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Domain 5 of cd163 for use in antiviral compositions against prrs, and transgenic animals

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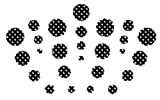
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Patents ADP number (*if you know it*)

3. Title of the invention **Antiviral Compositions, Methods and Animals**

4. Name of your agent (*if you have one*) **HARRISON GODDARD FOOTE LLP**
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Antiviral Compositions, Methods and Animals

The present invention relates to compositions and methods to prevent or treat porcine reproductive and respiratory syndrome virus (PRRSV) infection in animals.

5 The invention also relates to animals which have been modified to provide resistance to infection by PRRSV.

Introduction

10 Porcine reproductive and respiratory syndrome virus (PRRSV) is a virus that causes a disease of pigs, called porcine reproductive and respiratory syndrome (PRRS).

This economically important, pandemic disease causes reproductive failure in breeding stock and respiratory tract illness in young pigs. Initially referred to as
15 "mystery swine disease" and "mystery reproductive syndrome," it was first reported in 1987 in North America and Central Europe. It is estimated that the disease costs the United States swine industry around \$600 million annually.

PRRSV enters alveolar macrophages via a set of macrophage cell surface markers:
20 CD169 and CD163. The role of CD169/sialoadhesin was discovered by the group of Hans Nauwynck in Ghent. The role of CD163 was discovered by scientists working with Pfizer (Calvert et al. 2007). Calvert et al (2007) demonstrated that transfection of any non-susceptible cells with CD163 can turn the cells susceptible to PRRSV. That has allowed for the generation of vaccine strains without the need of using
25 Marc-145 cells.

Van Gorp et al. ("Susceptible cell lines for the production of porcine reproductive and respiratory syndrome virus by stable transfection of sialoadhesin and CD163",
BMC Biotechnology 2010, 10:48) have demonstrated that the domains 5 to 9 are
30 important for the PRRSV entry into non-susceptible cells. She has highlighted that domain 5 may be critical.

Das et al. ("The Minor Envelope Glycoproteins GP2a and GP4 of Porcine Reproductive and Respiratory Syndrome Virus Interact with the Receptor CD163",

JOURNAL OF VIROLOGY, Feb. 2010, p. 1731–1740) have demonstrated that that the PRRSV glycoprotein GP2A and GP4 interact physically with CD163.

5 US 20050271685 held by Pfizer (Zoetis) suggests that the use of CD163 molecule can make cells susceptible to PRRSV and ASFV.

WO 2012158828 describes PRRS resistant animals in which the SIGLEC1 and/or CD163 genes have been inactivated. CD163 is, however, has roles in normal physiological activities. It is therefore undesirable to inactivate this gene as it may have
10 undesirable and unforeseeable knock-on effects on the animal.

There remains a need for improvements in the prevention and treatment of PRRSV.

Statements of the Invention

15

According to a first aspect of the invention, there is provided an isolated or synthetic protein comprising CD163 or a biologically active fragment or variant thereof for the prevention or treatment of PRRSV in an animal.

20

Preferably the animal is a porcine animal, more preferably a pig (*Sus scrofa*), and most preferably a domestic pig (*Sus scrofa domesticus* or *Sus domesticus*).

Suitably the protein comprises one or more extracellular domains of CD163, but excludes transmembrane domains and/or intracellular domains.

25

Preferably the isolated or synthetic protein comprises a fragment of CD163, wherein said fragment comprises domain 5 of CD163, or a biologically active fragment or variant thereof.

30

It is believed that domain 5 is the critical portion of CD163 that mediates PRRS infection into cells. Accordingly, provision of a protein comprising domain 5 (or a functional equivalent) provides a protein which can bind to PRRS and prevent infection of cells.

It has surprisingly been found that fragments of CD163, rather than the complete protein, are more effective as decoys to prevent PRRS infection.

5 Suitably the protein comprises a fragment of CD163, wherein said fragment consists of at least a portion of domains 5 to 9 of CD163, or a biologically active fragment or variant thereof. Preferably said protein comprises domain 5 or a biologically active fragment or variant thereof.

10 In a particularly preferred embodiment the fragment consists of domain 5 alone, or a biologically active fragment or variant thereof. It has been found that fragments of CD163 which consist of domain 5 without additional domains (e.g. 4 or 7) are especially effective when used as decoys to prevent PRRS infection of susceptible cells.

15 Domain 5 of CD163 has the amino acid sequence:

PRLVGGDIPCSGRVEVQHGDTWGTVCDSDFSLEAASVLCRELQCGTVVSLGGAH
FGE GSGQIWAE EFQCEGHESHLSLCPVAPRPDGTCSHSRDVGVVCS (SEQ ID NO
3)

20

For the avoidance of doubt, it is emphasised that the present invention include variants of this sequence or fragments thereof which retain the ability to bind PRRS. Details of various types of variants are described below, but include deletions, additions, substitutions, amino acid modifications and the like, provided that they do
25 not prevent binding of the protein to PRRS.

The present invention includes biologically-active proteins comprising an amino acid sequences which is at least 75%, 80%, 85%, 90%, 95% or 99% identical to SEQ ID
30 NO 3.

30

"% identity" defines the relation between two or more polypeptides on the basis of a comparison between their aligned sequences.

Identity can be calculated by known methods. Identity, or homology, percentages as mentioned herein are those that can be calculated with the GAP program, running under GCG (Genetics Computer Group Inc., Madison, WI, USA). Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Needleman & Wunsch, *J. Mol. Biol.* 48:443, 1970; Pearson & Lipman, *Proc. Nat. Acad Sci. USA* 85:2444, 1988; Higgins & Sharp, *Gene*, 73:23744, 1988; Higgins & Sharp, *CABIOS* 5:151-3, 1989; Corpet et al., *Nuc. Acids Res.* 16:10881-90, 1988; Huang et al. *Computer Appls. in the Biosciences* 8, 155-65, 1992; and Pearson et al., *Meth Mol. Bio.* 24:307-31, 1994. Altschul et al., *J. Mol. Biol.* 215:403-10, 1990, presents a detailed consideration of sequence alignment methods and homology calculations.

The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI, National Library of Medicine, Building 38A, Room 8N805, Bethesda, Md. 20894,US) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx. Additional information can be found at the NCBI web site.

Parameters for polypeptide sequence comparison included the following:

Algorithm: Needleman and Wunsch, *J. Mol. Biol.* 1970, 48: 443-453.

As a comparison matrix for amino acid alignments the Blosum62 matrix is used (Henikoff and Henikoff, *supra*) using the following parameters:

- Gap penalty: 8
- Gap length penalty: 2
- No penalty for end gaps.

The present invention thus, in certain embodiments, provides the protein described above, which is suitable for use in the prevention (e.g. prophylaxis) or treatment of PRRS in pigs.

Accordingly, the present invention provides an isolated or synthetic protein comprising the sequence:

PRLVGGDIPCSGRVEVQHGDTWGTVCDSDFSLEAASVLCRELQCGTVVSLGGAH
5 FGEGSGQIWAEFQCEGHESHLSLCPVAPRPDGTCSHSRDVGWCS (SEQ ID NO
3)

or a biologically active fragment or variant thereof for the prevention or treatment of PRRSV in an animal.

10 It will be apparent to the skilled person that the entire polypeptide of domain 5/SEQ ID NO 3 may not be required to bind to CD163, and thus fragments representing a portion of domain 5 may be suitable for the present invention.

Suitably the protein of the present invention is a fusion protein.

15

In one embodiment the protein is a fusion of a CD163 or a biologically active fragment or variant thereof and an immunoglobulin (antibody) Fc fragment. Addition of an immunoglobulin Fc fragment can add stability and deliverability to proteins of the present invention. The Fc fragment can be a complete Fc region or a portion
20 thereof. For example, the Fc fragment can be the Fc region of an IgG, such as IgG2bFc.

The protein of the present invention suitably comprises the addition of a signal peptide, e.g. a secretory peptide which promotes secretion of the protein from the
25 cell in which translation occurs. Suitable secretory peptides are well-known in the art, and include the IL2 secretory domain.

Various signal peptides, and means for their optimization are set out in Kober L, Zehe C, Bode J (April 2013), "Optimized signal peptides for the development of high
30 expressing CHO cell lines". *Biotechnol. Bioeng.* **110** (4): 1164–73, and von Heijne G (Jul 1985). "Signal sequences: The limits of variation". *J Mol Biol* **184** (1): 99–105. Such signal peptides can be used in the present invention.

Preferably the signal peptide is suitable to direct secretion from the cell type or species of animal in which translation occurs.

5 The fusion protein can comprise a peptide to allow identification and/or purification of the protein. For example, the fusion protein can comprise a GST protein, FLAG peptide, or a hexa-his peptide (6xHis-tag) which can be isolated using affinity chromatography with nickel or cobalt resins.

10 A fusion protein according to the present invention may comprise one or more linker peptides. A suitable linker peptide has the sequence GSGSSRGGSGSGGGGGSKL (SEQ ID NO 13), but many other sequences would be suitable. Suitable linkers are well known in the art, and are typically rich in glycine for flexibility, and may comprise serine and/or threonine for solubility. Further information can be found, *inter alia*, at George RA and Heringa J. 'An analysis of
15 protein domain linkers: their classification and role in protein folding'. Protein Eng, 2002 Nov; 15(11) 871-9.

In one embodiment of the invention the protein comprises a fusion of an IL2 signal peptide, domain 5 of CD163 (or a biologically active fragment or variant thereof) and
20 the Fc fragment of an immunoglobulin. Suitably the Fc fragment is the Fc fragment of IgG. For example it can be the fragment IgG2bFc, e.g. rat IgG2bFc.

