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(54) Title: PEPTIDES FOR USE IN ASSESSING THE STATUS OF THE IMMUNE SYSTEM

(57) Abstract: The present invention concerns a mixture of peptides that are derivable from a virus and wherein the mixture includes peptides corresponding to peptides encoded by Open Reading Frame (ORF) 1 and Open Reading Frame (ORF) 9 of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) genome. The peptides may be used in methods for testing samples to assess the status of the immune system in a subject from which the sample has been taken. The peptides are particularly useful in tests for examining samples from subjects who may have been exposed to a virus; are concerned about being exposed to a virus; and may be used to determine if a subject has inherent protection from a virus.

PEPTIDES FOR USE IN ASSESSING THE STATUS OF THE IMMUNE SYSTEM

Provided herein are peptides that may most suitably be used in methods for testing samples to assess the status of the immune system in a subject from which the sample has been taken. The peptides are useful in tests for examining samples from subjects who may have been exposed to a virus; are concerned about being exposed to a virus; and may be used to determine if a subject has inherent protection from a virus.

There is a need to develop tests that will give insight into the status of the immune system and how that status may impact upon the ability of a subject to be resistant to viral infection. This need is clear when the impact of the COVID 19 pandemic is taken into account. Some individuals are asymptomatic when exposed to Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-Cov-2) whereas, as of January 2022, over 5.5 million people have died from COVID 19. A better understanding of a subject's ability to "fight off" viruses such as SARS-Cov-2 will enable individuals to make lifestyle choices and will be very useful in the clinical management of such an individual should they subsequently become infected or reinfected.

Viral infection elicits a response in the immune system to fight the infection. This consists of a short term and longer term immune response. After infection, T-cells reactive to a virus can be present in the blood long after recovery, whereas antibodies to the virus may only be present for months after disease recovery. For example, T-cells to the SARS virus have been found in survivors 17 years after the original infection and this kind of long-term protection may be expected for other viruses and including other coronaviruses such as SARS-CoV-2.

The inventor recognized that there is a need to develop a mixture of peptides that may be employed in tests that determine T cell responsiveness. Such tests may be useful for determining inherent protection from viruses in a subject who has avoided, or at least believes they have avoided, viral infection. Tests that determine T cell responsiveness are also useful for determining the degree of long-term protection a subject may have after recovering from a viral infection. Protection from re-infection or serious disease may be conferred by T cells which may be present long after antibodies have disappeared from the blood. Thus examining T-cell responses can be particularly useful for assessing the degree of long term protection from a viral threat.

Tests examining T-cell responsiveness to viruses are known in the art. For instance, MyriadRBM market a trueculture® test (for research use only) which examines T-Cell activation in response to peptides from the SARS-CoV-2 Spike protein. MyriadRBM employs ELISA to measure cytokines produced by the T Cells. However the MyriadRBM test only employs peptides from the Spike Protein and this protein can be highly variable and poorly conserved. Furthermore, Miltenyi market a SARS-CoV-2 T cell Analysis Kit that examines T-Cell activation in response to a mixture of peptides generated from across the SARS-CoV-2 genome (from their Peptivator® Peptide Pools) and employ flow cyometry to detect cytokine production from the activated T Cells. However, Peptivator® Peptide Pools appear to be randomly generated across the SARS-CoV-2 genome.

It is therefore an object of the present invention to provide an improved peptide mix for use in activating T cells.

According to a first aspect of the present invention there is provided a mixture of peptides derivable from a virus wherein the mixture includes peptides corresponding to peptides encoded by Open Reading Frame (ORF) 1 and Open Reading Frame (ORF) 9 of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) genome

According to a second aspect of the present invention there is provided a method of determining T cell activation in response to exposure to viral peptides, comprising:

(a) Stimulating a sample containing T cells that has been obtained from a subject with a mixture of peptides according to the first aspect of the invention;
(b) Fixing and permeabilizing cells from the sample;
(c) Staining the fixed and permeabilized cells with a labelled anti-CD3 antibody and a labelled anti-cytokine antibody or antibodies against at least one cytokine;
(d) Conducting an assay to identify cells that are CD3 + and which express the at least one cytokine.

The mixture of peptides and methods according to the invention are useful for determining inherent protection from viruses in a subject who has avoided, or at least believes they have avoided, viral infection. The mixture of peptides and methods are also useful for determining the degree of long-term protection a subject may have after recovering from a viral infection. The mixture of peptides and methods are particularly useful for determining the degree of long-term protection present in

a subject after recovery from COVID-19 or for assessing whether or not a subject has inherent protection from COVID-19 (e.g. following vaccination).

The assay used according to step (d) of the method according to the second aspect of the invention may be any assay that determines antigen specific (i.e. peptide induced) cytokine production by cells (e.g. peripheral blood cells). Example of assays that may be used include ELISA and ELISPOT. It is most preferred that cells which are CD3 + and which express the at least one cytokine are detected by employing flow cytometry. The use of ELISA, ELISPOT and particularly flow cytometry has the advantage that the methods of the invention may employ laboratory equipment that is easy to use and standard in both the developed and developing world. This increases the scope of the test beyond wealthy nations.

