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(54) **Title:** METHOD FOR THE GENERATION OF NON-TRANSFORMED MACROPHAGE CELL LINE

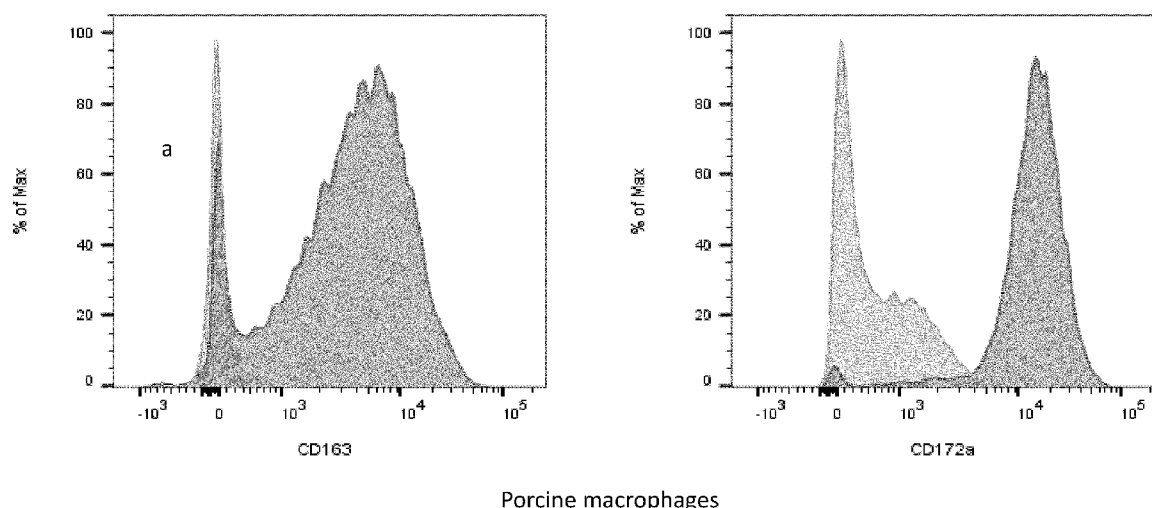


Fig. 1

(57) **Abstract:** A method for producing continuously replicating, non-transformed pig macrophages, comprising culturing a cell preparation from an organ obtained from a pig in culture medium to which GM-CSF has been added thereby differentiating the cell population into self-renewing, non-transformed macrophages.

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METHOD FOR THE GENERATION OF NON-TRANSFORMED MACROPHAGE CELL LINE

The present invention relates generally to macrophages and particularly, although not
5 exclusively, to the establishment of GM-CSF dependent, self-renewing, non-
transformed pig macrophages.

Introduction

10 Macrophages are specialised cells involved in the detection, phagocytosis and
destruction of bacteria and other harmful organisms. In addition, they can also present
antigens to T cells and initiate inflammation by releasing molecules (known as
cytokines) that activate other cells.

15 Macrophages are crucial in defence against infection and studies with these cells *in*
vitro are key to understanding host-pathogen interactions and to underpin vaccine
development.

Depending on their development and organ distribution, macrophages have very
20 distinct properties. Previously, all tissue resident macrophages, including alveolar
macrophages (AMs) were believed to be bone-marrow hematopoietic stem cell (HSC)
derived cells with a limited life-span. Recent studies however, demonstrated that most
tissue resident macrophages represent a separate, functionally distinct, embryonic
macrophage-derived lineage (1). Most tissue-resident macrophages are macrophage
25 colony-stimulating factor (M-CSF) driven self-renewing cells, while AMs are
granulocyte-macrophage colony-stimulating factor (GM-CSF) dependent,
autonomously growing cells. AMs have unique characteristics among macrophages
including sensitivity and responses to pathogens (2).

30 Macrophages are best investigated using primary cells because transformed cell lines
may not accurately represent the primary cells. Macrophage pathogenesis studies
mostly use cells isolated directly from organs or use *ex vivo* produced M-CSF derived
macrophages from bone marrow progenitors or peripheral blood mononuclear cells
(PBMC) (3). GM-CSF derived macrophages from human PBMCs can be used to
35 model human lung AMs (4). Nevertheless, these methods provide cells with a limited
life-span, restricted availability, are difficult to genetically manipulate and significant
inter-donor variability limits their use.

Mouse models including macrophage systems are often used to study human and veterinary infections because of the relatively easy access to tissues and experiments *in vivo*. However, there are significant differences regarding macrophage responses and/or intracellular pathogen replication between different mammalian species, which
5 limits the utility of murine studies (5).

Recently we established a novel, non-transformed, continuously growing, murine macrophage model (MPI cells) from foetal liver providing unlimited amounts of primary
10 cells. This robust system faithfully reproduces murine AM specific responses to various respiratory pathogens *in vitro* (6).

Macrophage studies in pigs are very important (5). Pigs are highly susceptible to various viral (e.g. African swine fever virus [ASFV], porcine reproductive and
15 respiratory syndrome virus [PRRSV] and influenza A virus [IAV]) and bacterial (e.g. *Mycobacterium avium*, and *Salmonella spp.*) pathogens that replicate within macrophages and/or profoundly alter macrophage functions (7-9). Some of these pathogens spread from pigs to humans and cause serious diseases in them (e.g. IAV) (9). Furthermore, swine models are used increasingly in biomedical research as pig
20 and human physiology, such as the anatomy and function of the airways and disease susceptibility are much closer to each other than to those of mice (10).

M-CSF can be used to differentiate pig macrophages from monocytes and detailed analysis of LPS stimulated macrophages showed a close functional relationship
25 between pig and human MCSF derived macrophages (5). However, these MCSF derived macrophages need to be differentiated freshly from bone marrow and similarly to mouse and human cells they have a limited life-span, which makes them unsuitable to produce viral vaccines and suitable, robust primary, non-transformed continuous pig macrophage cell lines are not available for research and vaccine production.

30

Such macrophage lines would be required to study important pathogens such as IAV, ASFV and PRRSV.

Humans and pigs are natural hosts for IAV and the disease can spread in both
35 directions between these species (9). IAV is the leading infectious cause of human death. AMs and innate immune responses play key roles in the pathogenesis of IAV infection (11). Pigs are susceptible to a wide range of IAV strains and are considered

to be the “mixing vessel” of these pathogens as both avian and human IAV strains replicate in pigs and coinfection may lead to new viruses such as the triple (bird-human-pig) reassortant H1N1 strain responsible for the most recent pandemic in 2009.