Suitably the protein is the fusion protein described as D55, D55-FC, of IL2-D55-FC
25 below.

According to a second aspect of the invention there is provided a method of treating or preventing an infection in an animal comprising administration to said animal a composition comprising an isolated or synthetic protein comprising CD163 or a biologically active fragment or variant thereof.

30 Such a synthetic protein can act as a decoy for the PRRS virus. The decoy binds to the virus and therefore the virus does not bind to membrane bound CD163 and thus infect cells.

Accordingly, it is preferred that the synthetic CD163 protein is adapted to be soluble in plasma.

- 5 By providing a fusion protein of CD163 and Fc, for example, solubility and stability *in vivo* can be improved.

The composition can be administered via any suitable route. Such routes include, but are not limited to, oral and parenteral routes, such as intravenous (iv),
10 intraperitoneal (ip), rectal, topical, ophthalmic, nasal, and transdermal.

The invention provides formulations comprising an active of the disclosure formulated for pharmaceutical use and optionally further comprising a pharmaceutically acceptable diluent, excipient and/or carrier.

15

The invention therefore includes pharmaceutical formulations which may include, in addition to active ingredient, a pharmaceutically acceptable diluent, excipient and/or carrier. Such formulations may be used in the methods of the disclosure.

Additionally or alternatively, pharmaceutical formulations may include a buffer,
20 stabiliser and/or other material well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be any suitable route, for example by a parenteral route and particularly by infusion or injection (with or without a needle). The route of
25 administration may be subcutaneous injection. The route of administration may be intravenous injection or infusion. Other routes of administration which may be used include administration by inhalation or intranasal administration.

Compositions are provided that include one or more of the actives that are disclosed
30 herein in a carrier. The compositions can be prepared in unit dosage form for administration to a subject. The amount and timing of administration are at the discretion of the treating physician to achieve the desired purposes. The active may be formulated for systemic or local administration. In one example, the active

formulated for parenteral administration, such as subcutaneous or intravenous administration.

5 Actual methods for preparing administrable compositions, whether for intravenous or subcutaneous administration or otherwise, will be known or apparent to those skilled in the art and are described in more detail in such publications as Remington's Pharmaceutical Science, 19th ed., Mack Publishing Company, Easton, Pa. (1995).

10 In embodiments of the present invention the administration of the composition may comprise delivering an expression vector to a subject, the vector being adapted for expression in the subject, and thereby causing the composition to be synthesized by the subject.

Various suitable expression vectors are described below.

15 According to a third aspect of the invention there is provided an isolated or synthetic nucleic acid construct which encodes the above-mentioned isolated or synthetic protein comprising CD163 or a biologically active fragment or variant thereof.

20 Said isolated or synthetic nucleic acid suitably comprises at least one expression control sequence operably linked to said nucleotide sequence encoding CD163 or a biologically active fragment or variant thereof to drive expression of CD163 or a biologically active fragment or variant thereof. Such expression control sequences generally comprise a promoter sequence and additional sequences which regulate transcription and translation and/or enhance expression levels. Suitable expression control sequences are well known in the art and include eukaryotic, prokaryotic, or viral promoter or poly-A signal. Expression control and other sequences will, of course, vary depending on the host cell selected or can be made inducible.

25 Examples of useful promoters are the SV-40 promoter (Science 1983, 222: 524-527), the metallothionein promoter (Nature 1982, 296: 39-42), the heat shock promoter (Voellmy *et al.*, P.N.A.S. USA 1985,82: 4949-4953), the PRV gX promoter (Mettenleiter and Rauh, J. Virol. Methods 1990, 30: 55-66), the human CMV IE promoter (US 5,168,062), the Rous Sarcoma virus LTR promoter (Gorman *et al.*, P.N.A.S. USA 1982, 79: 6777-6781), or human elongation factor 1 alpha or ubiquitin

promoter. Suitable control sequences to drive expression in animals, e.g. pigs, are well known in the art. The expression control sequences can drive ubiquitous expression or tissue- or cell-specific expression. The expression control sequence can be, for example, a viral or porcine promoter. A suitable promoter can be

5 ubiquitous (e.g. the CAG promoter) tissue restricted (e.g. the CMV immediate early promoter which is known to be expressed predominantly in exocrine cells) or tissue specific (e.g. CSF1 receptor promoter which is specific to cells of the mononuclear macrophage lineage, or a synthetic liver specific promoter). Further exemplary promoters for porcine expression can be found in, for example, Aigner *et al.*,

10 'Transgenic pigs for xenotransplantation: selection of promoter sequences for reliable transgene expression'. *Curr Opin Organ Transplant*. 2010 Apr;15(2):201-6. Many other suitable control sequences are known in the art, and it would be routine for the skilled person to select suitable sequences for the expression system being used.

15

Suitably the nucleic acid construct comprises a sequence encoding fusion protein IL2-D55-FC (D55 encoding region in lower case), such as:

ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCCTTGTCCAGG
 20 AATTCGATATCcaggaaaccaggctggttgaggaggacattccctgctctggtcgtgtgaagtacaacatg
 gagacacgtggggcaccgtctgtgattctgacttctctctggaggcggccagcgtgctgtgcagggaaactacagtgcg
 gcactgtggtttccctcctggggggagctcactttggagaaggaagtggacagatctgggctgaagaattccagtgtg
 aggggcacgagtcccaccttcactctgcccagtagcaccocgacctgacgggacatgtagccacagcaggggacg
 tcggcgtagtctgctcaagatacacCATGGTTAGATCTCCTACATGCCCTACATGTCACAAA
 25 TGCCCAGTTCCTGAACTCTTGGGTGGACCATCTGTCTTCATCTTCCCGCCAAAG
 CCCAAGGACATCCTCTTGATCTCCCAGAACGCCAAGGTCACGTGTGTGGTGGT
 GGATGTGAGCGAGGAGGAGCCGGACGTCCAGTTCAGCTGGTTTGTGAACAAC
 GTAGAAGTACACACAGCTCAGACACAACCCCGTGAGGAGCAGTACAACAGCAC
 CTTCAGAGTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGCGGCA
 30 AGGAGTTCAAATGCAAGGTCAACAACAAAGCCCTCCCAAGCCCCATCGAGAAA
 ACCATCTCAAACCCAAAGGGCTAGTCAGAAAACCCACAGGTATACGTCATGGGT
 CCACCGACAGAGCAGTTGACTGAGCAAACGGTCAGTTTGGCTGCTTGACCTC
 AGGCTTCCTCCCTAACGACATCGGTGTGGAGTGGACCAGCAACGGGCATATAG
 AAAAGAACTACAAGAACCCGAGCCAGTGATGGACTCTGACGGTTCTTTCTTCA

TGTACAGCAAGCTCAATGTGGAAAGGAGCAGGTGGGATAGCAGAGCGCCCTTC
GTCTGCTCCGTGGTCCACGAGGGTCTGCACAATCACCACGTGGAGAAGAGCAT
CTCCCGGCCTCCGGGTAAATGA (SEQ ID NO 14)

- 5 Suitably the nucleic acid construct comprises a sequence encoding construct D55-Fc (D55 encoding region in lower case), such as:

cccaggctggttgaggggacattccctgctctggtcgtgtgaagtacaacatggagacacgtggggcaccgtctgt
gattctgacttctctctggaggcggccagcgtgctgtgcaggaactacagtgcggcactgtggttccctcctggggg
10 gagctcactttggagaaggaagtggacagatctgggctgaagaattccagtgtgaggggcacgagtcccaccttca
ctctgccagtagcaccgcccctgacgggacatgtagccacagcagggacgtcggcgtagtctgctcaagataca
cCATGGTTAGATCTCCTACATGCCCTACATGTCACAAATGCCCAGTTCCTGAACT
CTTGGGTGGACCATCTGTCTTCATCTTCCCGCCAAAGCCCAAGGACATCCTCTT
GATCTCCCAGAACGCCAAGGTCACGTGTGTGGTGGTGGATGTGAGCGAGGAG
15 GAGCCGGACGTCCAGTTCAGCTGGTTTGTGAACAACGTAGAAGTACACACAGC
TCAGACACAACCCCGTGAGGAGCAGTACAACAGCACCTTCAGAGTGGTCAGTG
CCCTCCCCATCCAGCACCCAGGACTGGATGAGCGGCAAGGAGTTCAAATGCAAG
GTCAACAACAAAGCCCTCCCAAGCCCATCGAGAAAACCATCTCAAACCCAAA
GGGCTAGTCAGAAAACCACAGGTATACGTCATGGGTCCACCGACAGAGCAGTT
20 GACTGAGCAAACGGTCAGTTTGACCTGCTTGACCTCAGGCTTCCTCCCTAACGA
CATCGGTGTGGAGTGGACCAGCAACGGGCATATAGAAAAGAACTACAAGAACA
CCGAGCCAGTGATGGACTCTGACGGTTCTTTCTTCATGTACAGCAAGCTCAATG
TGAAAGGAGCAGGTGGGATAGCAGAGCGCCCTTCGTCTGCTCCGTGGTCCA
CGAGGGTCTGCACAATCACCACGTGGAGAAGAGCATCTCCCGGCCTCCGGGT
25 AAATGA (SEQ ID NO 15)

D55-Fc lacks the secretory signal peptide.

