Proposal and protocol for the construction of pEF1-ACE2-IGG2-SMAR vector for gene therapy of SARS-CoV-2

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INTRODUTION

The Coronavirus SARS-CoV-2, the etiological agent of COVID-19. Utilizes cellular ACE2 receptors for the entry of cells. This receptor binds to the receptor binding motif(RBM) of the SARS-CoV-2 S protein with affinity of around 100nM—comparable to a very high affinity monoclonal antibody. Fusion proteins consisting of the ectodomain of ACE2 fused to the Fc region of a human Antibody have been proven to neutralize the SARS-CoV-2 virus in vitro[1][2], and displayed extended serum half-life and lack of toxicity in-vivo[1][2][3]. In addition, this fusion protein have been proven to significantly attenuate SARS-CoV-2 infection In-Vivo using a murine model[1]. Such recombinant proteins have been proposed as a form of therapeutic for COVID-19 patients.

PROPOSAL

We propose a construct of a full-length (713AA) ectodomain of the Human Angiotensin Converting Enzyme 2 (ACE2), with the critical catalytic residues R273 and H505 mutated to K and N which abolishes Angiotensin I Converting activity, eliminating the undesirable side effect on blood pressure[4]; Fused to the Fc domain of the human Immunoglobin IGG2 subtype with the mutation K326W and E333S, which rescues the Complement-dependent cytotoxicity(CDC) activity of this subtype, but does not rescue binding to Fcry receptors or the activity of inducing Antibody dependent cytotoxicity(ADCC) or antibody-dependent cellular pnagocytosis(ADCP) [5][6], enabling safe neutralization and selective, complement dependent destruction of the SARS-CoV-2 virions, thus functioning both as a prophylactic and a therapeutic. Finally, in light of several papers claiming of insufficient neutralizing efficiency of the Wild-type sACE2, We propose to introduce several mutations on the ACE2 ectodomain which markedly enhances the binding affinity of the ACE2-Fc construct[7], increasing the efficacy of the proposed therapeutic.
In addition, an S/MAR element is included in the construct, allowing stable, long-term expression of the DNA construct and recombinant proteins in human cells, therefore conferring to extended immunity after admission of plasmid DNA using a chitosan nanoparticle-based delivery method.

PROTOCOL

Acquisition of the plasmids encoding ACE2, hIgG2 and cloning of the human S/MAR region from chromosome 9

Human ACE2, either Wild-type, or with the select mutation T27Y,L79T,N330Y can be acquired from https://www.addgene.org/browse/article/28211097/ with catalogue ID from 154098 to 154100. The ACE2 is cloned from the Amino acid 1 to 732 using the following pair of primers out of the plasmid:

Table 1: Primers for the cloning of ACE2 from the plasmid(s)

Name	Sequence(5'-3')	Primer concentration	Salt condition
ACE2_fwd	tccaccggtcgccaccatgtcaagctcttc	500nM	25nM
ACE2_rev	ggcactccactccgccgcttccgcctccaagtgttggctgtatc	500nM	25nM

Human IgG2 can only be obtained by cloning from the plasmid pFUSE-hIgG2-Fc https://www.invivogen.com/pfuse-higg2-fc using the following pair of primers:

Table 2: Primers for the isolation of human igG2-Fc

Name	Sequence(5'-3')	Primer concentration	Salt condition
Fc from pFUSE/hIGG2_fwd	aacacttggaggcggaagcggcggagtggagtgcccaccttgc	500nM	25nM
Fc from pFUSE/hIGG2_rev	aacagtcttgccagctagccgtggcact	500nM	25nM

The S/MAR element can be obtained from a conserved part of human chromosomal DNA, using the following pair of primers:

Table 3: Primers for the isolation of the human Interferon beta MAR element from chromosome 9

Name	Sequence(5'-3')	Primer concentration	Salt condition
S/MAR from human chr.9_fwd	ggctagctggcaagactgttacccagaaaac	500nM	25nM
S/MAR from human chr.9_rev	tgcggccgctaatgcccagatgggttcac	500nM	25nM

Cloning and modification of ACE2 into a mammalian expression vector

The vector pEF, catalogue https://www.addgene.org/11154/ , is isolated as linear DNA using the

following pair of primers:

Table 4: Primers for isolating the vector backbone:

Name	Sequence(5'-3')	Primer concentration	Salt condition
pEF backbone_fwd	tctgggcattagcggccgcactcctcag	500nM	25nM
pEF backbone_rev	acatggtggcgaccggtggatcccgggc	500nM	25nM

Primer annealing temperatures are provided in the provided supplementary material pEF_Episomal_Vector_ACE2_IGG2_K326W_E333S_summary.pdf

After the cloning of these 4 fragments, the fragments are assembled and circularized into a complete plasmid, using the NEBuilder® HiFi DNA Assembly Cloning Kit, catalogue https://international.neb.com/products/e5520-nebuilder-hifi-dna-assembly-cloning-kit#Product%20Information

Reaction conditions are provoded in the provided supplementary material pEF_Episomal_Vector_ACE2_IGG2_K326W_E333S_summary.pdf

Note: Gel purification should be conducted with the PCR products before use in the NEBassembly process, by cutting the band of DNA products out of the Agarose gel, and extracting the DNA using the miniprep kit https://www.the-odin.com/miniprep-kit-w-50-preps/

The plasmid should be transfected into a strain of dam+[8] E.Coli, streaked onto an agar plate containing Ampicillin and verified using colony PCR with the following primers before proceeding to the mutagenesis step.

Table 5: Primers for validating the insert sequence and the correct transformant E.Coli

Name	Sequence(5'-3')	Primer concentration	Salt condition
ACE2_fwd	tccaccggtcgccaccatgtcaagctcttc	500nM	25nM
S/MAR from human chr.9_rev	tgcggccgctaatgcccagatgggttcac	500nM	25nM

Plasmids should be isolated, after overnight culture of the PCR positive colony, using https://www.the-odin.com/miniprep-kit-w-50-preps/

And verified using PCR, before the QuikChange mutagenesis step is carried out.

Table 6: Primers for the mutagenesis of ACE2 and hIgG-Fc:

Name	Sequence(5'-3')	Tm
R273K SENSE	gcttggtgatatgtggggtaAattttggacaaatctgtactctttgac	81.6
R273K ANTISENSE	gtcaaagagtacagatttgtccaaaatTtaccccacatatcaccaagc	81.6
H505N SENSE	ctgtgaccccgcatctctgttcAatgtttctaatgattactcattc	81.6
H505N ANTISENSE	gaatgagtaatcattagaaacatTgaacagagatgcggggtcacag	81.6
K326W/E333S SENSE	aaggtctccaactggggcctcccagcccccatctcgaaaaccatctccaaaa	81.86
K326W/E333S ANTISENSE	ttttggagatggttttcgagatgggggctgggaggccccagttggagacctt	81.86

A single reaction using the QuikChange multi Site directed mutagenesis kit, Catalogue

https://www.agilent.com/cs/library/usermanuals/public/200514.pdf is carried out, to introduce the desired mutations.

Only 3 oligonucleotides, either the SENSE oligonucleotides, or the ANTISENSE nucleotides, should be used in the reaction.

Three(3) separate colony PCR reactions Should be carried out per colony, each using one of the forward primers on select colonies, in order to verify that the mutations are being properly introduced. Alternatively, a single PCR reaction with all 4 primers could be done on each colony. The product should generate 3 bands of the correct size

Table 7: Primers used to verify the success of QuikChange mutagenesis

Forward primers (5'-3')	Tm
acaagtgcaaggtctccaactgg	61C
gtgaccccgcatctctgttcA	61C
ctcatttgcttggtgatatgtggggtaA	61C
Reverse primer	
aacagtcttgCCAGCTAGCcg	61C

Bands of 2120bp,1418bp and 416bp should be expected on the gel electrophoresis results, before expanded culturing of the colony. Bacteria from each colony could be inoculated into LB medium w/Ampicillin sodium to keep them as stock until the PCR results are generated.

Alternatively, the provided .gb file Episomal_vector_ACE2RKHN_WT_hIGG2_K326W_E333S_MAR.gb with the insert Core_insert ACE2RKHN_WT_hIGG2_K326W_E333S_Tm_81.2C.gb could be synthesized and cloned into the appropriate vector containing an EF-1 α promoter.

