGGTRLAQEAASKLTRAPDSPSSVGRP
SSDPGLSPTQPCPEGSGDCRKQCEPDYYLDEAGRCTACVSCSRDDLVEKTPCAWN

SSRTCECRPGMICATSATNSCARCVPYPICAAETVTKPQDMAEKDTTFEAPPLGTQ
PDCNPTPENGEAPASTSPTQSLLVDSQASKTLPIPTSAPVALSSTGKPVLDAGPVLF
WVILVLVVVVGSSAFLLCHRRACRKRIRQKLHLCYPVQTSQPKLELVDSRPRRSST
QLRSGASVTEPVAEERGLMSQPLMETCHSVGAAYLESLPLQDASPAGGPSSPRDL
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PHYPEQETEPPLGSCSDVMLSVEEEGKEDPLPTAASGK

SEQ ID NO: 10
MGNSCYNIVATLLLVLNFERTRSLQDPCSNCPAGTFCDNNRNQICSPCPPNSFSSA
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ELTKKGCKDCCFGTFNDQKRGICRPWTNCSLDGKSVLVNGTKERDVVCGPSPAD
LSPGASSVTPPAPAREPGHSPQIISFFLALTSTALLFLLFFLTLRFSVVKRGRKKLLYI
FKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

SEQ ID NO: 11
MAPPPARVHLGAFLAVTPNPGSAASGTEAAAATPSKVWGSSAGRIEPRGGGRGAL
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IKLHDQSIGTQQWEHSPLGELCPPGSHRSEHPGACNRCTEGVGYTNASNNLFACLP
CTACKSDEEERSPCTTTRNTACQCKPGTFRNDNSAEMCRKCSRGCPRGMVKVKD
CTPWSDIECVHKESGNGHNIWVILVVTLVVPLLLVAVLIVCCCIGSGCGGDPKCM
DRVCFWRLGLLRGPGAEDNAHNEILSNADSLSTFVSEQQMESQEPADLTGVTVQS
PGEAQCLLGPAEAEGSQRRRLLVPANGADPTETLMLFFDKFANIVPFDSWDQLMR
QLDLTKNEIDVVRAGTAGPGDALYAMLMKWVNKTGRNASIHTLLDALERMEER
HAREKIQDLLVDSGKFIYLEDGTGSAVSLE

SEQ ID NO: 12
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LITQQDLAPQQRAAPQQKRSSPSEGLCPPGHHISEDGRDCISCKYGQDYSTHWNDL
LFCLRCTRCDSGEVELSPCTTTRNTVCQCEEGTFREEDSPEMCRKCRTGCPRGMV
KVGDCTPWSDIECVHKESGTKHSGEVPAVEETVTSSPGTPASPCSLSGIIIGVTVAA
VVLIVAVFVCKSLLWKKVLPYLKGICSGGGGDPERVDRSSQRPGAEDNVLNEIVSI
LQPTQVPEQEMEVQEPAEPTGVNMLSPGESEHLLEPAEAERSQRRRLLVPANEGD

PTETLRQCFDDFADLVPFDSWEPLMRKLGLMDNEIKVAKAEAAGHRDTLYTMLI
KWVNKTGRDASVHTLLDALETLGERLAKQKIEDHLLSSGKFMYLEGNADSAMS

SEQ ID NO: 13
MARIPKTLKFVVVIVAVLLPVLAYSATTARQEEVPQQTVAPQQQRHSFKGEECPA
GSHRSEHTGACNPCTEGVDYTNASNNEPSCFPCTVCKSDQKHKSSCTMTRDTVCQ
CKEGTFRNENSPEMCRKCSRCPSGEVQVSNCTSWDDIQCVEEFGANATVETPAAE
ETMNTSPGTPAPAAEETMNTSPGTPAPAAEETMTTSPGTPAPAAEETMTTSPGTPA
PAAEETMITSPGTPASSHYLSCTIVGIIVLIVLLIVFV

SEQ ID NO: 14
MGLWGQSVPTASSARAGRYPGARTASGTRPWLLDPKILKFVVFIVAVLLPVRVDS
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LPSCLLCTVCKSGQTNKSSCTTTRDTVCQCEKGSFQDKNSPEMCRTCRTGCPRGM
VKVSNCTPRSDIKCKNESAASSTGKTPAAEETVTTILGMLASPYHYLIIIVVLVIILA
VVVVGFSCRKKFISYLKGICSGGGGGPERVHRVLFRRRSCPSRVPGAEDNARNETL
SNRYLQPTQVSEQEIQGQELAELTGVTVESPEEPQRLLEQAEAEGCQRRRLLVPVN
DADSADISTLLDASATLEEGHAKETIQDQLVGSEKLFYEEDEAGSATSCL

SEQ ID NO: 15
MAPRARRRRPLFALLLLCALLARLQVALQIAPPCTSEKHYEHLGRCCNKCEPGKY
MSSKCTTTSDSVCLPCGPDEYLDSWNEEDKCLLHKVCDTGKALVAVVAGNSTTP
RRCACTAGYHWSQDCECCRRNTECAPGLGAQHPLQLNKDTVCKPCLAGYFSDAF
SSTDKCRPWTNCTFLGKRVEHHGTEKSDAVCSSSLPARKPPNEPHVYLPGLIILLLF
ASVALVAAIIFGVCYRKKGKALTANLWHWINEACGRLSGDKESSGDSCVSTHTA
NFGQQGACEGVLLLTLEEKTFPEDMCYPDQGGVCQGTCVGGGPYAQGEDARML
SLVSKTEIEEDSFRQMPTEDEYMDRPSQPTDQLLFLTEPGSKSTPPFSEPLEVGEND
SLSQCFTGTQSTVGSESCNCTEPLCRTDWTPMSSENYLQKEVDSGHCPHWAASPS
PNWADVCTGCRNPPGEDCEPLVGSPKRGPLPQCAYGMGLPPEEEASRTEARDQPE
DGADGRLPSSARAGAGSGSSPGGQSPASGNVTGNSNSTFISSGQVMNFKGDIIVVY
VSQTSQEGAAAAAEPMGRPVQEETLARRDSFAGNGPRFPDPCGGPEGLREPEKAS
RPVQEQGGAKA

SEQ ID NO: 16
MNNLLCCALVFLDISIKWTTQETFPPKYLHYDEETSHQLLCDKCPPGTYLKQHCT
AKWKTVCAPCPDHYYTDSWHTSDECLYCSPVCKELQYVKQECNRTHNRVCECK
EGRYLEIEFCLKHRSCPPGFGVVQAGTPERNTVCKRCPDGFFSNETSSKAPCRKHT
NCSVFGLLLTQKGNATHDNICSGNSESTQKCGIDVTLCEEAFFRFAVPTKFTPNWL
SVLVDNLPGTKVNAESVERIKRQHSSQEQTFQLLKLWKHQNKDQDIVKKIIQDIDL
CENSVQRHIGHANLTFEQLRSLMESLPGKKVGAEDIEKTIKACKPSDQILKLLSLW
RIKNGDQDTLKGLMHALKHSKTYHFPKTVTQSLKKTIRFLHSFTMYKLYQKLFLE
MIGNQVQSVKISCL

SEQ ID NO: 17
MARGSLRRLLRLLVLGLWLALLRSVAGEQAPGTAPCSRGSSWSADLDKCMDCAS
CRARPHSDFCLGCAAAPPAPFRLLWPILGGALSLTFVLGLLSGFLVWRRCRRREKF
TTPIEETGGEGCPAVALIQ

SEQ ID NO: 18
MSGLGRSRRGGRSRVDQEERFPQGLWTGVAMRSCPEEQYWDPLLGTCMSCKTIC
NHQSQRTCAAFCRSLSCRKEQGKFYDHLLRDCISCASICGQHPKQCAYFCENKLR
SPVNLPPELRRQRSGEVENNSDNSGRYQGLEHRGSEASPALPGLKLSADQVALVY
STLGLCLCAVLCCFLVAVACFLKKRGDPCSCQPRSRPRQSPAKSSQDHAMEAGSP
VSTSPEPVETCSFCFPECRAPTQESAVTPGTPDPTCAGRWGCHTRTTVLQPCPHIPD
SGLGIVCVPAQEGGPGA

SEQ ID NO: 19
MRRGPRSLRGRDAPAPTPCVPAECFDLLVRHCVACGLLRTPRPKPAGASSPAPRT
ALQPQESVGAGAGEAALPLPGLLFGAPALLGLALVLALVLVGLVSWRRRQRRLR
GASSAEAPDGDKDAPEPLDKVIILSPGISDATAPAWPPPGEDPGTTPPGHSVPVPAT
ELGSTELVTTKTAGPEQQ

SEQ ID NO: 20

MEPPGDWGPPPWRSTPKTDVLRLVLYLTFLGAPCYAPALPSCKEDEYPVGSECCP
KCSPGYRVKEACGELTGTVCEPCPPGTYIAHLNGLSKCLQCQMCDPAMGLRASR
NCSRTENAVCGCSPGHFCIVQDGDHCAACRAYATSSPGQRVQKGGTESQDTLCQ
NCPPGTFSPNGTLEECQHQTKCSWLVTKAGAGTSSSHWVWWFLSGSLVIVIVCST
VGLIICVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETIP
SFTGRSPNH

SEQ ID NO: 21
MGAGATGRAMDGPRLLLLLLLGVSLGGAKEACPTGLYTHSGECCKACNLGEGV
AQPCGANQTVCEPCLDSVTFSDVVSATEPCKPCTECVGLQSMSAPCVEADDAVCR
CAYGYYQDETTGRCEACRVCEAGSGLVFSCQDKQNTVCEECPDGTYSDEANHVD
PCLPCTVCEDTERQLRECTRWADAECEEIPGRWITRSTPPEGSDSTAPSTQEPEAPP
EQDLIASTVAGVVTTVMGSSQPVVTRGTTDNLIPVYCSILAAVVVGLVAYIAFKR
WNSCKQNKQGANSRPVNQTPPPEGEKLHSDSGISVDSQSLHDQQPHTQTASGQAL
KGDGGLYSSLPPAKREEVEKLLNGSAGDTWRHLAGELGYQPEHIDSFTHEACPVR
ALLASWATQDSATLDALLAALRRIQRADLVESLCSESTATSPV

SEQ ID NO: 22
MLQMAGQCSQNEYFDSLLHACIPCQLRCSSNTPPLTCQRYCNASVTNSVKGTNAI
LWTCLGLSLIISLAVFVLMFLLRKINSEPLKDEFKNTGSGLLGMANIDLEKSRTGDE
IILPRGLEYTVEECTCEDCIKSKPKVDSDHCFPLPAMEEGATILVTTKTNDYCKSLP
AALSATEIEKSISAR

SEQ ID NO: 23
MAQHGAMGAFRALCGLALLCALSLGQRPTGGPGCGPGRLLLGTGTDARCCRVH
TTRCCRDYPGEECCSEWDCMCVQPEFHCGDPCCTTCRHHPCPPGQGVQSQGKFSF
GFQCIDCASGTFSGGHEGHCKPWTDCTQFGFLTVFPGNKTHNAVCVPGSPPAEPL
GWLTVVLLAVAACVLLLTSAQLGLHIWQLRSQCMWPRETQLLLEVPPSTEDARS
CQFPEEERGERSAEEKGRLGDLWV

SEQ ID NO: 24

MALKVLLEQEKTFFTLLVLLGYLSCKVTCESGDCRQQEFRDRSGNCVPCNQCGPG
MELSKECGFGYGEDAQCVTCRLHRFKEDWGFQKCKPCLDCAVVNRFQKANCSA
TSDAICGDCLPGFYRKTKLVGFQDMECVPCGDPPPPYEPHCASKVNLVKIASTASS
PRDTALAAVICSALATVLLALLILCVIYCKRQFMEKKPSWSLRSQDIQYNGSELSC
FDRPQLHEYAHRACCQCRRDSVQTCGPVRLLPSMCCEEACSPNPATLGCGVHSA
ASLQARNAGPAGEMVPTFFGSLTQSICGEFSDAWPLMQNPMGGDNISFCDSYPEL
TGEDIHSLNPELESSTSLDSNSSQDLVGGAVPVQSHSENFTAATDLSRYNNTLVES
ASTQDALTMRSQLDQESGAVIHPATQTSLQVRQRLGSL

SEQ ID NO: 25
MGTSPSSSTALASCSRIARRATATMIAGSLLLLGFLSTTTAQPEQKASNLIGTYRHV
DRATGQVLTCDKCPAGTYVSEHCTNTSLRVCSSCPVGTFTRHENGIEKCHDCSQP
CPWPMIEKLPCAALTDRECTCPPGMFQSNATCAPHTVCPVGWGVRKKGTETEDV
RCKQCARGTFSDVPSSVMKCKAYTDCLSQNLVVIKPGTKETDNVCGTLPSFSSSTS
PSPGTAIFPRPEHMETHEVPSSTYVPKGMNSTESNSSASVRPKVLSSIQEGTVPDNT
SSARGKEDVNKTLPNLQVVNHQQGPHHRHILKLLPSMEATGGEKSSTPIKGPKRG
HPRQNLHKHFDINEHLPWMIVLFLLLVLVVIVVCSIRKSSRTLKKGPRQDPSAIVEK
AGLKKSMTPTQNREKWIYYCNGHGIDILKLVAAQVGSQWKDIYQFLCNASEREV
AAFSNGYTADHERAYAALQHWTIRGPEASLAQLISALRQHRRNDVVEKIRGLME
DTTQLETDKLALPMSPSPLSPSPIPSPNAKLENSALLTVEPSPQDKNKGFFVDESEPL
LRCDSTSSGSSALSRNGSFITKEKKDTVLRQVRLDPCDLQPIFDDMLHFLNPEELRV
IEEIPQAEDKLDRLFEIIGVKSQEASQTLLDSVYSHLPDLL

SEQ ID NO: 26
MEQRPRGCAAVAAALLLVLLGARAQGGTRSPRCDCAGDFHKKIGLFCCRGCPAG
HYLKAPCTEPCGNSTCLVCPQDTFLAWENHHNSECARCQACDEQASQVALENCS
AVADTRCGCKPGWFVECQVSQCVSSSPFYCQPCLDCGALHRHTRLLCSRRDTDC
GTCLPGFYEHGDGCVSCPTSTLGSCPERCAAVCGWRQMFWVQVLLAGLVVPLLL
GATLTYTYRHCWPHKPLVTADEAGMEALTPPPATHLSPLDSAHTLLAPPDSSEKIC
TVQLVGNSWTPGYPETQEALCPQVTWSWDQLPSRALGPAAAPTLSPESPAGSPA
MMLQPGPQLYDVMDAVPARRWKEFVRTLGLREAEIEAVEVEIGRFRDQQYEML
KRWRQQQPAGLGAVYAALERMGLDGCVEDLRSRLQRGP

SEQ ID NO: 27
MDCQENEYWDQWGRCVTCQRCGPGQELSKDCGYGEGGDAYCTACPPRRYKSS
WGHHRCQSCITCAVINRVQKVNCTATSNAVCGDCLPRFYRKTRIGGLQDQECIPC
TKQTPTSEVQCAFQLSLVEADTPTVPPQEATLVALVSSLLVVFTLAFLGLFFLYCK
QFFNRHCQRGGLLQFEADKTAKEESLFPVPPSKETSAESQVSENIFQTQPLNPILED
DCSSTSGFPTQESFTMASCTSESHSHWVHSPIECTELDLQKFSSSASYTGAETLGGN
TVESTGDRLELNVPFEVPSP

SEQ ID NO: 28
MSTESMIRDVELAEEALPKKTGGPQGSRRCLFLSLFSFLIVAGATTLFCLLHFGVIG
PQREEFPRDLSLISPLAQAVRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANAL
LANGVELRDNQLVVPSEGLYLIYSQVLFKGQGCPSTHVLLTHTISRIAVSYQTKVN
LLSAIKSPCQRETPEGAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAESG
QVYFGIIAL

SEQ ID NO: 29
MGALGLEGRGGRLQGRGSLLLAVAGATSLVTLLLAVPITVLAVLALVPQDQGGL
VTETADPGAQAQQGLGFQKLPEEEPETDLSPGLPAAHLIGAPLKGQGLGWETTKE
QAFLTSGTQFSDAEGLALPQDGLYYLYCLVGYRGRAPPGGGDPQGRSVTLRSSLY
RAGGAYGPGTPELLLEGAETVTPVLDPARRQGYGPLWYTSVGFGGLVQLRRGER
VYVNISHPDMVDFARGKTFFGAVMVG

SEQ ID NO: 30
MERVQPLEENVGNAARPRFERNKLLLVASVIQGLGLLLCFTYICLHFSALQVSHRY
PRIQSIKVQFTEYKKEKGFILTSQKEDEIMKVQNNSVIINCDGFYLISLKGYFSQEVN
ISLHYQKDEEPLFQLKKVRSVNSLMVASLTYKDKVYLNVTTDNTSLDDFHVNGG
ELILIHQNPGEFCVL

SEQ ID NO: 31
MIETYNQTSPRSAATGLPISMKIFMYLLTVFLITQMIGSALFAVYLHRRLDKIEDER
NLHEDFVFMKTIQRCNTGERSLSLLNCEEIKSQFEGFVKDIMLNKEETKKENSFEM

QKVLQWAEKGYYTMSNNLVTLENGKQLTVKRQGLYYIYAQVTFCSNREASSQA
PFIASLCLKSPGRFERILLRAANTHSSAKPCGQQSIHLGGVFELQPGASVFVNVTDP
SQVSHGTGFTSFGLLKL

SEQ ID NO: 32
MQQPFNYPYPQIYWVDSSASSPWAPPGTVLPCPTSVPRRPGQRRPPPPPPPPLPPP
PPPPPLPPLPLPPLKKRGNHSTGLCLLVMFFMVLVALVGLGLGMFQLFHLQKELAE
LRESTSQMHTASSLEKQIGHPSPPPEKKELRKVAHLTGKSNSRSMPLEWEDTYGIV
LLSGVKYKKGGLVINETGLYFVYSKVYFRGQSCNNLPLSHKVYMRNSKYPQDLV
MMEGKMMSYCTTGQMWARSSYLGAVFNLTSADHLYVNVSELSLVNFEESQTFF
GLYKL

SEQ ID NO: 33
MEESVVRPSVFVVDGQTDIPFTRLGRSHRRQSCSVARVGLGLLLLLMGAGLAVQG
WFLLQLHWRLGEMVTRLPDGPAGSWEQLIQERRSHEVNPAAHLTGANSSLTGSG
GPLLWETQLGLAFLRGLSYHDGALVVTKAGYYYIYSKVQLGGVGCPLGLASTITH
GLYKRTPRYPEELELLVSQQSPCGRATSSSRVWWDSSFLGGVVHLEAGEKVVVR
VLDERLVRLRDGTRSYFGAFMV

SEQ ID NO: 34
MAEDLGLSFGETASVEMLPEHGSCRPKARSSSARWALTCCLVLLPFLAGLTTYLL
VSQLRAQGEACVQFQALKGQEFAPSHQQVYAPLRADGDKPRAHLTVVRQTPTQH
FKNQFPALHWEHELGLAFTKNRMNYTNKFLLIPESGDYFIYSQVTFRGMTSECSEI
RQAGRPNKPDSITVVITKVTDSYPEPTQLLMGTKSVCEVGSNWFQPIYLGAMFSLQ
EGDKLMVNVSDISLVDYTKEDKTFFGAFLL

SEQ ID NO: 35
MPEEGSGCSVRRRPYGCVLRAALVPLVAGLVICLVVCIQRFAQAQQQLPLESLGW
DVAELQLNHTGPQQDPRLYWQGGPALGRSFLHGPELDKGQLRIHRDGIYMVHIQ
VTLAICSSTTASRHHPTTLAVGICSPASRSISLLRLSFHQGCTIASQRLTPLARGDTL
CTNLTGTLLPSRNTDETFFGVQWVRP

SEQ ID NO: 36
MPKRSCPFADVAPLQLKVRVSQRELSRGVCAERYSQEVFEKTKRLLFLGAQAYLD
HVWDEGCAVVHLPESPKPGPTGAPRAARGQMLIGPDGRLIRSLGQASEADPSGVA
SIACSSCVRAVDGKAVCGQCERALCGQCVRTCWGCGSVACTLCGLVDCSDMYE
KVLCTSCAMFET

SEQ ID NO: 37
MDPGLQQALNGMAPPGDTAMHVPAGSVASHLGTTSRSYFYLTTATLALCLVFTV
ATIMVLVVQRTDSIPNSPDNVPLKGGNCSEDLLCILKRAPFKKSWAYLQVAKHLN
KTKLSWNKDGILHGVRYQDGNLVIQFPGLYFIICQLQFLVQCPNNSVDLKLELLIN
KHIKKQALVTVCESGMQTKHVYQNLSQFLLDYLQVNTTISVNVDTFQYIDTSTFP
LENVLSIFLYSNSD

SEQ ID NO: 38
MEYASDASLDPEAPWPPAPRARACRVLPWALVAGLLLLLLLAAACAVFLACPWA
VSGARASPGSAASPRLREGPELSPDDPAGLLDLRQGMFAQLVAQNVLLIDGPLSW
YSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYVFFQLELRRVVAGEGSGSVSL
ALHLQPLRSAAGAAALALTVDLPPASSEARNSAFGFQGRLLHLSAGQRLGVHLHT
EARARHAWQLTQGATVLGLFRVTPEIPAGLPSPRSE

SEQ ID NO: 39
MAMMEVQGGPSLGQTCVLIVIFTVLLQSLCVAVTYVYFTNELKQMQDKYSKSGI
ACFLKEDDSYWDPNDEESMNSPCWQVKWQLRQLVRKMILRTSEETISTVQEKQQ
NISPLVRERGPQRVAAHITGTRGRSNTLSSPNSKNEKALGRKINSWESSRSGHSFLS
NLHLRNGELVIHEKGFYYIYSQTYFRFQEEIKENTKNDKQMVQYIYKYTSYPDPIL
LMKSARNSCWSKDAEYGLYSIYQGGIFELKENDRIFVSVTNEHLIDMDHEASFFG
AFLVG

SEQ ID NO: 40
MRRASRDYTKYLRGSEEMGGGPGAPHEGPLHAPPPPAPHQPPAASRSMFVALLGL
GLGQVVCSVALFFYFRAQMDPNRISEDGTHCIYRILRLHENADFQDTTLESQDTKL
IPDSCRRIKQAFQGAVQKELQHIVGSQHIRAEKAMVDGSWLDLAKRSKLEAQPFA

HLTINATDIPSGSHKVSLSSWYHDRGWAKISNMTFSNGKLIVNQDGFYYLYANICF
RHHETSGDLATEYLQLMVYVTKTSIKIPSSHTLMKGGSTKYWSGNSEFHFYSINV
GGFFKLRSGEEISIEVSNPSLLDPDQDATYFGAFKVRDID

SEQ ID NO: 41
MAARRSQRRRGRRGEPGTALLVPLALGLGLALACLGLLLAVVSLGSRASLSAQEP
AQEELVAEEDQDPSELNPQTEESQDPAPFLNRLVRPRRSAPKGRKTRARRAIAAH
YEVHPRPGQDGAQAGVDGTVSGWEEARINSSSPLRYNRQIGEFIVTRAGLYYLYC
QVHFDEGKAVYLKLDLLVDGVLALRCLEEFSATAASSLGPQLRLCQVSGLLALRP
GSSLRIRTLPWAHLKAAPFLTYFGLFQVH

SEQ ID NO: 42
MPASSPFLLAPKGPPGNMGGPVREPALSVALWLSWGAALGAVACAMALLTQQT
ELQSLRREVSRLQGTGGPSQNGEGYPWQSLPEQSSDALEAWENGERSRKRRAVLT
QKQKKQHSVLHLVPINATSKDDSDVTEVMWQPALRRGRGLQAQGYGVRIQDAG
VYLLYSQVLFQDVTFTMGQVVSREGQGRQETLFRCIRSMPSHPDRAYNSCYSAGV
FHLHQGDILSVIIPRARAKLNLSPHGTFLGFVKL

SEQ ID NO: 43
MDDSTEREQSRLTSCLKKREEMKLKECVSILPRKESPSVRSSKDGKLLAATLLLAL
LSCCLTVVSFYQVAALQGDLASLRAELQGHHAEKLPAGAGAPKAGLEEAPAVTA
GLKIFEPPAPGEGNSSQNSRNKRAVQGPEETVTQDCLQLIADSETPTIQKGSYTFVP
WLLSFKRGSALEEKENKILVKETGYFFIYGQVLYTDKTYAMGHLIQRKKVHVFGD
ELSLVTLFRCIQNMPETLPNNSCYSAGIAKLEEGDELQLAIPRENAQISLDGDVTFF
GALKLL

SEQ ID NO: 44
MESMAVATDGGERPGVPAGSGLSASQRRAELRRRKLLMNSEQRINRIMGFHRPGS
GAEEESQTKSKQQDSDKLNSLSVPSVSKRVVLGDSVSTGTTDQQGGVAEVKGTQ
LGDKLDSFIKPPECSSDVNLELRQRNRGDLTADSVQRGSRHGLEQYLSRFEEAMK
LRKQLISEKPSQEDGNTTEEFDSFRIFRLVGCALLALGVRAFVCKYLSIFAPFLTLQ

LAYMGLYKYFPKSEKKIKTTVLTAALLLSGIPAEVINRSMDTYSKMGEVFTDLCV
YFFTFIFCHELLDYWGSEVP

SEQ ID NO: 45
MSMLFYTLITAFLIGIQAEPHSESNVPAGHTIPQAHWTKLQHSLDTALRRARSAPA
AAIAARVAGQTRNITVDPRLFKKRRLRSPRVLFSTQPPREAADTQDLDFEVGGAAP
FNRTHRSKRSSSHPIFHRGEFSVCDSVSVWVGDKTTATDIKGKEVMVLGEVNINN
SVFKQYFFETKCRDPNPVDSGCRGIDSKHWNSYCTTTHTFVKALTMDGKQAAWR
FIRIDTACVCVLSRKAVRRA

SEQ ID NO: 46
MTILFLTMVISYFGCMKAAPMKEANIRGQGGLAYPGVRTHGTLESVNGPKAGSR
GLTSLADTFEHVIEELLDEDQKVRPNEENNKDADLYTSRVMLSSQVPLEPPLLFLL
EEYKNYLDAANMSMRVRRHSDPARRGELSVCDSISEWVTAADKKTAVDMSGGT
VTVLEKVPVSKGQLKQYFYETKCNPMGYTKEGCRGIDKRHWNSQCRTTQSYVR
ALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR

SEQ ID NO: 47
MSILFYVIFLAYLRGIQGNNMDQRSLPEDSLNSLIIKLIQADILKNKLSKQMVDVKE
NYQSTLPKAEAPREPERGGPAKSAFQPVIAMDTELLRQQRRYNSPRVLLSDSTPLE
PPPLYLMEDYVGSPVVANRTSRRKRYAEHKSHRGEYSVCDSESLWVTDKSSAIDI
RGHQVTVLGEIKTGNSPVKQYFYETRCKEARPVKNGCRGIDDKHWNSQCKTSQT
YVRALTSENNKLVGWRWIRIDTSCVCALSRKIGRT

SEQ ID NO: 48
MLPLPSCSLPILLLFLLPSVPIESQPPPSTLPPFLAPEWDLLSPRVVLSRGAPAGPPLL
FLLEAGAFRESAGAPANRSRRGVSETAPASRRGELAVCDAVSGWVTDRRTAVDL
RGREVEVLGEVPAAGGSPLRQYFFETRCKADNAEEGGPGAGGGGCRGVDRRHW
VSECKAKQSYVRALTADAQGRVGWRWIRIDTACVCTLLSRTGRA

SEQ ID NO: 49

GHTANKPCLAKFELLTSKWQMTSRKPPCVNSLPEGKLKILQDGLYLIYGQVAPST
AYKGVAPFAVQLRKNEAMLQTLTSNSTIYDVGGTYEFHAGDIIDLIFDDEHQVLK
NNTYWGIVLLANLFIS

SEQ ID NO: 50
MGYPEVERRELLPAAAPRERGSQGCGCGGAPARAGEGNSCLLFLGFFGLSLALHL
LTLCCYLELRSELRRERGAESRLGGSGTPGTSGTLSSLGGLDPDSPITSHLGQPSPK
QQPLEPGEAALHSDSQDGHQMALLNFFFPDEKPYSEEESRRVRRNKRSKSNEGAD
GPVKNKKKGKKAGPPGPNGPPGPPGPPGPQGPPGIPGIPGIPGTTVMGPPGPPGPPG
PQGPPGLQGPSGAADKAGTRENQPAVVHLQGQGSAIQVKNDLSGGVLNDWSRIT
MNPKVFKLHPRSGELEVLVDGTYFIYSQVYYINFTDFASYEVVVDEKPFLQCTRSI
ETGKTNYNTCYTAGVCLLKARQKIAVKMVHADISINMSKHTTFFGAIRLGEAPAS

"Specific binding" or "specifically binds" or "binds" refers to an anti-TNFR superfamily member antibody binding to a particular TNFR superfamily member or an epitope within the particular TNFR superfamily member with greater affinity than for other antigens. Typically, the antibody "specifically binds" when the equilibrium dissociation constant (K_D) for binding is about $1x10^{-8}$ M or less, for example about $1x10^{-9}$ M or less, about $1x10^{-10}$ M or less, about $1x10^{-11}$ M or less, or about $1x10^{-12}$ M or less, typically with the K_D that is at least one hundred-fold less than its K_D for binding to a non-specific antigen (e.g., BSA, casein). The K_D may be measured using standard procedures. Anti-TNFR superfamily member antibodies that specifically bind to the particular TNFR superfamily member or an epitope within the particular TNFR superfamily member may, however, have cross-reactivity to other related antigens, for example to the same antigen from other species (homologs), such as human or monkey, for example *Macaca fascicularis* (cynomolgus, cyno), *Pan troglodytes* (chimpanzee, chimp) or *Callithrix jacchus* (common marmoset, marmoset). While a monospecific antibody specifically binds one antigen or one epitope, a bispecific antibody specifically binds two distinct antigens or two distinct epitopes.

"Antibodies" is meant in a broad sense and includes immunoglobulin molecules including monoclonal antibodies including murine, human, humanized and chimeric monoclonal antibodies, antibody fragments, bispecific or multispecific antibodies, dimeric,

tetrameric or multimeric antibodies, single chain antibodies, domain antibodies and any other modified configuration of the immunoglobulin molecule that comprises an antigen binding site of the required specificity. "Full length antibody molecules" are comprised of two heavy chains (HC) and two light chains (LC) inter-connected by disulfide bonds as well as multimers thereof (e.g. IgM). Each heavy chain is comprised of a heavy chain variable region (VH) and a heavy chain constant region (comprised of domains CH1, hinge, CH2 and CH3). Each light chain is comprised of a light chain variable region (VL) and a light chain constant region (CL). The VH and the VL regions may be further subdivided into regions of hyper variability, termed complementarity determining regions (CDR), interspersed with framework regions (FR). Each VH and VL is composed of three CDRs and four FR segments, arranged from amino-to-carboxyl-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4.

"Complementarity determining regions (CDR)" are "antigen binding sites" in an antibody. CDRs may be defined using various terms: (i) Complementarity Determining Regions (CDRs), three in the VH (HCDR1, HCDR2, HCDR3) and three in the VL (LCDR1, LCDR2, LCDR3) are based on sequence variability (Wu et al. (1970) *J Exp Med* 132: 211-50) (Kabat *et al.,* Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, Md., 1991). (ii) "Hypervariable regions", "HVR", or "HV", three in the VH (H1, H2, H3) and three in the VL (L1, L2, L3) refer to the regions of an antibody variable domains which are hypervariable in structure as defined by Chothia and Lesk (Chothia et al. (1987) *J Mol Biol* 196: 901-17). The International ImMunoGeneTics (IMGT) database (http://www_imgt_org) provides a standardized numbering and definition of antigen-binding sites. The correspondence between CDRs, HVs and IMGT delineations is described in (Lefranc et al. (2003) *Dev Comp Immunol* 27: 55-77). The term "CDR", "HCDR1", "HCDR2", "HCDR3", "LCDR1", "LCDR2" and "LCDR3" as used herein includes CDRs defined by any of the methods described *supra,* Kabat, Chothia or IMGT, unless otherwise explicitly stated in the specification.

Immunoglobulins may be assigned to five major classes, IgA, IgD, IgE, IgG and IgM, depending on the heavy chain constant region amino acid sequence. IgA and IgG are further sub-classified as the isotypes IgA1, IgA2, IgG1, IgG2, IgG3 and IgG4. Antibody light chains of any vertebrate species may assigned to one of two clearly distinct types,

namely kappa (κ) and lambda (λ), based on the amino acid sequences of their constant regions.