Peptides encoded by ORF 1 and ORF 9 of the SARS-CoV-2 genome are advantageously antigenic viral proteins and provide a way of stimulating T-cells to respond as if they have come in to contact with the virus in question, but without having to use viral particles within a test. The peptides also enable the methods of the invention to establish whether or not innate immunity to a specific viral threat or antigen is built up through: a) adaptive immunity after exposure to the virus or antigen in the past; b) adaptive immunity after exposure to a similar virus or antigen (providing a cross-reactive immune response to the virus or antigen in question); and c) adaptive immunity through vaccination to the virus in question or a similar virus. The peptides according to the first aspect of the invention may be used in methods to provide an indication as to whether or not the immune system of the subject can generate a T-cell response if infected with the specific virus or antigen in question, and also provides an indication as to how well the body would respond if infected in the future.

The method of the second aspect of the invention may also give an indication as to what disease protection strategies are required for an individual who is concerned about catching a specific virus (vaccination, social distancing, mask wearing, isolation/shielding, and additional pharmaceutical or nutraceutical strategies to boost the immune function where required). This provides additional information about immune function for, in particular, the vaccine hesitant to influence their vaccination decisions.

Data generated by the methods of the second aspect of invention may be used to help governments and healthcare groups prioritize vaccination more effectively by targeting those who are identified by the methods of the invention of having lower levels of adaptive immunity.

Data generated by the methods of the second aspect of invention may be used to establish a database of T-cell responses in individuals which allows for the prediction of future immune responses based on individuals with a similar immune profile. For instance, if an individual has been infected with, and recovered from Sars-Cov-1 in the past, it may be possible to predict that they will effectively mount a T-cell response to Sars-Cov-2.

Peptide Selection

The peptide mixtures according to the first aspect of the invention may be derived from, or derivable from, any virus (e.g. a torovirus) although it is preferred that the peptides are derived from Beta-Coronaviruses. It is more preferred that the peptides are derived from SARS-CoV, HCoV-OC43, HCov-HKU1, MERS-CoV or SARS-CoV-2. It is most preferred that the peptides are derived from SARS-CoV-2.

Coronaviruses, including SARS-CoV-2, has four structural proteins, known as the S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins. The N protein holds the RNA genome, and the S, E, and M proteins together create the viral envelope. The Spike protein is encoded by Open Reading Frame (ORF) 1 of the viral genome whereas the E, M and N proteins are encoded by ORF4, ORF5 and ORF9 respectively. Peptide mixes used according to the invention should include peptides derived from ORF1 and ORF 9.

It is preferred that peptide mixtures according to the first aspect of the invention are derived, or derivable, from regions of ORF1 and ORF 9 which the inventor has associated with (a) being conserved between Sars-Cov-2 variants and also other viruses (particularly coronaviruses); and (b) elicit T-cell and B-cell mediated immune responses after prior infection with Sars-Cov-2 and other related coronaviruses or with no prior Sars-Cov-2 infection.

The inventor has recognized that the ORF1 region shows conservation between both coronaviruses and toroviruses. Peptides derived from ORF1 are therefore useful because they show cross reactivity to T-cells after infection with different viruses.

It is advantageous that peptides according to the first aspect of the invention are encoded by these conserved regions because infection with many different viruses, particularly different coronaviruses, can produce a T-cell response to the peptides contained within these regions. Subsequent infection with a different virus that also contains peptide sequences that are the same or similar, can elicit a rapid immune response that is mediated by memory T-cells that were produced to the initial infection.

Methods according to the second aspect of the invention may be used to identify T cells that are: (i) reactive due to previous exposure to Sars-Cov-2; (ii) which are reactive due to exposure to another virus (e.g. another coronavirus) which have the same or similar conserved regions; (iii) which are reactive following vaccination; or (iv) are primed/reactive for an unknown reason which may represent natural immunity. It will be appreciated that knowledge of T cell activation in subjects for each of (i) – (iv) is extremely valuable for an individual; a clinician treating such an individual; and for making decisions for disease management for a population of subjects. For instance, the methods may be used to evaluate the length of time immunity will persist following vaccination and inform authorities when a booster vaccine would be appropriate.

In preferred embodiments the peptide mixtures according to the first aspect of the invention include peptides encoded by ORF 1 or ORF 9 of the SARS-CoV-2 genome and also peptides encoded by ORF2 (encoding the Spike Protein).

Peptides used according to the invention may be of any size that is suitable for activating a T cell. Typically a peptide may be 5 – 50 amino acids in length, is preferably 5- 25 amino acids in length and more preferably 6 -18 amino acids in length.

