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(54) Title: HUMAN METAPNEUMOVIRUS IMMUNOGENIC PEPTIDES, COMPOSITIONS, AND METHODS OF USE

(57) Abstract: Isolated immunogenic peptides of eight to ten contiguous amino acids of a hMPV protein and a variants thereof are provided herein. Typically, the isolated immunogenic peptides are T cell epitopes. Also provided are isolated proteins comprising at least one of the isolated immunogenic peptides of the invention for example, a chimeric protein comprising an amino acid sequence of an isolated immunogenic peptide and an amino sequence derived from an heterologous protein. Also provided are expression constructs, VLPs, compositions, vaccines and methods of treatment that may be useful in the prophylactic and/or therapeutic treatment of hMPV-associated diseases, disorders or conditions.



WO 2008/148170 A1

HUMAN METAPNEUMOVIRUS IMMUNOGENIC PEPTIDES, COMPOSITIONS, AND METHODS OF USE

FIELD OF THE INVENTION

THIS INVENTION relates to immunotherapy. More particularly, the present invention relates to immunogenic peptides derived from human metapneumovirus proteins and their use for production of pharmaceutical compositions including a vaccine.

BACKGROUND TO THE INVENTION

The respiratory pathogen human metapneumovirus (hMPV), first isolated from nasopharyngeal aspirates taken from infants with respiratory tract disease not attributable to other viral respiratory pathogens, has circulated in the human population for at least 40 years {van den Hoogen, 2001; Williams, 2006}. hMPV is characterised as a non-segmented, enveloped negative strand RNA virus, classified as belonging to the family *Paramyxoviridae* {van den Hoogen, 2002}. The genomic organization is most closely related to avian metapneumovirus subtype C {Cook, 2002}, which causes severe upper respiratory tract infection in turkeys. Based on genomic organization, hMPV is classified within the *Pneumovirinae* sub-family along with other mammalian respiratory pathogens, including human respiratory syncytial virus (hRSV). Since the first isolation of hMPV in the Netherlands {van den Hoogen, 2001}, hMPV has been identified pan-globally {Williams, 2006; Laham, 2004; Peiris, 2003; Thanasugarn, 2003; Nissen, 2002}, particularly from infants, elderly patients and immunocompromised transplant patients {Boivin, 2002; van den Hoogen, 2003; Falsey, 2003}, similar to populations at risk from severe RSV infections. hMPV infection occurs in winter peaks in the northern hemisphere {Falsey, 2003}. The clinical syndrome associated with hMPV infection is similar to that observed for RSV infection, ranging from mild respiratory illness to bronchiolitis and pneumonia {van den Hoogen, 2001; Boivin, 2002; Williams, 2006}. Seven-to-twelve percent of respiratory tract illnesses in young children can be attributed to respiratory tract infection {Williams, 2006; van den Hoogen, 2003} in the absence of other known respiratory pathogens, although hMPV does co-circulate with other

respiratory viruses, particularly adenovirus, influenza A and B, and RSV {Osterhaus, 2003}. There is evidence of exacerbated disease in patients co-infected with hRSV and hMPV, compared with patients infected with hRSV alone {Semple, 2005; Bosis, 2005}.

Sequence analysis indicates two genotypes, A and B, of hMPV world-wide with 80-88% similarity between the two types, and 93-100% within each type {van den Hoogen, 2001}. Comparison of sequences from Europe, Asia and America showed no geographic clustering or antigen drift between the A and B viruses {van den Hoogen, 2004}.

The immunobiology of infection with hMPV is incompletely characterized both in animal models and humans, although progress has been made in animals in defining the hMPV-directed humoral response both during natural infection {Alvarez, 2004} and following vaccination {Tang, 2005}. While extensive literature describes sustained human humoral immune responses to both RSV {Baumeister, 2003; Ward, 1983} and hMPV {Ebihara, 2004; van den Hoogen, 2001}, clinical infection to RSV and MPV occurs throughout life, suggesting that neutralizing antibody is not sufficient to control infection.

SUMMARY OF THE INVENTION

Therapeutic approaches targeted against hMPV have yet to be developed. The present inventors have provided a first characterisation of hMPV-derived CTL epitopes that may be potentially suitable for use as immunotherapeutic treatments of respiratory disorders that are associated with hMPV infection.