"Antibody fragments" refers to a portion of an immunoglobulin molecule that retains the heavy chain and/or the light chain antigen binding site, such as heavy chain complementarity determining regions (HCDR) 1, 2 and 3, light chain complementarity determining regions (LCDR) 1, 2 and 3, a heavy chain variable region (VH), or a light chain variable region (VL). Antibody fragments include well known Fab, F(ab')2, Fd and Fv fragments as well as domain antibodies (dAb) consisting of one VH domain or one VL domain. VH and VL domains may be linked together via a synthetic linker to form various types of single chain antibody designs where the VH/VL domains may pair intramolecularly, or intermolecularly in those cases when the VH and VL domains are expressed by separate single chain antibody constructs, to form a monovalent antigen binding site, such as single chain Fv (scFv) or diabody; described for example in Int. Patent Publ. Nos. WO1998/44001, WO1988/01649, WO1994/13804 and WO1992/01047.

"Monoclonal antibody" refers to an antibody population with single amino acid composition in each heavy and each light chain, except for possible well known alterations such as removal of C-terminal lysine from the antibody heavy chain or alterations due to post-translational modification(s) of amino acids, such as methionine oxidation or asparagine or glutamine deamidation. Monoclonal antibodies typically specifically bind one antigenic epitope, except that bispecific or multispecific monoclonal antibodies specifically bind two or more distinct antigenic epitopes. Monoclonal antibodies may have heterogeneous glycosylation within the antibody population. Monoclonal antibody may be monospecific or multispecific, or monovalent, bivalent or multivalent. A bispecific antibody is included in the term monoclonal antibody.

"Isolated antibody" refers to an antibody or antibody fragment that is substantially free of other antibodies having different antigenic specificities (e.g., an anti-TNFR superfamily member antibody is substantially free of antibodies that specifically bind antigens other than the anti-TNFR superfamily member). "Isolated antibody" encompasses antibodies that are isolated to a higher purity, such as antibodies that are 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% pure.

"Humanized antibody" refers to an antibody in which the antigen binding sites are derived from non-human species and the variable region frameworks are derived from human immunoglobulin sequences. Humanized antibody may include substitutions in the framework so that the framework may not be an exact copy of expressed human immunoglobulin or human immunoglobulin germline gene sequences.

"Human antibody" refers to an antibody having heavy and light chain variable regions in which both the framework and the antigen binding site are derived from sequences of human origin and is optimized to have minimal immune response when administered to a human subject. If the antibody contains a constant region or a portion of the constant region, the constant region also is derived from sequences of human origin.

Human antibody comprises heavy or light chain variable regions that are "derived from" sequences of human origin if the variable regions of the antibody are obtained from a system that uses human germline immunoglobulin or rearranged immunoglobulin genes. Such exemplary systems are human immunoglobulin gene libraries displayed on phage, and transgenic non-human animals such as mice or rats carrying human immunoglobulin loci as described herein. "Human antibody" may contain amino acid differences when compared to the human germline immunoglobulin or rearranged immunoglobulin genes due to differences between the systems used to obtain the antibody and human immunoglobulin loci, introduction of somatic mutations or intentional introduction of substitutions into the framework or antigen binding site, or both. Typically, "human antibody" is at least about 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identical in amino acid sequence to an amino acid sequence encoded by human germline immunoglobulin or rearranged immunoglobulin genes. In some cases, "human antibody" may contain consensus framework sequences derived from human framework sequence analyses, for example as described in (Knappik et al. (2000) *J Mol Biol* 296: 57-86), or synthetic HCDR3 incorporated into human immunoglobulin gene libraries displayed on phage, for example as described in (Shi et al. (2010) *J Mol Biol* 397: 385-96), and in Int. Patent Publ. No. WO2009/085462.

Human antibodies derived from human immunoglobulin sequences may be generated using systems such as phage display incorporating synthetic CDRs and/or synthetic frameworks, or may be subjected to *in vitro* mutagenesis to improve antibody

properties, resulting in antibodies that are not expressed by the human antibody germline repertoire *in vivo*.

Antibodies in which antigen binding sites are derived from a non-human species are not included in the definition of "human antibody".

"Recombinant" refers to antibodies and other proteins that are prepared, expressed, created or isolated by recombinant means.

"Epitope" refers to a portion of an antigen to which an antibody specifically binds. Epitopes typically consist of chemically active (such as polar, non-polar or hydrophobic) surface groupings of moieties such as amino acids or polysaccharide side chains and may have specific three-dimensional structural characteristics, as well as specific charge characteristics. An epitope may be composed of contiguous and/or discontiguous amino acids that form a conformational spatial unit. For a discontiguous epitope, amino acids from differing portions of the linear sequence of the antigen come in close proximity in 3-dimensional space through the folding of the protein molecule. Antibody "epitope" depends on the methodology used to identify the epitope.

"Bispecific" refers to an antibody that specifically binds two distinct antigens or two distinct epitopes within the same antigen. The bispecific antibody may have cross-reactivity to other related antigens, for example to the same antigen from other species (homologs), such as human or monkey, for example *Macaca fascicularis* (cynomolgus, cyno), *Pan troglodytes* (chimpanzee, chimp) or *Callithrix jacchus* (common marmoset, marmoset), or may bind an epitope that is shared between two or more distinct antigens.

"Multispecific" refers to an antibody that specifically binds two or more distinct antigens or two or more distinct epitopes within the same antigen.

"Vector" refers to a polynucleotide capable of being duplicated within a biological system or that can be moved between such systems. Vector polynucleotides typically contain elements, such as origins of replication, polyadenylation signal or selection markers, that function to facilitate the duplication or maintenance of these polynucleotides in a biological system, such as a cell, virus, animal, plant, and reconstituted biological systems utilizing biological components capable of duplicating a vector. The vector polynucleotide may be DNA or RNA molecules, cDNA, or a hybrid of these, single stranded or double stranded.

"Expression vector" refers to a vector that can be utilized in a biological system or in a reconstituted biological system to direct the translation of a polypeptide encoded by a polynucleotide sequence present in the expression vector.

"Polynucleotide" refers to a synthetic molecule comprising a chain of nucleotides covalently linked by a sugar-phosphate backbone or other equivalent covalent chemistry. cDNA is a typical example of a synthetic polynucleotide.

"Polypeptide" or "protein" refers to a molecule that comprises at least two amino acid residues linked by a peptide bond to form a polypeptide. Small polypeptides of less than 50 amino acids may be referred to as "peptides".

"About" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, i.e., the limitations of the measurement system. Unless explicitly stated otherwise within the Examples or elsewhere in the Specification in the context of a particular assay, result or embodiment, "about" means within one standard deviation per the practice in the art, or a range of up to 5%, whichever is larger.

"Valent" refers to the presence of a specified number of binding sites specific for an antigen in a molecule. As such, the terms "monovalent", "bivalent", "tetravalent", and "hexavalent" refer to the presence of one, two, four and six binding sites, respectively, specific for an antigen in a molecule.

"Agonist" refers to an antibody that induces at least one biological activity of the TNFR superfamily member the antibody binds to that is induced by a natural ligand of the TNFR superfamily member. Exemplary agonistic activities include induction of production of a secreted embryonic alkaline phosphatase (SEAP) expressed under the control of NFκB-inducible promoter in an *in vitro* assay, induction of dendritic cell (DC) differentiation assessed by increased CD80, CD83, CD86 and HLA-DR surface expression on DC, activation of B cells assessed by increased B cell proliferation or increased CD23, CD80, CD83, CD86 and HLA-DR surface expression on B cells, induction of antigen-specific T cell recall responses assessed by production of interferon-γ (IFN-γ) by PBMCs isolated from patients previously exposed to the antigen, and induction of $CD4^+$ or $CD8^+$ T cell proliferation. Agonistic activity (*e.g.*, agonism) may be cross-linking dependent or independent of antibody cross-linking.

"Enhanced agonistic activity" or "enhanced agonism" refers to improvement in agonism of an engineered anti-TNFR superfamily member antibody when compared to the parental wild-type antibody, when agonistic activity is measured by anti-TNFR superfamily member antibody-induced production of secreted embryonic alkaline phosphatase (SEAP) expressed under the control of NFκB-inducible promoter. The engineered antibody has "enhanced agonistic activity" when it induces SEAP production at a level that is at least 20 % higher when compared to the wild-type parental antibody at antibody concentration of 1 μg/mL in either cross-linking dependent or cross-linking independent manner.

"Cross-linking" refers to the higher order multimerization of an anti-TNFR superfamily member antibody on cells expressing the TNFR superfamily member, induced by the antibody binding to FcγR, for example FcγRIIB *cis* or *trans*, and subsequent induction of TNFR agonistic activity. Cross-linking may be evaluated *in vitro* by using anti-human F(ab')2 as a cross-linker, or cells expressing FcγRIIB, such as Raji cells.

"Agonistic activity independent of antibody cross-linking" means that the antibody induces production of SEAP in a HEK-Blue™ reporter assay as described in Example 3 herein in solution in the absence of Raji cells expressing FcγR, for example FcγRIIB.

"Fc domain containing molecule" refers to a monomeric, dimeric or heterodimeric protein having at least an immunoglobulin CH2 and CH3 domain. Exemplary Fc domain containing molecules are fusion proteins containing an extracellular domain of a TNFR ligand such as those shown in **Table 1** linked to an Fc domain.

"Subject" includes any human or nonhuman animal. "Nonhuman animal" includes all vertebrates, *e.g.,* mammals and non-mammals, such as nonhuman primates, sheep, dogs, cats, horses, cows chickens, amphibians, reptiles, etc.

The numbering of amino acid residues in the antibody constant region throughout the specification is according to the EU index as described in Kabat *et al.,* Sequences of Proteins of Immunological Interest, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), unless otherwise explicitly stated.

Conventional one and three-letter amino acid codes are used herein as shown in **Table 2.**

Table 2.

Amino acid	Three-letter code	One-letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Gln	E
Glutamine	Glu	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Compositions of matter

The present invention provides engineered anti-tumor necrosis factor receptor (TNFR) superfamily member antibodies with enhanced agonistic activity, and optionally enhanced effector functions, and methods of using and making the antibodies. The invention is based, at least in part, on the identification that introducing certain mutations in the Fc region of anti-TNFR superfamily member antibodies results in engineered antibodies with enhanced agonism, and optionally with enhanced effector functions.

The present invention provides an engineered anti-tumor necrosis factor receptor (TNFR) superfamily member antibody, wherein the antibody comprises a T437R mutation when compared to a parental wild-type antibody, optionally further comprising a K248E mutation or a K338A mutation, residue numbering according to the EU Index.

The present invention provides an engineered anti-tumor necrosis factor receptor (TNFR) superfamily member antibody, wherein the antibody comprises a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation when compared to a parental wild-type antibody, residue numbering according to the EU Index.

In some embodiments, the antibody comprises a T437R mutation.

In some embodiments, the antibody comprises a T437R/K248E mutation.

In some embodiments, the antibody comprises a T437R/K338A mutation.

In some embodiments, the antibody comprises a heavy chain constant region (HC) of **SEQ ID NO: 63.**

In some embodiments, the antibody comprises a heavy chain constant region (HC) of **SEQ ID NO: 64.**

In some embodiments, the antibody comprises a heavy chain constant region (HC) of **SEQ ID NO: 65.**

SEQ ID NO: 63 (IgG1 antibody with a T437R mutation)
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC
PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY**R**QKSLSLSPGK

SEQ ID NO: 64 (IgG1 antibody with a T437R/K248E mutation)
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC
PAPELLGGPSVFLFPPKP**E**DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISK

AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY**R**QKSLSLSPGK

SEQ ID NO: 65 (IgG1 antibody with a T437R/K338A mutation)
ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC
PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTIS**A**
AKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT
TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY**R**QKSLSLSPGK

In some embodiments, the antibody has enhanced agonistic activity when compared to the parental wild-type antibody.

In some embodiments, agonistic activity is measured by measuring antibody-induced production of secreted embryonic alkaline phosphatase (SEAP) expressed under the control of NFκB-inducible promoter from Hek-293 cells.

The T437R mutation enhances agonistic activity of engineered anti-TNFR superfamily member antibodies.

The T437R/K248E mutation enhances agonistic activity of engineered anti-TNFR superfamily member antibodies.

The T437R/K338A mutation enhances agonistic activity of engineered anti-TNFR superfamily member antibodies.

In some embodiments, the antibody mediates antibody-dependent cellular cytotoxicity (ADCC).

In some embodiments, the antibody mediates antibody-dependent cell phagocytosis (ADCP).

In some embodiments, the antibody mediates CDC.

In some embodiments, the antibody is an IgG1 isotype, optionally further comprising a L234A/L235A mutation when compared to the wild-type IgG1.

In some embodiments, the antibody is an IgG1 isotype, optionally further comprising a L234F/L235E/D265A mutation when compared to the wild-type IgG1.

In some embodiments, the antibody is an IgG1 isotype, optionally further comprising a K214T/E233P/L234V/L235A/G236-deleted/A327G/P331A/D365E/L358M mutation when compared to the wild-type IgG1.

In some embodiments, the antibody is an IgG1 isotype, optionally further comprising a L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation when compared to the wild-type IgG1.

In some embodiments, the antibody is an IgG2 isotype, optionally further comprising a V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the antibody is an IgG2 isotype, optionally further comprising a V234A/G237A mutation when compared to the wild-type IgG2.

In some embodiments, the antibody is an IgG2 isotype, optionally further comprising a H268Q/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the antibody is an IgG3 isotype.

In some embodiments, the antibody is an IgG4 isotype, optionally further comprising a F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody is an IgG4 isotype, optionally further comprising a S228P/F234A/L235A/G237A/P238S mutation when compared to the wild-type IgG4.

In some embodiments, the antibody is an IgG4 isotype, optionally further comprising a S228P/F234A/L235A/G236-deleted/G237A/P238S mutation when compared to the wild-type IgG4

In some embodiments, the antibody is an IgG4 isotype, optionally further comprising a S228P/F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody is an IgG4 isotype and comprises the S228P/F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody has agonistic activity independent of antibody cross-linking, wherein agonistic activity is measured by measuring antibody-induced production of secreted embryonic alkaline phosphatase (SEAP) expressed under the control of NFκB-inducible promoter from Hek-293 cells.

In some embodiments, the anti-TNFR superfamily member antibody of the invention has agonistic activity independent of FcγR-mediated antibody cross-linking. Therefore, the antibodies of the invention may not be dependent on the bioavailability and density of cells expressing FcγR in the tumor microenvironment for their agonistic activity and can induce TNFR signaling in environments lacking sufficient FcγR cell infiltration.

The anti-TNFR superfamily member antibodies of the invention may demonstrate level of agonism less than that of the native ligand, and therefore may provide an improved safety profile.

In some embodiments, the TNFR superfamily member is tumor necrosis factor receptor 1 (SEQ ID NO: 1), Tumor necrosis factor receptor 2 (SEQ ID NO: 2) , lymphotoxin beta receptor (SEQ ID NO: 3), OX40 (SEQ ID NO: 4), CD40 (SEQ ID NO: 5), Fas receptor (SEQ ID NO: 6), decoy receptor 3 (SEQ ID NO: 7), CD27 (SEQ ID NO: 8), CD30 (SEQ ID NO: 9), CD137 (SEQ ID NO: 10), death receptor 4 (SEQ ID NO: 11), death receptor 5 (SEQ ID NO: 12), decoy receptor 1 (SEQ ID NO: 13), decoy receptor 2 (SEQ ID NO: 14), RANK (SEQ ID NO: 15), osteoprotegerin (SEQ ID NO: 16), TWEAK receptor (SEQ ID NO: 17), TACI (SEQ ID NO: 18), BAFF receptor (SEQ ID NO: 19), herpesvirus entry mediator (SEQ ID NO: 20), nerve growth factor receptor (SEQ ID NO: 21), B-cell maturation antigen (SEQ ID NO: 22), GITR (SEQ ID NO: 23), TROY (SEQ ID NO: 24), death receptor 6 (SEQ ID NO: 25), death receptor 3 (SEQ ID NO: 26) or ectodysplasin A2 receptor (SEQ ID NO: 27).

In some embodiments, the TNFR superfamily member is OX40 (SEQ ID NO: 4), CD27 (SEQ ID NO: 8), CD40 (SEQ ID NO: 5), CD137 (SEQ ID NO: 10), or GITR (SEQ ID NO: 23).

In some embodiments, the TNFR superfamily member is OX40 (SEQ ID NO: 4).

In some embodiments, the TNFR superfamily member is CD27 (SEQ ID NO: 8).

In some embodiments, the TNFR superfamily member is CD40 (SEQ ID NO: 5).

In some embodiments, the TNFR superfamily member is CD137 (SEQ ID NO: 10).

In some embodiments, the TNFR superfamily member is GITR (SEQ ID NO: 23).

In some embodiments, the antibody comprises the T437R mutation.

In some embodiments, the anti-TNFR superfamily member antibody comprises a constant region of SEQ ID NO: 63.

In some embodiments, the antibody comprises the T437R mutation and mediates ADCC.

In some embodiments, the antibody comprises the T437R mutation and an IgG1 isotype and mediates ADCC.

In some embodiments, the antibody comprises the T437R mutation and mediates ADCP.

In some embodiments, the antibody comprises the T437R mutation and is an IgG1 isotype and mediates ADCP.

In some embodiments, the antibody comprises the T437R mutation and mediates CDC.

In some embodiments, the antibody comprises the T437R mutation and is an IgG1 isotype and mediates CDC.

The reported Fc engineering efforts to enhance agonistic activity of the anti-TNFR superfamily member antibodies by introducing a S267E/L328F mutation (Chu et al. (2008) *Mol Immunol* 45: 3926-33) or an E233D/G237D/P238D/H268D/P271G/A330R mutation (Mimoto et al. (2013) *Protein Eng Des Sel* 26: 589-98) resulted in antibodies with abolished ADCC. Contrary to the antibodies described by Chu and Mimoto, the IgG1 antibodies of the present invention comprising the T437R mutation may be used in instances in which depletion of the TNFR expressing cells is desirable. Exemplary such instances are depletion of GITR and/or OX-40 expressing Treg cells in the tumor microenvironment to enhance anti-tumor immunity.

In some embodiments, the antibody of the invention comprising the T437R mutation may further comprise a second mutation which reduces or abolishes antibody Fc mediated effector functions. The antibodies of the present invention comprising the T437R mutation and a second mutation that reduces or abolishes antibody Fc mediated effector functions may therefore be used in instances in which depletion of the TNFR expressing cells is not desirable. Exemplary such instances are therapeutic treatments with anti-CD40 or anti-CD27 antibodies.

In some embodiments, the antibody comprises the T437R mutation and is an IgG1 isotype, optionally further comprising the L234A/L235A mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R mutation and is an IgG1 isotype, optionally further comprising the L234F/L235E/D265A mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R mutation and is an IgG1 isotype, optionally further comprising the K214T/E233P/L234V/L235A/G236-deleted/A327G/P331A/D365E/L358M mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R mutation and is an IgG1 isotype, optionally further comprising the L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R mutation and is n IgG1 isotype, and further comprises the L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation when compared to the wild-type IgG1.

In some embodiments, the anti-TNFR superfamily member antibody comprises a constant region of SEQ ID NO: 66.

SEQ ID NO: 66 IgG1sigma with T437R

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC
PAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPSSIEKTISKA
KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY**R**QKSLSLSPGK

In some embodiments, the antibody comprises the T437R mutation and is an IgG2 isotype, optionally further comprising the V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the anti-TNFR superfamily member antibody comprises a constant region of SEQ ID NO: 67.

SEQ ID NO: 67 IgG2sigma with T437R

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAP
PAAASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVQFNWYVDGVEVHNAK
TKPREEQFNSTFRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKTKGQPR
EPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLD
SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY**R**QKSLSLSPGK

In some embodiments, the antibody comprises the T437R mutation and is an IgG2 isotype, and further comprising the V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

Antibodies of the invention comprising the T437R mutation and the L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation retained their cross-linking independent agonistic activity but were unable to mediate ADCC. Antibodies with the T437R mutation and the L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation may therefore be used in instances in which depletion of the TNFR expressing cells is undesired.

In some embodiments, the antibody comprises the T437R mutation and is an IgG2 isotype, optionally further comprising the V234A/G237A mutation when compared to the wild-type IgG2.

In some embodiments, the antibody comprises the T437R mutation and is an IgG2 isotype, optionally further comprising the H268Q/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the antibody comprises the T437R mutation and is an IgG3 isotype.

In some embodiments, the antibody comprises the T437R mutation and is an IgG4 isotype, optionally further comprising the F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R mutation and is an IgG4 isotype, optionally further comprising the S228P/F234A/L235A/G237A/P238S mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R mutation and is an IgG4 isotype, optionally further comprising the S228P/F234A/L235A/G236-deleted/G237A/P238S mutation when compared to the wild-type IgG4

In some embodiments, the antibody comprises the T437R mutation and is an IgG4 isotype, optionally further comprising the S228P/F234A/L235A mutation when compared to the wild-type IgG4.

Antibodies of the invention comprising the T437R mutation and the S228P/F234A/L235A mutation retained their cross-linking independent agonistic activity but were unable to mediate ADCC. Antibodies with the T437R mutation and the S228P/F234A/L235A mutation may therefore be used in instances in which depletion of the TNFR expressing cells is undesired.

In some embodiments, the antibody comprises the T437R mutation and is an IgG4 isotype, and further comprises the S228P/F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the anti-TNFR superfamily member antibody comprises a constant region of SEQ ID NO: 68.

SEQ ID NO: 68 IgG4PAA with T437R

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAP
EAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHN
AKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
QPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHY**R**QKSLSLSLGK

In some embodiments, the antibody comprises the T437R mutation and has agonistic activity independent of antibody cross-linking, wherein agonistic activity is measured by measuring antibody-induced production of secreted embryonic alkaline phosphatase (SEAP) expressed under the control of NFκB-inducible promoter from Hek-293 cells.

In some embodiments, the antibody comprises the T437R mutation and binds TNFR superfamily member tumor necrosis factor receptor 1 (SEQ ID NO: 1), Tumor

necrosis factor receptor 2 (SEQ ID NO: 2) , lymphotoxin beta receptor (SEQ ID NO: 3), OX40 (SEQ ID NO: 4), CD40 (SEQ ID NO: 5), Fas receptor (SEQ ID NO: 6), decoy receptor 3 (SEQ ID NO: 7), CD27 (SEQ ID NO: 8), CD30 (SEQ ID NO: 9), CD137 (SEQ ID NO: 10), death receptor 4 (SEQ ID NO: 11), death receptor 5 (SEQ ID NO: 12), decoy receptor 1 (SEQ ID NO: 13), decoy receptor 2 (SEQ ID NO: 14), RANK (SEQ ID NO: 15), osteoprotegerin (SEQ ID NO: 16), TWEAK receptor (SEQ ID NO: 17), TACI (SEQ ID NO: 18), BAFF receptor (SEQ ID NO: 19), herpesvirus entry mediator (SEQ ID NO: 20), nerve growth factor receptor (SEQ ID NO: 21), B-cell maturation antigen (SEQ ID NO: 22), GITR (SEQ ID NO: 23), TROY (SEQ ID NO: 24), death receptor 6 (SEQ ID NO: 25), death receptor 3 (SEQ ID NO: 26) or ectodysplasin A2 receptor (SEQ ID NO: 27).

In some embodiments, the antibody comprises the T437R mutation and binds TNFR superfamily member OX40 (SEQ ID NO: 4), CD27 (SEQ ID NO: 8), CD40 (SEQ ID NO: 5), CD137 (SEQ ID NO: 10), or GITR (SEQ ID NO: 23).

In some embodiments, the antibody comprises the T437R mutation and binds TNFR superfamily member OX40 (SEQ ID NO: 4).

In some embodiments, the antibody comprises the T437R mutation and binds TNFR superfamily member CD27 (SEQ ID NO: 8).

In some embodiments, the antibody comprises the T437R mutation and binds TNFR superfamily member CD40 (SEQ ID NO: 5).

In some embodiments, the antibody comprises the T437R mutation and binds TNFR superfamily member CD137 (SEQ ID NO: 10).

In some embodiments, the antibody comprises the T437R mutation and binds TNFR superfamily member GITR (SEQ ID NO: 23).

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a solid tumor.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a melanoma.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a lung cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a squamous non-small cell lung cancer (NSCLC).

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a non-squamous NSCLC.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a lung adenocarcinoma.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a renal cell carcinoma (RCC) (e.g., A kidney clear cell carcinoma or a kidney papillary cell carcinoma), or a metastatic lesion thereof.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a mesothelioma.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a nasopharyngeal carcinoma (NPC).

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a colorectal cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a prostate cancer or castration-resistant prostate cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a stomach cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating an ovarian cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a gastric cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a liver cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating pancreatic cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a thyroid cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a squamous cell carcinoma of the head and neck.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a carcinomas of the esophagus or gastrointestinal tract.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a breast cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a fallopian tube cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a brain cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating an urethral cancer.

In some embodiments of the invention described herein, and in some embodiments of each and every one of the numbered embodiments listed below, the solid tumor is a genitourinary cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating an endometriosis.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a cervical cancer.

The antibody comprising the T437R mutation is suitable for use in therapy, for example in treating a metastatic lesion of the cancer.

In some embodiments, the antibody comprises the T437R/K248E mutation.

In some embodiments, the antibody comprises the HC of SEQ ID NO: 64.

In some embodiments, the antibody comprises the T437R/K248E mutation and mediates ADCC.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG1 isotype and mediates ADCC.

In some embodiments, the antibody comprises the T437R/K248E mutation and mediates ADCP.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG1 isotype and mediates ADCP.

In some embodiments, the antibody comprises the T437R/K248E mutation and mediates CDC.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG1 isotype and mediates CDC.

The reported Fc engineering efforts to enhance agonistic activity of the anti-TNFR superfamily member antibodies by introducing a S267E/L328F mutation (Chu et al. (2008) *Mol Immunol* 45: 3926-33) or an E233D/G237D/P238D/H268D/P271G/A330R mutation (Mimoto et al. (2013) *Protein Eng Des Sel* 26: 589-98) resulted in antibodies with abolished ADCC. Contrary to the antibodies described by Chu and Mimoto, the IgG1 antibodies of the present invention comprising the T437R/K248E mutation may be used in instances in which depletion of the TNFR expressing cells is desirable. Exemplary such instances are depletion of GITR and/or OX-40 expressing Treg cells in the tumor microenvironment to enhance anti-tumor immunity effects.

In some embodiments, the antibody of the invention comprising the T437R/K248E mutation may further comprise a second mutation which reduces or abolishes antibody Fc mediated effector functions. The antibodies of the present invention comprising the T437R/K248E mutation and a second mutation that reduces or abolishes antibody Fc mediated effector functions may therefore be used in instances in which depletion of the TNFR expressing cells is not desirable. Exemplary such instances are therapeutic treatments with anti-CD40 or anti-CD27 antibodies.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG1 isotype, optionally further comprising the L234A/L235A mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG1 isotype, optionally further comprising the L234F/L235E/D265A mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG1 isotype, optionally further comprising the K214T/E233P/L234V/L235A/G236-deleted/A327G/P331A/D365E/L358M mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG1 isotype, optionally further comprising the

L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG1 isotype, and further comprises the L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation when compared to the wild-type IgG1.

In some embodiments, the anti-TNFR superfamily member antibody comprises a constant region of SEQ ID NO: 69.

SEQ ID NO: 69 IgG1sigma with T437R/K248E

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC
PAPEAAGASSVFLFPPKP**E**DTLMISRTPEVTCVVVDVSAEDPEVKFNWYVDGVEV
HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPSSIEKTISKA
KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY**R**QKSLSLSPGK

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG2 isotype, optionally further comprising the V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG2 isotype, and further comprising the V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the anti-TNFR superfamily member antibody comprises a constant region of SEQ ID NO: 70.

SEQ ID NO: 70 IgG2sigma with T437R/K248E

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAP
PAAASSVFLFPPKP**E**DTLMISRTPEVTCVVVDVSAEDPEVQFNWYVDGVEVHNAK

TKPREEQFNSTFRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKTKGQPR
EPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLD
SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY**R**QKSLSLSPGK

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG2 isotype, optionally further comprising the V234A/G237A mutation when compared to the wild-type IgG2.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG2 isotype, optionally further comprising the H268Q/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG3 isotype.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG4 isotype, optionally further comprising the F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG4 isotype, optionally further comprising the S228P/F234A/L235A/G237A/P238S mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG4 isotype, optionally further comprising the S228P/F234A/L235A/G236-deleted/G237A/P238S mutation when compared to the wild-type IgG4

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG4 isotype, optionally further comprising the S228P/F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG4 isotype, and further comprises the S228P/F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the anti-TNFR superfamily member antibody comprises a constant region of SEQ ID NO: 71.

SEQ ID NO: 71 IgG4PAA with T437R/K248E

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAP
EAAGGPSVFLFPPKP**E**DTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNA
KTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQ
PREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPV
LDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHY**R**QKSLSLSLGK

In some embodiments, the antibody comprises the T437R/K248E mutation and is an IgG4 isotype and comprises the S228P/F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R/K248E mutation and has agonistic activity independent of antibody cross-linking, wherein agonistic activity is measured by measuring antibody-induced production of secreted embryonic alkaline phosphatase (SEAP) expressed under the control of NFκB-inducible promoter from Hek-293 cells.

In some embodiments, the antibody comprises the T437R/K248E mutation and binds TNFR superfamily member tumor necrosis factor receptor 1 (SEQ ID NO: 1), Tumor necrosis factor receptor 2 (SEQ ID NO: 2) , lymphotoxin beta receptor (SEQ ID NO: 3), OX40 (SEQ ID NO: 4), CD40 (SEQ ID NO: 5), Fas receptor (SEQ ID NO: 6), decoy receptor 3 (SEQ ID NO: 7), CD27 (SEQ ID NO: 8), CD30 (SEQ ID NO: 9), CD137 (SEQ ID NO: 10), death receptor 4 (SEQ ID NO: 11), death receptor 5 (SEQ ID NO: 12), decoy receptor 1 (SEQ ID NO: 13), decoy receptor 2 (SEQ ID NO: 14), RANK (SEQ ID NO: 15), osteoprotegerin (SEQ ID NO: 16), TWEAK receptor (SEQ ID NO: 17), TACI (SEQ ID NO: 18), BAFF receptor (SEQ ID NO: 19), herpesvirus entry mediator (SEQ ID NO: 20), nerve growth factor receptor (SEQ ID NO: 21), B-cell maturation antigen (SEQ ID NO: 22), GITR (SEQ ID NO: 23), TROY (SEQ ID NO: 24), death receptor 6 (SEQ ID NO: 25), death receptor 3 (SEQ ID NO: 26) or ectodysplasin A2 receptor (SEQ ID NO: 27).

In some embodiments, the antibody comprises the T437R/K248E mutation and binds TNFR superfamily member OX40 (SEQ ID NO: 4), CD27 (SEQ ID NO: 8), CD40 (SEQ ID NO: 5), CD137 (SEQ ID NO: 10), or GITR (SEQ ID NO: 23).

In some embodiments, the antibody comprises the T437R/K248E mutation and binds TNFR superfamily member OX40 (SEQ ID NO: 4).

In some embodiments, the antibody comprises the T437R/K248E mutation and binds TNFR superfamily member CD27 (SEQ ID NO: 8).

In some embodiments, the antibody comprises the T437R/K248E mutation and binds TNFR superfamily member CD40 (SEQ ID NO: 5).

In some embodiments, the antibody comprises the T437R/K248E mutation and binds TNFR superfamily member CD137 (SEQ ID NO: 10).

In some embodiments, the antibody comprises the T437R/K248E mutation and binds TNFR superfamily member GITR (SEQ ID NO: 23).

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a solid tumor.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a melanoma.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a lung cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a squamous non-small cell lung cancer (NSCLC).

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a non-squamous NSCLC.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a lung adenocarcinoma.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a renal cell carcinoma (RCC) (e.g., A kidney clear cell carcinoma or a kidney papillary cell carcinoma), or a metastatic lesion thereof.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a mesothelioma.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a nasopharyngeal carcinoma (NPC).

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a colorectal cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a prostate cancer or castration-resistant prostate cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a stomach cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating an ovarian cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a gastric cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a liver cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating pancreatic cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a thyroid cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a squamous cell carcinoma of the head and neck.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a carcinomas of the esophagus or gastrointestinal tract.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a breast cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a fallopian tube cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a brain cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating an urethral cancer.

In some embodiments of the invention described herein, and in some embodiments of each and every one of the numbered embodiments listed below, the solid tumor is a genitourinary cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating an endometriosis.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a cervical cancer.

The antibody comprising the T437R/K248E mutation is suitable for use in therapy, for example in treating a metastatic lesion of the cancer.

In some embodiments, the antibody comprises the T437R/K338A mutation.

In some embodiments, the antibody comprises the HC of SEQ ID NO: 65.

In some embodiments, the antibody comprises the T437R/K338A mutation and mediates ADCC.