5 ASFV and PRRSV are pathogens of huge economic importance. These viruses replicate almost exclusively in macrophages, thus, for their efficient study and vaccine development establishment of a continuously growing permissive macrophage system would be necessary (7, 8). ASFV causes a devastating, Ebola disease-like haemorrhagic syndrome in pigs. It kills almost all infected animals and in 2018 and
10 2019 resulted in the death of almost 1.5 million pigs and 10,000 wild boar. The lack of a vaccine limits disease control and research is directed to better understand how the virus interacts with and modulates macrophage functions. This is important, as potential vaccine strains deleted of anti-interferon viral genes elicit a vigorous innate response that restrict viral growth and prevent efficient vaccine production in normal
15 macrophages (7).

PRRSV is one of the most economically significant swine pathogens, endemic in the majority of pork producing countries, and lung AMs are primary targets of the virus in natural infections (8). There are two distinct PRRSV species which are rapidly
20 diversifying and the emerging highly pathogenic strains spread with high speed and cause devastating effects. Vaccination is key to control the disease, however, available vaccines are not sufficiently protective and there is a great need to develop safer and more immunogenic vaccines to which a suitable *in vitro* system is crucially important.

25

The present invention relates to the establishment of a GM-CSF derived, continuous, non-transformed pig macrophage system.

A method for producing continuously replicating, non-transformed porcine
30 macrophages, comprising culturing a cell preparation from an organ obtained from a pig in culture medium to which GM-CSF has been added thereby differentiating the cell population into self-renewing, non-transformed macrophages.

An aspect of the present invention provides a method for producing continuously
35 replicating, non-transformed pig macrophages, comprising culturing a cell preparation from an organ obtained from a pig in culture medium to which GM-CSF has been

added thereby differentiating the cell population into self-renewing, non-transformed macrophages.

5 The cell preparation may be cultured with feeder cells such as mesenchymal feeder cells.

Macrophages prepared using the method may be characterised by the expression of pig macrophage markers CD163 and CD172a.

10 The cell preparation may be based on pig foetal spleen cells.

The present invention also provides macrophages obtained or obtainable by the method as described and/or defined herein.

15 The present invention also provides macrophages obtainable by the method described herein for use in medicine and/or medical/pharmaceutical research.

The present invention also provides macrophages obtainable by the method described herein for use in the production of a vaccine.

20

The present invention also provides a GM-CSF derived, continuously replicating, non-transformed pig macrophage cell line.

25 The macrophages may be characterised by the expression of pig macrophage markers CD163 and CD172a.

The present invention also provides a continuously replicating, non-transformed pig macrophage system.

30 The system may be characterised by the expression of pig macrophage markers CD163 and CD172a.

35 Some aspects and embodiments of the present invention are based on the principle or observation that continuously replicating non-transformed macrophages can be obtained from pig haematopoietic organs by using GM-CSF and suitable mesenchymal feeder cells.

The following data has been obtained related to this system:

1. We cultured pig foetal spleen cells with 20 µg/ml GM-CSF in order to differentiate macrophages. After regular media changes and replenishment of GM-CSF cultures containing two different cell types developed. Flat, mesenchymal or fibroblast like cells on which round, loosely attached macrophage like cells grew in clumps and some of these latter cells also floated in the medium. GM-CSF was required to the development of both cells as spleen cell cultures without it did not produce viable cells. These cultures could be maintained for at least a year with regular media changes.
2. The floating macrophage-like cells were tried to culture separately with GM-CSF. These cells while stayed alive for weeks did not proliferate further. Separated floating cells were also cultured with MCSF or GM-CSF and MCSF. These MCSF cultures showed initial robust cell proliferation; however, after a week of growth cells stop to multiply.
3. The floating macrophage-like cells have been transferred to embryonic pig lung fibroblast cultures and similarly to the original cultures with the spleen derived fibroblasts, they attached and proliferated on the top the lung fibroblast feeder cells. They grew in clumps and many of these cells were floating in the medium. These cultures were maintained for more than year with regular media changes.
4. The floating cells from the lung fibroblast feeder culture have been cultured with GM-CSF but without feeder cells. These cells survive for extended time intervals but don't grow further significantly.
5. Isolated floating cells from feeder containing cultures strongly express the pig macrophage markers CD163 and CD172a.
6. Isolated floating macrophages from feeder containing cultures produce a strong cytokine response to bacterial endotoxin (LPS) stimulation.
7. Isolated floating macrophages from feeder containing cultures can be serially passaged on Mitomycin treated STO fibroblast line feeder cells with pig

GM-CSF for at least 4 passages. They grow continuously and have a doubling time of approximately 3-4 days.

5 8. When culturing pig foetal bone marrow with GM-CSF, a culture similar to the one from spleen develops with mesenchymal/fibroblast like adherent cells and floating macrophage like cells. The floating cells proliferate in the presence of GM-CSF when transferred to mytomycin treated STO fibroblast cells, however, more slowly than similar cells from the spleen.

10 9. Isolated floating macrophages from the feeder cultures have been transduced with a 2nd generation lentivirus expressing the SV40 Large T antigen tsA58 temperature sensitive mutant (Jat, P. S. & Sharp, P. A. (1989) Mol. Cell. Biol. 9, 1672-1681.). The tsA58 mutant of the SV40 Large T antigen is thermosensitive and can be used to generate conditionally immortalized cell lines (Jat, P. S. & Sharp, P. A. (1989) Mol. Cell. Biol. 9, 1672-1681.) The
15 obtained cells (known as PLTA58 cells) grow robustly and without the need of feeder cells. These data represent a significant difference to previous data by Takenouchi et al, Front Vet Sci 2017 Aug 21;4:132, where unconditional cellular immortalisation of pig kidney derived macrophages could only be achieved by
20 the combined expression of the telomerase protein and the wild type SV40 large T antigen.

25 10. PLTA58 cells are continuously growing without feeder cells and we have carried out at least 20 passages since their establishment in December 2021. We can regularly obtain at least 10 million cells from a T75 culture flask in one week of culture. We can freeze down the cells and culture them efficiently again after thawing.

30 11. PLTA58 cells are factor dependent and their growth is enhanced by GMCSF and/or MCSF.