- 30 Due to the degeneracy of the genetic code, polynucleotides encoding an identical or substantially identical amino acid sequence may utilize different specific codons. All polynucleotides encoding the proteins as defined above are considered to be part of the invention.

According to a fourth aspect of the present invention there is provided an expression vector, said vector comprising a nucleotide sequence encoding CD163 or a biologically active fragment or variant thereof.

- 5 Such vectors suitably comprise an isolated or synthetic nucleic acid construct as described above.

The vectors according to the invention are suitable for transforming a host cell. Examples of suitable cloning vectors are plasmid vectors such as pBR322, the
10 various pUC, pEMBL and Bluescript plasmids, or viral vectors such as HVT (Herpes Virus of Turkeys), MDV (Marek Disease Virus), ILT (Infectious Laryngotracheitis Virus), FAV (Fowl Adenovirus), FPV (FowlpoxVirus), or NDV (Newcastle Disease Virus). pcDNA3.1 is a particularly preferred vector for expression in animal cells.

- 15 After the polynucleotide has been cloned into an appropriate vector, the construct may be transferred into the cell, bacteria, or yeast by means of an appropriate method, such as electroporation, CaCl₂ transfection or lipofectins. When a baculovirus expression system is used, the transfer vector containing the polynucleotide may be transfected together with a complete baculo genome.

20 These techniques are well known in the art and the manufacturers of molecular biological materials (such as Clontech, Stratagene, Promega, and/or Invitrogen) provide suitable reagents and instructions on how to use them. Furthermore, there are a number of standard reference text books providing further information on this,
25 e.g. Rodriguez, R. L. and D. T. Denhardt, ed., "Vectors: A survey of molecular cloning vectors and their uses", Butterworths, 1988; Current protocols in Molecular Biology, eds.: F. M. Ausubel *et al.*, Wiley N. Y. , 1995; Molecular Cloning: a laboratory manual, supra; and DNA Cloning, Vol. 1-4, 2nd edition 1995, eds.: Glover and Hames, Oxford University Press).

30 Details of preferred proteins according to the present invention for expression via the vector are described above.

The vector may be adapted to provide transient expression in a host cell or stable expression. Stable expression can be achieved, for example, through integration of the nucleotide sequence encoding CD163 or a biologically active fragment or variant thereof into the genome of the host cell.

5

Suitable viral vectors include retroviral vectors (including lentiviral vectors), adenoviral vectors, adeno-associated viral (AAV) vectors, and alphaviral vectors.

The vector of the present invention can be present in a virion.

10

Vectors according to the present invention can be used in transforming cells for expression of a protein according to the present invention. This can be done in cell culture to produce recombinant protein for harvesting, or it can be done in vivo to deliver a protein according to the present invention to an animal.

15

In a fifth aspect the present invention provides a cell population which comprises a synthetic genetic construct adapted express a protein according to the present invention.

20

Preferably said cell population is present in a cell-culture system in a suitable medium to support cell growth.

The cells can be eukaryotic or prokaryotic.

25

Polynucleotides of the present invention may be cloned into any appropriate expression system. Suitable expression systems include bacterial expression system (e.g. *Escherichia coli* DH5 α), a viral expression system (e.g. Baculovirus), a yeast system (e.g. *Saccharomyces cerevisiae*) or eukaryotic cells (e.g. COS-7, CHO, BHK, HeLa, HD11, DT40, CEF, or HEK-293T cells). A wide range of suitable expression systems are available commercially. Typically the polynucleotide is cloned into an appropriate vector under control of a suitable constitutive or inducible promoter and then introduced into the host cell for expression.

30

Suitably the cells are animal cells, more preferably they are mammalian cells, and most preferably pig cells.

5 Suitably said synthetic genetic construct is adapted to express a secretory variant of CD163.

Suitably such cells comprise a vector as set out above.

10 Preferably the cells are adapted such that expression of the protein according to the present invention is inducible.

In a sixth aspect the present invention provides a genetically edited non-human animal comprising a genetic modification which alters the expression or activity of CD163.

15

Preferably the animal is a porcine animal, more preferably a pig (*Sus scrofa*), and most preferably a domestic pig (*Sus scrofa domesticus* or *Sus domesticus*).

20 In a first embodiment, at least a portion of the CD163 gene has been altered to abrogate its ability to bind to PRRSV.

Preferably the region of the gene encoding domain 5 of CD163 has been altered. More preferably alterations to the CD163 gene are only present in the region encoding domain 5 (exon 7) or flanking non-coding regions.

25

CD163 is a protein with a role in normal cellular functions. Thus, completely knocking out the function of GD163 is undesirable as it will have knock on effects on the animal. Accordingly, the present invention provides a solution to this by targeting modifications to domain 5 of CD163. Such modifications can thus prevent PRRSV from infecting cells, because it cannot bind to domain 5, but does not completely abrogate its normal cellular functions, which are not restricted to domain 5.

30

In a second embodiment the animal is modified to contain a synthetic genetic construct adapted to express a protein according to the present invention.

Suitably the CD163 is a secretory form, i.e. it is not membrane-bound.

5 Suitably biologically-active variants of CD163 (including fragments focussed on domain 5) are discussed above in more detail.

Animals comprising such a modification will express a decoy form of CD163 which will bind to PRRSV and thus prevent infection or reduce its severity.

10 Editing of the CD163 gene sequence can suitably be achieved by any one or more of:

- Deleting at least a portion of the CD163 gene;
 - Inserting a sequence into the CD163 gene; and
 - Replacing at least a portion of the CD163 gene. Such a replacement is
- 15 termed an 'introgression' or substitution.

Genetically edited animals according to the present invention preferably demonstrate one or more of the following phenotypes:

- an altered, especially increased, disease resilience or tolerance;
- 20 – an altered immune response; and
- an altered stress response.

More particularly, beneficial effects of the genetic modification may include improved tolerance or resistance to:

- 25 – Virus infection, e.g. PRRS infection in pigs.
- Pathogen infection, other than viral infection; and
- General or specific stressors.

In a particularly preferred aspect of the present invention the genetically edited

30 animal is a pig which has improved tolerance to PRRS infection.

An animal can be said to be more tolerant to infection when the mortality rate, morbidity rate, the proportion of animals showing significant morbidity (e.g. weight

loss or decreased growth rate), the level of morbidity or the duration of morbidity is reduced. Any statistically significant reduction (e.g. 95% confidence, or 99% confidence using an appropriate test) in the mortality or morbidity between a population of genetically edited pigs and a population of equivalent non-edited pigs when exposed to PRRS of the same virulence level (ideally the same isolate) demonstrates improved tolerance.

According to a seventh aspect the invention provides a method of producing a genetically edited non-human animal comprising the steps of:

- Providing a non-human animal cell;
- Editing the genetic content of the cell to create a modification which alters the expression or activity of CD163 or a biologically active fragment or variant thereof as set out above; and
- Generating an animal from said cell.

Preferably the animal is a porcine animal, more preferably a pig (*Sus scrofa*), and most preferably a domestic pig (*Sus scrofa domesticus* or *Sus domesticus*).

Suitably the modification alters the region of the CD163 gene which codes for domain 5 of CD163, i.e. exon 7, or flanking intronic regions.

Alternatively the modification introduces a sequence which encodes CD163 or a biologically active fragment or variant thereof as set out above which is able to act as a decoy.

The editing step suitably comprises:

- Introducing a site specific nuclease to the cell, the nuclease being adapted to bind to a suitable target sequence in the CD163 gene;
- Incubating said cell under suitable conditions for said nuclease to act upon the DNA at or near to said target sequence; and
- Thereby induce recombination, homology-directed repair (HDR) or non-homologous end joining (NHEJ) at or near the target site.

The non-human animal cell can be a somatic cell, a gamete, a germ cell, a gametocyte, a stem cell (e.g. a totipotent stem cell or pluripotent stem cell) or a zygote.

5 The method can optionally involve cloning, e.g. somatic cell nuclear transfer (SCNT). In such an embodiment the genetic editing event is carried out on a somatic cell, after which the edited nucleus is transferred to an enucleated egg cell. Typically a population of somatic cells will be edited and cells in which a desired editing event has occurred will be used to provide donor nuclei for SCNT. Processes for SCNT
10 have been well described in the art and would be known to the skilled person.

However, it is an advantage of the present invention that editing can be performed without the need for cloning.

15 Preferably the method is performed on a zygote. The term 'zygote' can be used in a strict sense to refer to the single cell formed by the fusion of gametes. However, it can also be used more broadly in the present context to refer to the cell bundle resulting from the first few divisions of the true zygote – this is more properly known as the morula.
20

It is preferred that the present method is at least initiated, and preferably completed, in the zygote at the single cell stage. This should result in all cells of the animal containing the same edit. It is, however, possible that the zygote may divide while the editing process is occurring. Depending on when the cell division occurs relative
25 to the stage of the editing process, it is possible that one of the following will occur:

- All cells will contain the same edit because they are derived from the a single cell which was edited before division occurred;
- All cells will contain the same edit because identical editing events occurred in the daughter cells after division occurred;
- 30 – A mosaic of cells with and without editing events is created because the cell divided before the editing event occurs and only one daughter cell was edited; and

- A mosaic of cells with different edits is created because the cell divided and differing editing events happened in the daughter cells.

Editing can also be conducted at after the first cell division, and the results may be of interest. However, this is generally not preferred where the desired result is a non-mosaic animal.

Accordingly, in a preferred embodiment the method comprises the steps of:

- Providing a zygote of the non-human animal;
- 10 – Introducing a site specific nuclease to the zygote which is adapted to bind to a suitable target sequence in the CD163 gene;
- Incubating said zygote under suitable conditions for said nuclease to act upon the DNA at or near to said target sequence; and
- Generating an animal from said genetically edited zygote.