In some embodiments, the antibody comprises the T437R/K338A mutation and is of IgG1 isotype and mediates ADCC.

In some embodiments, the antibody comprises the T437R/K338A mutation and mediates ADCP.

In some embodiments, the antibody comprises the T437R/K338A mutation and is of IgG1 isotype and mediates ADCP.

In some embodiments, the antibody comprises the T437R/K338A mutation and mediates CDC.

In some embodiments, the antibody comprises the T437R/K338A mutation and is of IgG1 isotype and mediates CDC.

The reported Fc engineering efforts to enhance agonistic activity of the anti-TNFR superfamily member antibodies by introducing a S267E/L328F mutation (Chu et al. (2008) *Mol Immunol* 45: 3926-33) or an E233D/G237D/P238D/H268D/P271G/A330R mutation (Mimoto et al. (2013) *Protein Eng Des Sel* 26: 589-98) resulted in antibodies with abolished ADCC. Contrary to the antibodies described by Chu and Mimoto, the IgG1 antibodies of the present invention comprising the T437R/K338A mutation may be used in instances in which depletion of the TNFR expressing cells is desirable. Exemplary

such instances are depletion of GITR and/or OX-40 expressing Treg cells in the tumor microenvironment to enhance anti-tumor immunity.

In some embodiments, the antibody of the invention comprising the T437R/K338A mutation may further comprise a second mutation which reduces or abolishes antibody Fc mediated effector functions. The antibodies of the present invention comprising the T437R/K338A mutation and a second mutation that reduces or abolishes antibody Fc mediated effector functions may therefore be used in instances in which depletion of the TNFR expressing cells is not desirable. Exemplary such instances are therapeutic treatments with anti-CD40 or anti-CD27 antibodies.

In some embodiments, the antibody comprises the T437R/K338A mutation and is of IgG1 isotype, optionally further comprising the L234A/L235A mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG1 isotype, optionally further comprising the L234F/L235E/D265A mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG1 isotype, optionally further comprising the K214T/E233P/L234V/L235A/G236-deleted/A327G/P331A/D365E/L358M mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG1 isotype, optionally further comprising the L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG1 isotype, and further comprises the L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation when compared to the wild-type IgG1.

In some embodiments, the antibody comprises the HC of SEQ ID NO: 72.

SEQ ID NO: 72: IgG1sigma with T437R/K338A

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPC
PAPEAAGASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVKFNWYVDGVEV

HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPSSIEKTIS**A**A
KGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTT
PPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY**R**QKSLSLSPGK

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG2 isotype, optionally further comprising the V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the antibody comprises the HC of SEQ ID NO: 73.

SEQ ID NO: 73: IgG2sigma with T437R/K338A

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAP
PAAASSVFLFPPKPKDTLMISRTPEVTCVVVDVSAEDPEVQFNWYVDGVEVHNAK
TKPREEQFNSTFRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTIS**A**TKGQPR
EPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLD
SDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY**R**QKSLSLSPGK

In some embodiments, the antibody comprises the T437R/K338A mutation and is of IgG2 isotype, and further comprising the V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG2 isotype, optionally further comprising the V234A/G237A mutation when compared to the wild-type IgG2.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG2 isotype, optionally further comprising the H268Q/V309L/A330S/P331S mutation when compared to the wild-type IgG2.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG3 isotype.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG4 isotype, optionally further comprising the F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG4 isotype, optionally further comprising the S228P/F234A/L235A/G237A/P238S mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG4 isotype, optionally further comprising the S228P/F234A/L235A/G236-deleted/G237A/P238S mutation when compared to the wild-type IgG4

In some embodiments, the antibody comprises the T437R/K338A mutation and is an IgG4 isotype, optionally further comprising the S228P/F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the HC of SEQ ID NO: 74.

SEQ ID NO: 74: IgG4PAA with T437R/K338A

ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAV
LQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAP
EAAGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHN
AKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTIS**A**AKG
QPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP
VLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHY**R**QKSLSLSLGK

In some embodiments, the antibody comprises the T437R/K338A mutation and is of IgG4 isotype, and further comprises the S228P/F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R/K338A mutation and is of IgG4 isotype and comprises the S228P/F234A/L235A mutation when compared to the wild-type IgG4.

In some embodiments, the antibody comprises the T437R/K338A mutation and has agonistic activity independent of antibody cross-linking, wherein agonistic activity is measured by measuring antibody-induced production of secreted embryonic alkaline phosphatase (SEAP) expressed under the control of NFκB-inducible promoter from Hek-293 cells.

In some embodiments, the antibody comprises the T437R/K338A mutation and binds TNFR superfamily member tumor necrosis factor receptor 1 (SEQ ID NO: 1),

Tumor necrosis factor receptor 2 (SEQ ID NO: 2) , lymphotoxin beta receptor (SEQ ID NO: 3), OX40 (SEQ ID NO: 4), CD40 (SEQ ID NO: 5), Fas receptor (SEQ ID NO: 6), decoy receptor 3 (SEQ ID NO: 7), CD27 (SEQ ID NO: 8), CD30 (SEQ ID NO: 9), CD137 (SEQ ID NO: 10), death receptor 4 (SEQ ID NO: 11), death receptor 5 (SEQ ID NO: 12), decoy receptor 1 (SEQ ID NO: 13), decoy receptor 2 (SEQ ID NO: 14), RANK (SEQ ID NO: 15), osteoprotegerin (SEQ ID NO: 16), TWEAK receptor (SEQ ID NO: 17), TACI (SEQ ID NO: 18), BAFF receptor (SEQ ID NO: 19), herpesvirus entry mediator (SEQ ID NO: 20), nerve growth factor receptor (SEQ ID NO: 21), B-cell maturation antigen (SEQ ID NO: 22), GITR (SEQ ID NO: 23), TROY (SEQ ID NO: 24), death receptor 6 (SEQ ID NO: 25), death receptor 3 (SEQ ID NO: 26) or ectodysplasin A2 receptor (SEQ ID NO: 27).

In some embodiments, the antibody comprises the T437R/K338A mutation and binds TNFR superfamily member OX40 (SEQ ID NO: 4), CD27 (SEQ ID NO: 8), CD40 (SEQ ID NO: 5), CD137 (SEQ ID NO: 10), or GITR (SEQ ID NO: 23).

In some embodiments, the antibody comprises the T437R/K338A mutation and binds TNFR superfamily member OX40 (SEQ ID NO: 4).

In some embodiments, the antibody comprises the T437R/K338A mutation and binds TNFR superfamily member CD27 (SEQ ID NO: 8).

In some embodiments, the antibody comprises the T437R/K338A mutation and binds TNFR superfamily member CD40 (SEQ ID NO: 5).

In some embodiments, the antibody comprises the T437R/K338A mutation and binds TNFR superfamily member CD137 (SEQ ID NO: 10).

In some embodiments, the antibody comprises the T437R/K338A mutation and binds TNFR superfamily member GITR (SEQ ID NO: 23).

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a solid tumor.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a melanoma.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a lung cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a squamous non-small cell lung cancer (NSCLC).

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a non-squamous NSCLC.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a lung adenocarcinoma.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a renal cell carcinoma (RCC) (e.g., A kidney clear cell carcinoma or a kidney papillary cell carcinoma), or a metastatic lesion thereof.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a mesothelioma.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a nasopharyngeal carcinoma (NPC).

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a colorectal cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a prostate cancer or castration-resistant prostate cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a stomach cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating an ovarian cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a gastric cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a liver cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating pancreatic cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a thyroid cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a squamous cell carcinoma of the head and neck.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a carcinomas of the esophagus or gastrointestinal tract.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a breast cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a fallopian tube cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a brain cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating an urethral cancer.

In some embodiments of the invention described herein, and in some embodiments of each and every one of the numbered embodiments listed below, the solid tumor is a genitourinary cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating an endometriosis.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a cervical cancer.

The antibody comprising the T437R/K338A mutation is suitable for use in therapy, for example in treating a metastatic lesion of the cancer.

"Antibody-dependent cellular cytotoxicity", "antibody-dependent cell-mediated cytotoxicity" or “ADCC" is a mechanism for inducing cell death that depends upon the interaction of antibody-coated target cells with effector cells possessing lytic activity, such as natural killer cells, monocytes, macrophages and neutrophils via Fc gamma receptors (FcγR) expressed on effector cells. For example, NK cells express FcγRIIIA, whereas monocytes express FcγRI, FcγRII and FcγRIIIA. Death of the antibody-coated target cell, such as TNFR expressing cells, occurs because of effector cell activity through the secretion of membrane pore-forming proteins and proteases. To assess ADCC activity of the antibodies of the invention, the antibodies may be added to cells expressing the target the antibody binds to in combination with immune effector cells, which may be activated by the antigen antibody complexes resulting in cytolysis of the target cell. Cytolysis may

be detected by the release of label (e.g. radioactive substrates, fluorescent dyes or natural intracellular proteins) from the lysed cells. Exemplary effector cells for such assays include peripheral blood mononuclear cells (PBMC) and NK cells. In an exemplary assay, target cells are used with a ratio of 1 target cell to 50 effector cells. Target cells are pre-labeled with BATDA (PerkinElmer) for 20 minutes at 37°C, washed twice and resuspended in DMEM, 10% heat-inactivated FBS, 2mM L-glutamine (all from Invitrogen). Target ($1x10^4$ cells) and effector cells ($0.5x10^6$ cells) are combined and 100 µl of cells are added to the wells of 96-well U-bottom plates. An additional 100 µl is added with or without the test antibodies. The plates are centrifuged at 200g for 3 minutes, incubated at 37°C for 2 hr, and then centrifuged again at 200g for 3 minutes. A total of 20 µl of supernatant is removed per well and cell lysis is measured by the addition of 200 µl of the DELPHIA Europium-based reagent (PerkinElmer). Data is normalized to maximal cytotoxicity with 0.67% (w/v) Triton X-100 (Sigma Aldrich) and minimal control determined by spontaneous release of BATDA from target cells in the absence of any antibody. Alternatively, ADCC activity may be assessed by evaluating activation of FcγRIIIA in a reporter gene assay in which activation of the receptor leads to expression of a luciferase reporter as described herein.

"Antibody-dependent cellular phagocytosis" ("ADCP") refers to a mechanism of elimination of antibody-coated target cells by internalization by phagocytic cells, such as macrophages or dendritic cells. ADCP may be evaluated by using monocyte-derived macrophages as effector cells and Daudi cells (ATCC® CCL-213™) or B cell leukemia or lymphoma or tumor cells expressing the target the antibody binds to as target cells engineered to express GFP or another labeled molecule. Effector to target cell ratio may be for example 4:1. Effector cells may be incubated with target cells for 4 hr with or without the test antibody. After incubation, cells may be detached using accutase. Macrophages may be identified with anti-CD11b and anti-CD14 antibodies coupled to a fluorescent label, and percent phagocytosis may be determined based on % GFP fluorescence in the $CD11^+CD14^+$ macrophages using standard methods.

The effector functions, for example ADCC, ADCP and/or CDC of the antibodies of the invention may further be enhanced by introducing additional mutations into the antibody Fc which enhances binding of the antibody to an activating Fcγ receptor (FcγR) or complement.

Fc positions that may be mutated to increase binding of the antibodies of the invention to the activating FcγR and/or to enhance antibody effector functions are those described for example in U.S. Patent No. 6,737,056, U.S. Patent Publ. No. 2015/0259434, Shields *et al.,* (Shields et al. (2001) *J Biol Chem* 276: 6591-604) (Lazar et al. (2006) *Proc Natl Acad Sci U S A* 103: 4005-10) (Stavenhagen et al. (2007) *Cancer Res* 67: 8882-90) (Richards et al. (2008) *Mol Cancer Ther* 7: 2517-27) (Diebolder et al. (2014) *Science* 343: 1260-3), and include positions 236, 239, 243, 256, 290, 292, 298, 300, 305, 312, 326, 330, 332, 333, 334, 360, 339, 378, 396 or 430 (residue numbering according to the EU index). Exemplary mutations that may be made singularly or in combination are G236A, S239D, F243L, T256A, K290A, R292P, S298A, Y300L, V305L, K326A, A330K, I332E, E333A, K334A, A339T and P396L mutations. Exemplary combination mutations that result in antibodies with increased ADCC or ADCP are S239D/I332E, S298A/E333A/K334A, F243L/R292P/Y300L, F243L/R292P/Y300L/P396L, F243L/R292P/Y300L/V305I/P396L and G236A/S239D/I332E mutations on IgG1.

Fc positions that may be mutated to enhance CDC of the antibodies of the invention are those described for example in Int. Patent Appl. WO2014/108198, (Idusogie et al. (2001) *J Immunol* 166: 2571-5) and (Moore et al. (2010) *MAbs* 2: 181-9), and include positions 267, 268, 324, 326, 333, 345 and 430. Exemplary mutations that may be made singularly or in combination are S267E, H268F, S324T, K326A, K326W, E333A, E430S, E430F and E430T mutations. Exemplary combination mutations that result in antibodies with increased CDC are K326A/E333A, K326W/E333A, H268F/S324T, S267E/H268F, S267E/S324T and S267E/H268F/S324T mutations on IgG1.

"Complement-dependent cytotoxicity", or" CDC", refers to a mechanism for inducing cell death in which the Fc effector domain of a target-bound antibody binds and activates complement component C1q which in turn activates the complement cascade leading to target cell death. Activation of complement may also result in deposition of complement components on the target cell surface that facilitate ADCC by binding complement receptors (e.g., CR3) on leukocytes. CDC of cells may be measured for example by plating Daudi cells at 1×10^5 cells/well (50 μL/well) in RPMI-B (RPMI supplemented with 1% BSA), adding 50 μL of test antibodies to the wells at final concentration between 0-100 μg/ml, incubating the reaction for 15 min at room temperature, adding 11 μl of pooled human serum to the wells, and incubation the reaction

for 45 min at 37° C. Percentage (%) lysed cells may be detected as % propidium iodide stained cells in FACS assay using standard methods.

The ability of the antibodies of the invention to induce ADCC may also be enhanced by engineering their oligosaccharide component. Human IgG1 or IgG3 are N-glycosylated at Asn297 with most the glycans in the well-known biantennary G0, G0F, G1, G1F, G2 or G2F forms. Antibodies produced by non-engineered CHO cells typically have a glycan fucose content of about at least 85%. The removal of the core fucose from the biantennary complex-type oligosaccharides attached to the Fc regions enhances the ADCC of antibodies via improved FcγRIIIa binding without altering antigen binding or CDC activity. Such mAbs may be achieved using different methods reported to lead to the successful expression of relatively high defucosylated antibodies bearing the biantennary complex-type of Fc oligosaccharides such as control of culture osmolality (Konno et al. (2012) *Cytotechnology* 64: 249-65), application of a variant CHO line Lec13 as the host cell line (Shields et al. (2002) *J Biol Chem* 277: 26733-40), application of a variant CHO line EB66 as the host cell line (Olivier et al. (2010) *MAbs* 2: 405-15), application of a rat hybridoma cell line YB2/0 as the host cell line (Shinkawa et al. (2003) *J Biol Chem* 278: 3466-73), introduction of small interfering RNA specifically against the α 1,6-fucosyltrasferase (*FUT8)* gene (Mori et al. (2004) *Biotechnol Bioeng* 88: 901-8), or co-expression of β-1,4-*N*-acetylglucosaminyltransferase III and Golgi α-mannosidase II or a potent alpha-mannosidase I inhibitor, kifunensine (Ferrara et al. (2006) *Biotechnol Bioeng* 93: 851-61; Ferrara et al. (2006) *J Biol Chem* 281: 5032-6).

In some embodiments, the antibody of the invention comprises a second mutation that enhances ADCC, ADCP and/or CDC of the antibody.

In some embodiments, the antibody of the invention comprises a second mutation that enhances ADCC, ADCP and/or CDC of the antibody selected from the group consisting of a G236A mutation, a S239D mutation, a F243L mutation, a T256A mutation, a K290A mutation, a R292P mutation, a S298A mutation, a Y300L mutation, a V305L mutation, a K326A mutation, a A330K mutation, a I332E mutation, an E333A mutation, a K334A mutation, an A339T mutation, a P396L mutation, a S267E mutation, a H268F mutation, a S324T mutation, a K326A mutation, a K326W mutation, an E333A mutation, an E430S mutation, an E430F mutation and an E430T mutation.

In some embodiments, the antibody of the invention comprises a second mutation that enhances ADCC, ADCP and/or CDC of the antibody selected from the group consisting of a S239D/I332E mutation, a S298A/E333A/K334A mutation, a F243L/R292P/Y300L mutation, a F243L/R292P/Y300L/P396L mutation, a F243L/R292P/Y300L/V305I/P396L mutation, a G236A/S239D/I332E mutation, a K326A/E333A mutation, a K326W/E333A mutation, a H268F/S324T mutation, a S267E/H268F mutation, a S267E/S324T mutation and a S267E/H268F/S324T mutation.

In some embodiments, the antibodies of the invention have a biantennary glycan structure with fucose content of about between 0% to about 15%, for example 15%, 14%, 13%, 12%, 11% 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0%.

In some embodiments, the antibodies of the invention have a biantennary glycan structure with fucose content of about 50%, 40%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 14%, 13%, 12%, 11% 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0%.

"Fucose content" means the amount of the fucose monosaccharide within the sugar chain at Asn297. The relative amount of fucose is the percentage of fucose-containing structures related to all glycostructures. These may be characterized and quantified by multiple methods, for example: 1) using MALDI-TOF of N-glycosidase F treated sample (e.g. complex, hybrid and oligo- and high-mannose structures) as described in Intl. Patent Publ. No. WO2008/077546; 2) by enzymatic release of the Asn297 glycans with subsequent derivatization and detection/ quantitation by HPLC (UPLC) with fluorescence detection and/or HPLC-MS (UPLC-MS); 3) intact protein analysis of the native or reduced mAb, with or without treatment of the Asn297 glycans with Endo S or other enzyme that cleaves between the first and the second GlcNAc monosaccharides, leaving the fucose attached to the first GlcNAc; 4) digestion of the mAb to constituent peptides by enzymatic digestion (e.g., trypsin or endopeptidase Lys-C), and subsequent separation, detection and quantitation by HPLC-MS (UPLC-MS) or 5) separation of the mAb oligosaccharides from the mAb protein by specific enzymatic deglycosylation with PNGase F at Asn 297. The oligosaccharides released may be labeled with a fluorophore, separated and identified by various complementary techniques which allow fine characterization of the glycan structures by matrix-assisted laser desorption ionization (MALDI) mass spectrometry by comparison of the experimental masses with the theoretical masses, determination of the degree of sialylation by ion exchange HPLC

(GlycoSep C), separation and quantification of the oligosaccharide forms according to hydrophilicity criteria by normal-phase HPLC (GlycoSep N), and separation and quantification of the oligosaccharides by high performance capillary electrophoresis-laser induced fluorescence (HPCE-LIF).

"Low fucose" or "low fucose content" refers to antibodies with fucose content of about 0% - 15%.

"Normal fucose" or 'normal fucose content" refers to antibodies with fucose content of about over 50%, typically about over 60%, 70%, 80% or over 85%.

In instances where effector functionality is not desired, the antibodies of the invention may further be engineered to introduce at least one mutation in the antibody Fc that reduces binding of the antibody to an activating Fcγ receptor (FcγR) and/or reduces Fc effector functions such as C1q binding, complement dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) or phagocytosis (ADCP).

Fc positions that may be mutated to reduce binding of the antibody to the activating FcγR and subsequently to reduce effector functions are those described for example in (Shields et al. (2001) *J Biol Chem* 276: 6591-604), Intl. Patent Publ. No. WO2011/066501, U.S. Patent Nos. 6,737,056 and 5,624,821, (Xu et al. (2000) *Cell Immunol* 200: 16-26), (Alegre et al. (1994) *Transplantation* 57: 1537-43)A, (Bolt et al. (1993) *Eur J Immunol* 23: 403-11), (Cole et al. (1999) *Transplantation* 68: 563-71), (Rother et al. (2007) *Nat Biotechnol* 25: 1256-64), (Ghevaert et al. (2008) *J Clin Invest* 118: 2929-38), (An et al. (2009) *MAbs* 1: 572-9) and include positions 214, 233, 234, 235, 236, 237, 238, 265, 267, 268, 270, 295, 297, 309, 327, 328, 329, 330, 331 and 365. Exemplary mutations that may be made singularly or in combination are K214T, E233P, L234V, L234A, deletion of G236, V234A, F234A, L235A, G237A, P238A, P238S, D265A, S267E, H268A, H268Q, Q268A, N297A, A327Q, P329A, D270A, Q295A, V309L, A327S, L328F, A330S and P331S mutations on IgG1, IgG2, IgG3 or IgG4. Exemplary combination mutations that may be made to reduced ADCC are L234A/L235A on IgG1, V234A,/G237A/P238S/H268A/V309L/A330S/P331S on IgG2, F234A/L235A on IgG4, S228P/F234A/L235A on IgG4, N297A on IgG1, IgG2, IgG3 or IgG4, V234A/G237A on IgG2, K214T/E233P/L234V/L235A/G236-deleted/A327G/P331A/D365E/L358M on IgG1, H268Q/V309L/A330S/P331S on IgG2, S267E/L328F on IgG1, L234F/L235E/D265A on IgG1,

L234A/L235A/G237A/P238S/H268A/A330S/P331S on IgG1, S228P/F234A/L235A/G237A/P238S on IgG4, and S228P/F234A/L235A/G236-deleted/G237A/P238S on IgG4. Hybrid IgG2/4 Fc domains may also be used, such as Fc with residues 117-260 from IgG2 and residues 261-447 from IgG4.

A S228P mutation may be made into IgG4 antibodies to enhance IgG4 stability.

In some embodiments, the antibodies of the invention comprise a second mutation selected from the group consisting of a K214T mutation, a E233P mutation, a L234V mutation, a L234A mutation, deletion of a G236, a V234A mutation, a F234A mutation, a L235A mutation, a G237A mutation, a P238A mutation, a P238S mutation, a D265A mutation, a S267E mutation, a H268A mutation, a H268Q mutation, a Q268A mutation, a N297A mutation, a A327Q mutation, a P329A mutation, a D270A mutation, a Q295A mutation, a V309L mutation, a A327S mutation, a L328F mutation, a A330S mutation and a P331S mutation, wherein residue numbering is according to the EU Index.

The antibodies of the invention may be further engineered to further modulate antibody half-life by introducing additional Fc mutations, such as those described for example in (Dall'Acqua et al. (2006) *J Biol Chem* 281: 23514-24), (Zalevsky et al. (2010) *Nat Biotechnol* 28: 157-9), (Hinton et al. (2004) *J Biol Chem* 279: 6213-6), (Hinton et al. (2006) *J Immunol* 176: 346-56), (Shields et al. (2001) *J Biol Chem* 276: 6591-604), (Petkova et al. (2006) *Int Immunol* 18: 1759-69), (Datta-Mannan et al. (2007) *Drug Metab Dispos* 35: 86-94), (Vaccaro et al. (2005) *Nat Biotechnol* 23: 1283-8), (Yeung et al. (2010) *Cancer Res* 70: 3269-77) and (Kim et al. (1999) *Eur J Immunol* 29: 2819-25), and include positions 250, 252, 253, 254, 256, 257, 307, 376, 380, 428, 434 and 435. Exemplary mutations that may be made singularly or in combination are T250Q, M252Y, I253A, S254T, T256E, P257I, T307A, D376V, E380A, M428L, H433K, N434S, N434A, N434H, N434F, H435A and H435R mutations. Exemplary singular or combination mutations that may be made to increase the half-life of the antibody are M428L/N434S, M252Y/S254T/T256E, T250Q/M428L, N434A and T307A/E380A/N434A mutations. Exemplary singular or combination mutations that may be made to shorten the half-life of the antibody are H435A, P257I/N434H, D376V/N434H, M252Y/S254T/T256E/H433K/N434F, T308P/N434A and H435R mutations.

Antibodies of the invention further comprising conservative modifications are within the scope of the invention.

"Conservative modifications" refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody containing the conservative modifications. Conservative modifications include amino acid substitutions, additions and deletions. Conservative substitutions are those in which the amino acid is replaced with an amino acid residue having a similar side chain. The families of amino acid residues having similar side chains are well defined and include amino acids with acidic side chains (e.g., aspartic acid, glutamic acid), basic side chains (e.g., lysine, arginine, histidine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), uncharged polar side chains (e.g., glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine, tryptophan), aromatic side chains (e.g., phenylalanine, tryptophan, histidine, tyrosine), aliphatic side chains (e.g., glycine, alanine, valine, leucine, isoleucine, serine, threonine), amide (e.g., asparagine, glutamine), beta-branched side chains (e.g., threonine, valine, isoleucine) and sulfur-containing side chains (cysteine, methionine). Furthermore, any native residue in the polypeptide may also be substituted with alanine, as has been previously described for alanine scanning mutagenesis. Amino acid substitutions to the antibodies of the invention may be made by known methods for example by PCR mutagenesis. Alternatively, libraries of variants may be generated for example using random (NNK) or non-random codons, for example DVK codons, which encode 11 amino acids (Ala, Cys, Asp, Glu, Gly, Lys, Asn, Arg, Ser, Tyr, Trp). The resulting antibody variants may be tested for their characteristics using assays described herein.

The antibodies of the invention may be post-translationally modified by processes such as glycosylation, isomerization, deglycosylation or non-naturally occurring covalent modification such as the addition of polyethylene glycol moieties (pegylation) and lipidation. Such modifications may occur *in vivo* or *in vitro*. For example, the antibodies of the invention may be conjugated to polyethylene glycol (PEGylated) to improve their pharmacokinetic profiles. Conjugation may be carried out by techniques known to those skilled in the art. Conjugation of therapeutic antibodies with PEG has been shown to enhance pharmacodynamics while not interfering with function.

Antibodies of the invention may be modified to improve stability, selectivity, cross-reactivity, affinity, immunogenicity or other desirable biological or biophysical property are within the scope of the invention. Stability of an antibody is influenced by a

number of factors, including (1) core packing of individual domains that affects their intrinsic stability, (2) protein/protein interface interactions that have impact upon the HC and LC pairing, (3) burial of polar and charged residues, (4) H-bonding network for polar and charged residues; and (5) surface charge and polar residue distribution among other intra- and inter-molecular forces. Potential structure destabilizing residues may be identified based upon the crystal structure of the antibody or by molecular modeling in certain cases, and the effect of the residues on antibody stability may be tested by generating and evaluating variants harboring mutations in the identified residues. One of the ways to increase antibody stability is to raise the thermal transition midpoint (T_m) as measured by differential scanning calorimetry (DSC). In general, the protein T_m is correlated with its stability and inversely correlated with its susceptibility to unfolding and denaturation in solution and the degradation processes that depend on the tendency of the protein to unfold. A number of studies have found correlation between the ranking of the physical stability of formulations measured as thermal stability by DSC and physical stability measured by other methods (Maa et al. (1996) *Int. J. Pharm.* 140: 155-68; Remmele et al. (1997) *Pharm. Res.* 15: 200-8; Gupta et al. (2003) *AAPS PharmSci.* 5E8: 2003; Bedu-Addo et al. (2004) *Pharm. Res.* 21: 1353-61; Zhang et al. (2004) *J. Pharm. Sci.* 93: 3076-89). Formulation studies suggest that a Fab T_m has implication for long-term physical stability of a corresponding mAb.

C-terminal lysine (CTL) may be removed from injected antibodies by endogenous circulating carboxypeptidases in the blood stream (Cai et al. (2011) *Biotechnol Bioeng* 108: 404-12). During manufacturing, CTL removal may be controlled to less than the maximum level by control of concentration of extracellular Zn^{2+}, EDTA or EDTA – Fe^{3+} as described in U.S. Patent Publ. No. US20140273092. CTL content in antibodies can be measured using known methods.

In some embodiments, the antibodies of the invention have a C-terminal lysine content of about 10% to about 90%, about 20% to about 80%, about 40% to about 70%, about 55% to about 70%, or about 60%.

In some embodiments, the antibodies of the invention have a C-terminal lysine content of about 0%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%.

Methods of generating antibodies of the invention

The antibodies of the invention with engineered Fc domains may be generated using standard cloning and expression technologies using wild type IgG1, IgG2, IgG3 or IgG4 sequences as templates. For example, site-directed mutagenesis or PCR-mediated mutagenesis may be performed to introduce the mutation(s) in the antibody Fc and the effect on antibody binding to FcγR, agonistic activity or other property of interest, may be evaluated using the methods described herein.

The VH and the VL domains of the anti-TNFR superfamily member antibodies may be generated *de novo*.

For example, the hybridoma method of (Kohler et al. (1975) *Nature* 256: 495-7) may be used to generate monoclonal antibodies. In the hybridoma method, a mouse or other host animal, such as a hamster, rat or monkey, is immunized with human TNFR or an extracellular domain of a TNFR followed by fusion of spleen cells from immunized animals with myeloma cells using standard methods to form hybridoma cells (Goding, Monoclonal Antibodies: Principles and Practice, pp.59-103 (Academic Press, 1986)). Colonies arising from single immortalized hybridoma cells are screened for production of antibodies with desired properties, such as specificity of binding, cross-reactivity or lack thereof, and affinity for the antigen.

Various host animals may be used to produce the anti-TNFR superfamily member antibodies of the invention. For example, Balb/c mice may be used to generate mouse anti-human TNFR superfamily member antibodies. The antibodies made in Balb/c mice and other non-human animals may be humanized using various technologies to generate more human-like sequences.

Exemplary humanization techniques including selection of human acceptor frameworks are known and include CDR grafting (U.S. Patent No. 5,225,539), SDR grafting (U.S. Patent No. 6,818,749), Resurfacing (Padlan (1991) *Mol Immunol* 28: 489-98), Specificity Determining Residues Resurfacing (U.S. Patent Publ. No. 2010/0261620), human framework adaptation (U.S. Patent No. 8,748,356) or superhumanization (U.S. Patent No. 7,709, 226). In these methods, CDRs of parental antibodies are transferred onto human frameworks that may be selected based on their overall homology to the parental frameworks, based on similarity in CDR length, or canonical structure identity, or a combination thereof.

Humanized antibodies may be further optimized to improve their selectivity or affinity to a desired antigen by incorporating altered framework support residues to preserve binding affinity (back mutations) by techniques such as those described in Int. Patent Publ. Nos. WO1090/007861 and WO1992/22653, or by introducing variation at any of the CDRs for example to improve affinity of the antibody.

Transgenic animals, such as mice or rat carrying human immunoglobulin (Ig) loci in their genome may be used to generate human antibodies against a target protein, and are described in for example U.S. Patent No. 6,150,584, Int. Patent Publ. No. WO99/45962, Int. Patent Publ. Nos. WO2002/066630, WO2002/43478, WO2002/043478 and WO1990/04036, (Lonberg et al. (1994) *Nature* 368: 856-9); (Green et al. (1994) *Nat Genet* 7: 13-21); (Lonberg et al. (1995) *Int Rev Immunol* 13: 65-93); (Bruggemann et al. (1991) *Eur J Immunol* 21: 1323-6). The endogenous immunoglobulin loci in such animal may be disrupted or deleted, and at least one complete or partial human immunoglobulin locus may be inserted into the genome of the animal using homologous or non-homologous recombination, using transchromosomes, or using minigenes. Companies such as Regeneron (http://_www_regeneron_com), Harbour Antibodies (http://_www_harbourantibodies_com), Open Monoclonal Technology, Inc. (OMT) (http://_www_omtinc_net), KyMab (http://_www_kymab_com), Trianni (http://_www.trianni_com) and Ablexis (http://_www_ablexis_com) may be engaged to provide human antibodies directed against a selected antigen using technologies as described above.

Human antibodies may be selected from a phage display library, where the phage is engineered to express human immunoglobulins or portions thereof such as Fabs, single chain antibodies (scFv), or unpaired or paired antibody variable regions. The antibodies of the invention may be isolated for example from phage display library expressing antibody heavy and light chain variable regions as fusion proteins with bacteriophage pIX coat protein as described in (Shi et al. (2010) *J Mol Biol* 397: 385-96), and Int. Patent Publ. No. WO09/085462). The libraries may be screened for phage binding to human and/or cyno TNFR and the obtained positive clones may be further characterized, the Fabs isolated from the clone lysates, and expressed as full length IgGs. Such phage display methods for isolating human antibodies are described in for example: U.S. Patent Nos.

5,223,409, 5,403,484, 5,571,698, 5,427,908, 5, 580,717, 5,969,108, 6,172,197, 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081.

Preparation of immunogenic antigens and monoclonal antibody production may be performed using any suitable technique, such as recombinant protein production. The immunogenic antigens may be administered to an animal in the form of purified protein, or protein mixtures including whole cells or cell or tissue extracts, or the antigen may be formed *de novo* in the animal's body from nucleic acids encoding said antigen or a portion thereof.