It is most preferred that a mixture of peptides according to the first aspect of the invention comprises between 10 and 100 different peptides (with at least one peptide from ORF 1 and ORF 9 and preferably at least one peptide from ORF2); more preferably the mixture comprises between 15 and 50 peptides (with at least one peptide from ORF 1 and ORF 9 and preferably at least one peptide from ORF2); and more preferably the mixture comprises between 20 and 40 peptides (with all of the peptides derived from ORF 1, ORF 9 and ORF2). It is most preferred that the mixture comprises about 30 peptides (e.g. 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 peptides) and with all

of the peptides derived from ORF 1, ORF 9 and ORF2. A most preferred mixture of peptides according to the first aspect of the invention is listed in Table 1.

Peptide mixtures according to the first aspect of the invention activate T cells such that they start producing cytokines. It is preferred that the peptides induce T cells to produce at least one of Tumour Necrosis Factor Alpha (TNFα), Interleukin 2 (IL-2) and Interferon Gamma (IFN-γ). It is more preferred that the T cells are induced to produce at last two of these cytokines and most preferred that the T cell produce all three.

In a preferred embodiment peptides according to the first aspect of the invention are chosen with one or more positive antigenicity characteristics, as determined by the three assessment methods outlined below:

(1) Solvent Accessible Surface Areas (SASA (Å2)) were assessed using the Euclidean Distance Transform Surface (EDTSurf) algorithm. This was used to predict the accessible area for potential protein docking. Peptides that had a SASA >900 Å2 were favoured for selection. For a 15 aa peptide a SASA of > 900 Å2 transforms into a solvent accessible surface area of > 20%. An amino acid with SASA < 20% is considered not solvent accessible according to the EDTSurf algorithm used in the peptide analysis. > 800 Å2 was accepted for peptides where they scored highly on the SVMTriP and KT metrics.

(2) The SVMTriP score - predicts antigenic epitopes, surface regions of protein that are preferentially recognised by antibodies or T-cells for receptor binding. In this method, Support Vector Machine (SVM) was used by combining the Tri-peptide similarity and Propensity scores (SVMTriP). SVMTriP scores of >0.90 were preferred for peptide selection.

(3) Peptides may also be selected on the basis of Kolaskar and Tongaonkar Antigenicity Analysis. These analyses provides a useful tool to predict antigenic determinants on proteins by analysing the occurrence of hydrophobic residues Cys, Leu and Val. Peptides from ORF 1 or ORF9 comprising hydrophobic residues Cys, Leu and Val are preferred peptides for use according to the invention.

It is preferred that peptide mixtures according to the first aspect of the invention include peptides derived from ORF 1 and ORF 9 of the SARS-CoV-2 genome and have the characteristics identified

in at least one of (1) – (3) above; preferably two out of three of (1) – (3) above; and most preferably each of (1) – (3) above.

The method according to the second aspect of the invention may employ the steps outlined below.

Sample Selection

Samples used according to the methods of the second aspect of the invention should be derived from a mammal and preferably from a Human.

The sample may be any tissue sample that contains T cells. Preferably the sample is blood or a fraction thereof (e.g a buffy coat). Preferably the sample is whole blood. A suitable amount of blood should be used. Given that T cell numbers are relatively low in blood it is preferred that samples comprise at least one milliliter of blood. Whole blood is preferably treated with heparin, according to conventional techniques, to prevent coagulation.

Stimulation of Samples

T cells in samples are preferably stimulated by adding a suitable amount of the peptides according to the first aspect of the invention and incubating for a suitable length of time.

A sufficient amount of a peptide mixture according to the first aspect of the invention should be added to the sample to stimulate cytokine production from activated T cells. It will be appreciated that individual laboratories may need to optimize the amount used and this will at least in part depend up the specific peptides in the mixture. By way of example, the inventor has found, when using the peptide mix specified in Table 1, that an end concentration of about 1 μg/mL of total peptides in a whole blood sample is suitable.

Stimulated samples are preferably incubated at $37^{O}C$ and for 1-24 hours, preferably 1-12 hours, more preferably 2-8 hour and in one embodiment for about 6 hours.

The secretion of cytokines from a cell can be disadvantageous when employing certain assays according to the second aspect of the invention and particularly when flow cytometry is used. When this is the case, it is preferred that a cytokine secretion inhibitor is included that retains a

higher concentration of the cytokine in the T cells. It is therefore preferred that a secretion inhibitor is used during stimulation of the samples in order that the amount of detectable cytokine is maximized. A preferred secretion inhibitor for use according to the invention is Brefaldin A. It is preferred that Brefaldin A is used at a concentration of about 1 μg/mL in a whole blood sample.

In a preferred embodiment a sample is first incubated with the peptides for about an hour. A sufficient amount of a secretion inhibitor is then added to the sample and the sample is incubated at 37^{O}C and for a further 2-7 hour and in one embodiment for about a further 5 hours.

A preferred procedure for stimulating the cells is described in 2.4 of Example 2.

Fixing and permeabilization

After the stimulation of a sample the T cells should be isolated from the sample. When the sample is whole blood, red blood cells should be lysed and the white cells fixed and permeabilized.