12. PLTA58 cells express pig macrophage markers.

35 13. PLTA58 cells can be efficiently stimulated with various innate response elicited ligands such as LPS (TLR4 ligand), Fsl-1 (TLR2 ligand), polyI:C (TLR3 ligand) and R848 (TLR7/8 ligand).

14. PLTA58 cells support the replication of the African Swine Fever Virus.

Different aspects and embodiments of the invention may be used separately or together.

5

Further particular and preferred aspects of the present invention are set out in the accompanying independent and dependent claims. Features of the dependent claims may be combined with the features of the independent claims as appropriate, and in combinations other than those explicitly set out in the claims.

10

The present invention is more particularly shown and described, by way of example, in the accompanying drawings.

15

The example embodiments are described in sufficient detail to enable those of ordinary skill in the art to embody and implement the systems and processes herein described. It is important to understand that embodiments can be provided in many alternative forms and should not be construed as limited to the examples set forth herein.

20

Accordingly, while embodiments can be modified in various ways and take on various alternative forms, specific embodiments thereof are shown in the drawings and described in detail below as examples. There is no intent to limit to the particular forms disclosed. On the contrary, all modifications, equivalents, and alternatives falling within the scope of the appended claims should be included.

25

Unless otherwise defined, all terms (including technical and scientific terms) used herein are to be interpreted as is customary in the art. It will be further understood that terms in common usage should also be interpreted as is customary in the relevant art and not in an idealised or overly formal sense unless expressly so defined herein.

30

In the description, all orientational terms, such as upper, lower, radially and axially, are used in relation to the drawings and should not be interpreted as limiting on the invention.

Fig.1

35

Continuously growing porcine GMCSF derived cells express markers characteristic for pig macrophages.

Surface markers were detected on porcine macrophages with FACS using the antibodies for the scavenger receptor CD163 and signal regulatory protein alpha CD172a. Histograms show unstained (blue) and stained cells (red).

5

Fig. 2

In response to smooth and rough form LPS the levels of pig macrophage produced TNF α is similar to those of human macrophages.

10

Fig. 3

TNF- α response in human GMDMs and pig macrophages is highly dependant on LBP present in Foetal Bovine Serum (FBS). Cells were stimulated with S-LPS (100 ng/ml) and R-LPS (100 ng/ml). At 16h post-infection supernatants were collected and TNF- α was measured by ELISA. Bars represent the mean from three sample \pm S.E.M.

15

Fig. 4

Reduced production of IL-6 and TNF- α in MPI and pig macrophages respectively to repeated LPS stimulations (LPS tolerance). (a) cells were unstimulated (N) or stimulated with 50 ng/ml S-LPS for 16h (T), washed with PBS and replenished with media (N) or 50 ng/ml LPS (N+L or T+L) for 24h. Arrows depict stimulation with LPS. Supernatants from MPI (b) and pig macrophages (c) were analysed by ELISA.

20

25

Fig. 5

Comparable TNF- α cytokine production upon S-LPS and IAV in pig macrophages cultured under various conditions. Pig cells were stimulated with Influenza A virus strain Perth/16/09 at MOI 3 and S-LPS (100 ng/ml). At 16h post-infection supernatants were collected and TNF- α were measured by ELISA. n=1

30

Similarities between the responses of M-CSF, GM-CSF and GM-CSF/M-CSF-cultured porcine macrophages after obtaining them from lung fibroblast feeder cultures with GM-CSF.

35

The first set of questions involving a newly developed model of pig macrophages aimed to compare those cells growing in different conditions. The data demonstrate that these pig macrophages can be efficiently stimulated when cultured without feeder cells with the growth factors used.

5

Fig. 6

Pig macrophages produce an early TNF- α response after challenge with lipopolysaccharide. Pig cells were stimulated with IAV strain Perth/16/09 at MOI 3 and S-LPS (100 ng/ml). At 16 h post-infection supernatants were collected and TNF- α were measured by ELISA. n=1

10

TNF- α cytokine production in pig GM-CSF-derived macrophages stimulated with S-LPS and IAV is time-dependent.

15

The next experiment was concerned about significant proinflammatory cytokine production in porcine macrophages challenged with S-LPS and IAV at various time points. Fig. 6 shows early TNF- α production in pig macrophages stimulated with S-LPS but not IAV. Additionally, the IAV responses are lower compared to those elicited by endotoxin.

20

Fig. 7

Pig and MPI macrophages transduced with RFP expressing lentivirus. These data demonstrate that the pig macrophages can be efficiently targeted for recombinant protein expression by lentiviral vectors similarly to mouse MPI macrophages.

25

Fig. 8

Extended culture of pig macrophages with GM-CSF separated from feeder cells. Multinuclear giant cells, characteristic for macrophages can be seen among regular sized cells.

30

Fig. 9

35

Pig macrophage cells growing on lung fibroblast feeder cells (A) and transferred separated floating macrophages without feeder (B).

Fig. 10

Pig macrophages grown on the STO fibroblast cell line.

5

Fig. 11

Factor dependency of PLTA58 cells shown by cultures with or without growth factors (GMCSF and/or MCSF).

10

Fig. 12

FACS data on the immortalized pig cells.

15 PLTA58 cells express typical pig macrophage markers.

Fig. 13

PLTA58 cells stimulated with bacterial lipopolysaccharide (LPS)

20

Fig. 14

PLTA58 cells stimulated with poly I:C, Fsl-1 or R848.

25 Although illustrative embodiments of the invention have been disclosed in detail herein, with reference to the accompanying drawings, it is understood that the invention is not limited to the precise embodiments shown and that various changes and modifications can be effected therein by one skilled in the art without departing from the scope of the invention as defined by the appended claims and their equivalents.