The high-affinity variant, Core_insert ACE2RKHN_V2.4_hIGG2_K326W_E333S_Tm_81.2C.gb and Episomal_vector_ACE2RKHN_V2.4_hIGG2_K326W_E333S_MAR.gb can be synthesized as an alternative, but this file can impact the subsequent generation of libraries of select mutants with high affinity to SARS-CoV-2 S.

Generation of polyclonal mutants containing select mutations that enhances binding to the SARS-CoV-2 S

Using the protocol provided above, or with the vector Episomal_vector_ACE2RKHN_V2.4_hIGG2_K326W_E333S_MAR.gb synthesized, the next step is the introduction of a polyclonal mutant library containing ACE2 variant that carries select mutation(s) which enhances binding to the SARS-CoV-2 S.

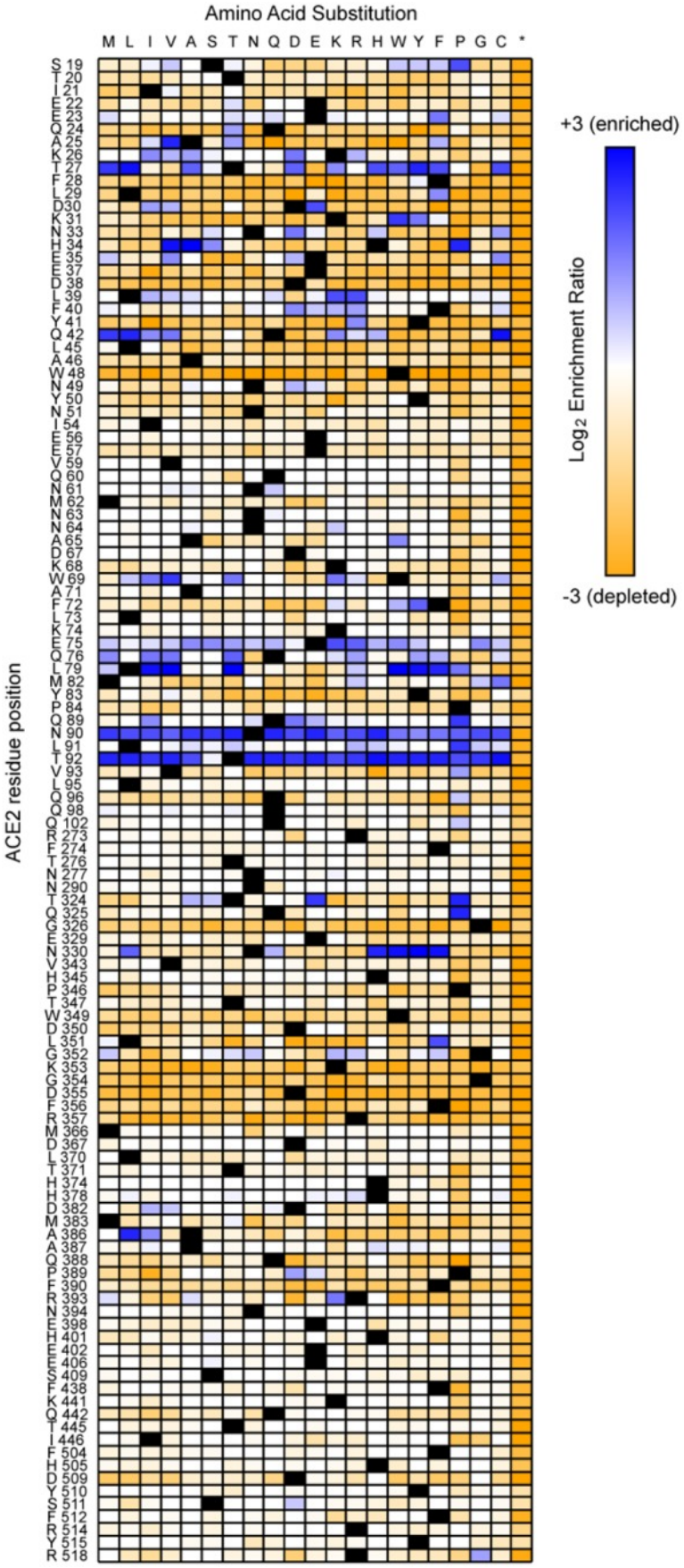

Figure 2 from [7]: A list of all possible ACE2 mutations in the term of binding affinity to

SARS-CoV-2 S. blue represent enhancement to the binding affinity, while orange represent reduction of binding affinity.