15

It should be noted that the site specific nuclease can be introduced to a cell in any suitable form. For example, the nuclease can be provided directly into the cell as a functional protein. Alternatively, the nuclease can be provided into the cell in the form of a precursor or template from which the active nuclease is produced by the cell. In a preferred embodiment an mRNA encoding the nuclease is introduced into the cell, e.g. by injection. The mRNA is then expressed by the cell to form the functioning protein. Using mRNA in this way allows rapid but transient expression of the nuclease within the cell, which is ideal for the purposes of genetic editing.

25 It should also be noted that the term 'nuclease' is intended to cover any biological enzyme which creates a single or double stranded cut of a target nucleic acid. Accordingly, the term includes nickases and recombinases, as well as more conventional nucleases which cause single or double stranded breaks.

30 The method may comprise inserting a heterologous sequence in the CD163 gene at the target site. Such a heterologous DNA sequence can replace and/or disrupt the endogenous DNA sequence. This can be achieved by introducing a suitable template DNA molecule to the cell, such as single or double-stranded DNA molecule,

which will be inserted by the cell's DNA repair mechanisms or an exogenous recombinase. Exemplary DNA sequences for insertion are described above, but many others could of course be used.

5 The genetically edited zygote can be grown to become an embryo and eventually an adult animal. As discussed above, if the editing event occurs in the single-cell zygote then all cells of this animal will therefore comprise the modified CD163 gene as all cells of the animal are derived from a single genetically edited cell. If the editing event occurs after one or more cell divisions then the resultant animal will likely be a
10 mosaic for the editing event, in that it will have some cells derived from the edited cell and some cells derived from unedited cells.

The method may involve characterising the genetic modification which has occurred. Suitable methods to achieve this are set out below.

15

The method can be performed on a plurality of zygotes and the method may involve selecting zygotes in which the desired genetic modification has been achieved.

20 Preferably the nuclease comprises a pair of transcription activator-like effector nucleases (TALENs) or zinc finger nucleases (ZFNs). Such nucleases are well known in the art and comprise a nuclease moiety fused to a sequence-specific DNA binding moiety. The nuclease activity requires a pair of the nuclease moieties to form the active nuclease dimer. Such nucleases are well adapted to site-specific cleaving of DNA molecules, and techniques to target said nucleases to any desired
25 sequences are known to the skilled person and described below. The TALENs or ZFNs can be tailored to target suitable sequences to achieve the desired DNA cut. By inducing a cut in the DNA the cell repairs the cut by NHEJ or HDR. The former is an error prone system and therefore can be used to introduce edits as a result of errors. The latter can be used to introduce a heterologous sequence into the cell.

30

Alternatively, the nuclease may comprise a nickase. Nickases are like TALENs or ZFNs in many ways, but they cause only a single strand break. This can be an advantage in inducing accurate homology-directed repair, which is particularly useful in the present invention to create a desired introgression. Nickases are described in

Ramirez et al. 'Engineered zinc finger nickases induce homology-directed repair with reduced mutagenic effects' *Nucleic Acids Research*, 2012, 1–9
doi:10.1093/nar/gks179.

5 Another option is that the nuclease comprises a recombinase. Recombinases are a group of enzymes which allow very precise manipulation and editing of DNA. Although they are not currently as versatile as TALENs, ZFNs and nickases, they have significant potential to allow very tightly controlled editing events. Recombination controlled by recombinases can be used to accurately paste a
10 sequence of interest into the CD163 gene.

Another option is that the nuclease comprises an RNA-guided site-specific nuclease, such as the CRISPR/Cas system described in Cong et al. 'Multiplex Genome Engineering Using CRISPR/Cas Systems', *Science*, 15 February 2013: Vol. 339 no.
15 6121 pp. 819-823. Such systems use an RNA molecule to target a specific sequence in to be cleaved by the nuclease, in the case of the CRISPR/Cas system 'spacer' sequences are used to target the nuclease. Suitable spacers can be created and used to target the Cas nuclease to the desired location in the CD163 gene and thus cause double-stranded breaks in the DNA whereupon NHEJ would
20 result in the introductions of indels.

Of course, in this rapidly developing field, other techniques for genetic editing are likely to become available. Such techniques could in many cases be readily adapted for use in the present invention.

25 Preferably the nuclease is adapted to target sequences in the region of the CD163 gene. The regions of particular interest are discussed in more detail above.

The site specific nuclease can be adapted to target a sequence which encodes
30 domain 5 of CD163 or flanking intronic sequences (i.e. introns 6 and 7). For example the nuclease can target a sequence within exon 7 of the CD163 gene. Non-homologous end joining and/or homology-directed repair can then be utilised to edit the CD163 gene.

Two or more pairs of TALENs, ZFNs or other such nucleases can be adapted to excise a region of DNA.

5 For example, a suitable strategy involves delivering two TALEN pairs at the same time to engineer deletion (removal) of the 7th exon of the CD163 gene (which contains domain 5)

In certain embodiments the site specific nucleases are TALENs or ZFNs and are adapted to target the sequences:

10 – 5'-

TCCCTCACCGAAATGCTATTTTCAGCCCACAGGAAACCCAGGCTGGTTGGA-
3' (CD163 intron 6 target sequence) (SEQ ID NO 4)

– 5'-

15 TCGGCGTAGTCTGCTCAAGTGAGACCCAGGGAATGTGTTCACTTTGTTCCCA
-3' (CD163 intron 7 target sequence) (SEQ ID NO 5)

However, site specific nucleases adapted to target other suitable target sequences could of course be used.

20 In the above example, the following primers can suitably be used to detect editing events:

– forward 5'-AAACCAAGAGGCATGAATGG-3' (SEQ ID NO 6)

– reverse 5'-TGTCACAGTGAGAGTTGCAG-3' (SEQ ID NO 7)

25 The method may comprise one or more of the step of testing the ability of the animal to tolerate challenge with a pathogen, e.g. a virus. For example, where the animal is a pig, the method may involve testing the ability of the genetically edited animal to survive infection with a highly virulent PRRS.

30 Relevant Definitions:

'Biologically active' in the present context refers to the ability of a fragment or variant to be bind to PRRSV. Fragments or variants may bind to PRRSV with higher or

lower affinity than the bond between PRRSV and wild-type, membrane-bound CD163. It is preferred that the binding of PRRSV to the fragment or variant of CD163 is at least 50% as strong as between PRRSV and wild-type, membrane-bound CD163, more preferably at least 70%, 80%, 90%, 95% or 100% as strong.

5 Bond strength in this case can be equated to the equilibrium constant K_d of the relevant interaction in a suitable medium, e.g. serum or serum equivalent.

'Conservative' changes or substitutions, as used herein, refer to one or more amino acid substitutions (for example of 1, 2, 5 or 10 residues) for amino acid residues
10 having similar biochemical properties. Typically, conservative substitutions have little to no impact on the activity of a resulting polypeptide. For example, a conservative substitution in a contact phase factor inhibitory peptide may be an amino acid substitution that does not substantially affect the ability of the peptide to inhibit a contact phase factor or combination thereof. Screening of variants can be used to
15 identify which amino acid residues can tolerate an amino acid substitution.

In one example, one conservative substitution is included in the protein. In another example, 10 or fewer conservative substitutions are included in the protein, such as five or fewer. A protein of the invention may therefore include 1, 2, 3, 4, 5, 6, 7, 8, 9
20 10 or more conservative substitutions. A polypeptide can be produced to contain one or more conservative substitutions by manipulating the nucleotide sequence that encodes that polypeptide using, for example, standard procedures such as site-directed mutagenesis or PCR. Alternatively, a polypeptide can be produced to contain one or more conservative substitutions by using peptide synthesis methods,
25 for example as known in the art.

Substitutional variants are those in which at least one residue in the amino acid sequence has been removed and a different residue inserted in its place. Examples of amino acids which may be substituted for an original amino acid in a protein and
30 which are regarded as conservative substitutions include: Ser for Ala; Lys for Arg; Gln or His for Asn; Glu for Asp; Asn for Gln; Asp for Glu; Pro for Gly; Asn or Gln for His; Leu or Val for Ile; Ile or Val for Leu; Arg or Gln for Lys; Leu or Ile for Met; Met, Leu or Tyr for Phe; Thr for Ser; Ser for Thr; Tyr for Trp; Trp or Phe for Tyr; and Ile or Leu for Val. In one embodiment, the substitutions are among Ala, Val Leu and Ile;

among Ser and Thr; among Asp and Glu; among Asn and Gln; among Lys and Arg; and/or among Phe and Tyr. Further information about conservative substitutions can be found in, among other locations, Ben-Bassat et al., (J. Bacteriol. 169:751-7, 1987), O'Regan et al., (Gene 77:237-51, 1989), Sahin-Toth et al., (Protein Sci. 3:240-7, 1994), Hochuli et al., (Bio/Technology 6:1321-5, 1988), WO 00/67796 (Curd et al.) and in standard textbooks of genetics and molecular biology.

By the term "Fc" or "Fc fragment" it is intended to refer to a region of an antibody molecule that binds to antibody receptors on the surface of cells such as macrophages and mast cells, and to complement protein. Fc (50,000 daltons) fragments contain the CH2 and CH3 region and part of the hinge region held together by one or more disulfides and noncovalent interactions. Fc and Fc5 μ fragments are produced from fragmentation of IgG and IgM, respectively. The term Fc is derived from the ability of these antibody fragments to crystallize. Fc fragments are generated entirely from the heavy chain constant region of an immunoglobulin. The Fc fragment cannot bind antigen, but it is responsible for the effector functions of antibodies, such as complement fixation.