Therefore the invention is broadly directed to new immunogenic peptides to a human respiratory virus wherein the immunogenic peptides can induce a cellular immune response.

In a first aspect, the invention provides an isolated immunogenic peptide comprising or consisting essentially of eight to ten contiguous amino acids of an hMPV protein.

Preferably, the hMPV protein is selected from the group consisting of a nucleocapsid (N) protein, a matrix (M) protein, a small hydrophobic (SH) protein, a fusion (F) protein and an M2-1 protein.

Preferably, the isolated immunogenic peptide is an isolated hMPV immunogenic epitope consisting essentially of an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and SEQ ID NO:8.

Preferably, the isolated immunogenic peptide is a T cell epitope.

More preferably, the T cell epitope is a CTL epitope.

In preferred embodiments, the isolated immunogenic peptide is a variant.

More preferably, the variant has an amino acid sequence according to any one of SEQ ID NOs:204-206, SEQ ID NOs:234-244 and SEQ ID NO:56.

In a second aspect, the invention provides an isolated protein comprising at least one isolated immunogenic peptide of the first aspect, wherein the isolated protein is not a hMPV protein.

Preferably, the isolated protein is a chimera comprising an amino acid sequence of the at least one isolated immunogenic peptide and an amino acid sequence derived from an heterologous protein.

More preferably, the heterologous protein is a HBsAg.

In preferred embodiments, the isolated protein comprises in addition to the at least one isolated immunogenic peptide, one or a plurality of immunogenic epitopes of one or more pathogens other than hMPV.

It is envisaged that the one or more pathogens other than hMPV is a virus and in particular a respiratory virus.

Preferably, the one or more pathogens other than hMPV is selected from the group consisting of respiratory syncytial virus and parainfluenza virus.

In preferred embodiments, the one or a plurality of immunogenic epitopes of respiratory syncytial virus are selected from the group consisting of SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:58 and SEQ ID NO:248.

In a third aspect, the invention provides an isolated nucleic acid encoding the isolated immunogenic peptide, variant or isolated protein of any of the aforementioned aspects.

Suitably, although not limited thereto, said isolated nucleic acid is DNA.

In a fourth aspect, the invention provides a genetic construct comprising the isolated nucleic acid of the third aspect operably linked to one or more regulatory nucleotide sequences.

In one preferred embodiment, the genetic construct is an expression construct.

The genetic construct may comprise a nucleotide sequence encoding at least one isolated immunogenic peptide and/or at least one isolated protein according to any of the aforementioned aspects, wherein the peptide and/or protein is capable of forming a virus-like particle.

In a fifth aspect, the invention provides a host cell comprising the genetic construct of the fourth aspect.

In one preferred embodiment, the host cell is of eukaryotic origin.

In a sixth aspect, the invention provides a virus-like particle (VLP) comprising the isolated immunogenic peptide and/or isolated protein according to any of the aforementioned aspects.

Preferably, the VLP is a HBsAg-derived VLP.

In preferred embodiments, the VLP comprises the isolated nucleic acid according to any of the aforementioned aspects.

In a seventh aspect, the invention provides a pharmaceutical composition comprising the isolated immunogenic peptide, isolated protein, isolated nucleic acid and/or VLP according to any of the aforementioned aspects, together with a pharmaceutically-acceptable diluent, excipient or carrier.

Preferably, the pharmaceutical composition of the invention is an immunotherapeutic composition.

More preferably, the pharmaceutical composition is a vaccine.

Preferably, the pharmaceutical composition is particularly useful for treating diseases, disorders and conditions which depend upon a cytotoxic T-lymphocyte and/or Th1 response.

Compositions according to this aspect may be used either prophylactically and/or therapeutically.

In an eighth aspect, the invention provides a method of immunizing an animal including the step of administering the pharmaceutical composition according to the seventh aspect to said animal to thereby induce immunity in said animal.

In a ninth aspect, the invention provides a method of treating an animal including the step of administering the pharmaceutical composition according to the seventh aspect to said animal to thereby modulate an immune response in said animal to prophylactically and/or therapeutically treat an hMPV-associated disease, disorder or condition.