The VH/VL regions of the anti-TNFR superfamily member antibodies of the invention may also be obtained from existing anti-TNFR superfamily receptor antibodies.

The VH and the VL regions of anti-OX40 antibodies described in U.S. Patent No. US8133983, U.S. Patent No. US7960515, U.S. Patent Publ. No. US2013/0280275, Intl. Patent Publ. No. WO2013/028231 and U.S. Patent Publ. No. US2014/0377284 may be used to engineer antibodies of the invention. Further, the VH/VL regions of anti-OX40 antibodies MEDI-6469, BMS-986178, MOXR-0916, MEDI-6383, MEDI-0562, PF-04518600 or GSK-3174998 may be used. Exemplary VH and VL regions that may be used to generate engineered anti-OX40 antibodies of the invention are:

SEQ ID NO: 51 (VH of antibody SF2 described in US2014/0377284)
QVQLVQSGAEVKKPGSSVKVSCKASGYTFKDYTMHWVRQAPGQGLEWIGGIYP
NNGGSTYNQNFKDRVTLTADKSTSTAYMELSSLRSEDTAVYYCARMGYHGPHLD
FDVWGQGTTVTVSS

SEQ ID NO: 52 (VL of antibody SF2 described in US2014/0377284)
DIQMTQSPSSLSASVGDRVTITCKASQDVGAAVAWYQQKPGKAPKLLIYWASTR
HTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYINYPLTFGGGTKVEIK

SEQ ID NO: 53 (VH of 12H3VH1VL1 described in US2014/0377284)
QVQLVQSGAEVKKPGSSVKVSCKASGYTFKDYTMHWVRQAPGQGLEWMGGIYP
NNGGSTYNQNFKDRVTITADKSTSTAYMELSSLRSEDTAVYYCARMGYHGPHLD
FDVWGQGTTVTVSS

SEQ ID NO: 54 (VL of 12H3VH1VL1 described in US2014/0377284)
DIQMTQSPSSLSASVGDRVTITCKASQDVGAAVAWYQQKPGKAPKLLIYWASTR
HTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQYINYPLTFGGGTKVEIK

SEQ ID NO: 55 (VH of 20E5VH3VL2 described in US2014/0377284)
QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYVMHWVRQAPGQRLEWIGYINP
YNDGTKYNEKFKGRATLTSDKSASTAYMELSSLRSEDTAVYYCANYYGSSLSMD
YWGQGTLVTVSS

SEQ ID NO: 56 (VL of 20E5VH3VL2 described in US2014/0377284)
DIQMTQSPSSLSASVGDRVTITCRASQDISNYLNWYQQKPGKAVKLLIYYTSRLHS
GVPSRFSGSGSGTDYTLTISSLQPEDFATYFCQQGNTLPWTFGQGTKVEIK

The VH and the VL regions of anti-CD40 antibodies that may be used to engineer antibodies of the invention are those of CP-870,893 and humanized S2C6 described in U.S. Patent No. 7,288,251 (antibody 21.4.1) and U.S. Patent No. 8,303,955, respectively, and anti-CD40 antibodies described in Int. Patent Publ. Nos. WO2001/056603, WO2001/083755, WO2013/034904 and WO2014/070934. Exemplary VH and VL regions that may be used to generate engineered anti-CD40 antibodies of the invention are:

SEQ ID NO: 57 (VH of M9 antibody)
QLQLQESGPGLVKPSEILSLTCTVSGGSISSSSYYWGWIRQPPGKGLEWIGNIYYRG
DTYYSPSLKSRVTISVDTSKNQFSLKLNSVTAADTAVYYCAKGFRFDYWGQGTLV
TVSS

SEQ ID NO: 58 (VL of M9 antibody)
QSALTQPPSASGSPGQSVTISCTGTSSDVGGYNYVSWYQQHPGKAPKLMIYEVSK
RPSGVPDRFSGSKSGNTASLTVSGLQAEDEADYYCSSYAGSNNLVFGGGTKLTVL

The VH and the VL regions of anti-GITR antibodies that may be used to engineer antibodies of the invention are those of described in U.S. Patent Nos. 7,812,135, 8,591,886

and 7,618,632, or in Int. Patent Publ. Nos. WO2011/028683, WO2013/039954, WO2005/007190 and WO2007/133822.

The VH and the VL regions of anti-CD27 antibodies that may be used to engineer antibodies of the invention are those of described in U.S. Patent No. US9169325 and U.S. Pat. Publ. No. US20130183316.

The VH and the VL regions of anti-CD137 antibodies that may be used to engineer antibodies of the invention are those of described in U.S. Patent Nos. US7288638, US8716452 and US8821867.

Antibodies of the invention engineered into full length bispecific antibodies are within the scope of the invention.

"Full length antibody" refers to an antibody having two full length antibody heavy chains and two full length antibody light chains. A full-length antibody heavy chain (HC) consists of well-known heavy chain variable and constant domains VH, CH1, hinge, CH2, and CH3. A full-length antibody light chain (LC) consists of well-known light chain variable and constant domains VL and CL. The full-length antibody may be lacking the C-terminal lysine (K) in either one or both heavy chains.

Full length bispecific antibodies may be generated for example using Fab arm exchange (or half molecule exchange) between two monospecific bivalent antibodies by introducing substitutions at the heavy chain CH3 interface in each half molecule to favor heterodimer formation of two antibody half molecules having distinct specificity either *in vitro* in cell-free environment or using co-expression. "Fab-arm" or "half molecule" refers to one heavy chain-light chain pair that specifically binds an antigen.

The Fab arm exchange reaction is the result of a disulfide-bond isomerization reaction and dissociation-association of CH3 domains. The heavy chain disulfide bonds in the hinge regions of the parental monospecific antibodies are reduced. The resulting free cysteines of one of the parental monospecific antibodies form an inter heavy-chain disulfide bond with cysteine residues of a second parental monospecific antibody molecule and simultaneously CH3 domains of the parental antibodies release and reform by dissociation-association. The CH3 domains of the Fab arms may be engineered to favor heterodimerization over homodimerization. The resulting product is a bispecific antibody having two Fab arms or half molecules which each bind a distinct epitope, i.e. an epitope on TNFR and an epitope on a second antigen.

"Homodimerization" refers to an interaction of two heavy chains having identical CH3 amino acid sequences. "Homodimer" refers to an antibody having two heavy chains with identical CH3 amino acid sequences.

"Heterodimerization" refers to an interaction of two heavy chains having non-identical CH3 amino acid sequences. "Heterodimer" refers to an antibody having two heavy chains with non-identical CH3 amino acid sequences.

The anti-TNFR superfamily member antibodies of the invention may be engineered into bispecific format using Knob-in-Hole (Genentech), CrossMAbs (Roche) and the electrostatically-matched (Chugai, Amgen, NovoNordisk, Oncomed), the LUZ-Y (Genentech), the Strand Exchange Engineered Domain body (SEEDbody) (EMD Serono), the Biclonic (Merus).

In the Knob-in-Hole strategy (see, e.g., Intl. Publ. No. WO 2006/028936), selected amino acids forming the interface of the CH3 domains in human IgG can be mutated at positions affecting CH3 domain interactions to promote heterodimer formation. An amino acid with a small side chain (hole) is introduced into a heavy chain of an antibody specifically binding a first antigen and an amino acid with a large side chain (knob) is introduced into a heavy chain of an antibody specifically binding a second antigen. After co-expression of the two antibodies, a heterodimer is formed because of the preferential interaction of the heavy chain with a "hole" with the heavy chain with a "knob". Exemplary CH3 substitution pairs forming a knob and a hole are expressed as modified position in the first CH3 domain of the first heavy chain/ modified position in the second CH3 domain of the second heavy chain: T366Y/F405A, T366W/F405W, F405W/Y407A, T394W/Y407T, T394S/Y407A, T366W/T394S, F405W/T394S and T366W/T366S_L368A_Y407V.

In the CrossMAb technology, in addition to utilizing the "knob-in-hole" strategy to promoter Fab arm exchange, one of the half arms have the CH1 and the CL domains exchanged to ensure correct light chain pairing of the resulting bispecific antibody (see e.g. U.S. Patent No. 8,242,247).

Other cross-over strategies may be used to generate full length bispecific antibodies by exchanging variable or constant, or both domains between the heavy chain and the light chain or within the heavy chain in the bispecific antibodies, either in one or both arms. These exchanges include for example VH-CH1 with VL-CL, VH with VL,

CH3 with CL and CH3 with CH1 as described in Int. Patent Publ. Nos. WO2009/080254, WO2009/080251, WO2009/018386 and WO2009/080252.

Other strategies such as promoting heavy chain heterodimerization using electrostatic interactions by substituting positively charged residues at one CH3 surface and negatively charged residues at a second CH3 surface may be used to generate bispecific antibodies, as described in US Patent Publ. No. US2010/0015133; US Patent Publ. No. US2009/0182127; US Patent Publ. No. US2010/028637 or US Patent Publ. No. US2011/0123532. In other strategies, heterodimerization may be promoted by following substitutions expressed as modified position in the first CH3 domain of the first heavy chain/ modified position in the second CH3 domain of the second heavy chain: L351Y_F405A_Y407V/T394W, T366I_K392M_T394W/F405A_Y407V, T366L_K392M_T394W/F405A_Y407V, L351Y_Y407A/T366A_K409F, L351Y_Y407A/T366V_K409F, Y407A/T366A_K409F, or T350V_L351Y_F405A_Y407V/T350V_T366L_K392L_T394W as described in U.S. Patent Publ. No. US2012/0149876 or U.S. Patent Publ. No. US2013/0195849.

LUZ-Y technology may be utilized to generate bispecific antibodies. In this technology, a leucine zipper is added into the C terminus of the CH3 domains to drive the heterodimer assembly from parental mAbs that is removed post-purification as described in (Wranik et al. (2012) *J Biol Chem* 287: 43331-9).

SEEDbody technology may be utilized to generate bispecific antibodies. SEEDbodies have, in their constant domains, select IgG residues substituted with IgA residues to promote heterodimerization as described in U.S. Patent No. US20070287170.

Bispecific antibodies may be generated *in vitro* in a cell-free environment by introducing asymmetrical mutations in the CH3 regions of two monospecific homodimeric antibodies and forming the bispecific heterodimeric antibody from two parent monospecific homodimeric antibodies in reducing conditions to allow disulfide bond isomerization according to methods described in Int.Patent Publ. No. WO2011/131746 (DuoBody technology). In the methods, the first monospecific bivalent antibody and the second monospecific bivalent antibody are engineered to have certain substitutions at the CH3 domain that promoter heterodimer stability; the antibodies are incubated together under reducing conditions sufficient to allow the cysteines in the hinge region to undergo disulfide bond isomerization; thereby generating the bispecific antibody by Fab arm

exchange. The incubation conditions may optimally be restored to non-reducing. Exemplary reducing agents that may be used are 2- mercaptoethylamine (2-MEA), dithiothreitol (DTT), dithioerythritol (DTE), glutathione, tris(2-carboxyethyl) phosphine (TCEP), L-cysteine and beta-mercaptoethanol. For example, incubation for at least 90 min at a temperature of at least 20°C in the presence of at least 25 mM 2-MEA or in the presence of at least 0.5 mM dithiothreitol at a pH of from 5-8, for example at pH of 7.0 or at pH of 7.4 may be used.

Antibody domains and numbering are well known. "Asymmetrical" refers to non-identical substitutions in the two CH3 domains in two separate heavy chains in an antibody. An IgG1 CH3 region typically consists of residues 341-446 on IgG1 (residue numbering according to the EU index).

The antibodies of the invention may be engineered into various well known antibody forms.

Fc domain containing molecules

The invention also provides for an isolated Fc domain containing molecule comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation in the Fc domain.

In some embodiments, the Fc domain containing molecule comprises the T437R mutation.

In some embodiments, the Fc domain containing molecule comprises the T437R/K248E mutation.

In some embodiments, the Fc domain containing molecule comprises the T437R/K338A mutation.

In some embodiments, the Fc domain is an IgG1, IgG2, IgG3 or IgG4 isotype.

In some embodiments, the Fc domain is an IgG1 isotype.

In some embodiments, the Fc domain is an IgG2 isotype.

In some embodiments, the Fc domain is an IgG3 isotype.

In some embodiments, the Fc domain is an IgG4 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R mutation and is an IgG1 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R mutation and is an IgG2 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R mutation and is an IgG3 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R mutation and is an IgG4 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R/K248E mutation and is an IgG1 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R/K248E mutation and is an IgG2 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R/K248E mutation and is an IgG3 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R/K248E mutation and is an IgG4 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R/K338A mutation and is an IgG1 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R/K338A mutation and is an IgG2 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R/K338A mutation and is an IgG3 isotype.

In some embodiments, the Fc domain containing molecule comprises the T437R/K338A mutation and is an IgG4 isotype.

The constant region sequences of the mammalian IgG heavy chain are designated in sequence as CH1-hinge-CH2-CH3. The “hinge”, "hinge region" or "hinge domain" of an IgG is generally defined as including Glu216 and terminating at Pro230 of human IgG1 according to the EU Index but functionally, the flexible portion of the chain may be considered to include additional residues termed the upper and lower hinge regions, such as from Glu216 to Gly237 and the lower hinge has been referred to as residues 233 to 239 of the Fc region where FcgammaR binding was generally attributed. Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S-S bonds. Although boundaries may vary slightly, as numbered according to the EU Index, the CH1 domain is adjacent to the VH

domain and amino terminal to the hinge region of an immunoglobulin heavy chain molecule and includes the first (most amino terminal) constant region of an immunoglobulin heavy chain, e.g., from about EU positions 118-215. The Fc domain extends from amino acid 231 to amino acid 447; the CH2 domain is from about Ala231 to Lys340 or Gly341 and the CH3 from about Gly341 or Gln342 to Lys447. The residues of the IgG heavy chain constant region of the CH1 region terminate at Lys. The Fc domain containing molecule comprises at least the CH2 and the CH3 domains of an antibody constant region, and therefore comprises at least a region from about Ala231 to Lys447 of IgG heavy chain constant region. The Fc domain containing molecule may optionally comprise at least portion of the hinge region.

Exemplary Fc domain containing molecules are heterologous fusion proteins comprising at least the CH2 and the CH3 domains of an antibody constant region, coupled to a heterologous protein or portion of a protein, such as a peptide, a cytokine, a chemokine, or an extracellular domain of a membrane protein, such as an extracellular domain of a TNFR ligand, such as those listed in **Table 1**.

The Fc domain containing molecules of the invention may be made by standard molecular biology techniques.

The invention also provides for an isolated polynucleotide encoding the Fc domain containing molecule of the invention.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 75.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 76.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 77.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 78.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 79.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 80.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 81.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 82.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 83.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 84.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 85.

The invention also provides an isolated polynucleotide comprising the polynucleotide sequence of SEQ ID NO: 86.

SEQ ID NO: 75 cDNA encoding IgG1 T437R

GCCAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCAC
CTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAAC
CGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTC
CCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGT
GCCCAGCTCCTCCCTGGGAACCCAGACCTATATCTGCAACGTGAACCACAAGC
CCTCCAATACCAAGGTGGACAAGAAGGTGGAGCCCAAATCCTGCGACAAGAC
CCACACCTGCCCCCCTTGTCCTGCCCCTGAACTGCTGGGAGGACCCTCCGTGTT
CCTGTTCCCCCCCAAGCCCAAGGACACCCTGATGATCAGCAGGACCCCCGAAG
TGACCTGTGTGGTGGTGGATGTGAGCCACGAGGACCCCGAGGTGAAGTTCAAC
TGGTACGTGGACGGCGTGGAGGTGCATAACGCCAAGACCAAGCCCAGGGAGG
AGCAGTACAACAGCACCTACAGGGTGGTGTCCGTGCTGACCGTGCTCCATCAG
GACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGAGCAACAAGGCCCTGC
CCGCCCCCATCGAGAAGACAATCTCCAAAGCCAAGGGCCAGCCCAGGGAGCC
TCAGGTCTACACCCTGCCCCCCTCCAGAGAGGAGATGACCAAGAACCAGGTG
AGCCTGACCTGCCTGGTGAAGGGCTTCTACCCTAGCGACATCGCCGTGGAGTG
GGAGAGCAACGGCCAGCCCGAGAACAACTACAAGACAACCCCCCCTGTGCTG
GACTCCGACGGCTCCTTCTTCCTGTATTCCAAGCTCACAGTGGACAAGAGCAG

ATGGCAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCAC
AACCACTATAGGCAGAAAAGCCTGTCCCTGAGCCCCGGAAAG

SEQ ID NO: 76 cDNA encoding IgG1 T437R/K248E
GCCAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCAC
CTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAAC
CGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTC
CCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGT
GCCCAGCTCCTCCCTGGGAACCCAGACCTATATCTGCAACGTGAACCACAAGC
CCTCCAATACCAAGGTGGACAAGAAGGTGGAGCCCAAATCCTGCGACAAGAC
CCACACCTGCCCCCCTTGTCCTGCCCCTGAACTGCTGGGAGGACCCTCCGTGTT
CCTGTTCCCCCCCAAGCCCGAGGACACCCTGATGATCAGCAGGACCCCCGAAG
TGACCTGTGTGGTGGTGGATGTGAGCCACGAGGACCCCGAGGTGAAGTTCAAC
TGGTACGTGGACGGCGTGGAGGTGCATAACGCCAAGACCAAGCCCAGGGAGG
AGCAGTACAACAGCACCTACAGGGTGGTGTCCGTGCTGACCGTGCTCCATCAG
GACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGAGCAACAAGGCCCTGC
CCGCCCCCATCGAGAAGACAATCTCCAAAGCCAAGGGCCAGCCCAGGGAGCC
TCAGGTCTACACCCTGCCCCCCTCCAGAGAGGAGATGACCAAGAACCAGGTG
AGCCTGACCTGCCTGGTGAAGGGCTTCTACCCTAGCGACATCGCCGTGGAGTG
GGAGAGCAACGGCCAGCCCGAGAACAACTACAAGACAACCCCCCCTGTGCTG
GACTCCGACGGCTCCTTCTTCCTGTATTCCAAGCTCACAGTGGACAAGAGCAG
ATGGCAGCAGGGCAACGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCAC
AACCACTATAGGCAGAAAAGCCTGTCCCTGAGCCCCGGAAAG

SEQ ID NO: 77 cDNA encoding IgG1 T437R/K338A
GCCAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCTCCTCCAAGAGCAC
CTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTACTTCCCCGAAC
CGGTGACGGTGTCGTGGAACTCAGGCGCCCTGACCAGCGGCGTGCACACCTTC
CCGGCTGTCCTACAGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGT
GCCCAGCTCCAGCCTGGGCACCCAGACCTACATCTGCAACGTGAACCACAAGC
CCAGCAACACCAAGGTGGATAAGAAAGTGGAGCCCAAGTCCTGCGATAAGAC
ACACACATGCCCCCCCTGTCCTGCCCCTGAACTGCTGGGAGGCCCTTCCGTCTT

TCTGTTCCCCCCCAAGCCCAAGGATACCCTGATGATCTCCAGGACCCCTGAAG
TGACCTGCGTCGTGGTGGACGTGAGCCACGAGGACCCCGAGGTGAAGTTCAA
CTGGTACGTCGATGGCGTGGAGGTGCACAACGCCAAGACCAAGCCTAGGGAG
GAGCAGTATAACAGCACCTACAGGGTGGTCTCCGTGCTGACAGTGCTGCACCA
GGACTGGCTGAACGGCAAGGAGTACAAGTGCAAGGTGAGCAATAAGGCCCTG
CCCGCTCCCATCGAGAAGACCATTAGCGCTGCCAAGGGACAGCCCAGGGAAC
CCCAGGTGTACACCCTGCCCCCCTCCAGGGAGGAGATGACCAAGAATCAGGT
GAGCCTGACCTGTCTGGTGAAAGGCTTCTACCCCAGCGACATCGCCGTGGAGT
GGGAGTCCAACGGCCAGCCTGAGAACAACTACAAGACCACCCCCCCTGTGCT
GGATTCCGACGGCAGCTTCTTCCTGTACAGCAAGCTGACCGTGGATAAGAGCA
GGTGGCAGCAGGGCAACGTGTTCTCCTGCTCCGTCATGCACGAGGCCCTCCAC
AACCACTACAGGCAGAAGAGCCTGAGCCTGAGCCCCGGCAAG

SEQ ID NO: 78 cDNA encoding IgG1sigma T437R
GCCAGCACCAAGGGCCCAAGCGTGTTTCCCCTGGCCCCTAGCAGCAAGAGCAC
CTCCGGCGGAACAGCTGCTCTGGGCTGCCTGGTGAAAGATTACTTCCCCGAAC
CCGTGACCGTGTCCTGGAACAGCGGAGCCCTGACCAGCGGCGTGCATACCTTC
CCTGCTGTGCTGCAGAGCAGCGGACTGTACAGCCTGTCCAGCGTGGTGACCGT
GCCCAGCAGCTCCCTGGGAACCCAGACCTACATCTGCAACGTGAATCACAAGC
CCAGCAACACCAAGGTGGACAAGAAGGTGGAACCCAAGAGCTGCGATAAGAC
ACACACCTGCCCCCCCTGTCCTGCTCCTGAAGCTGCCGGCGCTAGCAGCGTGT
TTCTGTTCCCCCCTAAGCCCAAGGACACACTGATGATCAGCAGAACCCCCGAG
GTGACATGTGTGGTGGTGGACGTGTCCGCTGAGGACCCCGAGGTCAAGTTTAA
CTGGTACGTCGATGGCGTGGAGGTGCATAACGCCAAAACCAAGCCTAGGGAG
GAGCAGTACAACAGCACCTACAGAGTGGTCTCCGTCCTCACCGTGCTCCATCA
GGACTGGCTGAACGGCAAGGAGTATAAGTGCAAAGTGAGCAACAAGGCCCTG
CCCAGCTCCATCGAGAAGACCATTTCCAAGGCCAAGGGCCAGCCTAGGGAGC
CTCAGGTGTATACCCTGCCTCCCAGCAGAGAGGAGATGACCAAGAACCAGGT
GAGCCTCACCTGCCTGGTCAAGGGATTCTACCCCTCCGACATCGCCGTGGAAT
GGGAAAGCAACGGCCAGCCCGAGAATAACTACAAGACCACCCCTCCTGTGCT
GGATTCCGACGGCTCCTTCTTTCTGTACAGCAAGCTGACCGTGGACAAGAGCA

GGTGGCAGCAGGGCAATGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCA
CAACCACTACAGGCAGAAGTCCCTGAGCCTGAGCCCCGGCAAA

SEQ ID NO: 79 cDNA encoding IgG1sigma T437R/K248E
GCCAGCACCAAGGGCCCAAGCGTGTTTCCCCTGGCCCCTAGCAGCAAGAGCAC
CTCCGGCGGAACAGCTGCTCTGGGCTGCCTGGTGAAAGATTACTTCCCCGAAC
CCGTGACCGTGTCCTGGAACAGCGGAGCCCTGACCAGCGGCGTGCATACCTTC
CCTGCTGTGCTGCAGAGCAGCGGACTGTACAGCCTGTCCAGCGTGGTGACCGT
GCCCAGCAGCTCCCTGGGAACCCAGACCTACATCTGCAACGTGAATCACAAGC
CCAGCAACACCAAGGTGGACAAGAAGGTGGAACCCAAGAGCTGCGATAAGAC
ACACACCTGCCCCCCCTGTCCTGCTCCTGAAGCTGCCGGCGCTAGCAGCGTGT
TTCTGTTCCCCCCTAAGCCCGAGGACACACTGATGATCAGCAGAACCCCCGAG
GTGACATGTGTGGTGGTGGACGTGTCCGCTGAGGACCCCGAGGTCAAGTTTAA
CTGGTACGTCGATGGCGTGGAGGTGCATAACGCCAAAACCAAGCCTAGGGAG
GAGCAGTACAACAGCACCTACAGAGTGGTCTCCGTCCTCACCGTGCTCCATCA
GGACTGGCTGAACGGCAAGGAGTATAAGTGCAAAGTGAGCAACAAGGCCCTG
CCCAGCTCCATCGAGAAGACCATTTCCAAGGCCAAGGGCCAGCCTAGGGAGC
CTCAGGTGTATACCCTGCCTCCCAGCAGAGAGGAGATGACCAAGAACCAGGT
GAGCCTCACCTGCCTGGTCAAGGGATTCTACCCCTCCGACATCGCCGTGGAAT
GGGAAAGCAACGGCCAGCCCGAGAATAACTACAAGACCACCCCTCCTGTGCT
GGATTCCGACGGCTCCTTCTTTCTGTACAGCAAGCTGACCGTGGACAAGAGCA
GGTGGCAGCAGGGCAATGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCA
CAACCACTACAGGCAGAAGTCCCTGAGCCTGAGCCCCGGCAAA

SEQ ID NO: 80 cDNA encoding IgG1sigma T437R/K338A
GCCAGCACCAAGGGCCCAAGCGTGTTTCCCCTGGCCCCTAGCAGCAAGAGCAC
CTCCGGCGGAACAGCTGCTCTGGGCTGCCTGGTGAAAGATTACTTCCCCGAAC
CCGTGACCGTGTCCTGGAACAGCGGAGCCCTGACCAGCGGCGTGCATACCTTC
CCTGCTGTGCTGCAGAGCAGCGGACTGTACAGCCTGTCCAGCGTGGTGACCGT
GCCCAGCAGCTCCCTGGGAACCCAGACCTACATCTGCAACGTGAATCACAAGC
CCAGCAACACCAAGGTGGACAAGAAGGTGGAACCCAAGAGCTGCGATAAGAC
ACACACCTGCCCCCCCTGTCCTGCTCCTGAAGCTGCCGGCGCTAGCAGCGTGT

TTCTGTTCCCCCCTAAGCCCAAGGACACACTGATGATCAGCAGAACCCCCGAG
GTGACATGTGTGGTGGTGGACGTGTCCGCTGAGGACCCCGAGGTCAAGTTTAA
CTGGTACGTCGATGGCGTGGAGGTGCATAACGCCAAAACCAAGCCTAGGGAG
GAGCAGTACAACAGCACCTACAGAGTGGTCTCCGTCCTCACCGTGCTCCATCA
GGACTGGCTGAACGGCAAGGAGTATAAGTGCAAAGTGAGCAACAAGGCCCTG
CCCAGCTCCATCGAGAAGACCATTTCCGCTGCCAAGGGCCAGCCTAGGGAGCC
TCAGGTGTATACCCTGCCTCCCAGCAGAGAGGAGATGACCAAGAACCAGGTG
AGCCTCACCTGCCTGGTCAAGGGATTCTACCCCTCCGACATCGCCGTGGAATG
GGAAAGCAACGGCCAGCCCGAGAATAACTACAAGACCACCCCTCCTGTGCTG
GATTCCGACGGCTCCTTCTTTCTGTACAGCAAGCTGACCGTGGACAAGAGCAG
GTGGCAGCAGGGCAATGTGTTCAGCTGCAGCGTGATGCACGAGGCCCTGCAC
AACCACTACAGGCAGAAGTCCCTGAGCCTGAGCCCCGGCAAA

SEQ ID NO: 81 cDNA encoding IgG2sigma T437R
GCCAGCACCAAGGGCCCATCCGTGTTTCCCCTGGCTCCCTGTAGCAGGTCCAC
CAGCGAGAGCACAGCCGCCCTGGGATGTCTGGTGAAGGACTATTTCCCCGAAC
CTGTGACCGTCAGCTGGAACAGCGGCGCTCTGACAAGCGGCGTGCACACATTT
CCCGCCGTGCTGCAGTCCAGCGGCCTGTACAGCCTGTCCAGCGTGGTGACCGT
GCCTAGCAGCAATTTCGGCACCCAGACCTACACCTGCAACGTGGACCACAAGC
CTTCCAACACCAAGGTGGACAAGACCGTGGAGAGGAAGTGCTGCGTGGAATG
CCCTCCCTGTCCTGCTCCTCCTGCTGCTGCCAGCTCCGTGTTCCTGTTCCCCCCC
AAACCCAAGGACACCCTGATGATCAGCAGGACCCCTGAGGTCACCTGTGTGGT
GGTGGACGTGAGCGCCGAGGATCCCGAGGTGCAGTTTAACTGGTACGTGGAC
GGCGTGGAGGTGCACAACGCCAAGACAAAGCCCAGGGAGGAACAGTTCAACA
GCACCTTCAGGGTGGTCTCCGTGCTGACCGTGCTGCATCAGGACTGGCTGAAC
GGCAAGGAGTACAAATGCAAGGTGAGCAATAAGGGCCTCCCCAGCAGCATCG
AAAAGACCATCAGCAAAACCAAGGGCCAGCCTAGAGAGCCCCAGGTGTACAC
ACTCCCTCCCTCCAGGGAGGAGATGACCAAGAACCAGGTGAGCCTCACCTGCC
TGGTGAAAGGCTTCTACCCCAGCGATATCGCCGTGGAGTGGGAGTCCAATGGC
CAGCCCGAGAATAACTACAAAACCACCCCCCCCATGCTGGACAGCGACGGCT
CCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAGAGCAGATGGCAGCAGGG

AAACGTGTTCTCCTGCAGCGTGATGCACGAAGCCCTGCACAACCATTACAGAC
AGAAGAGCCTGAGCCTGAGCCCCGGCAAG

SEQ ID NO: 82 cDNA encoding IgG2sigma T437R/K248E
GCCAGCACCAAGGGCCCATCCGTGTTTCCCCTGGCTCCCTGTAGCAGGTCCAC
CAGCGAGAGCACAGCCGCCCTGGGATGTCTGGTGAAGGACTATTTCCCCGAAC
CTGTGACCGTCAGCTGGAACAGCGGCGCTCTGACAAGCGGCGTGCACACATTT
CCCGCCGTGCTGCAGTCCAGCGGCCTGTACAGCCTGTCCAGCGTGGTGACCGT
GCCTAGCAGCAATTTCGGCACCCAGACCTACACCTGCAACGTGGACCACAAGC
CTTCCAACACCAAGGTGGACAAGACCGTGGAGAGGAAGTGCTGCGTGGAATG
CCCTCCCTGTCCTGCTCCTCCTGCTGCTGCCAGCTCCGTGTTCCTGTTCCCCCCC
AAACCCGAGGACACCCTGATGATCAGCAGGACCCCTGAGGTCACCTGTGTGGT
GGTGGACGTGAGCGCCGAGGATCCCGAGGTGCAGTTTAACTGGTACGTGGAC
GGCGTGGAGGTGCACAACGCCAAGACAAAGCCCAGGGAGGAACAGTTCAACA
GCACCTTCAGGGTGGTCTCCGTGCTGACCGTGCTGCATCAGGACTGGCTGAAC
GGCAAGGAGTACAAATGCAAGGTGAGCAATAAGGGCCTCCCCAGCAGCATCG
AAAAGACCATCAGCAAAACCAAGGGCCAGCCTAGAGAGCCCCAGGTGTACAC
ACTCCCTCCCTCCAGGGAGGAGATGACCAAGAACCAGGTGAGCCTCACCTGCC
TGGTGAAAGGCTTCTACCCCAGCGATATCGCCGTGGAGTGGGAGTCCAATGGC
CAGCCCGAGAATAACTACAAAACCACCCCCCCCATGCTGGACAGCGACGGCT
CCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAGAGCAGATGGCAGCAGGG
AAACGTGTTCTCCTGCAGCGTGATGCACGAAGCCCTGCACAACCATTACAGAC
AGAAGAGCCTGAGCCTGAGCCCCGGCAAG

SEQ ID NO: 83 cDNA encoding IgG2sigma T437R/K338A
GCCAGCACCAAGGGCCCATCCGTGTTTCCCCTGGCTCCCTGTAGCAGGTCCAC
CAGCGAGAGCACAGCCGCCCTGGGATGTCTGGTGAAGGACTATTTCCCCGAAC
CTGTGACCGTCAGCTGGAACAGCGGCGCTCTGACAAGCGGCGTGCACACATTT
CCCGCCGTGCTGCAGTCCAGCGGCCTGTACAGCCTGTCCAGCGTGGTGACCGT
GCCTAGCAGCAATTTCGGCACCCAGACCTACACCTGCAACGTGGACCACAAGC
CTTCCAACACCAAGGTGGACAAGACCGTGGAGAGGAAGTGCTGCGTGGAATG
CCCTCCCTGTCCTGCTCCTCCTGCTGCTGCCAGCTCCGTGTTCCTGTTCCCCCCC