30

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CLAIMS

1. A method for producing continuously replicating, non-transformed pig macrophages, comprising culturing a cell preparation from an organ obtained from a pig in culture medium to which GM-CSF has been added thereby differentiating the cell population into self-renewing, non-transformed macrophages.
2. A method as claimed in claim 1, in which the cell preparation is cultured with feeder cells.
3. A method as claimed in claim 2, in which the feeder cells comprise mesenchymal feeder cells.
4. A method as claimed in any preceding claim, wherein macrophages are characterised by the expression of pig macrophage markers CD163 and CD172a.
5. A method as claimed in any preceding claim, wherein macrophages are characterised by the expression of Integrin alpha M (CD11b) and/or MHC class II cell surface receptor (HLA-DR).
6. A method as claimed in any preceding claim based on pig foetal spleen cells.
7. Macrophages obtainable by the method of any preceding claim.
8. The macrophages of claim 7 for use in medicine and/or medical/pharmaceutical research.
9. The macrophages of claim 7 for use in the production of a vaccine.
10. A GM-CSF derived, continuously replicating, non-transformed pig macrophage cell line.
11. A cell line as claimed in claim 10, wherein macrophages are characterised by the expression of pig macrophage markers CD163 and CD172a.
12. A continuously replicating, non-transformed pig macrophage system.

13. A system as claimed in claim 12 characterised by the expression of pig macrophage markers CD163 and CD172a.

14. A method for producing continuously replicating, non-transformed pig
5 macrophages, comprising:
- culturing pig cells with GM-CSF in order to differentiate macrophages; and
- selecting floating macrophages and transferring them to a feeder cell culture.

15. A method as claimed in claim 14, further comprising the step of transducing the
10 floating macrophages from the feeder culture with tsA58 to generate a conditionally immortalized cell line.

16. A method for producing continuously replicating, non-transformed pig
macrophages, comprising:
15 - culturing pig cells with GM-CSF in order to differentiate macrophages; and
- transducing macrophages with tsA58 to generate a conditionally immortalized cell line.

17. A method for producing continuously replicating, non-transformed pig
20 macrophages, comprising the steps of: culturing pig cells to differentiate macrophages; and transducing the macrophages to generate an immortalized cell line.

18. A method as claimed in claim 17, in which the macrophages are transduced
using tsA58.

25

19. A self-renewing, non-transformed macrophage cell line capable of supporting replication of the African Swine Fever Virus.

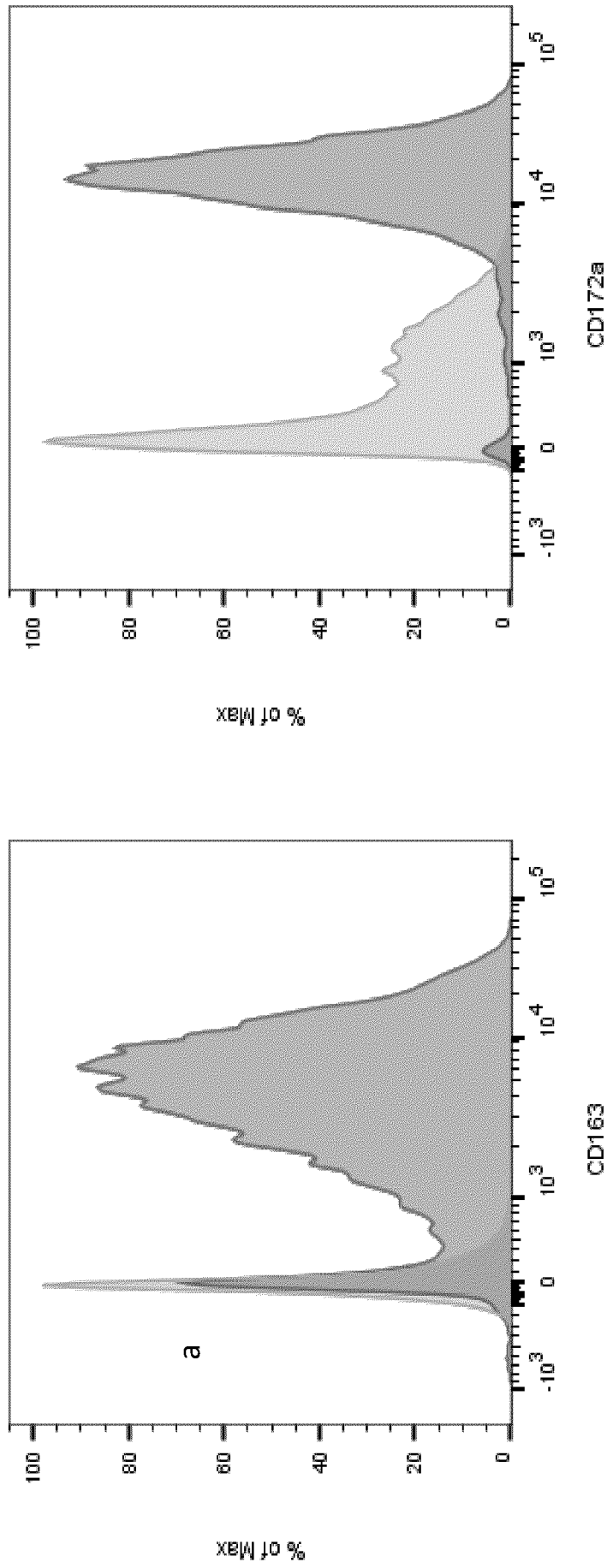
20. A cell line designated PLTA58.

30

21. A method for producing continuously replicating, non-transformed pig macrophages, comprising culturing a cell preparation from an organ obtained from a pig in culture medium to which GM-CSF has been added thereby differentiating the cell population into self-renewing, non-transformed macrophages.