Conventional fixatives may be used. In a preferred embodiment the fixative is formalin. A preferred procedure for fixing cells is described in 2.5 of Example 2.

Cells may be permeabilized prior to staining using conventional methods. In one embodiment cells may be permeabilized with Phosphate buffer saline containing 10% saponin (which may be added to a sample to 0.1% end concentration). A preferred procedure for permeablising cells is described in 2.6 of Example 2.

Staining

Step (c) of the method of the second aspect of the invention requires the fixed and permeabilized cells to be stained with a labelled anti-CD3 antibody and a labelled anti-cytokine antibody against at least one cytokine.

<u>Labelled anti-CD3 antibodies</u>

Cluster of Differentiation 3 (CD3), is a protein complex and T cell co-receptor that is involved in activating both the cytotoxic T cell (CD8+ naive T cells) and T helper cells (CD4+ naive T cells). It is composed of four distinct chains. In mammals, the complex contains a CD3γ chain, a CD3δ chain, and two CD3ε chains. These chains associate with the T-cell receptor (TCR) and

the CD3ζ chain to generate an activation signal in T lymphocytes. The TCR, CD3ζ, and the other CD3 molecules together constitute the TCR complex.

The inventor has found that the labelled anti- CD3 antibodies represent an ideal way of identifying T cells in a sample.

The anti-CD3 antibody may be raised against antigens derived from the CD3γ chain, CD3δ chain, CD3ε chain or TCR. A preferred anti-CD3 antibody is raised against CD3ε. Antibodies are preferably monoclonal antibodies and a most preferred antibody is clone UCHT1 from ThermoFisher.

It is preferred that a label is selected that is suitable for cell sorting by flow cytometry. It is preferred that the label for anti-CD3 antibodies is Fluorescein isothiocyanate (FITC). FITIC may be conjugated to the antibody via a succinimidyl ester.

Labelled anti-cytokine antibodies

Anti-cytokine antibodies used according to the invention, should be raised against at least one cytokine selected from TNFα, IL-2 and IFN-γ.

It has been reported (e.g. Le Bert *et al.* (2020) Nature 584 (7821) p457-462) that flow cytometry may be used to detect (specific) antigen induced cytokine production by peripheral blood cells (PBMC). However, such reports concern the detection of single cytokines and extremely low percentages (<1%) of cytokine positive lymphocytes were reported. The signals also strongly vary in the amount of cytokine per cell (signal strength), which makes it difficult to evaluate the data. Furthermore, signals cannot always clearly be discriminated from unstimulated samples (background signal). It is therefore preferred that the detection of cytokine positive T Cells is optimized by evaluation of at least two cytokines by using anti-cytokine antibodies raised against at least two cytokines selected from TNFα, IL-2 and IFN-γ. It is most preferred that anti-cytokine antibodies used according to the invention are raised against each of TNFα, IL-2 and IFN-γ.

When employing flow cytometry, the detection of multiple cytokines may be achieved by evaluation of all cytokines in the same fluorescence channel. This may be achieved by all cytokine antibodies being conjugated with the same fluorochrome. This strongly increases the sensitivity of

the assay, since there are cells that produce one, two or all three of the cytokines. All these events may be evaluated as one population. The percentage of detected cells is significantly higher and the fluorescent intensity is significantly increased and this makes it possible for cells producing low levels of cytokine to be distinguished over the background.

It is preferred that the fluorochrome used to label the cytokine antibodies is phycoerythrin (PE). PE is widely available and is useful for all commonly available flow cytometers. PE also advantageously has a high signal intensity.

It is preferred that monoclonal antibodies are used that are specific to individual cytokines.

According to one embodiment of the methods of the second aspect of the invention the anti-cytokine antibodies may be the following antibodies conjugated with R-PE from Becton Dickinson (bdbiosciences.com):

PE Rat Anti-Human IL-2 (554566)
PE Mouse Anti-Human TNF alpha (554513)
PE Mouse Anti-Human IFN-γ (554552)

According to another embodiment of the methods of the second aspect of the invention the anti-cytokine antibodies may be the following antibodies:

Mouse Anti-Human IL-2 (clone MQ1-17H12)
Mouse Anti-Human TNF alpha (clone B-C7)
Mouse Anti-Human IFN-γ (clone B-B1)

These antibodies are conjugated with R-PE by the applicant after delivery using standard conjugation protocols. After conjugation a size exclusion column (Sephadex) is used to separate free antibody and fluorochrome form the conjugate.

In a preferred embodiment the anti-CD3 antibody is labelled with a first fluorochrome; and the antibody or antibodies against the at least one cytokine is labelled with a different fluorochrome or fluorochromes. It is most preferred that all anti-cytokine antibodies are labelled with the same fluorochrome (e.g. PE)

Standard staining techniques are used to introduce the label into the fixed permeabilized cells. A preferred procedure for staining cells is described in 2.7 of Example 2.