"Fusion protein", as used herein, refers to a protein containing amino acid sequences from each of two distinct proteins; it is formed by the expression of a recombinant gene in which two coding sequences have been joined together such that their reading frames are in phase. Hybrid genes of this type may be constructed in vitro in order to label the product of a particular gene with a protein that can be more readily assayed. For example, a protein may be linked to a signal peptide to allow its secretion by the cell, or linked a peptide to promote stability of efficacy.

"Genetically edited" or "genetically modified" when used in relation to subject biological material, refers to the fact that the subject biological material has been treated to produce a genetic modification thereof compared to control, e.g. wild type, biological material.

The term "isolated" means a biological component (such as a nucleic acid molecule or protein) that has been substantially separated or purified away from other biological components in the cell of the organism in which the component naturally

occurs, i.e., other chromosomal and extrachromosomal DNA and RNA, and proteins. Nucleic acids and proteins that have been "isolated" include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as
5 chemically synthesized nucleic acids, proteins and peptides.

"Protein" and "peptide", as used herein, can be used interchangeably (unless the context suggests otherwise) and mean at least two covalently attached amino acids linked by a peptidyl bond. The term protein encompasses purified natural products,
10 or products which may be produced partially or wholly using recombinant or synthetic techniques. The terms peptide and protein may refer to an aggregate of a protein such as a dimer or other multimer, a fusion protein, a protein variant, or derivative thereof. A protein may comprise amino acids not encoded by a nucleic acid codon, i.e. non-natural amino acids.

15 The term "prophylaxis" includes reference to treatment therapies for the purpose of preserving health or inhibiting or delaying the initiation and/or progression of an event, state, disorder or condition, for example for the purpose of reducing the chance of an event, state, disorder or condition occurring. The outcome of the
20 prophylaxis may be, for example, preservation of health or delaying the initiation and/or progression of an event, state, disorder or condition. It will be recalled that, in any individual subject or even in a particular subject population, a treatment may fail, and this paragraph is to be understood accordingly.

25 "Protein modification", as used herein, is a type of protein variant and means an alteration to a moiety chemically linked to a protein. For example, a modification may be an altered carbohydrate or PEG structure attached to a protein. The proteins of the invention may include at least one such protein modification. The term includes modifications of the protein, for example, protein modified by glycosylation,
30 acetylation, phosphorylation, pegylation, ubiquitination, and so forth.

"Site-directed mutagenesis", as used herein, refers to the in-vitro induction of mutagenesis at a specific site in a given target nucleic acid molecule.

The term "treatment", and the therapies encompassed by this invention, include the following and combinations thereof: (1) inhibiting, e.g. delaying initiation and/or progression of, an event, state, disorder or condition, for example arresting, reducing or delaying the development of the event, state, disorder or condition, or a relapse thereof in case of maintenance treatment or secondary prophylaxis, or of at least one clinical or subclinical symptom thereof; (2) preventing or delaying the appearance of clinical symptoms of an event, state, disorder or condition developing in an animal (e.g. a pig) that may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; and/or (3) relieving and/or curing an event, state, disorder or condition (e.g., causing regression of the event, state, disorder or condition or at least one of its clinical or subclinical symptoms, curing a patient or putting a patient into remission). The benefit to a subject to be treated may be either statistically significant or at least perceptible to the subject or to the veterinarian. It will be understood that a medicament will not necessarily produce a clinical effect in each subject to whom it is administered; thus, in any individual subject or even in a particular patient population, a treatment may fail or be successful only in part, and the meanings of the terms "treatment", "prophylaxis" and "inhibitor" and of cognate terms are to be understood accordingly. The invention concerns, amongst other things, the treatment of PRRS. The compositions and methods described herein are of use for therapy and/or prophylaxis of the mentioned conditions.

"Variants" of a protein or peptide, as used herein, refers to a protein resulting when a polypeptide is modified by one or more amino acids (e.g. insertion, deletion or substitution), or which comprises a protein modification, or which contains modified or non-natural amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties. More rarely, a variant may have "non-conservative" changes, for example, replacement of a glycine with a tryptophan. Variants may also include sequences with amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or immunological activity may be found using computer programs well known in the art.

Protein variants according to the present invention can be modified by a variety of chemical techniques to produce derivatives having essentially the same activity as the unmodified peptides, and optionally having other desirable properties. For example, carboxylic acid groups of the protein, whether carboxyl-terminal or side chain, may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified, for example to form a C1-C6 alkyl ester, or converted to an amide, for example of formula CONR₁R₂ wherein R₁ and R₂ are each independently H or C1-C6 alkyl, or combined to form a heterocyclic ring, such as a 5- or 6-membered ring. Amino groups of the peptide, whether amino-terminal or side chain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to C1-C6 alkyl or dialkyl amino or further converted to an amide. Hydroxyl groups of the peptide side chains may be converted to alkoxy or ester groups, for example C1-C6 alkoxy or C1-C6 alkyl ester, using well-recognized techniques. Phenyl and phenolic rings of the peptide side chains may be substituted with one or more halogen atoms, such as F, Cl, Br or I, or with C1-C6 alkyl, C1-C6 alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide side chains can be extended to homologous C2-C4 alkylenes. Thiols can be protected with any one of a number of well-recognized protecting groups, such as acetamide groups. Those skilled in the art will also recognize methods for introducing cyclic structures into the peptides of this disclosure to select and provide conformational constraints to the structure that result in enhanced stability.

“Vector”, as used herein, refers to a self-replicating DNA or RNA molecule that transfers a nucleic acid segment between cells.

“Virion”, as used herein, refers to a particle composed of viral RNA and viral capsid protein.

30

The invention will now be further described, by way of example only, with reference to the accompanying figures in which:

– Figure 1 illustrates CD163 expressed on the cell surface of the alveolar macrophage

- Figure 1a is a schematic representation of CD163 protein
- Figure 2 shows the result of the RT-PCR amplification of domains D14, D55 and D59
- Figure 3 shows a schematic of the cloning vector pFUSE-rtIgG2B-Fc2
- 5 – Figure 4 shows a schematic representation of the Fc fusion proteins
- Figure 5 shows immunodetection of D55-Fc fusion protein in CHO (lane 1) and MARC-145 (lane 2) recombinant cells
- Figure 6 shows microscopic images PRRSV Infection of recombinant MARC-145 cells. PRRSV (pink staining) are detected in the cytosol of D14-Fc, D59-Fc and
- 10 pFuse control cells but not in 3 fields of D55-Fc cells. Nuclei are stained blue.
- Figure 7: PRRSV viral load in the growth medium 24, 48, 72 and 120 hours post infection. PRRSV GP5 RNA was detected using Sybrgreen RTPCR.

Examples

15

Example 1 - In vitro production and testing of decoy CD163 fragments

1 - RTPCR of porcine CD163 SRCR domains

- 20 To generate cDNA fragment that encodes domain of the scavenger receptor CD163 (Figure 1 and Figure 1a) we have used the published sequence GeneBank accession # ABV80230. Tables 1 and 2 show the primer sequences and their combination that were employed for the reverse transcriptase and PCR stages. Domains of cD163 were cloned using RNA prepared from porcine alveolar
- 25 macrophage isolated from lung macrophage of a Large White breed #131 (Ait-Ali *et al.* 2007). Reverse transcriptase and PCR were performed as described elsewhere (Ait-Ali *et al.* 2007) and the result is shown in Figure 2.

Table 1

Primers	Sequence
D1-FW	ACGAATTCCAGAATGGTGCTACATGAAACTCTGG (SEQ ID NO 8)
D5-FW	TCGATATCACACAGGAAACCCAGGCTGGTTGGAGG(SEQ ID NO 9)

D4-RV	GCCGATATCGCTGAGCAGGTAATTTGGCTTCGTC (SEQ ID NO 10)
D5-RV	TAACCATGGTGTATCTTGAGCAGACTACGCCGACG (SEQ ID NO 11)
D9-RV	CCGATATCGAGCACGTCACAGCAGCATCCTCCTTG (SEQ ID NO 12)

Table 2

Decoy domains	Size (bp)	Forward primer	Reverse primer	Restriction digestion
(D14) 1-2-3-4	1215	D1-FW	D4-RV	EcoR and EcoRV
(D55) 5	303	D5-FW	D5-RV	EcoRV and NcoI
(D59) 5-6-7-8-9	1521	D5-FW	D9-RV	EcoRV

2 - Cloning of CD163 domains

5

Decoy domains D14, D55 and D59 were cloned into the expression vector pFUSE-rtIgG2B-Fc2 (Figure 3, Novagen) into the multi-cloning site and between the IL2ss secretory sequence and the rat-IgG2B Fc region to generate soluble Fc fusion proteins as shown in Figure 4. IL2-D55-FC refers to a fusion IL2ss, domain 5 and rat-IgG2B Fc. The accuracy of the fusion constructs were verified using Sanger sequencing.