Using the same QuikChange multi site directed mutagenesis kit, a mixture of the set of primers, outlined below, should be used to randomly and specifically apply the select mutations that enhances binding to SARS-CoV-2 S, onto the ACE2-Fc fusion protein.

Table 8: list of primer pairs to be used in a single QuikChange site directed mutagenesis reaction, catalogue https://www.agilent.com/cs/library/usermanuals/Public/200523.pdf to generate the polyclonal library of ACE2-Fc containing mutations that enhances binding to the SARS-CoV-2 S. Note: each primer is a pair with it's antisense primer, labled _antisense_. Primer pairs should be used together.

Name	Sequence	Effect
a274c_c275a_a276g_T92Q	5'-actacaagaaattcagaatctccaggtcaagcttcagctgcaggctc-3'	Aglycosylated ACE2
a274c_c275a_a276g_antisense_T92Q	5'-gagcctgcagctgaagcttgacctggagattctgaatttcttgtagt-3'	Aglycosylated ACE2
a79t_c80a_a81t_T27Y	5'-caccattgaggaacaggccaagtatttttttggacaagtttaaccacg-3'	Strongly enhanced binding
a79t_c80a_a81t_antisense_T27Y	5'-cgtggttaaacttgtccaaaaaatacttggcctgttcctcaatggtg-3'	Strongly enhanced binding
c100g_a101c_antisense_H34A	5'-caggtcttcggcttcggcgttaaacttgtccaaaaatgtcttg-3'	Weakly enhanced binding
c100g_a101c_H34A	5'-caagacatttttggacaagtttaacgccgaagccgaagacctg-3'	Weakly enhanced binding
c235a_t236c_antisense_L79T	5'-gtggatacatttgggcagttgtggactgttcctttaaaaaggcag-3'	Unstable but strongly enhance binding
c235a_t236c_L79T	5'-ctgccttttttaaaggaacagtccacaactgcccaaatgtataccac-3'	Unstable but strongly enhance binding
a125t_antisense_Q42L	5'-caagaagcaagtgaacttagatagaacaggtcttcgg-3'	Weakly enhance binding
a125t_Q42L	5'-ccgaagacctgttctatctaagttcacttgcttcttg-3'	Weakly enhance binding

g1156c_c1157t_t1158a_antisense_A386L	5'-agcagaaaaggttgtgctagatatgccatatcatactggatatgccc-3'	Weakly enhance binding
g1156c_c1157t_t1158a_A386L	5'-gggcatatccagtatgatatggcatatctagcacaacctttctgct-3'	Weakly enhance binding

The last 2 primer pairs are incompatible with each other, and only one pair should be used in a single reaction.

Pair 1	Sequence	Effect
a988t_antisense_N330Y	5'-gtccgttagcatggaatattcccagaatccttgag-3'	Strongly enhances binding
a988t_N330Y	5'-ctcaaggattctgggaatattccatgctaacggac-3'	Strongly enhances binding
Pair 2	Sequence	Effect
a974c_antisense_Q325P	5'-ggaattttcccagaatcctggagtcatattaggaagacc-3'	Stabilized bound conformation
a974c_Q325P	5'-ggtcttcctaatatgactccaggattctgggaaaattcc-3'	Stabilized bound conformation

An aglycosylated version, using only T92Q, displays significantly enhanced binding to the SARS-CoV-2 S, and is predicted to be stable against viral escape. [7]

After reactionis finished, the resulting plasmids should be transformed into E.Coli cells and inoculated into LB medium containing Ampicillin sodium.

The mutagenesis step can be skipped if the mutated version of ACE2 is used.