The Assay

The assay used according to step (d) of the method of the second aspect of the invention may be any assay that determines antigen specific (i.e. peptide induced) cytokine production by cells (e.g. peripheral blood cells). Examples of assays that may be used include ELISA and ELISPOT.

It is most preferred that cells that are CD3 + and which express the at least one cytokine are detected by flow cytometry.

Flow cytometry may be conducted according to standard protocols. In a preferred embodiment the anti-CD3 antibody is labelled with a first fluorochrome and detected in a first channel; and the antibody or antibodies against the at least one cytokine is labelled with a different fluorochrome or fluorochromes and detected in a second channel.

Selecting the cells on the basis of their size and scatter profile, followed by the selection of the CD3 positive cells, makes it possible to determine the percentage of COVID-19 reactive cells by looking at the cytokine positive cells.

The use of 3 antibodies within one channel and all attached to the same fluorophore provides a higher signal for the detection of activated cells. This increases the sensitivity of the test and allows detection of activated T cells at lower thresholds than known to the art.

Use of Controls

A skilled person will appreciate that data obtained when cells are stimulated with peptide mixtures (e.g. according to step (a) of the method of the second aspect of the invention) may be best interpreted in view of data generated using positive and/or negative controls instead of the peptides. A negative control may involve a stimulation step which excludes the peptides (and may be useful to evaluate if any artificial T cell activation occurs e.g. caused by the blood draw). A positive control may involve stimulating the sample with a polyclonal stimulus instead of the peptides according to the invention (this may be used to demonstrate a generic ability of T cells to produce cytokines). PMA and/or Ionomycin may be used as a positive control as described in the

Example 2. Therefore, according to a preferred embodiment of the methods of the second aspect of the invention, two or three samples may be taken from a subject and T cells reactivity assessed in view of positive and/or negative controls.

EXAMPLE 1

The inventor analyzed the genome of Sars-Cov-2 and realized that conserved regions of ORF1 and ORF9 may be useful regions of the genome from which peptides may be derived that will elicit a T cell response. Furthermore, peptides may be selected that may be advantageously used as described above.

The inventor then further analyzed conserved regions of ORF1 and ORF9 and also the Spike protein (ORF2) by the following assessment methods:

(1) Solvent Accessible Surface Areas (SASA (Å2)) were assessed using the Euclidean Distance Transform Surface (EDTSurf) algorithm. This was used to predict the accessible area for potential protein docking. Peptides that had a SASA >900 Å2 were favoured for selection. For a 15 aa peptide a SASA of > 900 Å2 transforms into a solvent accessible surface area of > 20%. An amino acid with SASA < 20% is considered not solvent accessible according to the EDTSurf algorithm used in our peptide analysis. > 800 Å2 was accepted for peptides where they scored highly on the SVMTriP and KT metrics.

(2) The SVMTriP score - predicts antigenic epitopes, surface regions of protein that are preferentially recognised by antibodies or T-cells for receptor binding. In this method, Support Vector Machine (SVM) was used by combining the Tri-peptide similarity and Propensity scores (SVMTriP). SVMTriP scores of >0.90 were preferred for peptide selection.

(3) Peptides were also selected on the basis of Kolaskar and Tongaonkar Antigenicity Analysis. These analyses provides a useful tool to predict antigenic determinants on proteins by analysing the occurrence of hydrophobic residues Cys, Leu and Val. Peptides from ORF 1 or ORF9 comprising hydrophobic residues Cys, Leu and Val are preferred peptides for use according to the invention.

197 peptides were identified by analytical methods (1) – (3) as being antigenic coronavirus peptides which were from conserved regions of ORF 1, ORF 2 or ORF 9 and which would activate T-cells primed to such antigens. The inventor chose the most antigenic for use as peptides according to the first aspect of the invention. It will be appreciated that the total number of peptides and the precise selection of peptides may be varied within the spirit of the invention. Table 1 provides details of a most preferred, but exemplary, peptide mixture according to the first aspect of the invention.

Table 1:

SEQ ID	Peptide Sequence	Sars-Cov-2	Sequence	NCBI Reference Seq:
1	ILLNKHIDAYKTFPP	NP	351-365	NCBI Reference Seq: YP_009724397.2
2	LTQHGKEDLKFPRGQ	NP	56-70	NCBI Reference Seq: YP_009724397.2
3	PSGTWLTYTGAIKLD	NP	326-340	NCBI Reference Seq: YP_009724397.2
4	KRTATKAYNVTQAFG	NP	261-275	NCBI Reference Seq: YP_009724397.2
5	KAYNVTQAFGRRGPE	NP	266-280	NCBI Reference Seq: YP_009724397.2
6	YYRRATRRIRGGDGK	NP	86-100	NCBI Reference Seq: YP_009724397.2
7	NKDGIIWVATEGALN	NP	126-140	NCBI Reference Seq: YP_009724397.2
8	SKLWAQCVQLHNDIL	NSP7	26-40	NCBI Reference Seq: YP_009725303.1
9	VKCTSVVLLSVLQQL	NSP7	6-20	NCBI Reference Seq: YP_009725303.1
10	KMVSLLSVLLSMQGA	NSP7	51-65	NCBI Reference Seq: YP_009725303.1
11	VGDYFVLTSHTVMPL	NSP13-1	221-235	NCBI Reference Seq: YP_009725308.1
12	VLTSHTVMPLSAPTL	NSP13-1	226-240	NCBI Reference Seq: YP_009725308.1
13	VPQEHYVRITGLYPT	NSP13-1	241-255	NCBI Reference Seq: YP_009725308.1
14	TTADIVVFDEISMAT	NSP13-1	366-380	NCBI Reference Seq: YP_009725308.1
15	PIDKCSRIIPARARV	NSP13-1	326-340	NCBI Reference Seq: YP_009725308.1
16	SRIIPARARVECFDK	NSP13-1	331-345	NCBI Reference Seq: YP_009725308.1
17	CFKMFYKGVITHDVS	NSP13-2	471-485	NCBI Reference Seq: YP_009725308.1
18	MSDRDLYDKLQFTSL	NSP13-2	576-590	NCBI Reference Seq: YP_009725308.1