35

22. A GM-CSF dependent, self-renewing, non-transformed porcine macrophage cell line.



Porcine macrophages

Fig. 1

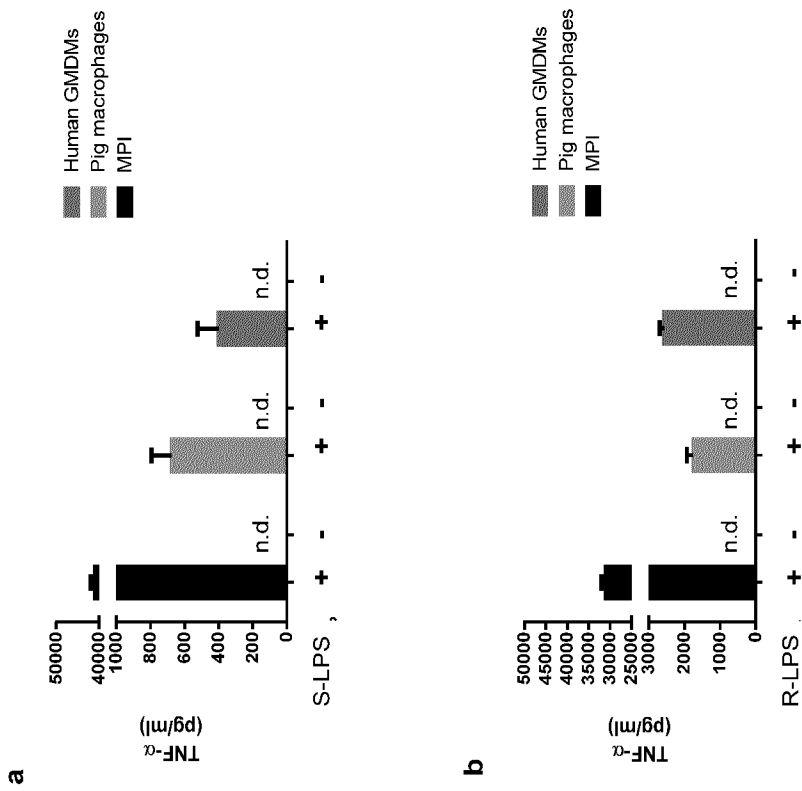


Fig. 2

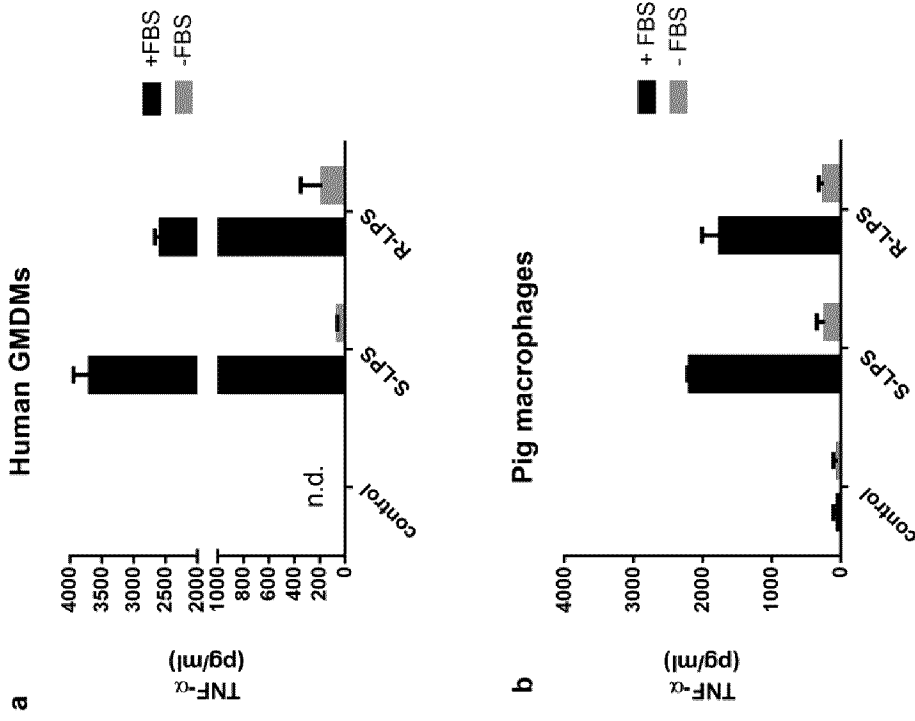


Fig. 3