AAACCCAAGGACACCCTGATGATCAGCAGGACCCCTGAGGTCACCTGTGTGGT
GGTGGACGTGAGCGCCGAGGATCCCGAGGTGCAGTTTAACTGGTACGTGGAC
GGCGTGGAGGTGCACAACGCCAAGACAAAGCCCAGGGAGGAACAGTTCAACA
GCACCTTCAGGGTGGTCTCCGTGCTGACCGTGCTGCATCAGGACTGGCTGAAC
GGCAAGGAGTACAAATGCAAGGTGAGCAATAAGGGCCTCCCCAGCAGCATCG
AAAAGACCATCAGCGCCACCAAGGGCCAGCCTAGAGAGCCCCAGGTGTACAC
ACTCCCTCCCTCCAGGGAGGAGATGACCAAGAACCAGGTGAGCCTCACCTGCC
TGGTGAAAGGCTTCTACCCCAGCGATATCGCCGTGGAGTGGGAGTCCAATGGC
CAGCCCGAGAATAACTACAAAACCACCCCCCCCATGCTGGACAGCGACGGCT
CCTTCTTCCTGTACAGCAAGCTGACCGTGGACAAGAGCAGATGGCAGCAGGG
AAACGTGTTCTCCTGCAGCGTGATGCACGAAGCCCTGCACAACCATTACAGAC
AGAAGAGCCTGAGCCTGAGCCCCGGCAAG

SEQ ID NO: 84 cDNA encoding IgG4PAA T437R
GCCAGCACCAAGGGCCCAAGCGTGTTCCCTCTGGCCCCCTGTAGCAGGAGCAC
CAGCGAGTCCACAGCCGCTCTGGGCTGCCTGGTGAAGGACTACTTCCCCGAGC
CTGTGACCGTGAGCTGGAACAGCGGAGCCCTGACAAGCGGAGTGCATACCTTC
CCCGCCGTGCTGCAATCCTCCGGACTGTACTCCCTGTCCTCCGTGGTGACCGTG
CCTAGCAGCAGCCTGGGAACCAAGACCTACACCTGCAACGTGGACCATAAGC
CCAGCAACACCAAGGTGGACAAGAGGGTGGAGAGCAAGTACGGCCCCCCTTG
TCCTCCTTGCCCTGCCCCTGAAGCTGCTGGAGGACCCAGCGTGTTCCTGTTCCC
CCCCAAGCCCAAGGACACCCTGATGATTAGCAGGACCCCCGAGGTGACCTGC
GTGGTGGTGGACGTGAGCCAGGAGGATCCCGAGGTGCAGTTTAACTGGTACGT
GGACGGCGTGGAGGTGCACAACGCTAAAACCAAACCCAGGGAGGAGCAGTTC
AACAGCACCTATAGGGTGGTGAGCGTGCTCACCGTGCTGCACCAGGACTGGCT
GAATGGCAAGGAGTACAAGTGCAAAGTGAGCAACAAGGGCCTGCCCTCCAGC
ATCGAGAAGACAATCTCCAAGGCCAAGGGCCAGCCCAGAGAGCCTCAGGTGT
ACACCCTGCCCCCCTCCCAGGAGGAAATGACCAAGAACCAGGTGTCCCTGACC
TGTCTGGTGAAGGGCTTCTACCCCTCCGATATCGCCGTGGAGTGGGAGTCCAA
CGGCCAGCCCGAGAACAACTACAAGACAACCCCCCCCGTGCTGGATTCCGAC
GGCTCCTTCTTTCTGTACAGCAGACTGACCGTGGACAAGTCCAGGTGGCAGGA

GGGCAATGTGTTCTCCTGTAGCGTGATGCACGAGGCCCTCCACAATCACTACA
GGCAGAAGAGCCTGAGCCTGTCCCTGGGCAAA

SEQ ID NO: 85 cDNA encoding IgG4PAA T437R/K248E
GCCAGCACCAAGGGCCCAAGCGTGTTCCCTCTGGCCCCCTGTAGCAGGAGCAC
CAGCGAGTCCACAGCCGCTCTGGGCTGCCTGGTGAAGGACTACTTCCCCGAGC
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CCCGCCGTGCTGCAATCCTCCGGACTGTACTCCCTGTCCTCCGTGGTGACCGTG
CCTAGCAGCAGCCTGGGAACCAAGACCTACACCTGCAACGTGGACCATAAGC
CCAGCAACACCAAGGTGGACAAGAGGGTGGAGAGCAAGTACGGCCCCCCTTG
TCCTCCTTGCCCTGCCCCTGAAGCTGCTGGAGGACCCAGCGTGTTCCTGTTCCC
CCCCAAGCCCGAGGACACCCTGATGATTAGCAGGACCCCCGAGGTGACCTGC
GTGGTGGTGGACGTGAGCCAGGAGGATCCCGAGGTGCAGTTTAACTGGTACGT
GGACGGCGTGGAGGTGCACAACGCTAAAACCAAACCCAGGGAGGAGCAGTTC
AACAGCACCTATAGGGTGGTGAGCGTGCTCACCGTGCTGCACCAGGACTGGCT
GAATGGCAAGGAGTACAAGTGCAAAGTGAGCAACAAGGGCCTGCCCTCCAGC
ATCGAGAAGACAATCTCCAAGGCCAAGGGCCAGCCCAGAGAGCCTCAGGTGT
ACACCCTGCCCCCCTCCCAGGAGGAAATGACCAAGAACCAGGTGTCCCTGACC
TGTCTGGTGAAGGGCTTCTACCCCTCCGATATCGCCGTGGAGTGGGAGTCCAA
CGGCCAGCCCGAGAACAACTACAAGACAACCCCCCCCGTGCTGGATTCCGAC
GGCTCCTTCTTTCTGTACAGCAGACTGACCGTGGACAAGTCCAGGTGGCAGGA
GGGCAATGTGTTCTCCTGTAGCGTGATGCACGAGGCCCTCCACAATCACTACA
GGCAGAAGAGCCTGAGCCTGTCCCTGGGCAAA

SEQ ID NO: 86 cDNA encoding IgG4PAA T437R/K338A
GCCAGCACCAAGGGCCCAAGCGTGTTCCCTCTGGCCCCCTGTAGCAGGAGCAC
CAGCGAGTCCACAGCCGCTCTGGGCTGCCTGGTGAAGGACTACTTCCCCGAGC
CTGTGACCGTGAGCTGGAACAGCGGAGCCCTGACAAGCGGAGTGCATACCTTC
CCCGCCGTGCTGCAATCCTCCGGACTGTACTCCCTGTCCTCCGTGGTGACCGTG
CCTAGCAGCAGCCTGGGAACCAAGACCTACACCTGCAACGTGGACCATAAGC
CCAGCAACACCAAGGTGGACAAGAGGGTGGAGAGCAAGTACGGCCCCCCTTG
TCCTCCTTGCCCTGCCCCTGAAGCTGCTGGAGGACCCAGCGTGTTCCTGTTCCC

CCCCAAGCCCAAGGACACCCTGATGATTAGCAGGACCCCCGAGGTGACCTGC
GTGGTGGTGGACGTGAGCCAGGAGGATCCCGAGGTGCAGTTTAACTGGTACGT
GGACGGCGTGGAGGTGCACAACGCTAAAACCAAACCCAGGGAGGAGCAGTTC
AACAGCACCTATAGGGTGGTGAGCGTGCTCACCGTGCTGCACCAGGACTGGCT
GAATGGCAAGGAGTACAAGTGCAAAGTGAGCAACAAGGGCCTGCCCTCCAGC
ATCGAGAAGACAATCTCCGCTGCCAAGGGCCAGCCCAGAGAGCCTCAGGTGT
ACACCCTGCCCCCCTCCCAGGAGGAAATGACCAAGAACCAGGTGTCCCTGACC
TGTCTGGTGAAGGGCTTCTACCCCTCCGATATCGCCGTGGAGTGGGAGTCCAA
CGGCCAGCCCGAGAACAACTACAAGACAACCCCCCCCGTGCTGGATTCCGAC
GGCTCCTTCTTTCTGTACAGCAGACTGACCGTGGACAAGTCCAGGTGGCAGGA
GGGCAATGTGTTCTCCTGTAGCGTGATGCACGAGGCCCTCCACAATCACTACA
GGCAGAAGAGCCTGAGCCTGTCCCTGGGCAAA

The invention also provides for a vector comprising the polynucleotide of the invention.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 75.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 76.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 77.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 78.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 79.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 80.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 81.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 82.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 83.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 84.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 85.

The invention also provides a vector comprising the polynucleotide of SEQ ID NO: 86.

The invention also provides a host cell comprising the vector of the invention.

The invention also provides a method of producing the Fc domain containing molecule of the invention, comprising culturing the host cell of the invention in conditions wherein the Fc domain containing molecule is expressed, and isolating the Fc domain containing molecule.

In some embodiments, the Fc domain containing molecule is an antibody.

Pharmaceutical compositions/Administration

The invention also provides for pharmaceutical compositions comprising the antibodies or the Fc domain containing molecules of the invention and a pharmaceutically acceptable carrier. For therapeutic use, the antibodies or the Fc domain containing molecules of the invention may be prepared as pharmaceutical compositions containing an effective amount of the antibody or the Fc domain containing molecule as an active ingredient in a pharmaceutically acceptable carrier. "Carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the antibody of the invention is administered. Such vehicles may be liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. For example, 0.4% saline and 0.3% glycine may be used. These solutions are sterile and generally free of particulate matter. They may be sterilized by conventional, well-known sterilization techniques (*e.g.*, filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, stabilizing, thickening, lubricating and coloring agents, etc. The concentration of the antibodies or the Fc domain containing molecules of the invention in such pharmaceutical formulation may vary, from less than

about 0.5%, usually to at least about 1% to as much as 15 or 20% by weight and may be selected primarily based on required dose, fluid volumes, viscosities, etc., according to the particular mode of administration selected. Suitable vehicles and formulations, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in e.g. Remington: The Science and Practice of Pharmacy, 21st Edition, Troy, D.B. ed., Lipincott Williams and Wilkins, Philadelphia, PA 2006, Part 5, Pharmaceutical Manufacturing pp 691-1092, See especially pp. 958-989.

The mode of administration for therapeutic use of the antibodies or the Fc domain containing molecules of the invention may be any suitable route that delivers the antibody to the host, such as parenteral administration, e.g., intradermal, intramuscular, intraperitoneal, intravenous or subcutaneous, pulmonary, transmucosal (oral, intranasal, intravaginal, rectal), using a formulation in a tablet, capsule, solution, powder, gel, particle; and contained in a syringe, an implanted device, osmotic pump, cartridge, micropump; or other means appreciated by the skilled artisan, as well known in the art. Site specific administration may be achieved by for example intratumoral, intrarticular, intrabronchial, intraabdominal, intracapsular, intracartilaginous, intracavitary, intracelial, intracerebellar, intracerebroventricular, intracolic, intracervical, intragastric, intrahepatic, intracardial, intraosteal, intrapelvic, intrapericardiac, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrarectal, intrarenal, intraretinal, intraspinal, intrasynovial, intrathoracic, intrauterine, intravascular, intravesical, intralesional, vaginal, rectal, buccal, sublingual, intranasal, or transdermal delivery.

The antibodies or the Fc domain containing molecules of the invention may be administered to a subject by any suitable route, for example parentally by intravenous *(i.v.)* infusion or bolus injection, intramuscularly or subcutaneously or intraperitoneally. *i.v.* infusion may be given over for example 15, 30, 60, 90, 120, 180, or 240 minutes, or from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 hr.

The dose given to a subject is sufficient to alleviate or at least partially arrest the disease being treated ("therapeutically effective amount") and may be sometimes 0.005 mg to about 100 mg/kg, e.g. about 0.05 mg to about 30 mg/kg or about 5 mg to about 25 mg/kg, or about 4 mg/kg, about 8 mg/kg, about 16 mg/kg or about 24 mg/kg, or for example about 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 mg/kg, but may even higher, for example about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 40, 50, 60, 70, 80, 90 or 100 mg/kg.

A fixed unit dose may also be given, for example, 50, 100, 200, 500 or 1000 mg, or the dose may be based on the patient's surface area, e.g., 500, 400, 300, 250, 200, or 100 mg/m^2. Usually between 1 and 8 doses, (e.g., 1, 2, 3, 4, 5, 6, 7 or 8) may be administered to treat the patient, but 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more doses may be given.

The administration of the antibodies or the Fc domain containing molecules of the invention may be repeated after one day, two days, three days, four days, five days, six days, one week, two weeks, three weeks, one month, five weeks, six weeks, seven weeks, two months, three months, four months, five months, six months or longer. Repeated courses of treatment are also possible, as is chronic administration. The repeated administration may be at the same dose or at a different dose. For example, the antibodies or the Fc domain containing molecules of the invention may be administered at 8 mg/kg or at 16 mg/kg at weekly interval for 8 weeks, followed by administration at 8 mg/kg or at 16 mg/kg every two weeks for an additional 16 weeks, followed by administration at 8 mg/kg or at 16 mg/kg every four weeks by intravenous infusion.

For example, the antibodies or the Fc domain containing molecules in the methods of the invention may be provided as a daily dosage in an amount of about 0.1-100 mg/kg, such as 0.5, 0.9, 1.0, 1.1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40, 45, 50, 60, 70, 80, 90 or 100 mg/kg, per day, on at least one of day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40, or alternatively, at least one of week 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 after initiation of treatment, or any combination thereof, using single or divided doses of every 24, 12, 8, 6, 4, or 2 hr, or any combination thereof.

The antibodies or the Fc domain containing molecules in the methods of the invention may also be administered prophylactically in order to reduce the risk of developing cancer, delay the onset of the occurrence of an event in cancer progression, and/or reduce the risk of recurrence when a cancer is in remission.

The antibodies or the Fc domain containing molecules of the invention may be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional protein preparations and well known lyophilization and reconstitution techniques can be employed.

Methods and Uses

The antibodies or the Fc domain containing molecules of the invention have *in vitro* and *in vivo* diagnostic, as well as therapeutic and prophylactic utilities. For example, the antibodies of the invention may be administered to cells in culture, *in vitro* or *ex vivo*, or to a subject to treat, prevent, and/or diagnose a variety of disorders, such as cancers and infectious disorders.

The invention provides for a method of enhancing an agonistic activity of an anti-TNFR superfamily member antibody in a subject, comprising providing the anti-TNFR superfamily member antibody, introducing a T437R mutation, a K248E mutation, a K338A mutation, a T437R/K248E mutation or a T437R/K338A mutation into the antibody to generate an engineered antibody specifically binding the TNFR superfamily member, and administering the engineered antibody to the subject.

The invention also provides for a method of treating a cancer in a subject, comprising administering to the subject an antibody specifically binding a TNFR superfamily member comprising a T437R mutation, a K248E mutation, a T437R/K338A mutation, or a T437R/K248E for a time sufficient to treat the cancer.

In the methods of the invention, the antibody mediates ADCC.

In the methods of the invention, the antibody mediates ADCP.

In the methods of the invention, the antibody mediates CDC.

In some methods of the invention, the antibody enhances the agonistic activity of an anti-TNFR superfamily member independent of antibody cross-linking, wherein agonistic activity is measured by measuring antibody-induced production of secreted embryonic alkaline phosphatase (SEAP) expressed under the control of NFκB-inducible promoter from Hek-293 cells.

In the methods of the invention, the antibody optionally further comprises a second mutation that reduces ADCC.

In the methods of the invention, the subject has a viral infection.

In the method of the invention, the subject has a cancer.

In the methods of the invention, the cancer is a solid tumor.

In the methods of the invention, the solid tumor is a melanoma, a lung cancer, a squamous non-small cell lung cancer (NSCLC), a non-squamous NSCLC, a colorectal cancer, a prostate cancer, a castration-resistant prostate cancer, a stomach cancer, an

ovarian cancer, a gastric cancer, a liver cancer, a pancreatic cancer, a thyroid cancer, a squamous cell carcinoma of the head and neck, a carcinoma of the esophagus or gastrointestinal tract, a breast cancer, a fallopian tube cancer, a brain cancer, an urethral cancer, a genitourinary cancer, an endometriosis, a cervical cancer or a metastatic lesion of the cancer.

In the methods of the invention, the TNFR is tumor necrosis factor receptor 1 (SEQ ID NO: 1), Tumor necrosis factor receptor 2 (SEQ ID NO: 2) , lymphotoxin beta receptor (SEQ ID NO: 3), OX40 (SEQ ID NO: 4), CD40 (SEQ ID NO: 5), Fas receptor (SEQ ID NO: 6), decoy receptor 3 (SEQ ID NO: 7), CD27 (SEQ ID NO: 8), CD30 (SEQ ID NO: 9), CD137 (SEQ ID NO: 10), death receptor 4 (SEQ ID NO: 11), death receptor 5 (SEQ ID NO: 12), decoy receptor 1 (SEQ ID NO: 13), decoy receptor 2 (SEQ ID NO: 14), RANK (SEQ ID NO: 15), osteoprotegerin (SEQ ID NO: 16), TWEAK receptor (SEQ ID NO: 17), TACI (SEQ ID NO: 18), BAFF receptor (SEQ ID NO: 19), herpesvirus entry mediator (SEQ ID NO: 20), nerve growth factor receptor (SEQ ID NO: 21), B-cell maturation antigen (SEQ ID NO: 22), GITR (SEQ ID NO: 23), TROY (SEQ ID NO: 24), death receptor 6 (SEQ ID NO: 25), death receptor 3 (SEQ ID NO: 26) or ectodysplasin A2 receptor (SEQ ID NO: 27).

In the methods of the invention, the TNFR is OX40 (SEQ ID NO: 4), CD27 (SEQ ID NO: 8), CD40 (SEQ ID NO: 5), CD137 (SEQ ID NO: 10), or GITR (SEQ ID NO: 23).

In the methods of the invention, the TNFR is OX40 (SEQ ID NO: 4).

In the methods of the invention, the TNFR is CD27 (SEQ ID NO: 8).

In the methods of the invention, the TNFR is CD40 (SEQ ID NO: 5).

In the methods of the invention, the TNFR is CD137 (SEQ ID NO: 10).

In the methods of the invention, the TNFR is GITR (SEQ ID NO: 23).

Many of the TNFR superfamily members and their ligands have been implicated as targets for cancer therapy, including TNFR1/2/TNF-α, CD70/CD27, CD137/4-1BB, OX40/OX40L, CD40/CD40L, GITR/GITRL and several agonistic antibodies targeting the TNFR superfamily members, such as anti-CD40, anti-OX-40, anti-GITR, anti-CD27, anti-CD137 antibodies are in clinical development for various solid tumors as well as heme malignancies such as non-Hodgkin's lymphoma and B-cell malignancies. It can be expected that anti-CD40, anti-OX40, anti-GITR, anti-CD27, anti-CD137 and other anti-TNFR superfamily member antibodies of the invention with improved properties in terms

of their enhanced agonistic activity optionally coupled with effector functionality will be therapeutically effective in the treatment of various cancers, including solid tumors.

While having described the invention in general terms, the embodiments of the invention will be further disclosed in the following examples that should not be construed as limiting the scope of the claims.

Example 1 Fc engineering approach to improve agonistic activity of anti-TNFR superfamily member antibodies

Agonistic antibodies directed against immunostimulatory receptors belonging to the tumor necrosis factor receptor (TNFR) superfamily are emerging as promising drug candidates for cancer immunotherapies. Several Fc engineering approaches were discovered recently that can augment the anti-tumor activities of anti-TNFR antibodies by enhancing their agonistic activities and/or effector functions.

Monoclonal antibodies that stimulate antitumor immunity are emerging as an important class of cancer therapeutics (Mellman et al. (2011) *Nature* 480: 480-9) (Chen et al. (2013) *Nat Rev Immunol* 13: 227-42). The antibodies targeting the immune checkpoint receptors CTLA-4 and PD-1 have been approved as monotherapies for advanced melanoma, lung cancer and evaluated for the treatment of other types of human cancer. Besides targeting the inhibitory pathways, agonistic antibodies directed against the immunostimulatory receptors on T cells and antigen presenting cells also can stimulate antitumor immunity and are emerging as a promising area of clinical development for cancer immunotherapies (Schaer et al. (2014) *J Immunother Cancer* 2: 7).

Many immunostimulatory receptors belong to the tumor necrosis factor (TNF) receptor superfamily. Of them, OX40, CD27, 4-1BB and GITR are expressed on effector T cells and their ligands and agonistic antibodies can activate these receptors to stimulate the proliferation and activation of T cells (Kanamaru et al. (2004) *J Immunol* 172: 7306-14) (Gramaglia et al. (1998) *J Immunol* 161: 6510-7) (Pollok et al. (1993) *J Immunol* 150: 771-81) (Ramakrishna et al. (2015) *J Immunother Cancer* 3: 37) . CD40 is expressed on antigen presenting cells and the activation of this receptor facilitates more efficacious presentation of tumor antigens to activated T cells (Khalil et al. (2007) *Update Cancer Ther* 2: 61-5) (Mangsbo et al. (2015) *Clin Cancer Res* 21: 1115-26). Many evidences indicated that the agonistic activities of therapeutic antibodies to these TNF

receptors are important for their anti-tumor activities (Mangsbo et al. (2015) *Clin Cancer Res* 21: 1115-26) (He et al. (2013) *J Immunol* 191: 4174-83) (Wilson et al. (2011) *Cancer Cell* 19: 101-13). On the other hand, several TNFR superfamily members, such as OX40 and GITR, have elevated expression on regulatory T cells (T_{reg}) which negatively modulate tumor immunity. Several studies have revealed that the anti-OX40 and anti-GITR antibodies may facilitate the selective elimination of regulatory T cells in tumor microenvironment by the effector functions of the antibody (Bulliard et al. (2013) *J Exp Med* 210: 1685-93) (Bulliard et al. (2014) *Immunol Cell Biol* 92: 475-80). Such antibody-mediated killing of regulatory T cells may be more important than the antibody-mediated activation of effector T cells for the anti-tumor activities of therapeutic anti-OX40 and anti-GITR antibodies.

Accumulating evidence indicated that immunomodulatory antibodies engage different types of Fc receptors for their agonistic activities and effector functions. To activate downstream signaling pathways, TNFR trimerization is required. However, one antibody molecule commonly is not sufficient to cluster enough TNF receptors; instead, antibody crosslinking is necessary for receptor activation in *in vitro* assays (Morris et al. (2007) *Mol Immunol* 44: 3112-21). Recent studies in mice indicated that the engagement to the inhibitory FcγRIIB receptor is critical for the agonistic activity of antibodies to a number of TNFR targets, including CD40 (Li et al. (2011) *Science* 333: 1030-4) (White et al. (2011) *J Immunol* 187: 1754-63), death receptor 5 (DR5) (Wilson et al. (2011) *Cancer Cell* 19: 101-13) (Li et al. (2012) *Cell Cycle* 11: 3343-4) and CD95 (Xu et al. (2003) *J Immunol* 171: 562-8). The crosslinking of IgG Fc to FcγRIIB receptors can multimerize more than one antibody molecule, which in turn can facilitate the clustering of enough TNFR for signaling pathway activation. On the other hand, the antibody effector functions, such as ADCC and ADCP depend on the interactions with various activating Fcγ receptors. Studies in mice revealed that activating Fcγ receptors contributed to the antitumor activities of immunomodulatory anti-OX40 and anti-GITR antibodies by selectively eliminating intratumoral regulatory T cells (Bulliard et al. (2013) *J Exp Med* 210: 1685-93) (Bulliard et al. (2014) *Immunol Cell Biol* 92: 475-80).

Human IgG antibodies have poor binding affinities to the majority of human Fc receptors except FcγRI (Guilliams et al. (2014) *Nat Rev Immunol* 14: 94-108). To optimize the antitumor activity of agonist antibodies for immunostimulatory TNF

receptors, one approach is to engineer the Fc region of the IgG antibody to improve its Fcγ receptor engagement, particularly the engagement with FcγRIIB receptor. In this regard, Chu et al. described S267E/L328F mutations in IgG1 Fc with enhanced FcγRIIB binding affinity (Chu et al. (2008) *Mol Immunol* 45: 3926-33). Anti-CD19 antibody engineered with such mutations showed improved inhibition of B cell receptor-mediated activation of primary human B cells. However, further study revealed that such Fc variant also has enhanced binding to R131 allotype of the activating FcγRIIA receptor (Mimoto et al. (2013) *Protein Eng Des Sel* 26: 589-98). Recently, Mimoto et al. reported a set of six mutations in IgG1 Fc, collectively named as V12 mutations, with selectively enhanced FcγRIIB engagement without associated increased binding to either H131 or R131 allotype of FcγRIIA receptor (Mimoto et al. (2013) *Protein Eng Des Sel* 26: 589-98). Anti-CD137 agonistic antibody with the engineered V12 mutation showed much enhanced agonistic activity dependent on FcγRIIB engagement.

Although optimizing FcγRIIB engagement is a viable approach, the agonistic activity of such engineered antibody depends heavily on the FcγR expression in the local microenvironment and the efficacy of such antibody may be limited to the anatomical site of action. If the purpose of crosslinking to FcγRIIB is solely to increase the clustering of agonistic antibodies for receptor activation, then we hypothesized those Fc mutations that can promote antibody multimerization may enhance the agonism of antibodies to TNF receptors without the need of FcγRIIB crosslinking. Diebolder et al. reported that selective Fc mutations can facilitate IgG antibody into the formation of a hexamer upon binding targets on cell surface (Diebolder et al. (2014) *Science* 343: 1260-3). While it was reported that such IgG hexamer can greatly activate complement-dependent cytotoxicity (CDC), we think another application may be that oligomerized antibodies to TNF receptors may activate the receptors by promoting receptor clustering.

This work describes evaluation of different Fc engineering approaches on the enhancement of the agonism of an anti-OX40 antibody. Besides, the effects of Fc mutations on ADCC and ADCP effector functions of the engineered antibodies were also evaluated. Such study may help to guide the design of engineered antibodies to OX40 and other TNF receptors with improved anti-tumor activity.

Example 2 Identifying mutations to human IgG that facilitating multimeric association of Fc domains

With the goal of identifying mutations to human IgG that might enhance agonistic activity by facilitating Fc-mediated multimerization, a sequence-based search of structures deposited in the RCSB Protein Data Bank (PDB) (Berman et al. (2000) *Nucleic Acids Res* 28: 235-42) was first performed to identify those entries containing an Fc domain. From that list, structures resulting from crystals belonging to the hexagonal crystal family were inspected with the anticipation that application of crystallographic symmetry would for a subset of the structures result in a closed, hexameric arrangement of Fc domains that might be used as a model to aid in the identification of mutations to promote multimerization. The crystal structure of an intact IgG1 molecule with specificity for HIV-1 gp120 (PDB 1HZH) (Saphire et al. (2001) *Science* 293: 1155-9) was thus identified in which Fc domains packed to form a closed-ring configuration.

Diebolder *et al.* had previously hypothesized that a hexameric association of IgG molecules similar to that observed in structure 1HZH might be important for CDC activation, and had used this model for the identification of mutations that facilitate hexamerization (Diebolder et al. (2014) *Science* 343: 1260-3). The multimeric model revealed that most contacts stabilizing the closed-ring arrangement of molecules were between CH3 domains of neighboring Fc molecules. Thus, it was postulated that one way to facilitate multimerization would be to mutate residues on the CH3 surface to optimize interaction with a neighboring CH3 surface if molecules were to pack as observed in the multimeric model. Upon overlaying CH3 domains of the IgG1 Fc present in crystal structure 3AVE onto those of the multimeric model, a clash of 3AVE CH2 domains from neighboring Fc domains was observed (**Figure 1**). Such a clash was also noted by Davies et. al, however it was suggested that a conformational change within the CH2 domain AB loop could prevent such a clash from occurring (Davies et al. (2014) *Mol. Immunol.* 62:46-53). Alternatively, an altered angle between CH2 and CH3 domains in the multimeric model allowed CH3 domains to pack as described without clash between CH2 domains of adjacent molecules.

Therefore, it was postulated that enhanced flexibility of the CH2 domain relative to the CH3 domain would allow Fc molecules to more easily assemble into a multimeric arrangement by allowing the CH2 to more facilely adopt a conformation that would avoid

steric clash and potentially contribute favorably to the packing interaction. Mutations to the CH2:CH3 interface have been shown to alter CH2 domain flexibility (Frank et al. (2014) *J Mol Biol* 426: 1799-811) (Teplyakov et al. (2013) *Mol Immunol* 56: 131-9). Thus, two categories of mutations were defined to promote IgG multimerization, those that enhance inter-Fc CH3:CH3 interactions through optimization of intermolecular contacts and those that weaken the intramolecular CH2:CH3 interface for promoting enhanced flexibility of the CH2 domain. The multimeric model was manually inspected using the programs Coot and PyMol, and a list of mutations anticipated to facilitate multimerization by at least one of the postulated mechanisms was devised. Various mutations were engineered on anti-OX40 antibody 20E5 and tested for their agonistic activity either in solution or cross-linked with Raji cells. From these initial experiments, Fc mutations K248E, K338A and T437R were selected for further studies.

Example 3 Materials and Methods

Fc engineering of anti-OX40 antibody

The VH and the VL regions of an anti-OX40 antibody 20E5VH3VL2 (herein called as 20E5) **(VH: SEQ ID NO: 55, VL: SEQ ID NO: 56)** were cloned onto human wild type IgG1 or IgG2 and select substitutions were engineered onto the Fc to evaluate the effect of the substitutions on agonistic activity of the antibody and effector functions. The names of the generated antibodies and their Fc substitutions are shown in **Table 3.**

Table 3.

Antibody name	Isotype	Fc mutations (residue numbering according to the EU Index)
OX4020E5IgG1	IgG1	Wild-type
OX4020E5IgG1K248E	IgG1	K248E
OX4020E5IgG1T437R	IgG1	T437R
OX4020E5IgG1K338A	IgG1	K338A
OX4020E5IgG1T437R/K248E	IgG1	T437R, K248E
OX4020E5IgG1T437R/K338A	IgG1	T437R, K338A
OX4020E5IgG1K248E/K338A	IgG1	K248E, K338A

OX40SF2IgG1	IgG1	Wild type
OX40SF2IgG1T437R	IgG1	T437R
OX40SF2IgG1T437R/K248E	IgG1	T437R, K248E
OX40SF2IgG1T437R/K338A	IgG1	T437R, K338A

Antibody Expression and Purification

Plasmids encoding antibody heavy chains (HC) and light chains (LC) were co-transfected at a 1:3 (HC: LC) molar ratio into Expi293F cells following the transfection kit instructions (Life Technologies). Cells were spun down five days post transfection and the supernatant were passed through a 0.2 μm filter. The titer of antibody expression was quantified using Octet (ForteBio). Antibody purification was carried out using prepacked Protein A spin columns following the kit instruction (GE Healthcare Life Sciences). The purified antibody was buffer-exchanged into DPBS, pH7.2 by dialysis and protein concentration was determined by UV absorbance at 280 nm. Quality was assessed by high-performance size-exclusion chromatography (HP-SEC) and SDS-PAGE of reduced and non-reduced samples.

NanoBRET protein-protein interaction assay

The coding sequence for the light chain of anti-OX40 SF2 antibody was cloned into pNLF-C and pHTC halotag vectors (Promega, Madison, WI) in frame with C-terminal Nanoluc and Halotag sequences respectively. These light chains were paired with the heavy chains to express Fc engineered SF2 antibodies with either Nanoluc or Halotag attached at the C-termini of the light chains. Standard Protein A spin column were employed to purify these modified antibodies.

To study antibody multimerization on the cell surface by the NanoBRET protein-protein interaction assay (Promega, Madison, WI), 0.25 x10^5 HEK-Blue: OX40 cells were seeded in each well of the 96-well assay plate and cultured at 37°C overnight. The next day, equal concentrations of Nanoluc-tagged antibody (donor) and Halotag-tagged antibody (acceptor) in 50 μl assay medium (Opti-MEM I reduced serum medium, no phenol red plus 4% FBS) were applied to the cells. Halotag 618 ligand diluted 1:1000 in 50 μl assay medium were added in experimental well, and a no ligand control well was

also set up by diluting DMSO 1:1000 in assay medium. After incubation at 37°C for 30 min, the cells were washed twice with assay medium and resuspended in 100 µl assay medium. 25 µl Nano-Glo substrate, diluted 1:200 in assay medium without FBS, was added to each well. After shaking for 30 seconds, the donor emission (460nm) and acceptor emission (618nm) were measured by Envision. Raw NanoBRET ratio values with milliBRET units (mBU) were calculated as RawBRET = $618nm_{Em}/460nm_{Em}$*1000. To factor in donor-contributed background or bleed through, Corrected NanoBRET ratio values with milliBRET units was calculated as CorrectedBRET = $RawBRET_{experimental\ sample}$ – $RawBRET_{no\text{-}ligand\ control\ sample}$, which reflects energy transfer from a bioluminescent protein donor to a fluorescent protein acceptor due to protein-protein interactions.