19	YFNSVCRLMKTIGPD	NSP13-2	421-435	NCBI Reference Seq: YP_009725308.1
20	CNVNRFNVAITRAKI	NSP13-2	556-570	NCBI Reference Seq: YP_009725308.1
21	FNVAITRAKIGILCI	NSP13-2	561-575	NCBI Reference Seq: YP_009725308.1
22	SVLNDILSR	Spike	1006-1014	NCBI Reference Seq: YP_009724390.1
23	VLNDILSRL	Spike	1007-1015	NCBI Reference Seq: YP_009724390.1
24	VQIDRLITGR	Spike	1022-1031	NCBI Reference Seq: YP_009724390.1
25	NLNESLIDL	Spike	1223-1231	NCBI Reference Seq: YP_009724390.1
26	SLIDLQELGK	Spike	1227-1236	NCBI Reference Seq: YP_009724390.1
27	MAYRFNGIGVTQNVLY	Spike	933-948	NCBI Reference Seq: YP_009724390.1
28	IDRLITGRLQSLQTY	Spike	1024-1038	NCBI Reference Seq: YP_009724390.1
29	GSFCTQLNR	Spike	788-796	NCBI Reference Seq: YP_009724390.1

EXAMPLE 2

The peptides selected in Example 1 were employed in a method according to the second aspect of the invention as described below.

2.1 Reagents Used

Table 2:

Reagent A Negative control	Dimethylsulfoxide (DMSO; pure)
Reagent B Positive control	Phorbol Myristate Acetate (PMA); used as 10 ng/mL end concentration Ionomycin; used as 1 ug/mL end concentration as mix in DMSO
Reagent C Peptide mixture	See 2.2
Reagent D DMSO	Dimethylsulfoxide (DMSO; pure)
Reagent E Secretion inhibitor	Brefeldin A in DMSO; used as 1 ug/mL end conc.
Reagent F Lysing solution (10x)	Ammonium chloride buffer (10x)
Reagent G Fixative	Formalin 4% (ready to use)
Reagent H Permeabilization buffer (10x)	Phosphate buffer saline with 10% fetal bovine serum
Reagent I Permeabilization reagent (100x)	Phosphate buffer saline with 10% saponin (used as 0.1% end concentration)
Reagent J Antibody cocktail (CD3/anti-cytokine antibodies)	PE Rat Anti-Human IL-2 (bdbiosciences.com) 554566

	PE Mouse Anti-Human TNF alpha (bdbiosciences.com) 554513 PE Mouse Anti-Human IFN-γ (bdbiosciences.com) 554552 FITC anti CD3 (UCHT1)
Reagent K Dilution buffer	Hanks' Balanced Salt Solution (10x)

2.2 Peptide Mix

The 29 peptides identified in Table 1 were used as a peptide mixture in the test.

Peptides of SEQ ID No. 1- 29 were synthesized using standard peptide protocols; mixed in equal mass ratio; and lyophilized. After storage the peptides were reconstituted in dimethylsulfoxide (DMSO) to get a concentrated stock. The stock solution was diluted with HBSS to a 22x concentrated work solution. Peptides are used in an end concentration of 1 µg/mL for each peptide.

2.3 Preparation of Reagents

2.3.1 Reagent A Negative control

Calculate the volume necessary for the number of donors (N) to be tested.

N x 60 µL = Z µL total volume

Dilute Reagent A 1:20 with 1x Dilution buffer (Reagent K) by mixing (3 x N) µL Reagent A with (57 x N) µL 1x Reagent K thoroughly in a sterile tube.

2.3.2 Reagent B Positive control

Calculate the volume necessary for the number of donors (N) to be tested.

N x 60 µL = Z µL total volume

Dilute Reagent B 1:20 with 1x Dilution buffer (Reagent K) by mixing (3 x N) µL Reagent A with (57 x N) µL 1x Reagent K thoroughly in a sterile tube

2.3.3 Reagent C Peptide mix

Add 100 µl Reagent D (DMSO) to the Reagent C vial, close vial and vortex thoroughly.