Assaying the efficacy of ACE2-Fc in neutralizing SARS-CoV-2 S

The plasmids, containing polyclonal ACE2-Fc with single to multiple mutations which enhances binding to SARS-CoV-2 S, as well as unmutated Wild-type ACE2-Fc, can be then extracted after overnight bacterial culture, transfected into HEK293T cells, and assayed for the ability of the cell culture supernatants to inhibit the fusion of cells induced by SARS-CoV-2 S and human ACE2.
Catalogue for SARS-CoV-2 S and hACE2:
Human ACE2: https://www.addgene.org/1786/
SARS-CoV-2 S G614: https://www.addgene.org/141347/
SARS-CoV-2 S D614: https://www.addgene.org/145032/
Co-transfect with the S and hACE2 plasmid to assay the presence of Syncytia.
A positive control should be used for Syncytia formation assaying, while the culture supernatants of the ACE2-Fc Plasmid-transfected cells added to different experimental groups to assay the

ability of the culture supernatants to inhibit syncytia formation.
If possible, a Pseudovirus entry inhibition assay, using pLenti-GFP and the plasmid encoding the S, could be used to more accurately assay the efficacy of the polyclonal ACE2-Fc.

Delivery of the ACE2-Fc plasmids into cells and human tissue In-Vivo

Chitosan-DNA nanoparticles[9][10][11], have emerged as an optimal mean of delivering genes both in-vitro and in-vivo. Nanoparticles are formed at at chitosan concentrations of 50–400 µg/mL and DNA concentrations of 40–80 µg/mL, where buffer solutions were at pH 5.5 and temperature was 55°C, Sodium sulfate (25 mM) was added as a desolvating agent to enhance the phase separation (Figure 3). Particle formation was dependent on the N/P ratio (amine groups on chitosan/phosphate groups on DNA) and N/P ratios of 3–8 yielded 150–250-nm particles with surface charges of approximately + 15 mV.

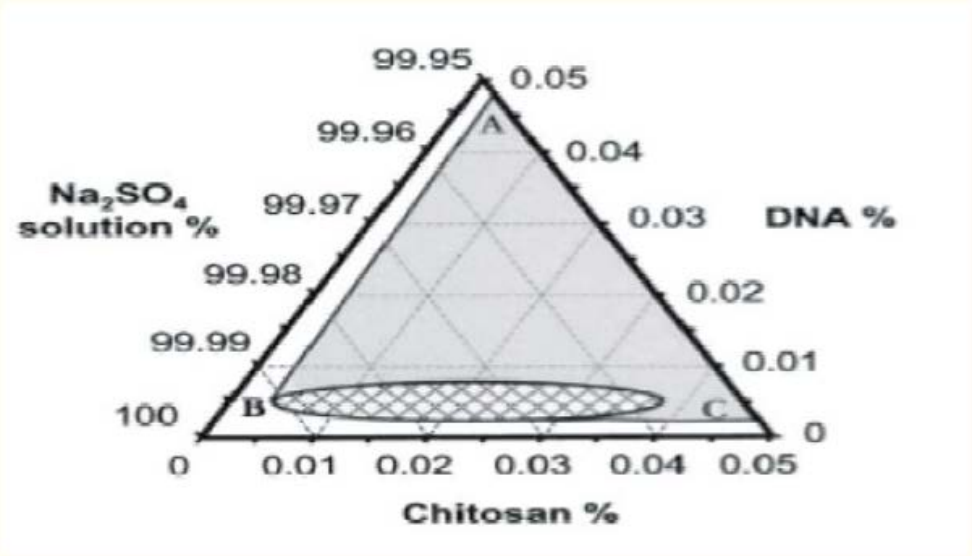

Figure 3

Ternary phase diagram of complex coacervation between pRE-luciferase plasmid and chitosan at 55°C in 50 mM Na_2SO_4. Sodium sulfate solution was regarded as one component, since the concentration change in the experiment range was minimal. The region to the right of line ABC depicts the conditions under which phase separation occurred. The concentration ranges in the small grid area yielded distinct particles as observed under a phase contrast microscope. Reprinted from Mao H-Q, Roy K, Troung-Le VL, et al. 2001. Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. *J Control Release*, 70:399–421. Copyright © 2001, with permission from Elsevier.