Flow Cytometry Staining

Plasmids expressing cDNAs encoding human FcγRI (NM_000566) (**SEQ ID NO: 59),** FcγRIIA (NM_021642) (**SEQ ID NO: 60**), FcγRIIB (NM_004001) (**SEQ ID NO: 61**), and FcγRIIIA (NM_000569) (**SEQ ID NO: 62)** (Origene) were transiently transfected into Expi293F cells by ExpiFectamine293 transfection kit (Life Technologies). Flow cytometry assays were performed 48 h after transfection. To confirm the expression of transfected Fc receptors, their specific antibodies, 10.1 (BD Pharmingen) for FcγRI, IV.3 (StemCell Technologies) for FcγRIIA, 2B6 (in house preparation) for FcγRIIB (Veri et al. (2007) *Immunology* 121: 392-404), and 3G8 (BD Pharmingen) for FcγRIIIA, were employed in flow cytometry staining as positive controls. Raji cells (ATCC: CCL-86) were also employed to test the binding of anti-OX40 antibody to FcγRIIB receptor.

2×10^5 cells per well were seeded in 96-well plate and blocked in BSA Stain Buffer (BD Biosciences, San Jose, USA) for 30 min at 4°C. Cells were incubated with test antibody on ice for 1.5 h at 4°C. After being washed twice with BSA stain buffer, the cells were incubated with R-PE labeled anti-human or anti-mouse IgG secondary antibody (Jackson Immunoresearch Laboratories) for 45 min at 4°C. The cells were washed twice in stain buffer and then resuspended in 150 µL of Stain Buffer containing 1:200 diluted DRAQ7 live/dead stain (Cell Signaling Technology, Danvers, USA). PE and DRAQ7 signals of the stained cells were detected by Miltenyi MACSQuant flow cytometer (Miltenyi Biotec, Auburn, USA) using B2 and B4 channel respectively. Live cells were gated on DRAQ7 exclusion and the geometric mean fluorescence signals were determined

for at least 10,000 live events collected. FlowJo software (Tree Star) was used for analysis. Data was plotted as the logarithm of antibody concentration versus mean fluorescence signals. Nonlinear regression analysis was performed by GraphPad Prism 6 (GraphPad Software, Inc.) and EC_{50} values were calculated.

SEQ ID NO: 59

MWFLTTLLLWVPVDGQVDTTKAVITLQPPWVSVFQEETVTLHCEVLHLPGSSSTQ
WFLNGTATQTSTPSYRITSASVNDSGEYRCQRGLSGRSDPIQLEIHRGWLLLQVSS
RVFTEGEPLALRCHAWKDKLVYNVLYYRNGKAFKFFHWNSNLTILKTNISHNGT
YHCSGMGKHRYTSAGISVTVKELFPAPVLNASVTSPLLEGNLVTLSCETKLLLQRP
GLQLYFSFYMGSKTLRGRNTSSEYQILTARREDSGLYWCEAATEDGNVLKRSPEL
ELQVLGLQLPTPVWFHVLFYLAVGIMFLVNTVLWVTIRKELKRKKKWDLEISLDS
GHEKKVISSLQEDRHLEEELKCQEQKEEQLQEGVHRKEPQGAT

SEQ ID NO: 60

MTMETQMSQNVCPRNLWLLQPLTVLLLLASADSQAAPPKAVLKLEPPWINVLQE
DSVTLTCQGARSPESDSIQWFHNGNLIPTHTQPSYRFKANNNDSGEYTCQTGQTSL
SDPVHLTVLSEWLVLQTPHLEFQEGETIMLRCHSWKDKPLVKVTFFQNGKSQKFS
HLDPTFSIPQANHSHSGDYHCTGNIGYTLFSSKPVTITVQVPSMGSSSPMGIIVAVVI
ATAVAAIVAAVVALIYCRKKRISANSTDPVKAAQFEPPGRQMIAIRKRQLEETNND
YETADGGYMTLNPRAPTDDDKNIYLTLPPNDHVNSNN

SEQ ID NO: 61

MGILSFLPVLATESDWADCKSPQPWGHMLLWTAVLFLAPVAGTPAAPPKAVLKL
EPQWINVLQEDSVTLTCRGTHSPESDSIQWFHNGNLIPTHTQPSYRFKANNNDSGE
YTCQTGQTSLSDPVHLTVLSEWLVLQTPHLEFQEGETIVLRCHSWKDKPLVKVTF
FQNGKSKKFSRSDPNFSIPQANHSHSGDYHCTGNIGYTLYSSKPVTITVQAPSSSPM
GIIVAVVTGIAVAAIVAAVVALIYCRKKRISALPGYPECREMGETLPEKPANPTNPD
EADKVGAENTITYSLLMHPDALEEPDDQNRI

SEQ ID NO: 62

MAEGTLWQILCVSSDAQPQTFEGVKGADPPTLPPGSFLPGPVLWWGSLARLQTEK
SDEVSRKGNWWVTEMGGGAGERLFTSSCLVGLVPLGLRISLVTCPLQCGIMWQL
LLPTALLLLVSAGMRTEDLPKAVVFLEPQWYRVLEKDSVTLKCQGAYSPEDNSTQ
WFHNESLISSQASSYFIDAATVDDSGEYRCQTNLSTLSDPVQLEVHIGWLLLQAPR
WVFKEEDPIHLRCHSWKNTALHKVTYLQNGKGRKYFHHNSDFYIPKATLKDSGS
YFCRGLFGSKNVSSETVNITITQGLAVSTISSFFPPGYQVSFCLVMVLLFAVDTGLY
FSVKTNIRSSTRDWKDHKFKWRKDPQDK

HEK-Blue NF-κB reporter assay

A stable HEK-Blue reporter cell line expressing human OX40 (HEK-Blue: OX40) was established by transfection OX40 expression plasmid (pUNO1-hOX40) into HEK-Blue™ Null 1 cells engineered to express a secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of NF-κB-inducible promoter (IFN-β minimal promoter). For the reporter assay, $1x10^5$ HEK-Blue: OX40 cells resuspended in 200 µl culture media were aliquoted in each well of the 96-well assay plate and the OX40 ligand or anti-OX40 antibodies were added. To test the crosslinking effect, either 1 µl of protein G magnetic beads (Pierce) or $1x10^5$ Raji cell was added in the same assay well. After incubation at 37°C overnight, the agonistic activities of the antibodies were evaluated by the quantification of the induced secreted alkaline phosphatase (SEAP) reporter gene expression using Quanti-Blue detection kit (Invivogen). Briefly, 40 µl cell culture supernatant was mixed with 160 µl Quanti-Blue reagent and incubated at 37°C until appropriate blue color developed. The OD at 650nm was measured using a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA). The agonistic activity of anti-OX40 antibody was normalized as percent activity relative to that induced by 1 µg/mL OX40 ligand.

ADCC assay

The ADCC activities of anti-OX40 antibodies were evaluated by an ADCC reporter bioassay as instructed by the manufacturer (Promega). Briefly, 25,000 HEK-Blue: OX40 cells per well plated in a 96-well plate overnight were mixed with the engineered effector cells in which the activation of FcγRIIIA receptor leads to the expression of a luciferase reporter. Anti-OX40 antibodies were added to the cells and incubated at 37°C

for 6 h. Then Bio-Glo luciferase reagent was added and the luciferase signals were quantitated by Envision. The ADCC activities of anti-OX40 antibodies were expressed as fold of activation of luciferase signals over that without test antibody added.

CDC assay

Complement-dependent cytotoxicity (CDC) activities of anti-OX40 antibodies were evaluated by a complement-mediated cell killing assay. Briefly, 100,000 HEK-Blue: OX40 cells were incubated with rabbit complement (Cedar Lane Labs) and test anti-OX40 antibodies in a 96-well plate for one hour. The activities of lactate dehydrogenase (LDH) released from the cytosol of lysed HEK-Blue: OX40 cells into the supernatant were quantitated by cytotoxicity detection kit (Roche). The complement-mediated cytotoxicities were expressed as percent cytotoxicity relative to that lysed by Triton X-100.

ADCP assay

An OX40 target cell line expressing GFP was established by infection HEK-Blue: OX40 cells with a Turbo GFP transduction particle (Sigma Aldrich). Stable GFP-expressing cells were selected with puromycin. The human $CD14^{+}CD16^{+}$ monocytes were isolated from PBMCs (Biologics Specialty) using a negative human monocyte enrichment kit without CD16 depletion (StemCell Technologies). Isolated monocytes were plated in X-VIVO-10 medium (Lonza) containing 10% FBS and macrophages were differentiated from monocytes by the addition of 25 ng/mL macrophage colony-stimulating factor (R&D Systems) for 7 days. IFNγ (50 ng/mL; R&D Systems) was added for the final 24 h of differentiation. For the ADCP assay, 1×10^5 cells/well differentiated macrophages were mixed with 0.25×10^5 cells/well GFP-expressing HEK-Blue: OX40 cells (4 : 1 ratio) in 200 μl medium (DMEM + 10% FBS) in 96-well U-bottom plates. The test antibodies were added and the plate was incubated in a 37°C incubator for 24 h. Then the cells were detached using Accutase (Sigma) and resuspended in BSA Stain Buffer. Macrophages were stained with anti-CD11b and anti-CD14 antibodies (BD Biosciences) coupled to Alexa Fluor 647 (Invitrogen). GFP positive HEK-Blue: OX40 target cells and Alexa647 positive macrophages were identified by flow cytometry using Miltenyi MACSQuant flow cytometer (Miltenyi Biotec, Auburn, USA). The data were analyzed using FlowJo software (Tree Star) and ADCP-mediated cell killing was determined by measuring the

reduction in GFP fluorescence using the following equation: Percentage of target cells killed = ((Percentage of GFP^+, $CD11b^-$, $CD14^-$ cells with the lowest concentration of antibody) − (Percentage of GFP^+, $CD11b^-$, $CD14^-$ cells with the test concentration of antibody))/ (Percentage of GFP^+, $CD11b^-$, $CD14^-$ cells with the lowest concentration of antibody) × 100.

Example 4 Characterization of anti-OX40 antibodies with singular or combination K248E, K338A and T437R mutations

Antibody agonism

Recent studies have indicated that FcγRIIB can provide the crosslinking activity and facilitate the agonistic activity of anti-TNFR superfamily member antibodies (Li et al. (2012) *Cell Cycle* 11: 3343-4). Therefore, effect of the mutations K248E, K338A and T437R singularly and in combination on agonistic properties of the generated antibodies OX4020E5IgG1K248E, OX4020E5IgG1T437R, OX4020E5IgG1K338A, OX4020E5IgG1T437R/K248E, OX4020E5IgG1T437R/K338A and OX4020E5IgG1K248E/K338A (as shown in **Table 3**) were tested in solution and after cross-linking with human B lymphoblastoid Raji cells, which predominantly express FcγRIIB (Rankin et al. (2006) *Blood* 108: 2384-91) in a HEK-Blue™ reporter assay.

Figure 2A shows that the agonistic activity of the singularly mutated antibodies OX4020E5IgG1K248E, OX4020E5IgG1T437R and OX4020E5IgG1K338A in solution, assessed as percent (%) activity relative to the OX40 ligand (OX40L). All antibodies demonstrated agonism in solution. Cross-linking with Daudi cells further increased agonism, the antibody with the T437R mutation demonstrating the highest agonistic activity (**Figure 2B**). All double mutated antibodies OX4020E5IgG1T437R/K248E, OX4020E5IgG1T437R/K338A and OX4020E5IgG1K248E/K338A demonstrated enhanced agonistic activity when compared to the wild type IgG1 antibody in solution (**Figure 2C**). The agonistic activity was substantially enhanced for OX4020E5IgG1T437R/K248E (**Figure 2D**) and OX4020E5IgG1T437R/K338A (**Figure 2E**) when cross-linked with Daudi cells, at higher level observed for the wild type antibody OX4020E5IgG1 (**Figure 2F**). Cross-linking OX4020E5IgG1T437R with Daudi cells also enhanced agonistic activity of that antibody (**Figure 2G**).

VH/VL regions of a second anti-OX40 antibody SF2 **(VH: SEQ ID NO: 51, VL: SEQ ID NO: 52)** was also engineered onto singularly or in combination mutated Fc domains to generate antibodies OX40SF2IgG1T437R, OX40SF2IgG1T437R/K248E and OX40SF2IgG1T437R/K338A. These antibodies were tested for their agonism in solution and upon cross-linking with Daudi cells. Similar to the engineered 20E5 antibody, SF2-derived antibodies with T437R or the T437R/K248E and the T437R/K338A mutations had enhanced agonism when compared to the wild type parental antibody in solution (i.e. cross-linking independent agonism) (**Figure 3A**). Cross-linking with Daudi cells enhanced agonism of OX40SF2IgG1T437R (**Figure 3B**) and OX40SF2IgG1T437R/K248E (**Figure 3C**).

Similar effects of the boost of agonism for engineered anti-OX40 SF2 antibody were observed when antibodies were cross-linked with Raji cells, another cell line derived from B cells that express FcγRIIB. Cross-linking was confirmed to be FcγRIIB-mediated, as an anti- FcγRIIB antibody 2B6 blocked the Raji-cell mediated boost in agonism for OX40SF2IgG1T437R (**Figure 4A**) and OX40SF2IgG1T437R/K248E (**Figure 4B**).

Antibody multimerization

The aggregation states of engineered anti-OX40 SF2 antibodies in solution were evaluated by Size Exclusion Chromatography. Briefly, the antibodies were injected onto a TSKgel G3SW column (Tosoh Bioscience LLC) and their sizes were resolved by chromatography. The engineered antibodies with Fc mutations had a major protein peak eluted at about 8.5 minutes similar as the antibody with native IgG1 Fc, indicating a dominant monomer form of the engineered antibodies in solution. Some of the antibodies showed minor fractions (< 5%) of high molecular weight protein peaks which may be the oligomer forms of the antibodies.

To evaluate whether the engineered antibodies multimerize upon binding antigens at the cell surface, NanoBRET protein-protein interaction (PPI) assay (Promega, Madison, WI) was performed on engineered anti-OX40 SF2 antibody. SF2 antibodies with native IgG1 and with T437R, T437R/K248E or E345R mutations were further engineered to have either the Nanoluc tag or the Halotag attached at the C-terminus of light chain as the donor and acceptor respectively. NanoBRET PPI assays were performed by applying the donor and acceptor antibodies to HEK-Blue™ cells stably

expressing OX40. The calculated corrected NanoBRET ratios reflect the association of multimerized antibody.

The tagged antibodies showed comparable functional activities as the corresponding un-tagged antibodies in the reporter assay, indicating that the tags at the light chains did not affect the functional properties of the antibodies. In NanoBRET PPI assay, OX40SF2IgG1 and OX40SF2IgG1T437R showed background corrected NanoBRET ratio (**Figure 5**). In contrast, the SF2 antibodies with either OX40SF2IgG1T437R/K248E showed much higher corrected NanoBRET ratio across concentrations from 10 ng/mL to 1000 ng/mL **(Figure 5)**, indicating that the antibody is multimerized at the cell surface.

Antibody binding to various FcγR

The binding of engineered anti-OX40 SF2 antibodies to various FcγR receptors expressed on transiently-transfected Expi293F cells were assessed by flow cytometry as described in Example 3.

Neither mutation T437R nor K248E affected binding of the variant antibody to FcγRI or FcγRIIIA, as the monomeric OX40SF2IgG1T437R and OX40SF2IgG1T437R/K248E antibodies in solution bound these receptors with similar binding properties when compared to the wild type antibody OX40SF2IgG1. The antibodies also showed similar binding potencies to FcγRIIA and FcγRIIB when compared to OX40SF2IgG1. **Table 4** shows the EC_{50} values for the binding. The binding of engineered anti-OX40 SF2 antibodies to Raji cells, a B cell line expressing FcγRIIB, were also assessed by flow cytometry. Although the expression of FcγRIIB can be detected by FcγRIIB antibody 2B6, it was observed that neither the engineered anti-OX40(SF2) nor OX40SF2IgG1 antibodies had significant binding to Raji cells, probably due to lower expression of FcγRIIB in Raji cells compared to ectopically transfected cells (Data not shown).

Table 4.

Antibody	EC_{50} (ng/mL)			
	FcγRI	FcγRIIA	FcγRIIB	FcγRIIIA
OX40SF2IgG1T437R	240	4128	>10,000	1136

OX40SF2IgG1T437R/K248E	248	3394	>10,000	1558
OX40SF2IgG1	326	4557	>10,000	744

Example 5. Characterization of anti-OX40 antibodies engineered into effector silent forms

Various antibodies were cloned onto effector silent Fc isoform, expressed, purified and characterized according to methods described in Example 3 and characterized. The generated antibodies are shown in **Table 5**.

Table 5.

Antibody name	Isotype	Fc mutation
OX40SF2IgG2sigma	IgG2	V234A, G237A, P238S, H268A, V309L, A330S, P331S
OX40SF2IgG2sigmaT437R/K248E	IgG2	V234A, G237A, P238S, H268A, V309L, A330S, P331S, T437R, K248E
OX40SF2IgG4S228PAA	IgG4	S228P, F234A, L235A
OX40SF2IgG4PAA/T437R/K248E	IgG4	S228P, F234A, L235A, T437R, K248E

Antibody aggregation

The aggregation states of the engineered antibodies in solution were evaluated by Size Exclusion Chromatography as described in Example 3. The engineered antibodies had a major protein peak eluted at about 8.5 minutes similar as the corresponding antibodies with native IgG1 Fc, indicating a dominant monomer form of the engineered antibodies in solution. Some of them, largely the IgG4PAA antibodies, showed minor fractions (<3%) of high molecular weight protein peaks which may be the oligomer forms of the antibodies.

T437R/K248E mutation rescues agonism in Fc effector function silent antibodies

The agonistic activity of OX40SF2IgG2sigma, OX40SF2IgG2sigmaT437R/K248E, OX40SF2IgG4PAA and OX40SF2IgG4PAA/T437R/K248E were evaluated using the HEK-Blue™ NFκB reporter assay either in solution or cross-linked with Raji cells. Neither OX40SF2IgG2sigma nor OX40SF2IgG4PAA had agonistic activity in solution, whereas T437R/K248E mutation rescued agonism in both OX40SF2IgG2sigmaT437R/K248E and OX40SF2IgG4PAA/T437R/K248E (**Figure 6**). Cross-linking with Raji cells was at best marginal at boosting agonistic activities (Data not shown).

Example 6. Effector functions of the engineered antibodies

The ability of the engineered anti-OX40 antibodies with T437R or T437R/K248E mutations to mediate ADCC, ADCP and CDC was evaluated as described in Example 3.

Both OX40SF2IgG1T437R and OX40SF2IgG1T437R/K248E mediated ADCC with improved potency when compared to the wild-type antibody OX40SF2IgG1 (**Figure 7**). The mutations did not rescue ADCC in already effector silent antibody, as both OX40SF2IgG2sigmaT437R/K248E and OX40SF2IgG4PAA/T437R/K248E remained unable to mediate ADCC (data not shown).

Both OX40SF2IgG1T437R and OX40SF2IgG1T437R/K248E mediated ADCP at comparable levels to that of mediated by the wild-type OX40SF2IgG1 (**Figure 8**). OX40SF2IgG1T437R and OX40SF2IgG1T437R/K248E also mediated CDC with improved potency when compared to the wild-type antibody OX40SF2IgG1 (**Figure 9A**). The mutations partially rescued ADCP in already effector silent antibody, as ADCP activity for OX40SF2IgG2sigmaT437R/K248E was elevated at higher concentrations relative to that observed for OX40FS2IgG2sigma (**Figure 9B**).

Example 7. Pharmacokinetic properties of antibodies with T437R and T437R/K248E mutations in Tg32 hemizygous mice

Methods Eighteen Tg32 hemizygous mice (Jackson Labs) were injected with test antibody (OX40SF2IgG1T437R, OX40SF2IgG1T437R/K248E or OX40SF2IgG1) intravenously via tail vein at a dose of 2 mg/kg into 5 animals per group. Time points were taken after injection at 1h, 1d, 3d, 7d, 14d and 21d. Serial retro-orbital bleeds were

obtained from CO_2-anesthesized mice at the indicated time points and terminal bleeds were taken by cardiac puncture. After 30 minutes at room temperature, blood samples were centrifuged at 2500 rpm for 15 minutes and serum collected for analyses.

Concentrations of human IgG in serum samples were determined by an electrochemiluminescent immunoassay. Streptavidin Gold multiarray 96-well plates (Meso Scale Discovery) were coated with 50 µL/well of 5 µg/mL biotinylated goat anti-human IgG (Jackson ImmunoResearch #109-055-088) overnight at 4°C, and washed with Tris-buffered saline with 0.05% Tween. Serum samples and standards were diluted in Starting Block (Thermo Scientific), added to plates and incubated for 2 hours on a shaker at room temperature. Bound antibody was detected using a Sulfo-TAG labeled mouse anti-human Fc antibody, R10Z8E9, at 1.5 µg/mL for 2 hr on a shaker. Plates were washed, Read buffer T (Meso Scale Discovery) was added and plates were read on the MSD Sector Imager 6000.

To determine whether the PK serum samples had notable immune titers that could affect the PK of the test samples, an ELISA was performed on 96 well plates (Nunc Maxisorb #446612) coated with the respective test article at 10 µg/mL, 4°C overnight. Serum samples were diluted in 1% BSA-PBS and incubated on the plates. Horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) was used to detect captured antibody; followed by OPD or TMB addition for substrate development. Plates were read and spectrophotometer readings that were three times greater than buffer or control sera values were considered positive, and expressed as 1:1 fold-dilution.

Terminal half-life ($t_{1/2}$) calculations of the elimination phase for PK studies were determined using the 1-phase exponential decay model fitted by linear regression of natural log concentration versus time using Prism version 6.02 software (GraphPad Software, Inc). The least squares nonlinear decay model was weighted by 1/fitted concentration. Half-life calculations of the elimination phase were determined using the formula $t_{1/2}= \ln 2/\beta$, where β is the –slope of the line fitted by the least square regression analysis starting after first dose. The terminal half-life value for an antibody was determined by taking the average of the $t_{1/2}$ values calculated for each animal within the test group.

Results

The serum IgG concentration versus time profiles of the antibodies show a decline over time on a semi-log plot (**Figure 10**). The immune responses of the animals were tested. Mice dosed with OX40SF2IgG1T437R/K248E showed significant immune titers (1:1000 – 1:14,000) at 7 d, 14 d and 21 d.

Serum levels were normalized to the first time point of the linear phase of the curve to highlight differences among the PK profiles of the antibodies. The first time point of 1h indicated that the mice were fully dosed. Only animals with three or more data points were used, and values from animals at day 7, 14, or 21 with immune titers were excluded.

Results showed that the antibodies containing the T437R mutation had a shorter half-life compared to the half-life of the wild-type antibody. Half-life values for the various antibodies were: OX40SF2IgG1T437R with $t_{1/2}$ = 3.9 $\pm$ 2.1 d; OX40SF2IgG1 T437RK248E with $t_{1/2}$ =2.4 $\pm$ 0.8 d, and OX40SF2IgG1 with $t_{1/2}$ = 11.3 $\pm$ 1.1 d.

In summary, the PK study indicated that OX40SF2IgG1T437and OX40SF2IgG1T437R/K248E half-life values were 3-4 fold shorter when compared to that of the wild-type IgG1 antibody with a half-life of 11 days, which is within the normal range for mouse studies. However, the interpretation of the PK results was compounded by the immune responses in test animals that significantly affected the serum IgG levels, especially the group for OX40SF2IgG1T437R/K248E. Since any immune response observed in these mice do not correspond to what is expected in humans, the shorter half-life values observed in mice may not reflect normal human IgG circulating serum half-life.

Example 8. Structural rationalization of the effect of K248E and T437R mutations on antibody multimerization and agonism in the context of the IgG2 and IgG4 isotypes

The sequence differences between IgG1 and IgG2, IgG1 and IgG3, and between IgG1 and IgG4 were mapped onto crystal structures of IgG2 Fc and IgG4 Fc, respectively, aligned to the multimeric model via their CH3 domains. Positions of sequence difference were identified to be structurally remote from the positions of both K248 and T437. Similar to IgG1, alignment of IgG2 and IgG4 Fc structures in the manner above described resulted in a clash between CH2 domains of juxtaposed Fc domains suggesting that these domains would have to reorient relative to their observed conformations in order for Fc domains to pack as in the multimeric model. For IgG1, it was hypothesized that disruption of an intramolecular salt bridge interaction between K248 in the CH2 domain and E380 in

the CH3 domain with a K248E mutant might weaken the CH2:CH3 interface facilitating reorientation of the CH2 domain and multimerization. This salt bridge interaction is conserved in structures of IgG2 and IgG4; therefore, introduction of the K248E mutation would be hypothesized to function similarly as in IgG1. Furthermore, as residues at the inter-Fc CH3:CH3 interface in the multimeric model are conserved among IgG1, IgG2, IgG3 and IgG4, a T437R mutation introduced into either IgG2, IgG3 or IgG4 is hypothesized to strengthen this interface by forming a salt bridge interaction with E382 in a neighboring Fc as was originally hypothesized for IgG1. Taken together, that the K248E and T437R mutations are found experimentally to multimerize and enhance agonism of anti-TNFR antibodies independent of the IgG subtype (IgG1, IgG2, IgG3 or IgG4) is consistent with observations made from structural modelling. Although these mutations are in the vicinity of the Fc-FcRn binding interface, these residues do not directly contact FcRn (Martin et al. (2001) *Mol Cell* 7: 867-77).

Example 9. Pharmacokinetic properties of antibodies with T437R/K248E mutations in FcRn transgenic SCID mice

Methods For the antibody PK studies, female Tg32 homozygous SCID mice, 4-8 weeks old (Stock 018441, Jackson Laboratory) were injected with test antibodies intravenously via tail vein at a dose of 2 mg/kg into 5 animals per group. Serial retro-orbital bleeds were obtained from CO_2-anesthesized mice at the indicated time points and terminal bleeds were taken by cardiac puncture. After 30 min at room temperature, blood samples were centrifuged 3,000 x g for 15 min and serum collected for analyses. The PK study was approved by the Institutional Animal Care and Use Committee at Janssen Research & Development, LLC.

Human IgG in mouse sera was determined using an electrochemiluminescent immunoassay. Streptavidin Gold 96 well plates (Meso Scale Diagnostics) were coated with 50 µL/well of 2.5 µg/mL biotinylated goat anti human IgG $F(ab')_2$ antibody (Jackson Immunoresearch Laboratories) in Starting Block T20 (Thermo Scientific) overnight at 4°C. Plates were washed with Tris-buffered saline with 0.5% Tween 20 (TBST); samples and standards diluted in 2% bovine serum albumin-TBST were added to plates and incubated for 2 h on a shaker at room temperature. Bound antibody was detected using Sulfo-TAG labeled R10Z8E9, an anti-human Fcγ-pan antibody. Plates were washed and

200 µL MSD Read Buffer was added and plates read on the MSD Sector Imager 6000 (Meso Scale Diagnostics). Serum concentrations of the Abs were determined from a standard curve using a 5-parameter non-linear regression program in GraphPad Prism 6 (GraphPad Software).

Terminal half-life ($t_{1/2}$) calculations of the elimination phase (β phase) for PK studies were determined using the 1-phase exponential decay model fitted by a non-linear regression of natural log concentration versus time using GraphPad Prism 6 (GraphPad Software). Half-life of the elimination phase (β phase) was calculated using the formula $t_{1/2} = \ln 2/\beta$, where β is the negative slope of the line fitted by the least square regression analysis starting after day 1. The terminal antibody half-life value was the average of the $t_{1/2}$ values within the test group. Values for each antibody vs IgG1 were compared by T-tests, and a p value <0.05 indicated a significant difference.

Results

The second PK study was performed to evaluate the OX40SF2IgG1T437R and OX40SF2IgG1T437R/K248E in FcRn transgenic SCID (severe combined immunodeficient) mice, which are deficient in functional B and T lymphocytes and hence have minimal immune responses to test antibodies. Tg32 homozygous SCID mice (5 mice/group) were injected intravenously with a 2 mg/kg dose of antibodies. Serial retro-orbital bleeds from each animal were obtained at 1h, and 1, 3, 7, 14 and 21 days after injection. Sera were prepared and the amounts of human IgG were determined by an electrochemiluminescent immunoassay. Mean serum concentrations for each antibody were shown in **Figure 11**. For all samples, there was a linear decline of serum concentration over the course of 21 days and no significant differences (p <0.05) were observed among the test groups. Half-life values, $t_{1/2}$, were estimated as follows: OX40SF2IgG1T437R, $t_{1/2} = 9.5 \pm 0.7$ d; OX40SF2IgG1T437R/K248E, $t_{1/2} = 8.3 \pm 0.5$ d; OX40SF2IgG1, $t_{1/2} = 9.2 \pm 0.6$ d. These data revealed comparable PK profiles of the engineered antibodies to that with native IgG1 Fc.

In this PK study, the use of SCID mice significantly reduced the mouse immune responses to test antibodies which compounded the results in previous PK study using non-SCID mice. Therefore, the comparable PK profiles observed in SCID mice would reflect normal human IgG circulating serum half-life

Example 10. Binding of the engineered antibodies to FcRn

Methods In vitro assay of competitive binding to FcRn

A competitive binding assay was used to assess relative affinities of different antibody samples to a recombinant human FcRn extracellular domain with a poly-histidine affinity tag (FcRn-His6). Ninety-six-well copper-coated plates (Thermo Scientific) were used to capture FcRn-His6 at 5 µg/mL in PBS, after which plates were washed with 0.15M NaCl, 0.02% Tween 20, and then incubated with blocking reagent (0.05M MES [2-(*N*-morpholino) ethanesulfonic acid], 0.025% bovine serum albumin (BSA), 0.001% Tween-20, pH 6.0, 10% chemiBLOCKER (Millipore)). Plates were washed as above, and then serial dilutions of competitor test antibodies in blocking reagent were added to the plate in the presence of a fixed 1 µg/mL concentration of an indicator antibody (a biotinylated human IgG1 monoclonal antibody). Plates were incubated at room temperature for 1 hour, washed three times as above, and then incubated with a 1: 10,000 dilution of streptavidin-horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories) at room temperature for 30 minutes to bind biotinylated antibody. Plates were washed five times as above, and bound streptavidin-HRP detected by adding TMB (3,3′,5,5′-tetramethylbenzidine) peroxidase substrate with stable stop (Fitzgerald Industries International) and incubating for 4 minutes. Color development was stopped by addition of 0.5 M HCl. Optical densities were determined with a SpectraMax Plus384 plate reader (Molecular Devices) at 450 nm wavelength.

Results

Since the T437R and K248E mutations are both located close to the known binding sites to FcRn receptor, possible interference from these mutations with the proper interaction of our variant antibodies with FcRn were evaluated using an *in vitro* competitive binding assay. Both OX40SF2IgG1T437R/K248E and OX40SF2IgG1T437R exhibited apparent similar potencies (IC50: 1.6 □g/ml and 1.1 □g/ml respectively) in competing biotinylated human IgG1 for FcRn binding relative to OX40SF2IgG1 (IC50: 1.5 □g/ml), indicating little to no apparent impact of the engineered mutations on FcRn binding. This result, in the absence of other potential PK-impacting influences, predicted

that the T437R and K248E mutations would not have a significant impact on serum half-life.

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We claim:

1. An isolated engineered anti-tumor necrosis factor receptor (TNFR) superfamily member antibody, wherein the antibody comprises a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation when compared to a parental wild-type antibody, residue numbering according to the EU Index.
2. The antibody of claim 1, wherein the antibody has enhanced agonistic activity when compared to the parental wild-type antibody.
3. The antibody of claim 2, comprising the T437R mutation.
4. The antibody of claim 3, comprising the T437R/K248E mutation.
5. The antibody of claim 4, comprising the T437R/K338A mutation.
6. The antibody of any one of claims 1-5, wherein the antibody is an IgG1, IgG2, IgG3 or IgG4 isotype.
7. The antibody of any one of claims 1-6, wherein the antibody mediates antibody-dependent cellular cytotoxicity (ADCC).
8. The antibody of any one of claims 1-7, wherein the antibody mediates antibody-dependent cell phagocytosis (ADCP).
9. The antibody of any one of claims 1-8, wherein the antibody mediates complement-dependent cytotoxicity (CDC).
10. The antibody of any one of claims 1-9, wherein the antibody further comprises a second mutation.
11. The antibody of claim 10, wherein the second mutation is a L234A/L235A mutation on IgG1, a V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation on IgG2, a F234A/L235A mutation on IgG4, a S228P/F234A/L235A mutation on IgG4, a N297A mutation on IgG1, IgG2, IgG3 or IgG4, a V234A/G237A mutation on IgG2, a K214T/E233P/L234V/L235A/G236-deleted/A327G/P331A/D365E/L358M mutation on IgG1, a H268Q/V309L/A330S/P331S mutation on IgG2, a L234F/L235E/D265A mutation on IgG1, a L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation on IgG1, a S228P/F234A/L235A/G237A/P238S mutation on IgG4, or a S228P/F234A/L235A/G236-deleted/G237A/P238S mutation on IgG4.