10

The nucleotide sequence encoding IL2-D55-FC (D55 encoding region in lower case) is:

15

ATGTACAGGATGCAACTCCTGTCTTGCATTGCACTAAGTCTTGCACCTTGTACAG
AATTCGATATCcaggaaaccaggctggttgaggggacattccctgctctggtcgtgtgaagtacaacatg
gagacacgtggggcaccgtctgtgattctgacttctctctggaggcggccagcgtgctgtgcagggaaactacagtgcg
gcactgtggtttccctcctggggggagctcactttggagaaggaagtggacagatctgggctgaagaattccagtgtg
aggggcacgagtcccaccttctcctgcccagtagcaccgcccctgacgggacatgtagccacagcagggacg
tcggcgtagtctgctcaagatacacCATGGTTAGATCTCCTACATGCCCTACATGTCACAAA
TGCCAGTTCCTGAACTCTTGGGTGGACCATCTGTCTTCATCTTCCCGCCAAAG
CCCAAGGACATCCTCTTGATCTCCCAGAACGCCAAGGTCACGTGTGTGGTGGT
GGATGTGAGCGAGGAGGAGCCGGACGTCCAGTTCAGCTGGTTTGTGAACAAC
GTAGAAGTACACACAGCTCAGACACAACCCCGTGAGGAGCAGTACAACAGCAC
CTTCAGAGTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGAGCGGCA

25

AGGAGTTCAAATGCAAGGTCAACAACAAAGCCCTCCCAAGCCCCATCGAGAAA
ACCATCTCAAACCCAAAGGGCTAGTCAGAAAACACAGGTATACGTCATGGGT
CCACCGACAGAGCAGTTGACTGAGCAAACGGTCAGTTTGACCTGCTTGACCTC
AGGCTTCCTCCCTAACGACATCGGTGTGGAGTGGACCAGCAACGGGCATATAG
5 AAAAGAACTACAAGAACACCGAGCCAGTGATGGACTCTGACGGTTCTTTCTTCA
TGACAGCAAGCTCAATGTGGAAAGGAGCAGGTGGGATAGCAGAGCGCCCTTC
GTCTGCTCCGTGGTCCACGAGGGTCTGCACAATCACCACGTGGAGAAGAGCAT
CTCCCGGCCTCCGGGTAAATGA (SEQ ID NO 14)

10 3 - Generation of CHO and MARC-145 recombinant cells

D14-Fc, D55-Fc, D59-Fc constructs and the empty pFUSE vector (negative control) were transfected into MARC-145 and CHO cells using Lipofectamine 2000 (Invitrogen™) and stable transfected cells were selected with Zeocin antibiotic (data
15 not shown).

4 - Detection of the expression of D55-Fc protein

D55-Fc expression was detected in growth medium of D55-Fc in recombinant CHO
20 and MARC-145 cells. Figure 5 shows the western blot detection of the D55-Fc using goat anti-rat IgG2b antibody. The integrity of the D55-Fc RNA expressed in the recombinant cells was confirmed using RT-PCR and Sanger sequencing (data not shown).

25 5 - PRRSV infectivity

Recombinant MARC-145 cells were challenged with the European PRRSV strain Olot/91 at multiplicity of infection (moi) of 1 for 40 hours. Cells were then fixed and PRRSV viral nucleoprotein was detected using antibody SDOW17 and the
30 secondary antibody goat anti-mouse IgG-Alexa Fluor-647 (Figure 6). This experiment was repeated 3 times using different batches of virus.

To test if the PRRSV virus RNA can be detected in the growth medium of infected cells, recombinant Marc cells were challenged with the European PRRSV strain

Olot/91 at moi of 1 and the virus RNA was monitored in the medium 24, 48, 72, 120 hours post infection using RTPCR. Figure 7 shows that in contrast to D55-Fc cells the Ct declined drastically in Pfuse, D14-Fc and D59-Fc cells. This experiment was repeated twice with independent virus preparation. Taken together these results suggest that the D55-Fc MARC145 cells were less susceptible to PRRSV than other cells.

6 - D55-Fc MARC cells transfection with a PRRSV infectious clone

To test that D55-Fc Marc cells reduced susceptibility is due to a blockade of the viral rather than an increase of immunity of the recombinant cells, D55-Fc MARC145 cells were transfected with an infectious clone SD01-08-GFP that expresses PRRSV-GFP. Results showed that the recombinant cells expressed GFP-PRRSV similarly to the non-recombinant cells. This experiment suggests strongly that the phenotype of recombinant D55-Fc cells is probably associated to a blockade of viral entry rather than an increase of immunity.

Example 2 - Creation of a pig over-expressing domain 5 using a lentiviral vector

Lentiviral vectors are produced by co-transfection of HEK293T cells with 3 plasmids; the first encodes a chosen promoter (this could be ubiquitous, e.g. the CAG promoter, tissue restricted, e.g. the CMV immediate early promoter which is known to be expressed predominantly in exocrine cells, or tissue specific, e.g. CSF1 receptor promoter which is specific to cells of the mononuclear macrophage lineage or a synthetic liver specific promoter) directing expression of a transgene comprised of exon7 (domain 5) fused to a secretory signal peptide such as that from IL-2. This promoter-exon 7 combination is flanked by HIV1 regulatory sequence elements and inverted terminal repeats. The second plasmid encodes HIV1 Gag and Pol elements, such as those encoded by plasmid psPAX2. The third plasmid encodes a version of the vesicular stomatitis virus G protein that is commonly used to pseudotype lentiviral particles due to its broad host tropism.

Once transfected into HEK293T cells these plasmids result in the production of replication defective lentiviral particles will be secreted from the cells into the culture medium. Lentiviral particles are recovered from the medium by (for example) ultracentrifugation.

5

Transgenic pigs are produced by injection of a suspension of the lentiviral particles into the perivitelline space of the fertilised egg (zygote). Typically embryos are produced from Large-White gilts that are approximately 9 months of age and weigh at least 120 kg at time of use. Super-ovulation is achieved by feeding, between day 10 11 and 15 following an observed oestrus, 20 mg altrenogest (Regumate, Hoechst Roussel Vet Ltd) once daily for 4 days and 20 mg altrenogest twice on the 5th day. On the 6th day, 1500 IU of eCG (PMSG, Intervet UK Ltd) is injected at 20:00hrs. Eighty three hours later 750 IU hCG (Chorulon, Intervet UK Ltd) is injected. Donor gilts are inseminated twice 6 hours apart after exhibiting heat generated following super-ovulation. Zygotes are surgically recovered from mated donors by mid-line 15 laparotomy under general anesthesia on day 1 following oestrus into NCSU-23 HEPES base medium. Zygotes are subjected to an injection of between 50-1000 pl injection of lentiviral particle suspension into the perivitelline space. Recipient females are treated identically to donor gilts but remain un-mated. Following lentiviral 20 injection, zygotes are transferred to recipient gilts following a mid-line laparotomy under general anesthesia. During surgery, the reproductive tract is exposed and zygotes transferred into the oviduct of recipients using a 3.5 French gauge tomcat catheter.

25 **Example 3 - Creation of a pig overexpressing domain 5 using DNA editor-mediated targeting**

DNA editors (TALENs, ZFNs or CRISPRs) to a target site in the porcine genome can be constructed through known techniques.

30

This could, for example, be the porcine ROSA26 locus or a similar locus where robust gene expression is anticipated (see, for example, CN102851279 for details of a pig ROSA26 locus).

A DNA molecule comprised of a promoter directing expression of exon7 (domain 5) fused to a secretory signal peptide as detailed above, flanked by sequence of 30-2000bp immediately upstream and downstream of the DNA editor target site in the porcine genome can be designed and constructed using known techniques (henceforth referred to as the targeting construct).

Porcine zygotes produced as above will be injected into either the cytoplasm or a pronucleus with a solution comprising the targeting construct together with the DNA editor supplied in either DNA, RNA or protein form. Injected zygotes will be transferred to recipient sows as above.

Example 4 - Editing of CD163 locus using genome editing technologies.

Example 4a: Editor Design and Construction

Editing of the CD163 locus can be achieved using DNA editor technology (e.g. TALEN, ZFN, CRISPR/Cas9). This is illustrated through TALEN methodology. TALENs are designed using the TALE-NT software and assembled using methods described in Cermak et al (2011). Briefly, intermediary arrays are produced for each TALEN pair that are compatible for Golden Gate cloning into pC-+63-TAL modified vector (although other vectors such as pC-+231-TAL, RCIscript-+231-TAL, pC-GoldyTALEN or RCIscript-GoldyTALEN can be used. Arrays are joined in the above vectors as follows; 150 ng each pFUS_A, pFUS_B, pLR-X and the desired backbone are mixed in a 20 µl digestion/ligation reaction including 50 units T4 DNA ligase (New England Biolabs) and 10 units Esp3I (Fermentas) in 1X T4 ligase buffer (New England Biolabs). The reaction is incubated in a thermocycler for 10 cycles of 5 min at 37°C and 10 min at 16°C, then heated to 50°C for 5 min and then 80°C for 5 min. Two microliters of each reaction is transformed into E.coli and plated on LB-carbenicillin plates. Plasmid DNA is purified and mRNA synthesized from SacI linearized RCIscript vectors using the mMessage Machine T3 Kit (Ambion).

Example 4b: Target site details

With the intention of deleting CD163 exon 7 from the pig genome, target sites located upstream (e.g. in intron 6) or downstream (e.g. in intron 7) are selected.