Figure 3 from [9]
To test transfection efficiency, Chitosan nanoparticles could be used to co-transfect hACE2 and SARS-CoV-2 S plasmids into HEK293T cells and assayed for syncytia formation. The more syncytia, the higher the efficiency. Alternatively, a GFP plasmid, https://www.addgene.org/11154/ could be used as a control.

Materials and Reagents

For the culture of human HEK293T cells, https://www.the-odin.com/human-cells/ Provides a complete kit.
For the performing of PCR and biochemical reactions,
https://www.the-odin.com/taq-polymerase-master-mix-2x-1ml-40-reactions/
And the thermal cycler
https://www.the-odin.com/mj-research-ptc-100-thermal-cycler-controller-dna-engine-60-well/
is available at https://www.the-odin.com/
The rest is supplied with the Kit https://www.the-odin.com/genetic-engineering-home-lab-kit/
DNA stain for visualizing the DNA, in preparation for cutting of bands:
https://www.the-odin.com/dna-stain-for-gels-10-000x/
Agarose for making DNA gels:
https://www.the-odin.com/agarose/
https://www.the-odin.com/tae-buffer-mix-10-l-50g/
and the DNA ladder necessary for verification of band size:
https://www.the-odin.com/dna-ladder-1kb/
And the loading dye:
https://www.the-odin.com/6x-dual-color-dna-loading-dye/
Chitosan for preparation of nanoparticles for transfection of the cells can be obtained at
https://chitolytic.com/chitosan-products/chitosan-low-molecular-weight-very-high-dda/
Lipofectamine, useful for testing, is found at
https://www.thermofisher.com/au/en/home/brands/product-brand/lipofectamine/lipofectamine-3000.html

DISCUSSIONS

Gene synthesis as an alternative

Despite the protocols listed above, the provided insert
ACE2RKHN_WT_hIGG2_K326W_E333S_Tm_81.2C.gb
And
Episomal_vector_ACE2RKHN_V2.4_hIGG2_K326W_E333S_MAR.gb
Could be more easily synthesized and cloned into an appropriate Eukaryotic expression vector with EF-1 alpha promotor. The additional ACE2 mutations could then be introduced using the QuikChange site-directed mutagenesis kit.
Commercial gene synthesis services, like
https://www.genscript.com/gene_synthesis.html?src=service
or
https://www.twistbioscience.com/products/genes?tab=overview
Could be used instead with minimal added cost and significantly higher success rate.

ACE2 mutations and necessity for a complement-dependent activity.

In light of the discovery in [1] which indicate the Fc-LALA mutation with ACE2-WT may not be able to efficiently suppress SARS-CoV-2 infections in WT mice, a good neutralizing ACE2-Fc should contain mutations on the ACE2 that increases the binding affinity to the Spike glycoprotein above WT. such mutations have been documented in detail in [7], and the corresponding QuikChange multi Site-directed mutagenesis primers have been supplied in the included .txt file
QuikChange Primers of high affinity mutant ACE2 for use after assembly Tm=78.10~79.37.txt
Alongside with an proven ACE2 variant that displays significantly enhanced binding affinity to the Spike glycoprotein in
Episomal_vector_ACE2RKHN_V2.4_hIGG2_K326W_E333S_MAR.gb
In light of other publications which suggest the efficacy of normal hIGG1-Fc in the neutralization of SARS-CoV-2 S [2][12], and the apparent efficacy of serum in the neutralization of SARS-CoV-2 both as pseudotyped virus and as real virus, an Complement-dependent pathway, of which the antibodies recruit c1q and initiate the complement activation cascade to punch holes in the viral envelope and destroying the virus by the assembly of the Membrane Attack Complex(MAC) and subsequent infiltration of serum and ambient RNAses appeared to be in play.
However, since the normal IGG1-Fc can also cause Antibody-dependent enhancement by binding to the Fc gamma receptors in cytotoxic T cells and phagocytotic cells, the naturally occouring Fc variant with attenuated activity of ADCC and ADCP, the human IGG2 subtype, should be used. Mutations on the IGG2 have been provided for the restoration of the CDC activity, but not the ADCC activity [5], in all constructs provided.

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