12. The antibody of claim 11, wherein the second mutation is the V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation on IgG2.
13. The antibody of claim 11, wherein the second mutation is the L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation on IgG1.
14. The antibody of claim 11, wherein the second mutation is the S228P/F234A/L235A mutation on IgG4.
15. The antibody of any one of claims 1-14, wherein the TNFR superfamily member is OX40 (SEQ ID NO: 4), CD27 (SEQ ID NO: 8), CD40 (SEQ ID NO: 5), CD137 (SEQ ID NO: 10), or GITR (SEQ ID NO: 23).
16. The antibody of any one of claims 1-15, wherein the antibody has agonistic activity independent of antibody cross-linking.
17. The antibody of any one of claims 1-16, wherein the antibody comprises a heavy chain constant region of SEQ ID NOs: 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73 or 74.
18. A pharmaceutical composition comprising the antibody of any one of claims 1-17 and a pharmaceutically acceptable carrier.

19. A method of enhancing an agonistic activity of an anti-TNFR superfamily member antibody in a subject, comprising providing the anti-TNFR superfamily member antibody, introducing aT437R mutation, a K248E mutation, a K338A mutation, a T437R/K248E mutation or a T437R/K338A mutation into the antibody to generate an engineered antibody specifically binding the TNFR superfamily member, and administering the engineered antibody to the subject.
20. The method of claim 19, wherein the antibody mediates ADCC, ADCP or CDC.
21. The method of claim 19 or 20, wherein the antibody further comprises a second mutation.
22. The method of claim 21, wherein the second mutation is a L234A/L235A mutation on IgG1, a V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation on IgG2, a F234A/L235A mutation on IgG4, a S228P/F234A/L235A mutation on IgG4, a N297A mutation on IgG1, IgG2, IgG3 or IgG4, a V234A/G237A mutation on IgG2, a K214T/E233P/L234V/L235A/G236-deleted/A327G/P331A/D365E/L358M mutation on IgG1, a

H268Q/V309L/A330S/P331S mutation on IgG2, a L234F/L235E/D265A mutation on IgG1, a L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation on IgG1, a S228P/F234A/L235A/G237A/P238S mutation on IgG4, or a S228P/F234A/L235A/G236-deleted/G237A/P238S mutation on IgG4.

23. The method of claim 21, wherein the second mutation is the V234A/G237A/P238S/H268A/V309L/A330S/P331S mutation on IgG2.
24. The method of claim 21, wherein the second mutation is the L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation on IgG1
25. The method of claim 21, wherein the second mutation is the S228P/F234A/L235A mutation on IgG4.
26. The method of any one of claims 19-25, wherein the antibody comprises the heavy chain constant region of SEQ ID NOs: 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73 or 74.
27. The method of any one of claims 19-26, wherein the subject has a cancer.
28. The method of claim 27, wherein the cancer is a solid tumor.
29. The method of claim 28, wherein the solid tumor is a melanoma, a lung cancer, a squamous non-small cell lung cancer (NSCLC), a non-squamous NSCLC, a colorectal cancer, a prostate cancer, a castration-resistant prostate cancer, a stomach cancer, an ovarian cancer, a gastric cancer, a liver cancer, a pancreatic cancer, a thyroid cancer, a squamous cell carcinoma of the head and neck, a carcinoma of the esophagus or gastrointestinal tract, a breast cancer, a fallopian tube cancer, a brain cancer, an urethral cancer, a genitourinary cancer, an endometriosis, a cervical cancer or a metastatic lesion of the cancer.
30. The method of any one of claims 19-29, wherein the TNFR superfamily member is OX40 (SEQ ID NO: 4), CD27 (SEQ ID NO: 8), CD40 (SEQ ID NO: 5), CD137 (SEQ ID NO: 10), or GITR (SEQ ID NO: 23).

31. A method of treating a cancer in a subject, comprising administering to the subject an antibody specifically binding a TNFR superfamily member comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation when compared to a parental wild-type antibody, residue numbering according to the EU Index, for a time sufficient to treat the cancer.

32. The method of claim 31, wherein the antibody comprises the T347R mutation.
33. The method of claim 31, wherein the antibody comprises the T437R/K248E mutation.
34. The antibody of claim 31, wherein the antibody comprises the T437R/K338A mutation.
35. The method of any one of claims 31-34, wherein the antibody has enhanced agonistic activity when compared to the parental antibody.
36. The method of any one of claims 31-35, wherein the antibody further comprises a second mutation.
37. The method of claim 36, wherein the second mutation is a L234A/L235A mutation on IgG1, a V234A/G237A/ P238S/H268A/V309L/A330S/P331S mutation on IgG2, a F234A/L235A mutation on IgG4, a S228P/F234A/ L235A mutation on IgG4, a N297A mutation on IgG1, IgG2, IgG3 or IgG4, a V234A/G237A mutation on IgG2, a K214T/E233P/ L234V/L235A/G236-deleted/A327G/P331A/D365E/L358M mutation on IgG1, a H268Q/V309L/A330S/P331S mutation on IgG2, a L234F/L235E/D265A mutation on IgG1, a L234A/L235A/G237A/P238S/H268A/A330S/P331S mutation on IgG1, a S228P/F234A/L235A/G237A/P238S mutation on IgG4, or a S228P/F234A/L235A/G236-deleted/G237A/P238S mutation on IgG4.
38. The method of any one of claims 31-36, wherein the antibody comprises the heavy chain constant region of SEQ ID NOs: 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73 or 74.
39. The method of any one of claims 31-38, wherein the cancer is a melanoma, a lung cancer, a squamous non-small cell lung cancer (NSCLC), a non-squamous NSCLC, a colorectal cancer, a prostate cancer, a castration-resistant prostate cancer, a stomach cancer, an ovarian cancer, a gastric cancer, a liver cancer, a pancreatic cancer, a thyroid cancer, a squamous cell carcinoma of the head and neck, a carcinoma of the esophagus or gastrointestinal tract, a breast cancer, a fallopian tube cancer, a brain cancer, an urethral cancer, a genitourinary cancer, an endometriosis, a cervical cancer or a metastatic lesion of the cancer.

40. The method of any one of claims 31-39, wherein the receptor of the TNFR family is OX40 (SEQ ID NO: 4), CD27 (SEQ ID NO: 8), CD40 (SEQ ID NO: 5), CD137 (SEQ ID NO: 10), or GITR (SEQ ID NO: 23).

41. An isolated Fc domain containing molecule comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation in the Fc domain.
42. The Fc domain containing molecule of claim 41, comprising the T437R mutation.
43. The Fc domain containing molecule of claim 41, comprising a T437R/K248E mutation.
44. The Fc domain containing molecule of claim 41, comprising a T437R/K338A mutation.
45. The Fc domain containing molecule of any one of claims 41-44, wherein the Fc domain is an IgG1, IgG2, IgG3 or IgG4 isotype.
46. The Fc domain containing molecule of any one of claims 41-45, wherein the Fc domain containing molecule is a monoclonal antibody.
47. The Fc domain containing molecule of any one of claims 41-46, comprising the amino acid sequence of SEQ ID NOs: 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73 or 74.
48. An isolated polynucleotide
    a. encoding the Fc domain containing molecule comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation in the Fc domain;
    b. encoding the Fc domain of SEQ ID NOs: 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73 or 74; or
    c. comprising the polynucleotide sequence of SEQ ID NOs: 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, or 86.
49. A vector comprising the polynucleotide of claim 48.
50. The vector of claim 49, which is an expression vector.
51. A host cell comprising the vector of claim 49 or 50.
52. A method of producing the Fc domain containing molecule of claim 41, comprising culturing the host cell of claim 51 in conditions wherein the Fc domain

containing molecule is expressed, and isolating the Fc domain containing molecule.

53. An isolated anti-tumor necrosis factor receptor (TNFR) superfamily member antibody comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation when compared to a parental wild-type antibody, residue numbering according to the EU Index, for use in the treatment of a cancer.
54. The antibody of claim 53 for use in the treatment of the cancer, wherein the cancer is a melanoma, a lung cancer, a squamous non-small cell lung cancer (NSCLC), a non-squamous NSCLC, a colorectal cancer, a prostate cancer, a castration-resistant prostate cancer, a stomach cancer, an ovarian cancer, a gastric cancer, a liver cancer, a pancreatic cancer, a thyroid cancer, a squamous cell carcinoma of the head and neck, a carcinoma of the esophagus or gastrointestinal tract, a breast cancer, a fallopian tube cancer, a brain cancer, an urethral cancer, a genitourinary cancer, an endometriosis, a cervical cancer or a metastatic lesion of the cancer.
55. Use of an isolated anti-tumor necrosis factor receptor (TNFR) superfamily member antibody comprising a T437R mutation, a T437R/K248E mutation or a T437R/K338A mutation, residue numbering according to the EU Index, in the manufacture of a medicament for the treatment of cancer.
56. The use of claim 35, wherein the cancer is a melanoma, a lung cancer, a squamous non-small cell lung cancer (NSCLC), a non-squamous NSCLC, a colorectal cancer, a prostate cancer, a castration-resistant prostate cancer, a stomach cancer, an ovarian cancer, a gastric cancer, a liver cancer, a pancreatic cancer, a thyroid cancer, a squamous cell carcinoma of the head and neck, a carcinoma of the esophagus or gastrointestinal tract, a breast cancer, a fallopian tube cancer, a brain cancer, an urethral cancer, a genitourinary cancer, an endometriosis, a cervical cancer or a metastatic lesion of the cancer.

Figure 1.

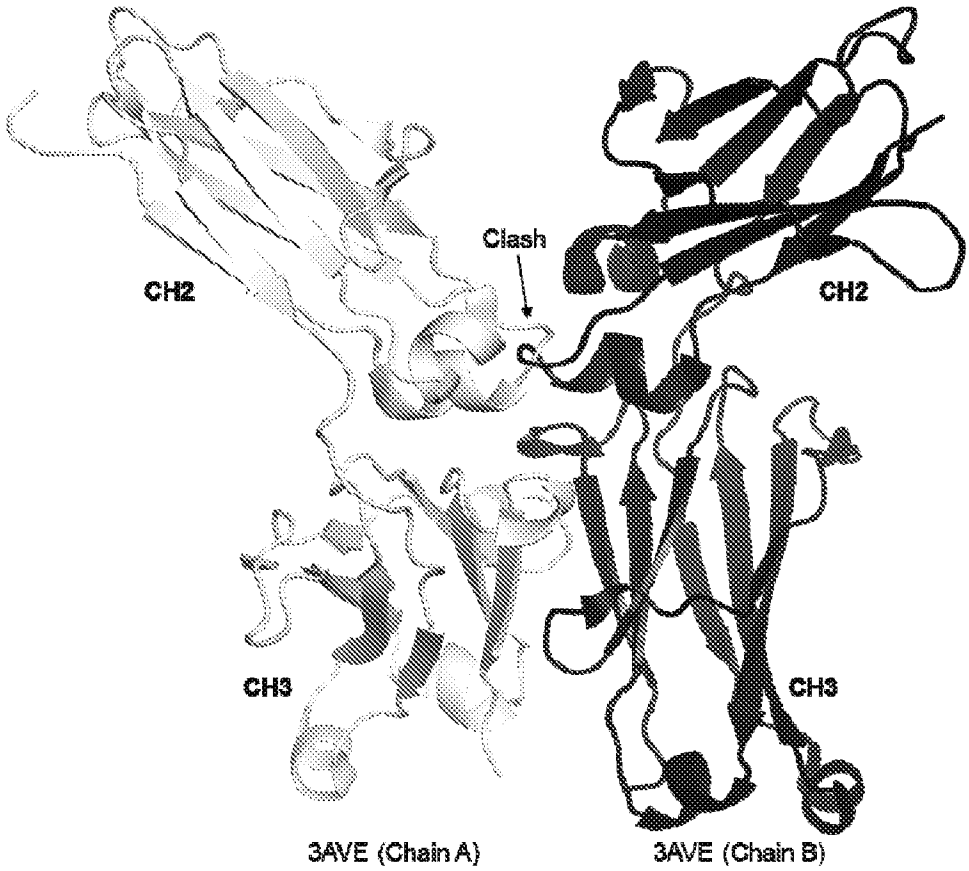


Figure 2A.

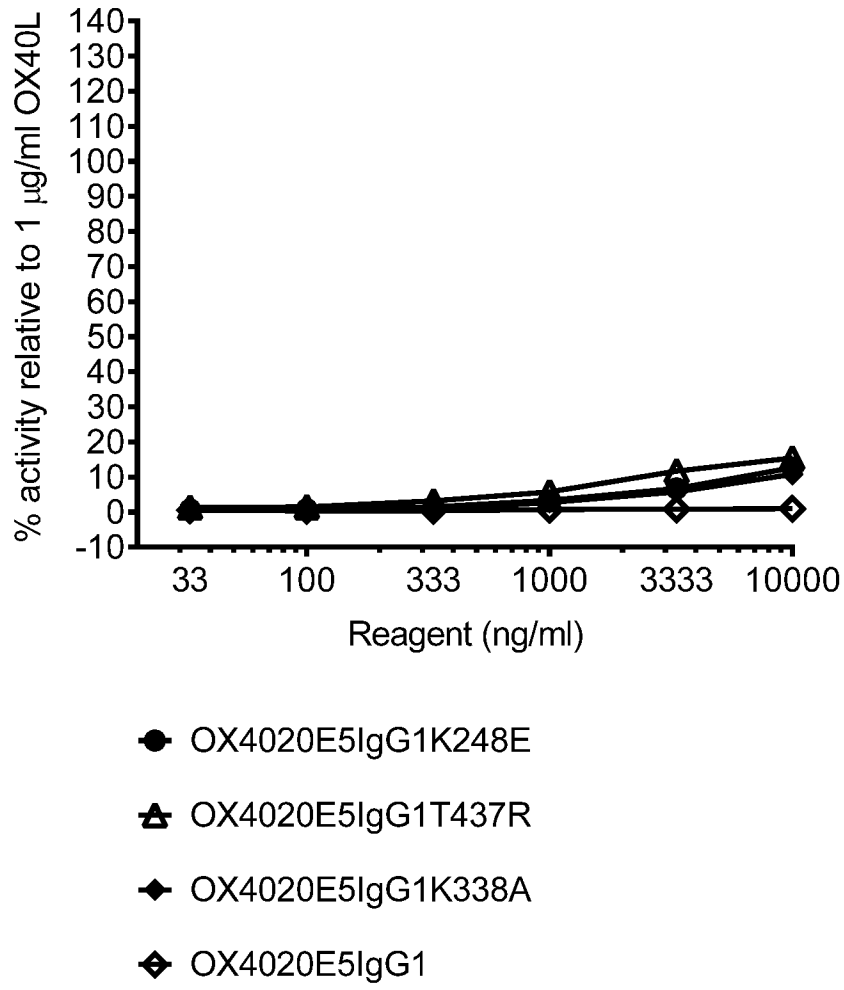

% activity relative to 1 μg/ml OX40L
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
33
100
333
1000
3333
10000
Reagent (ng/ml)
OX4020E5IgG1K248E
OX4020E5IgG1T437R
OX4020E5IgG1K338A
OX4020E5IgG1

Figure 2B.

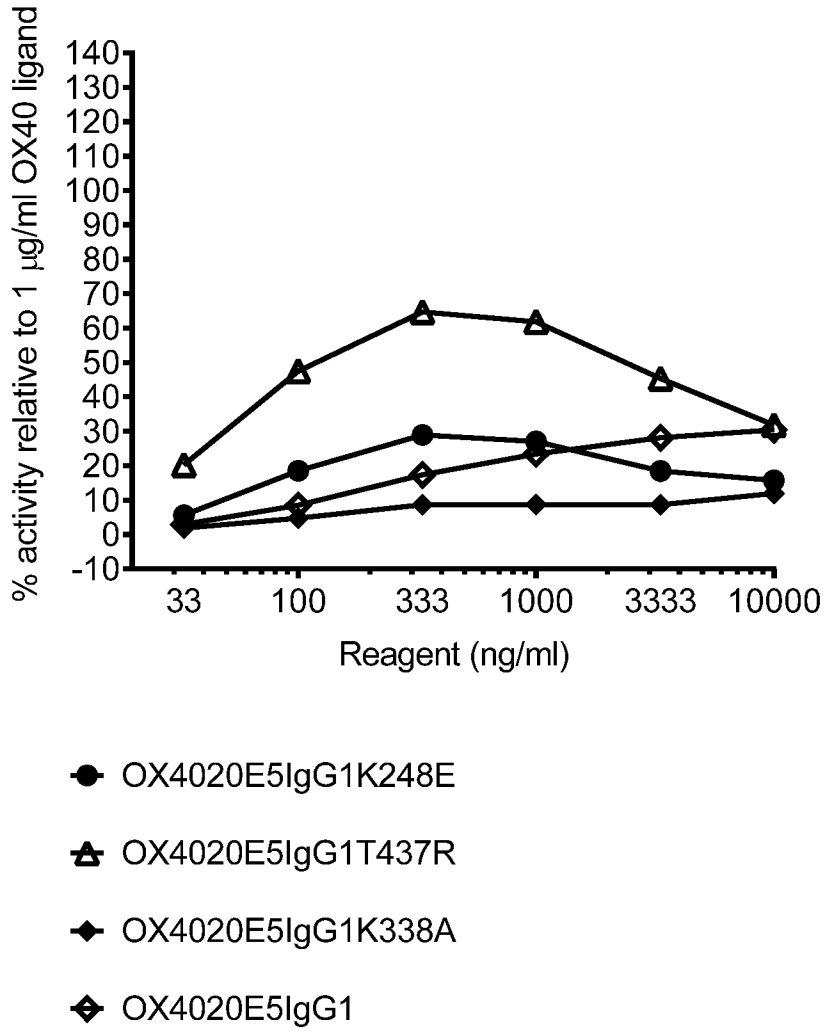

% activity relative to 1 μg/ml OX40 ligand
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
33
100
333
1000
3333
10000
Reagent (ng/ml)
OX4020E5IgG1K248E
OX4020E5IgG1T437R
OX4020E5IgG1K338A
OX4020E5IgG1

Figure 2C.

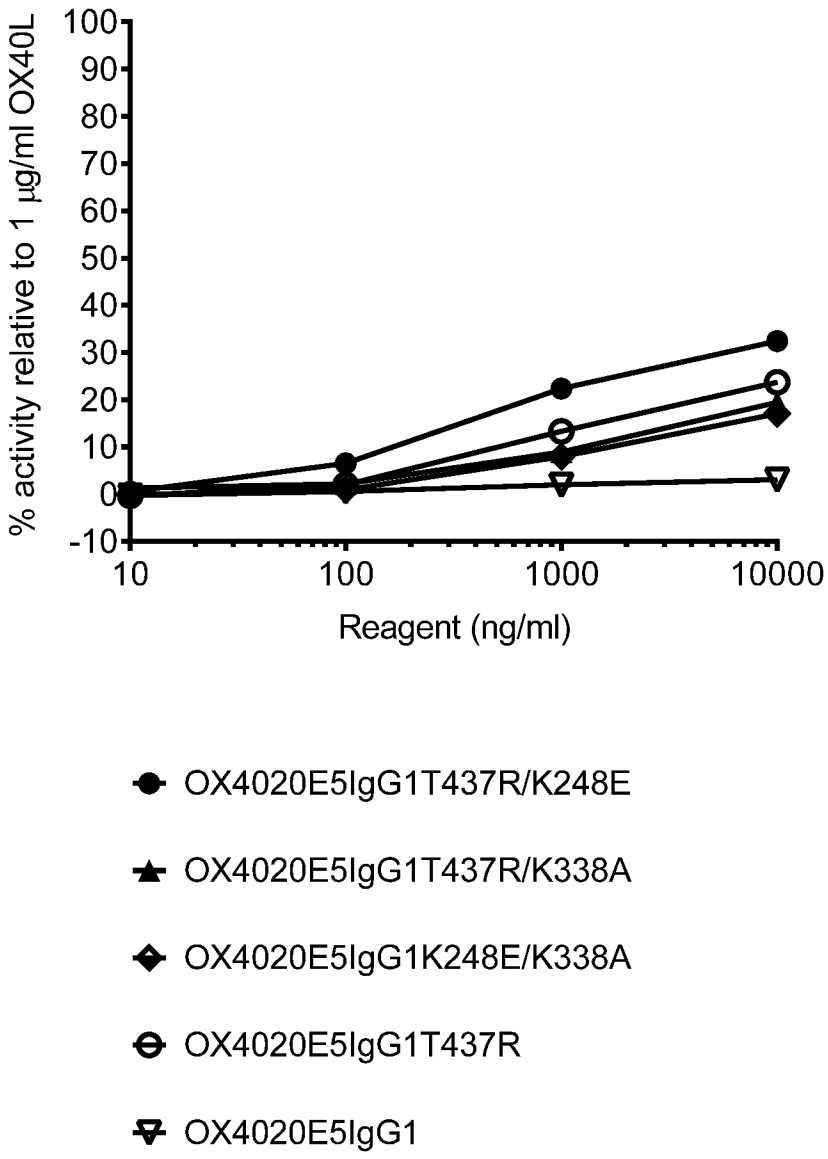

% activity relative to 1 µg/ml OX40L
100
90
80
70
60
50
40
30
20
10
0
-10
10
100
1000
10000
Reagent (ng/ml)
OX4020E5IgG1T437R/K248E
OX4020E5IgG1T437R/K338A
OX4020E5IgG1K248E/K338A
OX4020E5IgG1T437R
OX4020E5IgG1

Figure 2D.

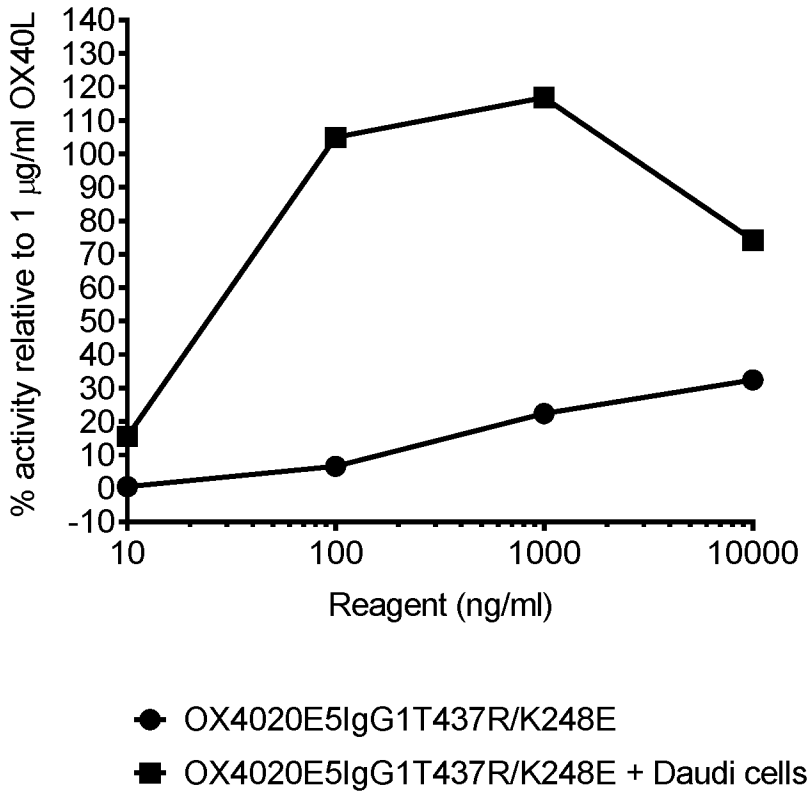

% activity relative to 1 μg/ml OX40L
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
10
100
1000
10000
Reagent (ng/ml)
OX4020E5IgG1T437R/K248E
OX4020E5IgG1T437R/K248E + Daudi cells

Figure 2E.

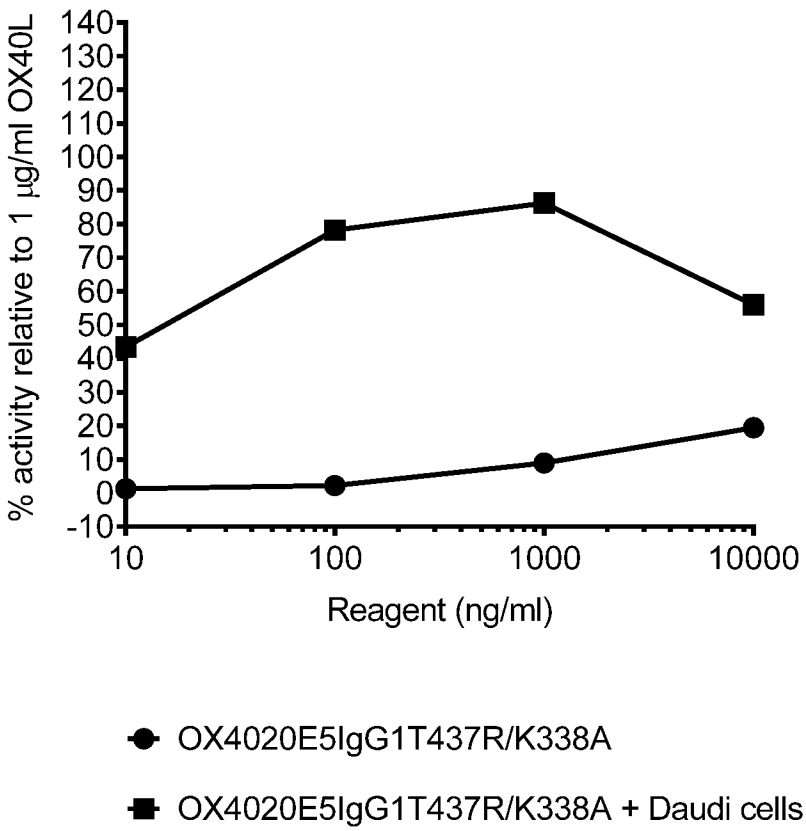

% activity relative to 1 µg/ml OX40L
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
10
100
1000
10000
Reagent (ng/ml)
OX4020E5IgG1T437R/K338A
OX4020E5IgG1T437R/K338A + Daudi cells

Figure 2F.

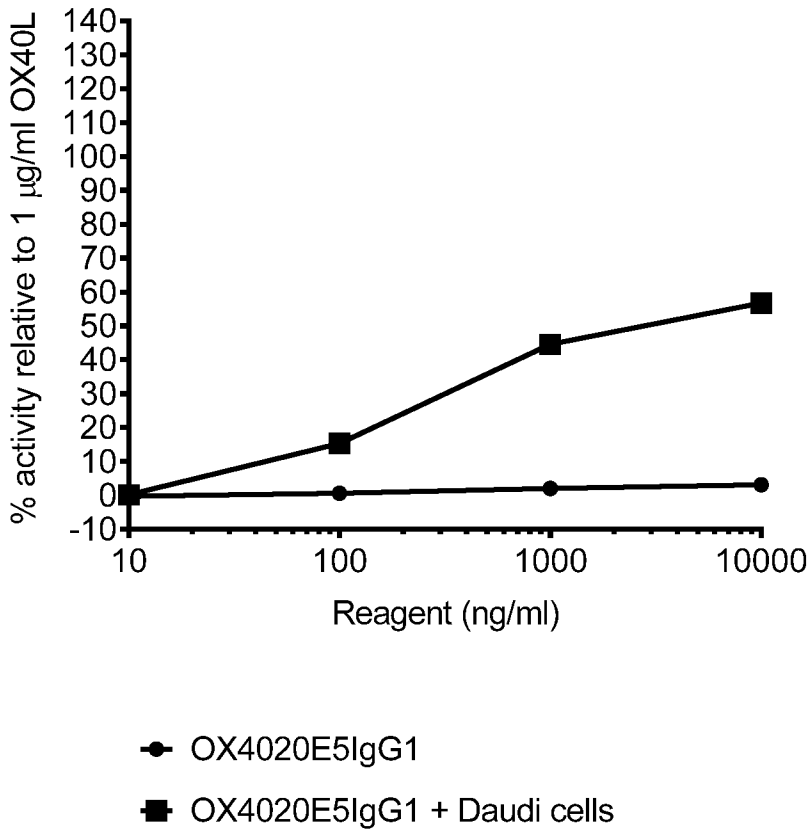

% activity relative to 1 μg/ml OX40L
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
10
100
1000
10000
Reagent (ng/ml)
OX4020E5IgG1
OX4020E5IgG1 + Daudi cells

Figure 2G.

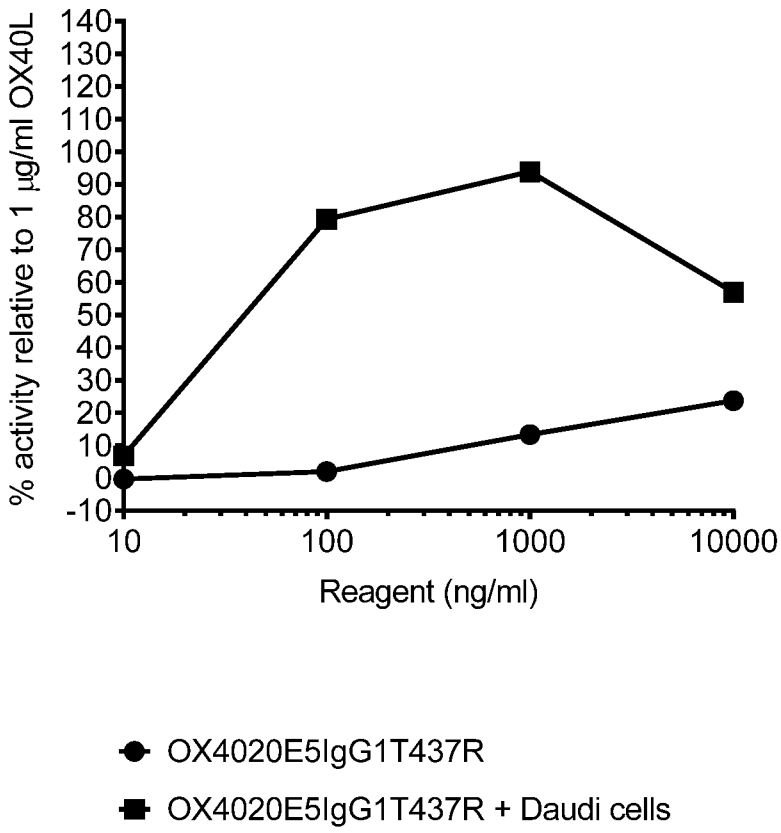

% activity relative to 1 μg/ml OX40L
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
10
100
1000
10000
Reagent (ng/ml)
OX4020E5IgG1T437R
OX4020E5IgG1T437R + Daudi cells

Figure 3A.

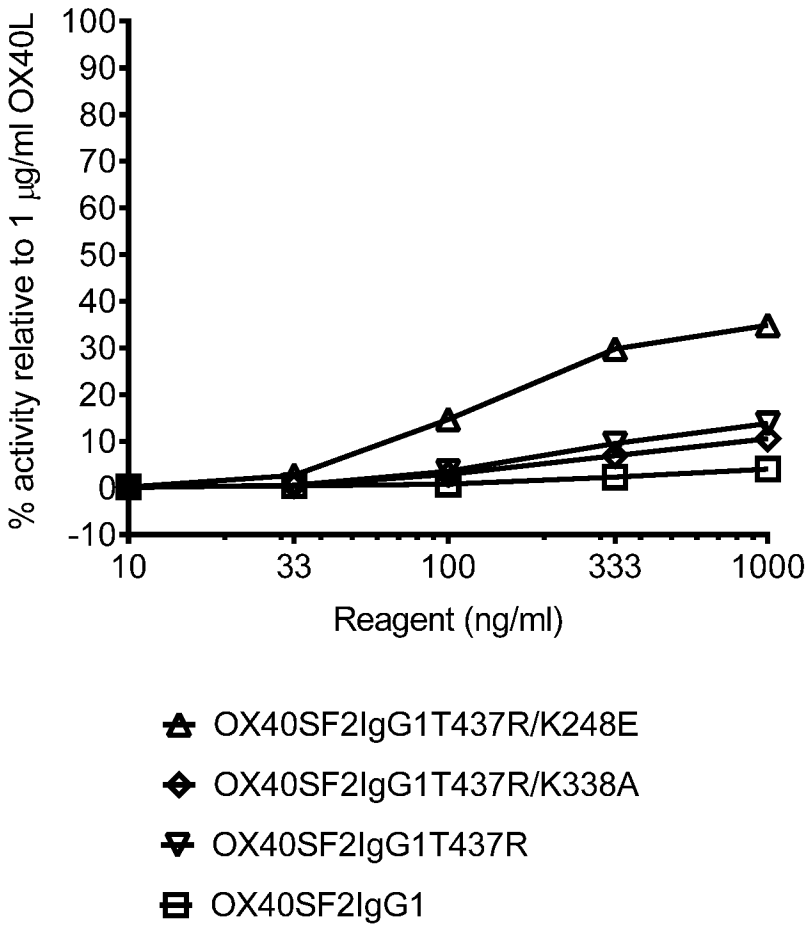

% activity relative to 1 μg/ml OX40L
100
90
80
70
60
50
40
30
20
10
0
-10
10
33
100
333
1000
Reagent (ng/ml)
OX40SF2IgG1T437R/K248E
OX40SF2IgG1T437R/K338A
OX40SF2IgG1T437R
OX40SF2IgG1

Figure 3B.