For example, the TALENs can target the following sequences:

– 5'-

TCCCTCACCGAAATGCTATTTTCAGCCCACAGGAAACCCAGGCTGGTTGGA-

5 3' (CD163 intron 6 target sequence) (SEQ ID NO 4)

– 5'-

TCGGCGTAGTCTGCTCAAGTGAGACCCAGGGAATGTGTTCACTTTGTTCCCA

-3' (CD163 intron 7 target sequence) (SEQ ID NO 5)

10 Other suitable target sequences could of course be used.

In the above example, the following primers can be used to detect and analyse editing events:

– forward 5'-AAACCAAGAGGCATGAATGG-3' (SEQ ID NO 6)

15 – reverse 5'-TGTCCCAGTGAGAGTTGCAG-3' (SEQ ID NO 7)

Example 4c: Generation of gene edited pigs

Gene edited pigs are produced through injection of the TALEN (or another DNA editor) into the fertilised egg (zygote). Delivery can be to the nucleus or cytoplasm of the zygote. The TALEN can be delivered as DNA, RNA or protein. To illustrate the approach the method of cytoplasmic delivery of a TALEN is given.

Embryos are produced from Large-White gilts that are approximately 9 months of age and weigh at least 120 kg at time of use. Super-ovulation is achieved by feeding, between day 11 and 15 following an observed oestrus, 20 mg altrenogest (Regumate, Hoechst Roussel Vet Ltd) once daily for 4 days and 20 mg altrenogest twice on the 5th day. On the 6th day, 1500 IU of eCG (PMSG, Intervet UK Ltd) is injected at 20:00hrs. Eighty three hours later 750 IU hCG (Chorulon, Intervet UK Ltd) is injected. Donor gilts are inseminated twice 6 hours apart after exhibiting heat generated following super-ovulation. Embryos are surgically recovered from mated donors by mid-line laparotomy under general anesthesia on day 1 following oestrus into NCSU-23 HEPES base medium. Embryos are subjected to a single 2-5 pl cytoplasmic injection of TALEN pair mRNA at 2 ng/μl. Recipient females are treated

identically to donor gilts but remained un-mated. Following TALEN injection, fertilized embryos are transferred to recipient gilts following a mid-line laparotomy under general anesthesia. During surgery, the reproductive tract is exposed and embryos are transferred into the oviduct of recipients using a 3.5 French gauge tomcat catheter.

Example 4d: Genotyping of editing events in pigs

Gene editing events in born piglets are identified by direct sequencing of amplified, isolated DNA (e.g. using the primers set out above) and through gel electrophoresis assay. The latter identified mismatch between the two alleles through digestion by the Cel1 enzyme.

The DNA is extracted from tissue samples (e.g. ear skin biopsy) using the Fast Tissue-to-PCR kit, Fermentas. The Fast tissue DNA sample is then used as a template for High fidelity PCR (AccuPrime™ Taq DNA Polymerase High Fidelity, Invitrogen™) using primers designed to amplify across CD163 exon 7 and neighbouring genomic region (as set out above, for example). The PCR product is then sent for sequence analysis to allow identification of editing events. Alternatively the PCR products are cloned into a plasmid and individual clones sequenced allowing heterozygous and mosaic editing events to be analysed separately. Alternatively the deletion of CD163 exon 7 can be identified by standard Southern blotting with appropriate restriction enzymes.

Nucleotide and Peptide sequences for CD163

GeneBank accession # ABV80230 – CD163 protein (SEQ ID NO 1) – exon 7 which encodes domain 5 is underlined.

```

1   mdklrmvlhe  nsgsadrfrcc  sahlssftfa  vvavlsaclv  tsslggkdke  lrltgge
30  nkc
   61   sgrvevkvqe  ewgtvcnngw  dmdvvsvvcr  qlgcptaika  tgwanfsags  griwmdhv
   sc
  121   rgnosalwdc  khdgwgkhnc  thqqdagvtc  sdgsdlemrl  vnggnrclgr  ievkfqgr
   wg
  35  181   tvcdnfnin  hasvvckqle  cgsavsfsgs  anfgegsgpi  wfddlvcngn  esalwnck
   he

```

241 gwgkhncdha edagviclng adlklrvvdg vtecsgrlev kfqqewgtic ddgwdsdd
 aa
 301 vackqlgcpt avtaigrvna segtghiwld svschghesa lwqcrhhewg khynchne
 da
 5 361 gvtcsdgsdl elrlkgggsh cagtveveiq klvgkvcdrs wglkeadvvc rqlgcgsa
 lk
 421 tsyqvysktk atntwlfvss cngnetsslwd cknwqwgglc cdhydeakit csahrkpr
lv
 481 ggdipcsgrv evqhgdtwgt vcdsdfslea asvlcrelqc gtvvslggga hfgegsqg
 10 iw
 541 aefqceghe shslcpvap rpdgtcshsr dvgvvcryt qirlvngktp cegrveln
 il
 601 gswgslcnsh wdmedahvlc qqlkcgvals iprgapfgkg seqvwrhmfh ctgtekhm
 gd
 15 661 csvtalgasl cssgqvasvi csgnqsqtls pcnsssdps ssiiseengv acigsgql
 rl
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 20 ev
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 dt
 901 lwqcpsspwk krlaspseet witcankirl qegntncsgr veiwyggswg tvcdsdsw
 le
 25 961 daqvvcrlqg cgsaleagke aafgqgtgpi wlnevkcckgn etslwdcpar swghsdcg
 hk
 1021 edaavtcsei aksreslhat grssfvalai fgvillacli afliwtkrr qrqlsvf
 sg
 1081 gensvhqiqy remnsclkad etdmlnpsgd hsevq
 30

NCBI Reference Sequence: NM_213976.1 – CD163 mRNA sequence (SEQ ID NO 2)

35 1 atggtgctac ttgaagactc tggatctgca gactttagaa gatgttctgc
 ccatttaagt
 61 tccttcactt ttgctgtagt cgctgttctc agtgctgctc tggtcactag
 ttctcttgga
 121 ggaaaagaca aggagctgag gctaacgggt ggtgaaaaca agtgctctgg
 40 aagagtggag
 181 gtgaaagtgc aggaggagtg gggaaactgtg tgtaataatg gctgggacat

ggatgtggtc
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5 gaatgagtca
361 gctctctggg actgcaaaca tgatggatgg ggaaagcata actgtactca
ccaacaggat
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10 481 cgggtgcttag gaagaataga agtcaaattt caagagcggg ggggaacagt
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ggacattccc
5 1441 tgctctggtc gtggtgaagt acaacatgga gacacgtggg gcaccgtctg
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tgtggtttcc
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10 agaattccag
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agtgtccagg
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25 cctcatttgg
3181 actcagaagc gaagacagag gcagcggctc tcagttttct caggaggaga
gaattctgtc
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ggatatgcta
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CD163