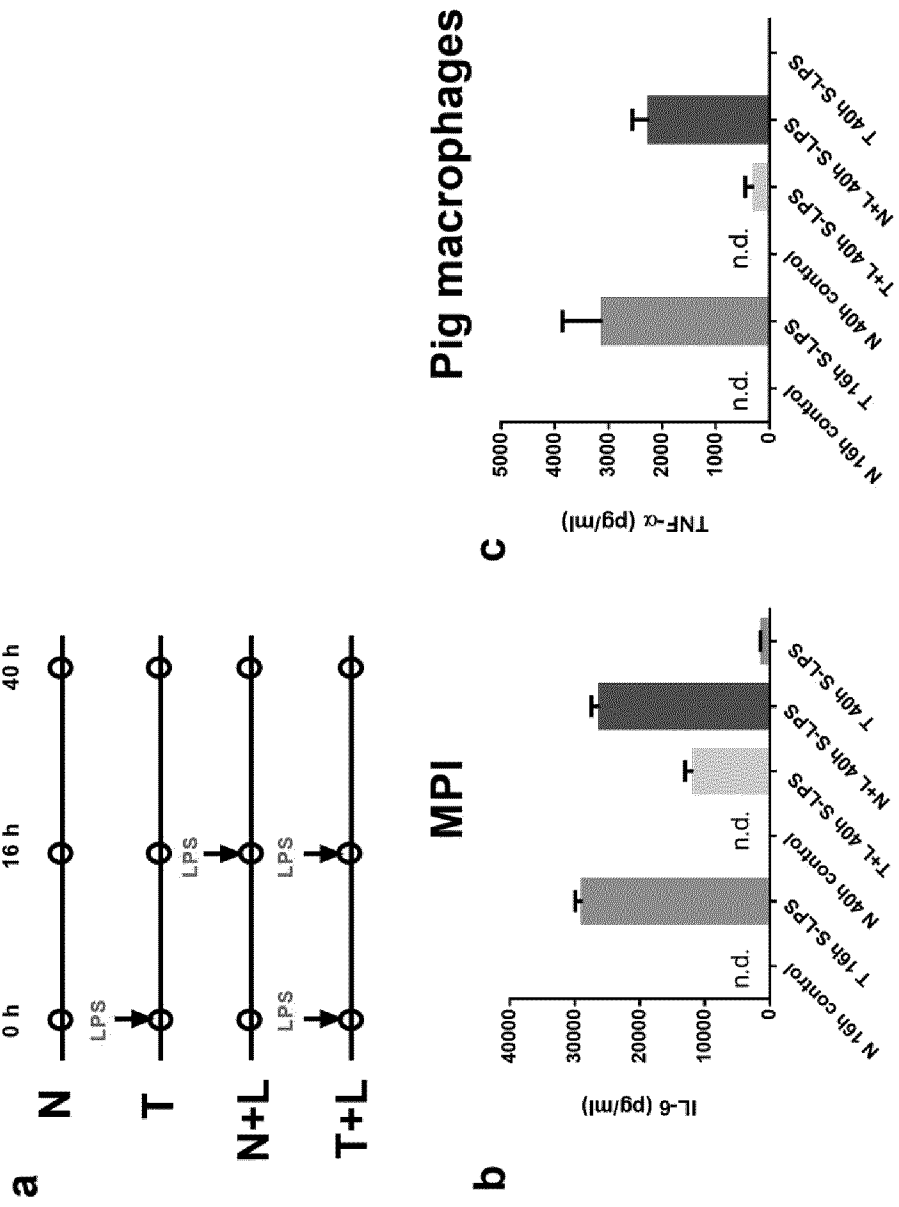


Fig. 4

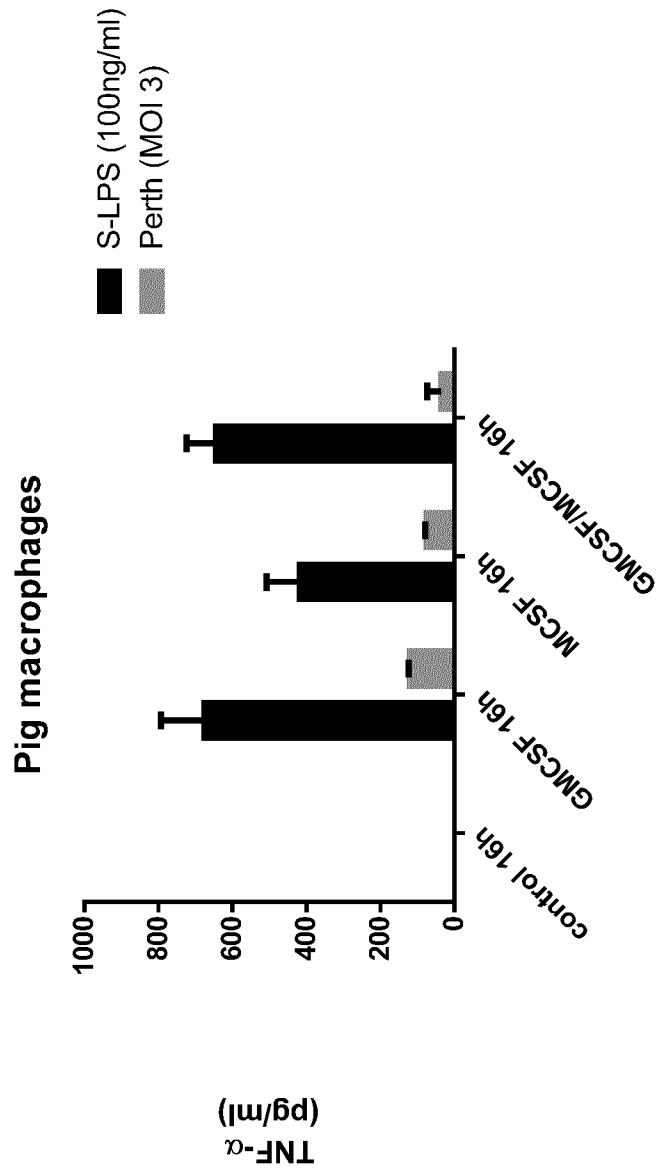


Fig. 5

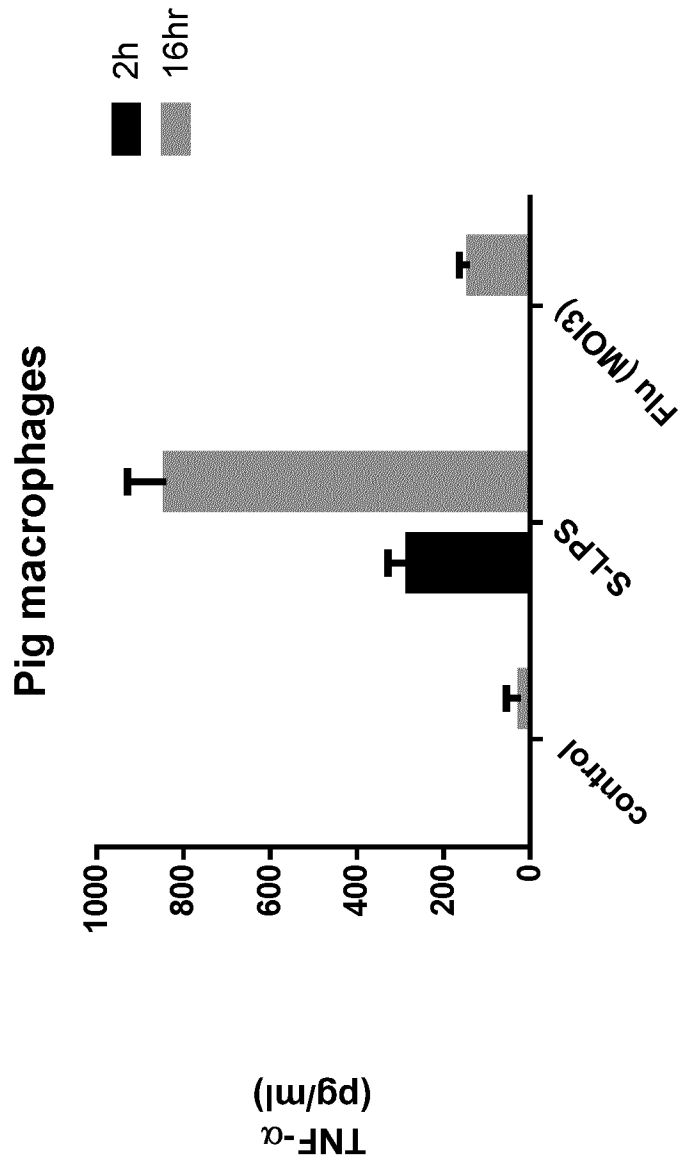
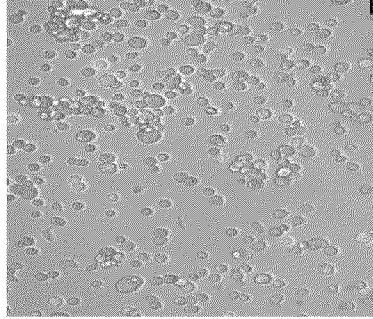
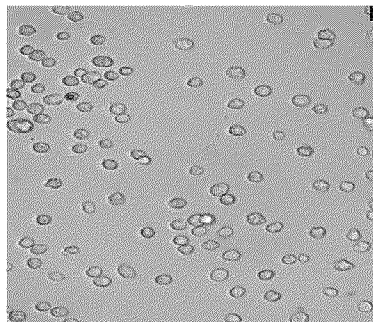