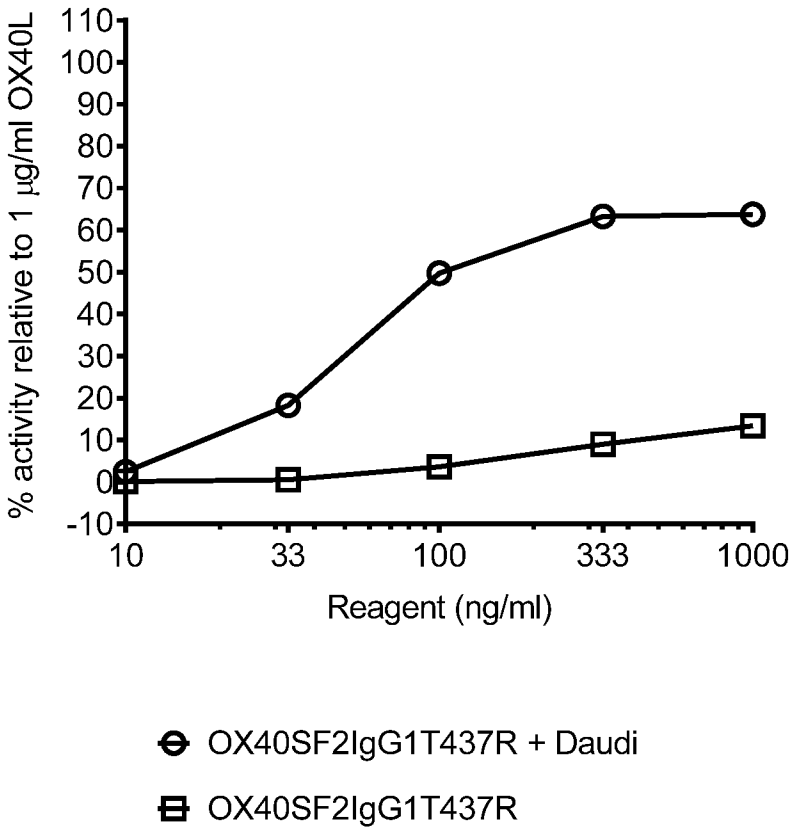

% activity relative to 1 μg/ml OX40L
110
100
90
80
70
60
50
40
30
20
10
0
-10
10
33
100
333
1000
Reagent (ng/ml)
OX40SF2IgG1T437R + Daudi
OX40SF2IgG1T437R

Figure 3C.

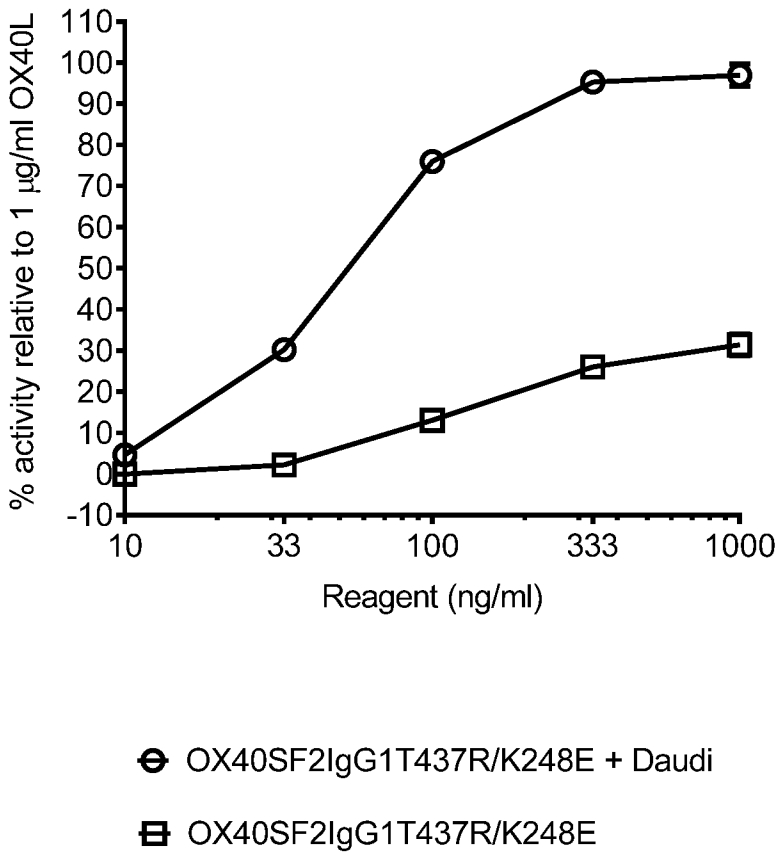

% activity relative to 1 µg/ml OX40L
110
100
90
80
70
60
50
40
30
20
10
0
-10
10
33
100
333
1000
Reagent (ng/ml)
OX40SF2IgG1T437R/K248E + Daudi
OX40SF2IgG1T437R/K248E

Figure 4A.

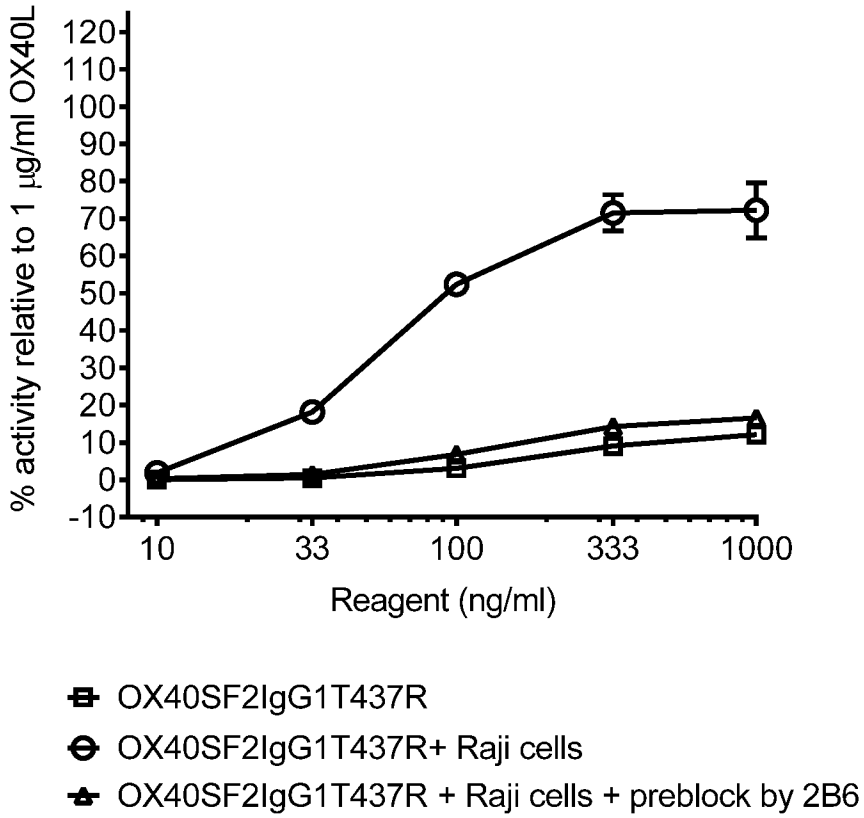

% activity relative to 1 μg/ml OX40L
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
10
33
100
333
1000
Reagent (ng/ml)
OX40SF2IgG1T437R
OX40SF2IgG1T437R+ Raji cells
OX40SF2IgG1T437R + Raji cells + preblock by 2B6

Figure 4B.

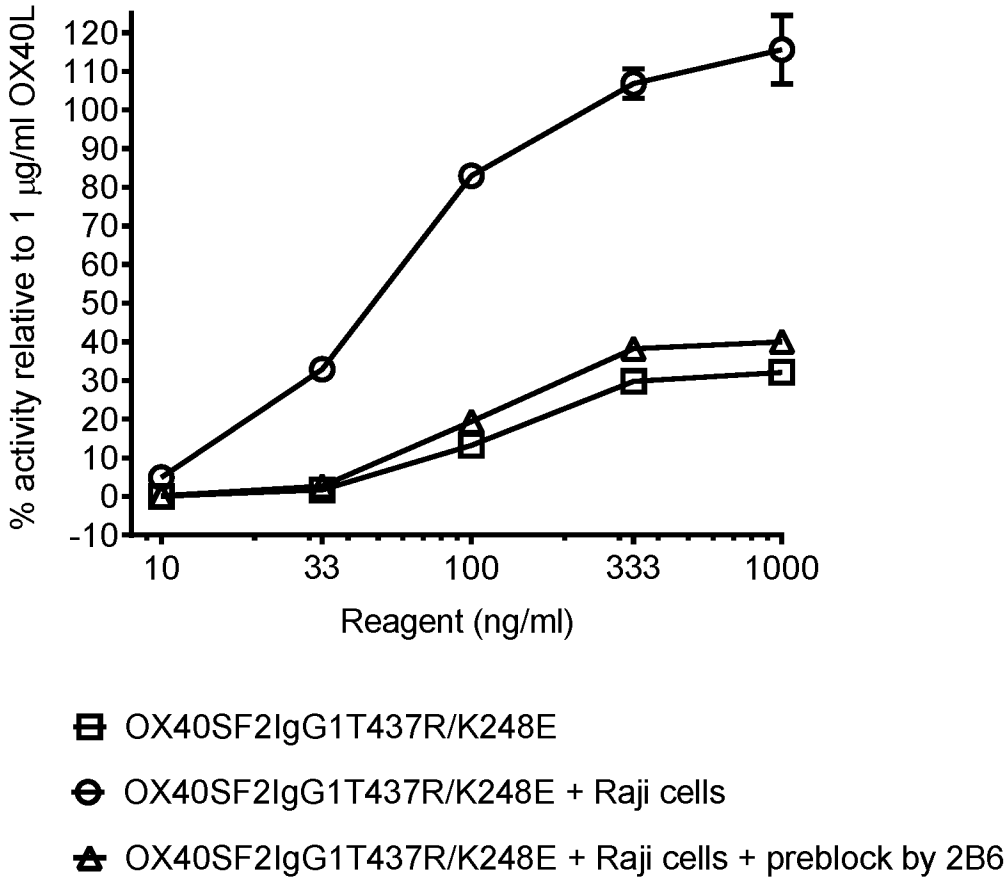

% activity relative to 1 µg/ml OX40L
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
10
33
100
333
1000
Reagent (ng/ml)
OX40SF2IgG1T437R/K248E
OX40SF2IgG1T437R/K248E + Raji cells
OX40SF2IgG1T437R/K248E + Raji cells + preblock by 2B6

Figure 5.

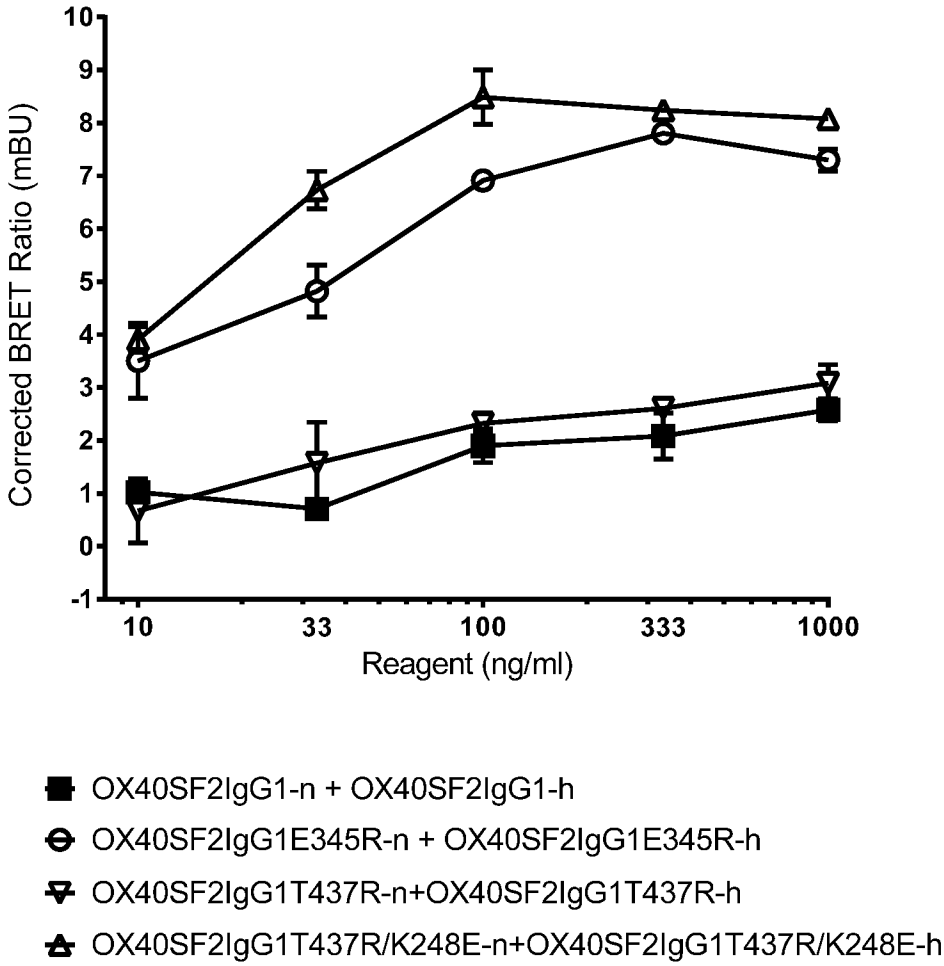

Corrected BRET Ratio (mBU)
10
9
8
7
6
5
4
3
2
1
0
-1
10
33
100
333
1000
Reagent (ng/ml)
OX40SF2IgG1-n + OX40SF2IgG1-h
OX40SF2IgG1E345R-n + OX40SF2IgG1E345R-h
OX40SF2IgG1T437R-n+OX40SF2IgG1T437R-h
OX40SF2IgG1T437R/K248E-n+OX40SF2IgG1T437R/K248E-h

Figure 6.

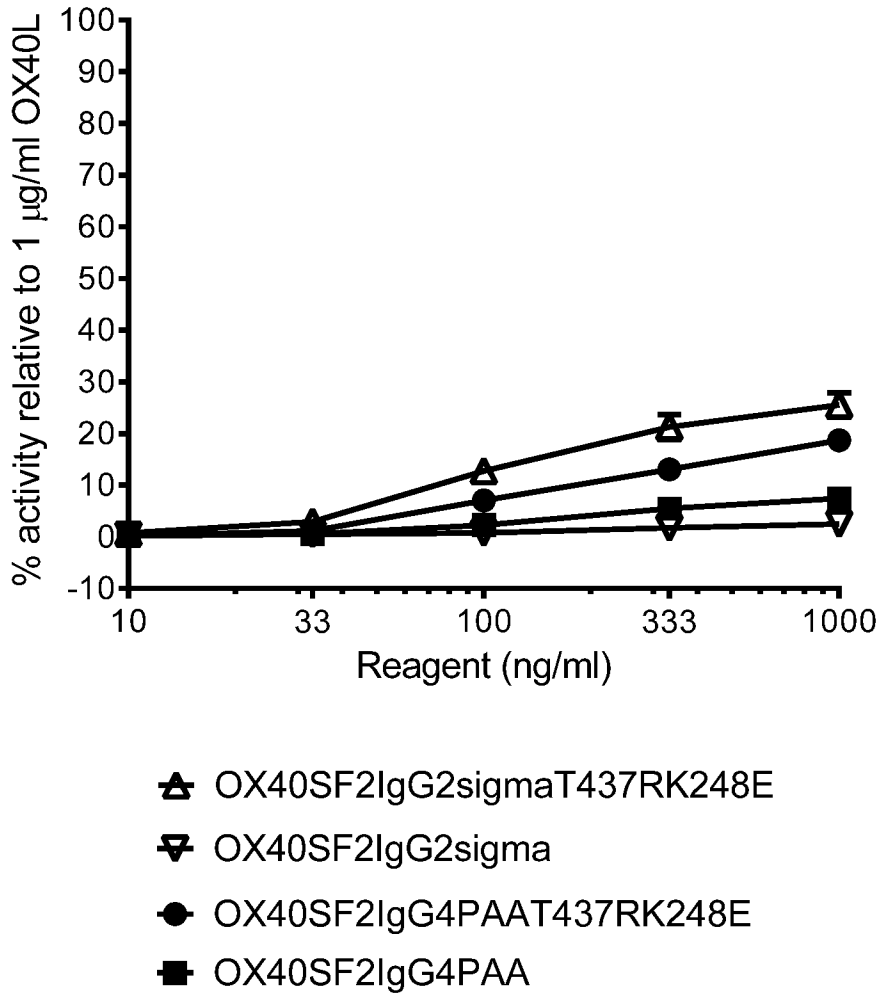

% activity relative to 1 µg/ml OX40L
100
90
80
70
60
50
40
30
20
10
0
-10
10
33
100
333
1000
Reagent (ng/ml)
OX40SF2IgG2sigmaT437RK248E
OX40SF2IgG2sigma
OX40SF2IgG4PAAT437RK248E
OX40SF2IgG4PAA

Figure 7.

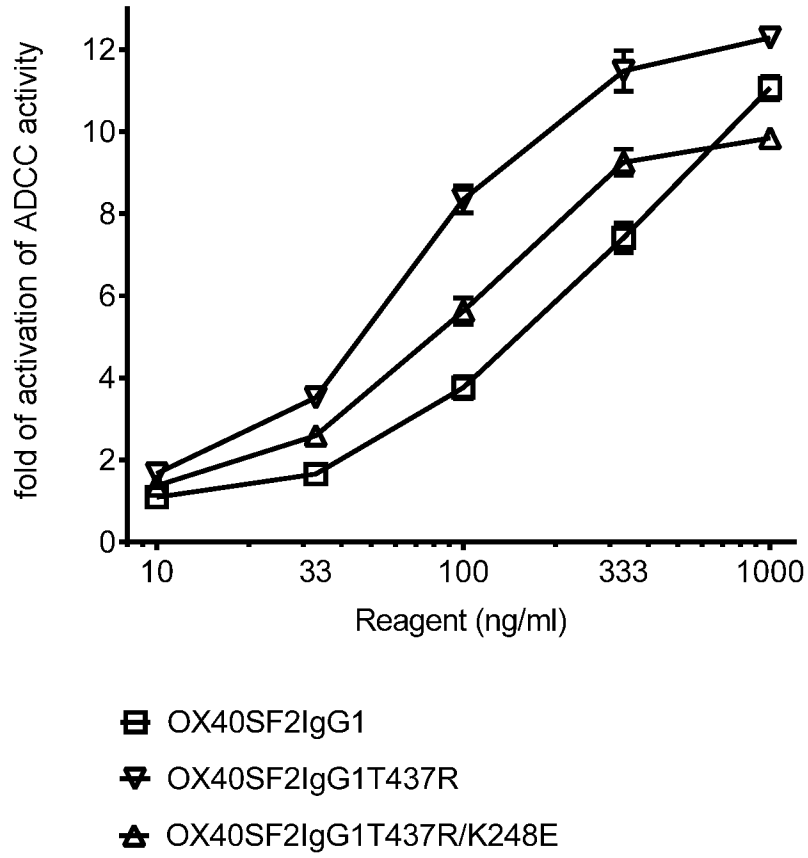

fold of activation of ADCC activity
0
2
4
6
8
10
12
10
33
100
333
1000
Reagent (ng/ml)
OX40SF2IgG1
OX40SF2IgG1T437R
OX40SF2IgG1T437R/K248E

Figure 8.

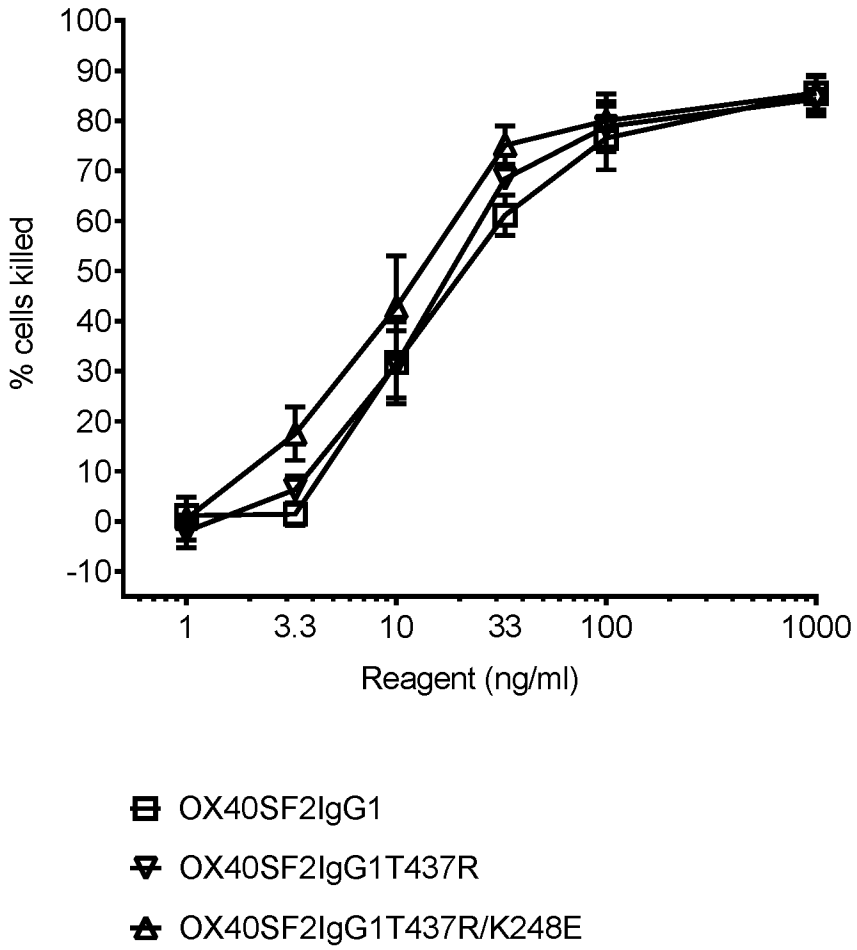

100
90
80
70
60
50
40
30
20
10
0
-10
% cells killed
1
3.3
10
33
100
1000
Reagent (ng/ml)
OX40SF2IgG1
OX40SF2IgG1T437R
OX40SF2IgG1T437R/K248E

Figure 9A.

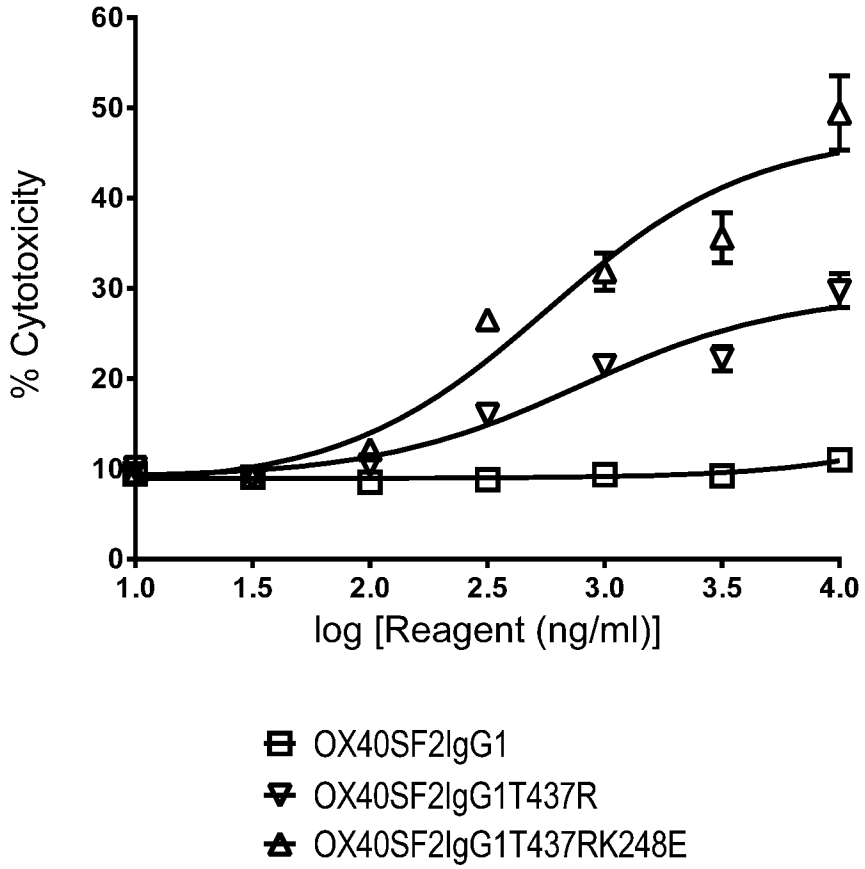

60
50
40
30
20
10
0
% Cytotoxicity
1.0
1.5
2.0
2.5
3.0
3.5
4.0
log [Reagent (ng/ml)]
OX40SF2IgG1
OX40SF2IgG1T437R
OX40SF2IgG1T437RK248E

Figure 9B.

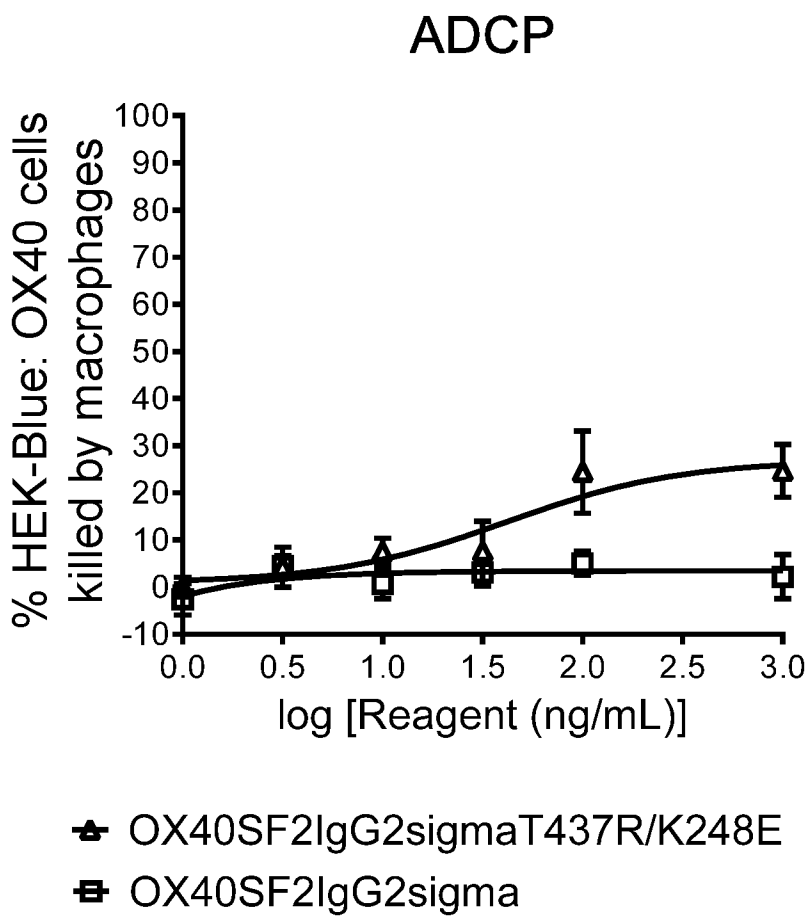

ADCP
% HEK-Blue: OX40 cells killed by macrophages
100
90
80
70
60
50
40
30
20
10
0
-10
0.0
0.5
1.0
1.5
2.0
2.5
3.0
log [Reagent (ng/mL)]
OX40SF2IgG2sigmaT437R/K248E
OX40SF2IgG2sigma

Figure 10.

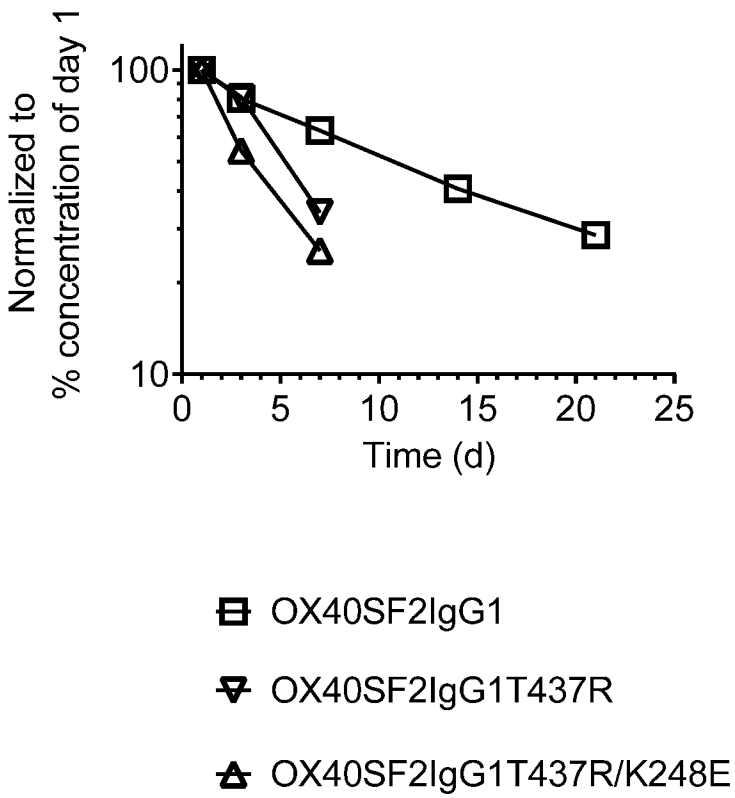
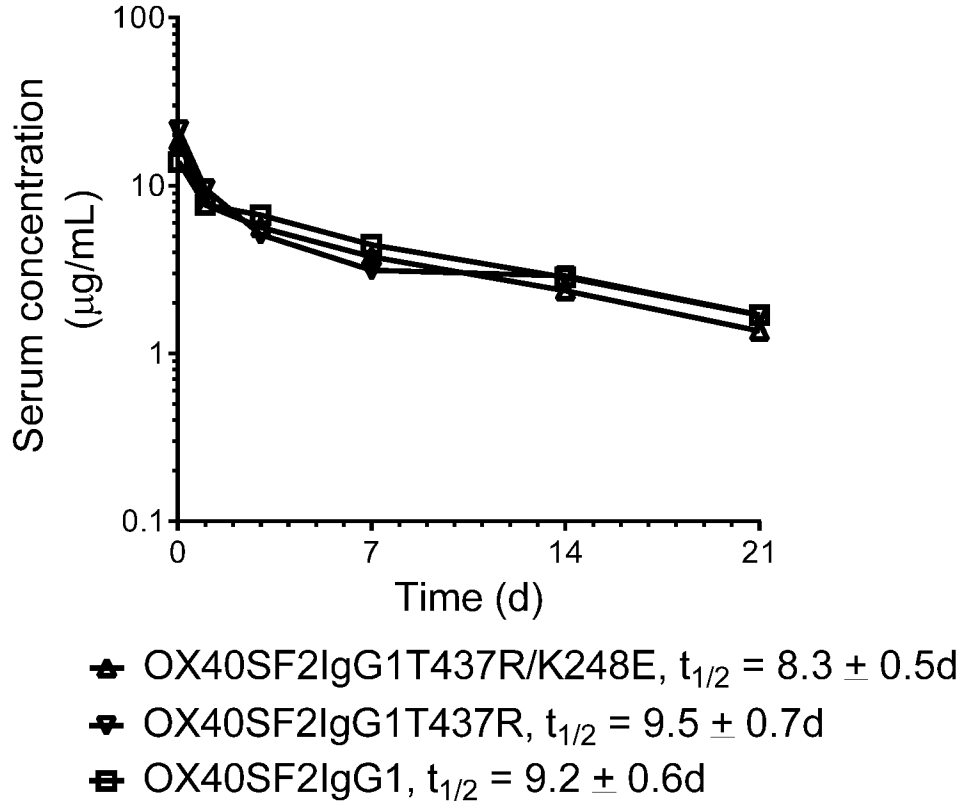

Normalized to
% concentration of day 1
100
10
0
5
10
15
20
25
Time (d)
OX40SF2IgG1
OX40SF2IgG1T437R
OX40SF2IgG1T437R/K248E

Figure 11.


100
10
1
0.1
Serum concentration (μg/mL)
0
7
14
21
Time (d)
OX40SF2IgG1T437R/K248E, t1/2 = 8.3 ± 0.5d
OX40SF2IgG1T437R, t1/2 = 9.5 ± 0.7d
OX40SF2IgG1, t1/2 = 9.2 ± 0.6d

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2017/044393

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/735 C07K16/18 C07K16/28
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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Date of the actual completion of the international search	Date of mailing of the international search report
12 October 2017	30/10/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Meyer, Wolfram

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2017/044393

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