Calculate the volume necessary for the number of donors (N) to be tested.

N x 60 µL = Z µL total volume

Dilute Reagent C 1:20 with 1x Dilution buffer (Reagent K) by mixing (3 x N) µL Reagent C with (57 x N) µL 1x Reagent K thoroughly in a sterile tube

2.3.4 Reagent E Secretion inhibitor (Two vials)
Calculate the volume necessary for the number of donors (N) to be tested.

N x 200 µL = Z µL total volume

Dilute Reagent E 1:20 with 1x Dilution buffer (Reagent K) by mixing (10 x N) µL Reagent E with (190 x N) µL 1x Reagent K thoroughly in a sterile tube

2.3.5 Reagent F Lysing solution (10x)

Dilute the 10x Reagent F 1:10 by mixing 1 part of the concentrated Reagent F with 9 parts sterile demineralized water. The final pH of this solution should be 7.40 +/- 0.05.

2.3.6 Reagent G Fixative

No sample preparation is needed

2.3.7 Reagent H & I Permeabilization solution

Dilute 1.5 mL Reagent H concentrate ten times with 13.5 mL sterile demineralized water for each donor tested (3 samples) and add 150 µl Reagent I (1:100).

2.3.8 Reagent J Antibody cocktail (Two vials)

No sample preparation is needed.

2.3.9 Reagent K Dilution buffer

Dilute 3.5 mL the 10x Dilution buffer (Reagent K) concentrate with 31.5 mL sterile demineralized water for each donor tested (3 samples) in a sterile tube.

2.4 Stimulation of Cells

Three Tubes are used per donor (negative control, positive control and peptide mix).

i. Label three 5 mL tubes per donor and add 1 mL Li-Heparin treated whole blood to each tube;
ii. Add 50µL diluted Reagent A to Tube 1 (negative control), add 50 µl diluted Reagent B to Tube 2 (positive control) and 50 µl diluted Reagent C to Tube 3 (peptide mix). Gently mix total volume of each tube well.
iii. Incubate for 1 hour at 37°C, 5% CO2.

iv. Add, after the initial hour of incubation, 50 µl diluted Reagent E to all tubes and gently mix total volume of each tube well
v. Incubate for 5 hours at 37°C, 5% CO2.
vi. Add 3 mL of 1x lysing solution (Reagent F) to all samples and mix immediately. Make sure buffer is at room temperature.
vii. Incubate 15 minutes at room temperature. Vortex every 2 minutes.
viii. Centrifuge the cell suspension for 5 minutes at 300x g.
ix. Remove supernatant, add 3 mL of 1x Dilution buffer (Reagent K) and centrifuge for 5 minutes at 300x g.
x. Remove supernatant and loosen pellet.

2.5 Fixation of Cells

i. Resuspend pellet in 500 µl cold fixative (2 to 8 °C, Reagent G), vortex and incubate for 10 minutes at room temperature.
ii. Add 4 ml of 1x Dilution buffer (Reagent K) and centrifuge at 300x g for 5 minutes.
iii. Remove supernatant and loosen pellet.

2.6 Permeabilization of cells

i. Add 1.5 ml of the prepared permeabilization solution (Reagent H/I), mix well and centrifuge at 300x g for 5 minutes.
ii. Remove the supernatant and resuspend the cells in 250 µl of prepared permeabilization solution (Reagent H/I).

2.7 Staining of intracellular antigens

i. Add 50 µl of conjugated monoclonal antibody mix (Reagent J) to the tube and mix well by vortexing.
ii. Incubate for 20 minutes at room temperature in the dark.
iii. Add 1.5 ml of prepared permeabilization solution (Reagent H/I) and centrifuge at 300x g for 5 minutes.
iv. Remove the supernatant and resuspend the cells in 1 mL of 1x Dilution buffer (Reagent K).

2.8 Analysis by flow cytometry

i. Calibrate flow cytometer.
ii. Collect all cells from each tube with the flow cytometer.
iii. Analyze all events of each measurement, but for convenience reasons only show 5000 events per dot plot.
iv. Make three dot plots,
    1. a Forward Scatter (FSC) vs. Sideward Scatter (SSC) dot plot; to select the lymphocyte population
    2. a FITC (FL-1) vs. SSC dot plot; to select T-cells
    3. a R-PE (FL-2) vs. SSC dot plot; to determine the cytokine positive T-cells.

    FSC and SSC are plotted linear and the fluorescence channels FL-1 and Fl-2 are plotted logarithmic.
v. Create gate 1 by selecting all lymphocytes based on FSC/SSC profile and exclude debris, background noise and other cell types.

vi. Make gate 1 active on the second dot plot and only show the events representing the lymphocytes. Create gate 2 by selecting the T-cell marker (FL-1) positive events.
vii. Make gate 2 active on the third dot plot and only show the T-cell marker positive events. Only select the cytokine positive events with a quadrant to determine the percentage CD3+/cytokine+ T cells. The correct evaluation is done with the use of a quadrant and use the negative and positive control to set appropriate gating.
viii. The percentage of activated T-cells is expressed as percentage of the complete $CD3^{pos}$ T-cell population.