Fig. 6



Mouse MPI cells



Pig macrophages

Fig. 7

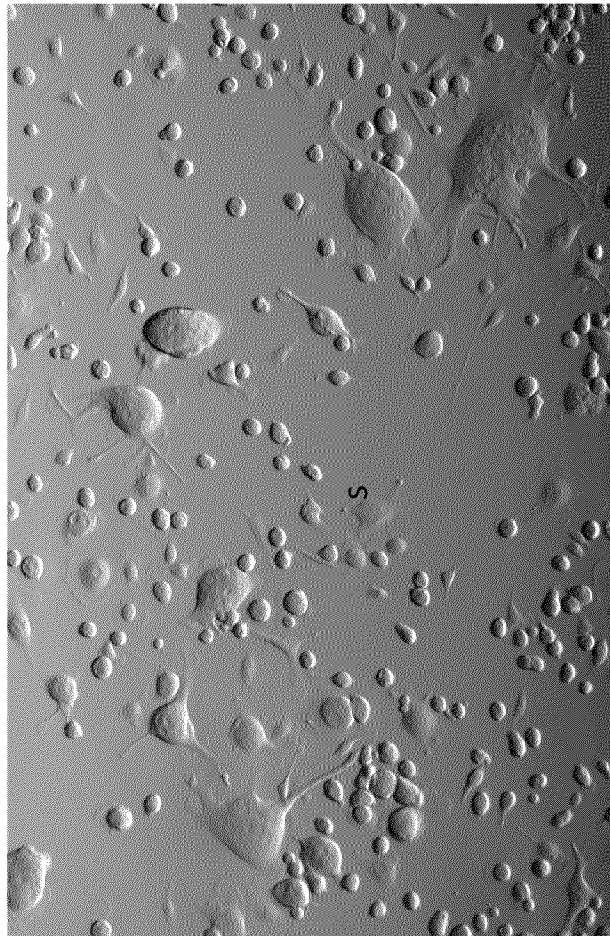


Fig. 8

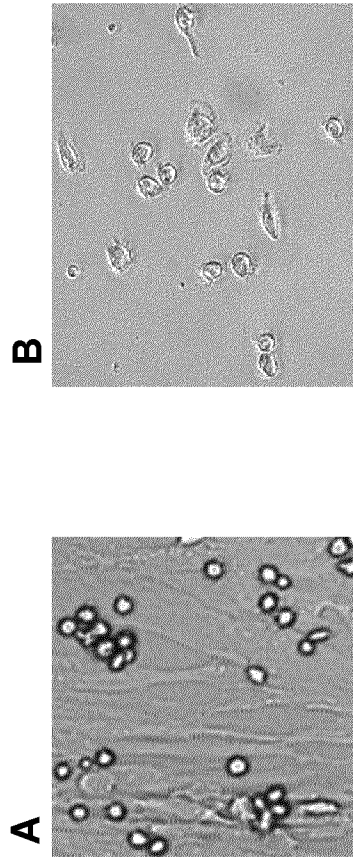


Fig. 9

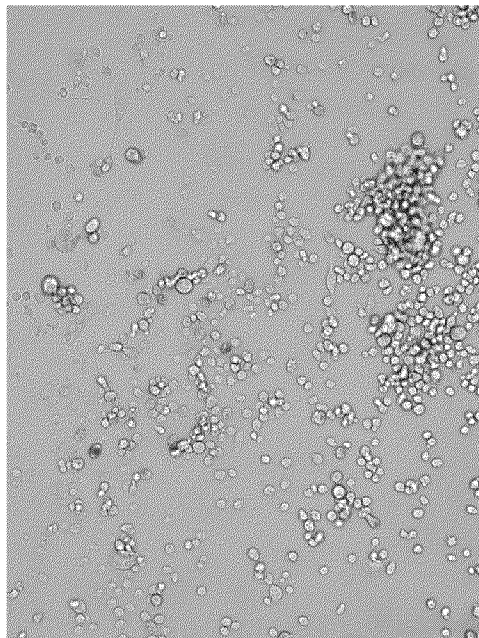


Fig. 10

PLTA58 cells express
typical pig macrophage markers

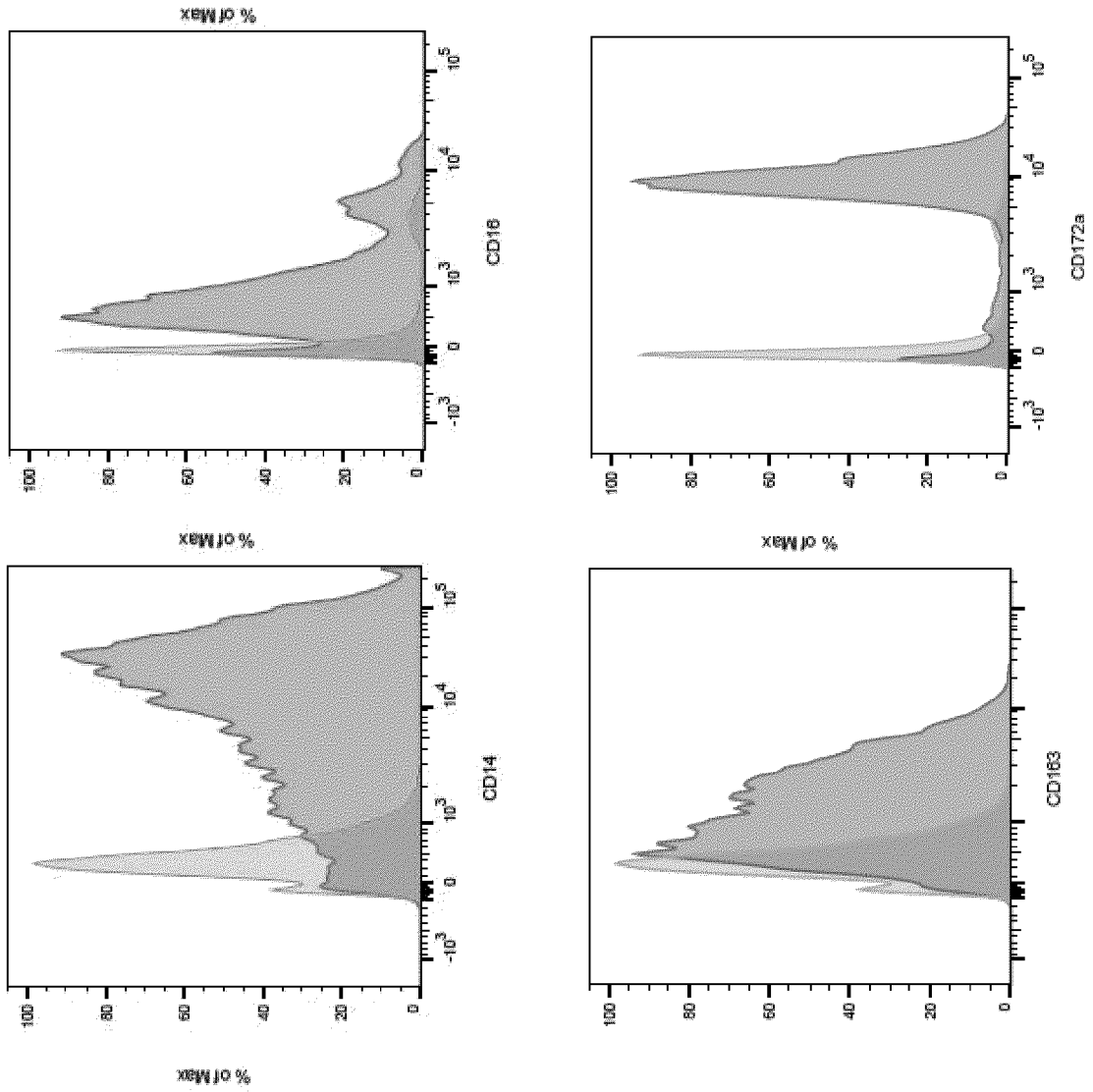
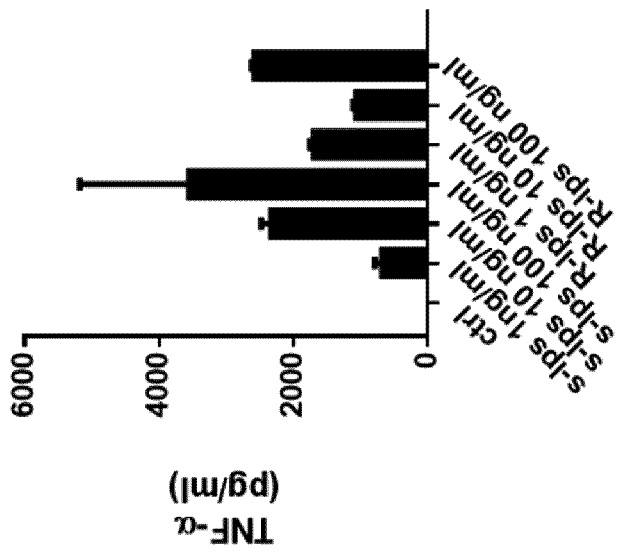
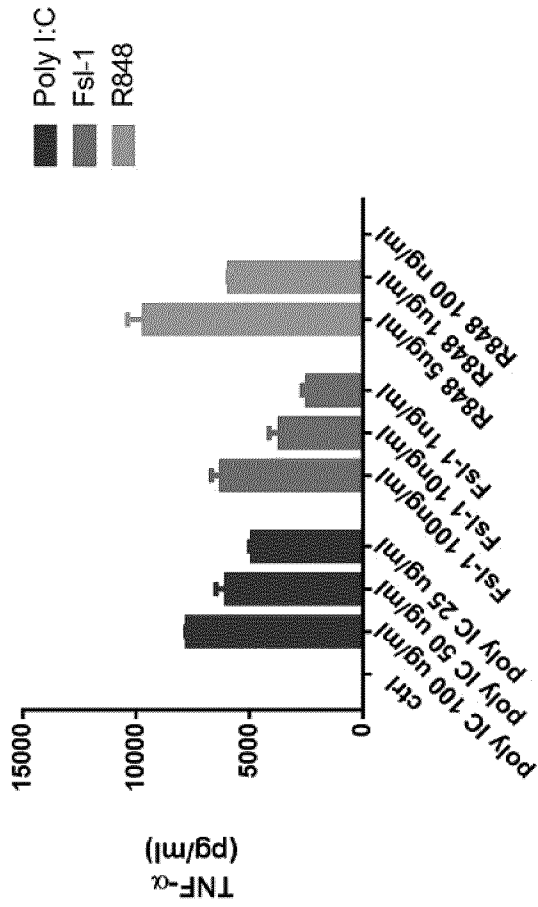


Fig. 12



PLTA58 cells stimulated with bacterial lipopolysaccharide (LPS)

Fig. 13



PLTA58 cells stimulated with poly I:C, Fsl-1 or R848

Fig. 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2022/076456

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0786
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)
C12N

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/012933 A1 (MAX PLANCK GESELLSCHAFT [DE]) 23 January 2014 (2014-01-23)	1-9, 12, 13, 21
Y	the whole document	10, 11, 14-19, 22

X	DENHAM S ET AL: "Granulocyte-macrophage colony stimulating factor promotes prolonged survival and the support of virulent infection by African swine fever virus of macrophages generated from porcine bone marrow and blood", JOURNAL OF GENERAL VIROLOGY,, vol. 77, no. PART 10, 1 October 1996 (1996-10-01), pages 2625-2630, XP002688768, ISSN: 0022-1317, DOI: 10.1099/0022-1317-77-10-2625	1-5, 7-9, 12
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Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents :

<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>
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Date of the actual completion of the international search 10 January 2023	Date of mailing of the international search report 18/01/2023
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Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Zuber Perez, C
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2022/076456

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	<p>----- CANDY LEE ET AL: "Generation of a conditionally immortalized myeloid progenitor cell line requiring the presence of both interleukin-3 and stem cell factor to survive and proliferate", BRITISH JOURNAL OF HAEMATOLOGY, JOHN WILEY, HOBOKEN, USA, vol. 122, no. 6, 3 September 2003 (2003-09-03), pages 985-995, XP071109479, ISSN: 0007-1048, DOI: 10.1046/J.1365-2141.2003.04537.X -----</p>	15-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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