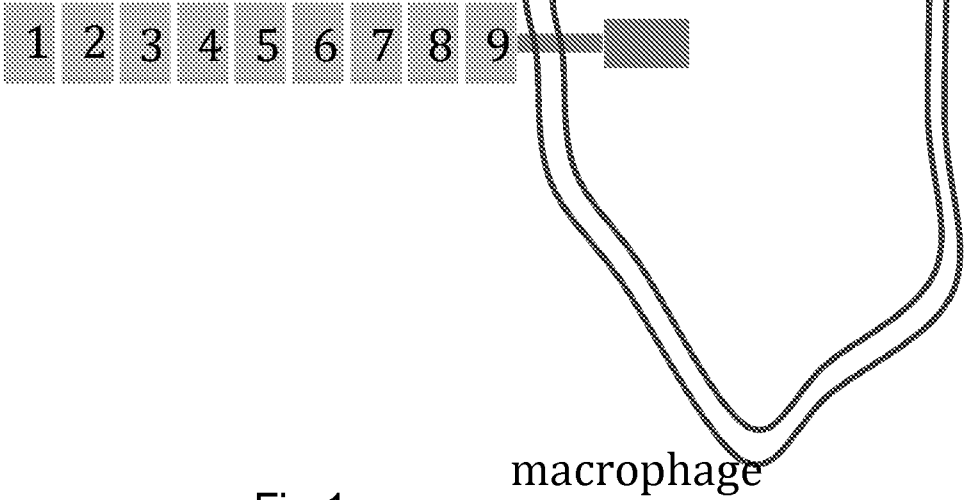


Fig 1

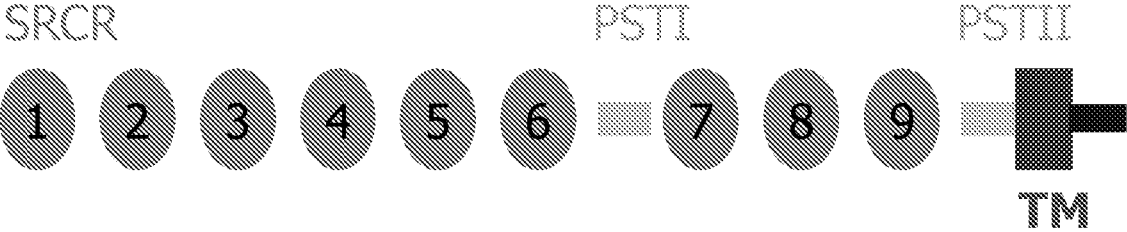


Fig 1a

D14 D55 D59



Fig 2

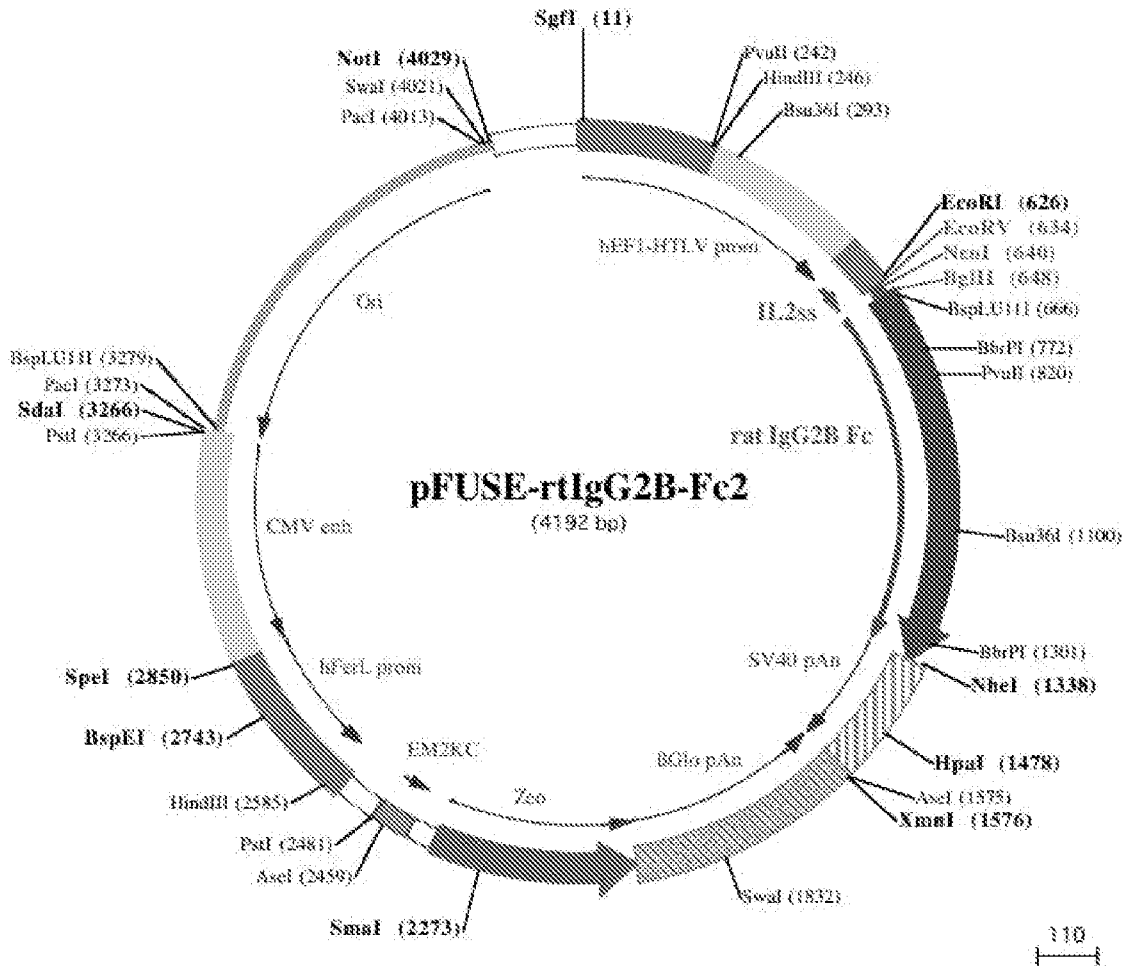


Fig 3

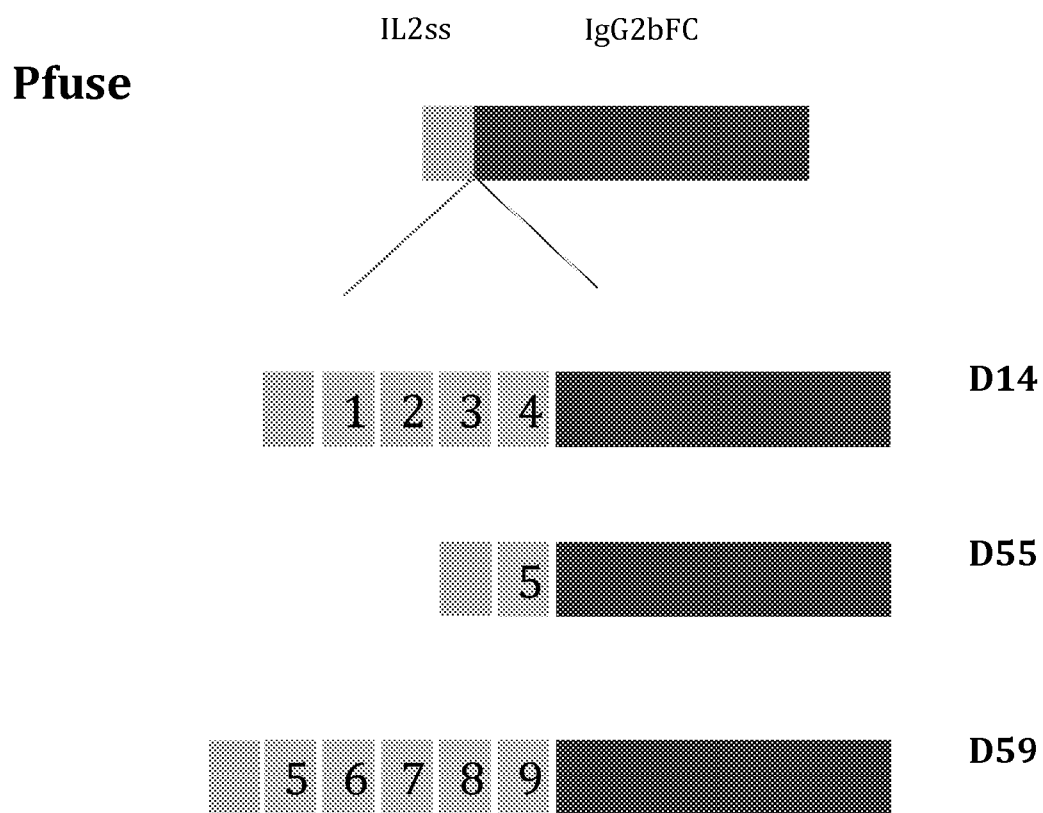


Fig 4

1

2

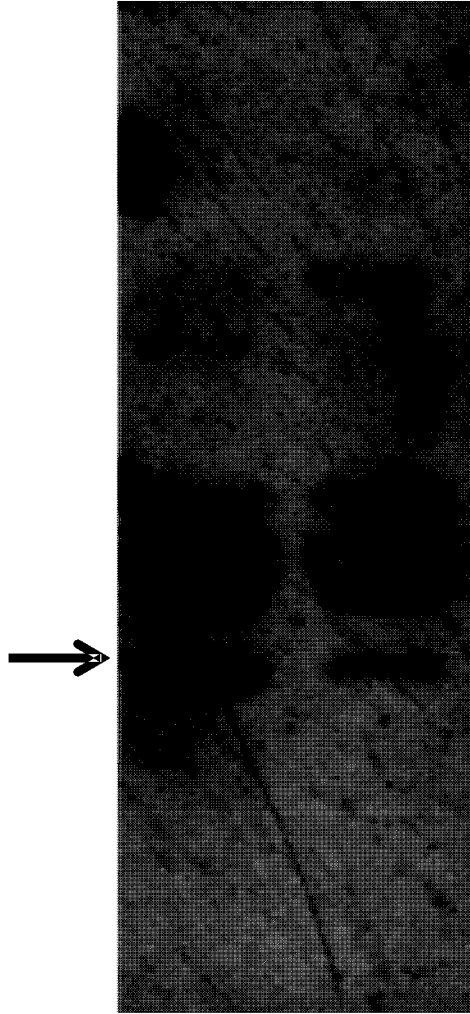
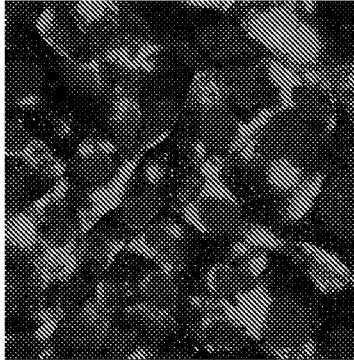
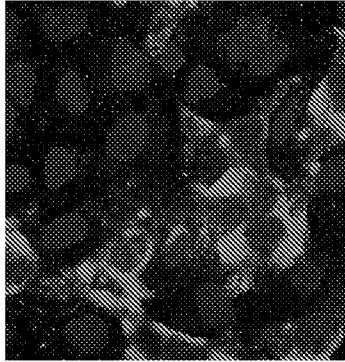


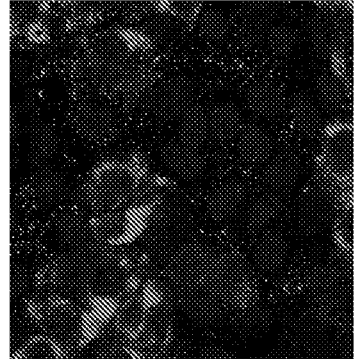
Fig 5



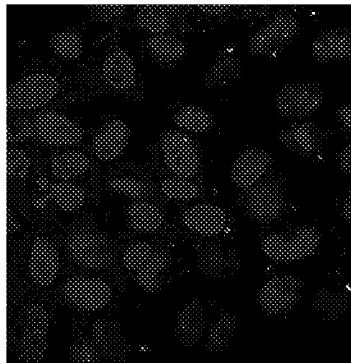
Pfuse-control



D14-Fc



D59-Fc



D55-Fc

Fig 6

Cycle threshold (Ct)

Time post infection	D14	D55	D59	pFUSE
24 H	26.06	30.03	25.24	25.69
48 H	15.15	30.37	15.45	15.31
72 H	15.46	29.59	14.80	14.18
120 H	no reading	30.14	16.11	16.24

Fig 7