EXAMPLE 3

The protocols of Example 2 were employed to evaluate the status of T Cells in blood samples taken from healthy volunteers as described below.

3.1 Volunteer Selection

Inclusion Criteria:

- Healthy volunteers men or women
- 21 to 65 years old (inclusive) on the day of consent
- Able and willing to provide written informed consent and comply with study procedures
- Fluent in the language of the study site and able to read in this language
- Using a form of contraception with at least a 98% success rate when used correctly

Exclusion Criteria:

- Individuals younger than 21 years or older than 65 years of age
- pregnant women
- Have not had a COVID-19 vaccination or contracted COVID-19 in the last three months
- Individuals taking immune suppressive medication or receiving chemotherapy (cancer treatment)
- Individuals that took non-steroid anti-inflammatory drugs, like ibuprofen, diclofenac, etc. during the last 48 hours.
- Individuals with underlying blood disorders, like leukaemia
- Individuals known to have human anti mouse antibodies (HAMA response)
- Individuals receiving cytokine or anti-cytokine therapy
- Individuals receiving anti-thrombotic medication.
- Subjects who, in the opinion of the Investigator, are not suitable for enrolment for another reason
- No participation in a clinical trial in the last 2 months prior giving consent

The following information was collected:

- Gender
- Age
- Background (race)
- Date & volume of the blood draw

3.2 Sample Collection

From each subject one to three (maximum) 9 ml heparinised tubes of blood were collected. The blood was processed immediately or stored for a defined period as specified in Example 2.

This samples were anonymised using a unique identification number and only the clinical site was able to link this code to a volunteer's name.

CLAIMS:

1. A mixture of peptides derivable from a virus wherein the mixture includes peptides corresponding to peptides encoded by Open Reading Frame (ORF) 1 and Open Reading Frame (ORF) 9 of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) genome.

2. The mixture according to claim 1 wherein the peptides include peptides corresponding to peptides encoded by Open Reading Frame (ORF) 2.

3 The mixture according to claim 1 or 2 wherein the peptides are encoded by regions conserved between viruses.

4 The mixture according to claim 3 wherein at least one peptide is encoded by a region of ORF1 conserved between viruses.

5 The mixture according to claim 3 or 4 wherein at least one peptide is encoded by a region of ORF9 conserved between viruses.

6 The mixture according to any one of claims 3 -5 wherein the conserved region is conserved between a coronavirus and a torovirus.

7 The mixture according to any one of claims 3 -5 wherein the conserved region is conserved between different coronaviruses.

8 The mixture according to any preceding claim wherein the peptides are capable of activating a T cell.

9. The mixture according to claim 8 wherein the peptides activate a T cell by causing the T cell to produce at least one cytokine selected from Tumour Necrosis Factor Alpha (TNFα), Interleukin 2 (IL-2) or Interferon Gamma (IFN-γ).

10. The mixture according to claim 9 wherein the peptides activate a T cell by causing the T cell to produce each of TNFα, IL-2 and IFN-γ.

11 The mixture according to any preceding claim wherein the mixture comprises between 15 and 50 peptides.

12 The mixture according to claim 11 wherein the mixture comprises between 20 and 40 peptides and all of the peptides are derivable from ORF 1, ORF 9 or ORF2.

13. The mixture according to any preceding claim wherein the mixture comprises peptides of between 5 and 25 amino acids in length.

14. The mixture according to claim 13 wherein the mixture comprises peptides of between 6 and 18 amino acids in length.

15. A method of determining T cell activation in response to exposure to viral peptides, comprising:

(a) Stimulating a sample containing T cells that has been obtained from a subject with a peptide mixture according to any one of claims 1-14;
(b) Fixing and permeabilizing cells from the sample;
(c) Staining the fixed and permeabilized cells with a labelled anti-CD3 antibody and a labelled anti-cytokine antibody against at least one cytokine; and
(d) Conducting an assay to identify cells that are CD3 + and which express the at least one cytokine.

16. The method according claim 15 wherein step (d) is conducted by flow cytometry.

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2023/050315

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/569 C07K14/00
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)
G01N C07K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2021/163371 A1 (LA JOLLA INST FOR IMMUNOLOGY [US]) 19 August 2021 (2021-08-19) tables 4-9; SQL; claim 1; chapter "flow cytometry" -----	1-16
X	WO 2021/188969 A2 (BIONTECH US INC [US]) 23 September 2021 (2021-09-23) SQL; examples; claims; paragraph [0507] ----- -/--	1-16

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

[x] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

"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)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"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

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 May 2023	24/05/2023

Name and mailing address of the ISA/	Authorized officer
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1

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2023/050315

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB2023/050315

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2023/050315

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