Preferably, the methods of the aforementioned aspects facilitate induction of a cell-mediated immune response, such as T cell mediated immune response.

More preferably, the T cell mediated immune response is a protective immune response.

Suitably, the animal is selected from the group consisting of humans, domestic livestock, laboratory animals, companion animals, performance animals, poultry and other animals of commercial importance, although without limitation thereto.

Preferably, the animal is a mammal.

More preferably, the animal is a human.

In a tenth aspect, the invention provides a method of determining whether an animal harbours, or has been exposed to, metapneumovirus, said method including the step of contacting one or more T cells isolated from said individual with one or more isolated immunogenic peptides of the first aspect, whereby a response to at least one of the one or more isolated immunogenic peptides by said one or more T cells indicates that the animal harbours, or has been exposed to, metapneumovirus.

Preferably, the animal is a human.

In an eleventh aspect, the invention provides an isolated antibody which binds and/or has been raised against, the isolated immunogenic peptide of the first aspect.

In a twelfth aspect, the invention provides a method of determining whether an animal harbours, or has been exposed to, metapneumovirus, said method including the step of contacting at least one isolated antibody of the eleventh aspect with a sera of said animal, whereby a response to at least one isolated antibody

indicates that the animal harbours, or has been exposed to, metapneumovirus.

Throughout this specification, unless the context requires otherwise, the words “comprise”, “comprises” and “comprising” will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

BRIEF DESCRIPTION OF THE FIGURES

In order that the invention may be readily understood and put into practical effect, preferred embodiments will now be described by way of example with reference to the accompanying figures wherein like reference numerals refer to like parts and wherein:

FIG. 1 T cell immunity to the M protein. T cell immunity of patients (#2, #9, #13, #16) was evaluated at various times (months) past hMPV diagnosis as indicated. (A, C); IFN- γ -secreting T cells were quantified by ELISPOT assay. PBMC (ex vivo) were incubated for 18 h with or without predictope peptide as shown. Results are expressed as IFN- γ -positive cells/ 10^6 PBMC. Cytotoxic T cells were measured by a ^{51}Cr release assay. T cell lines (restimulated 2 or 5 times) were tested against autologous target cells with or without epitope peptide as shown. Results are expressed as percent cytotoxicity (mean $\pm$ standard deviation) (SD was always <5%). Numbers represent peptide-specific cytotoxicity at an effector-to-target ratio of 10:1. (B, D); Protein sequence alignments for amino acids 1 to 40 (B) and 181 to 221 (D) of the M protein (254 amino acids) indicate that the identified CTL epitopes are present in type A and B hMPV strains. The conserved hexapeptide {Easton, 1997} is boxed and the two identified CTL epitopes, H6 (M12, IPYTAAVQV [SEQ ID NO:1]/HLA-B*07) and H7 (M¹⁹⁴, IAPYAGLIMI [SEQ ID NO:2]/HLA-B*07 and -B*51) are shaded. The amino acid sequence in FIG. 1B have been assigned sequence identifiers as follows: A1_NP690065_NDL 00-1 (SEQ ID NO:60), A1_AAS22100_JPS 03-180 (SEQ ID NO:61), A1_AAN52878_CAN 99-81 (SEQ ID NO:62), A1_AAN52883_CAN 00-14 (SEQ ID NO:63), A2_unsubmit_AUS -001 (SEQ ID NO:64), A2-AAN52880_CAN 97-83 (SEQ ID NO:65), A2_AAN52881_CAN 00-12 (SEQ ID NO:66), A2_AAN52885_CAN 00-16 (SEQ ID NO:67), A2_AAS22084_JPS 03-176 (SEQ ID NO:68), A2_AAS22092_JPS 03-

178 (SEQ ID NO:69), A2_AAS22108_JPS 03-187 (SEQ ID NO:70), A2_AAS22124_JPS 03-240 (SEQ ID NO:71), B1_AAN52879_CAN 97-82 (SEQ ID NO:72), B1_AAS22076_JPS 02-76 (SEQ ID NO:73), B1_AAS22116_JPS 03-194 (SEQ ID NO:74), B2_AAN52871_CAN 98-73 (SEQ ID NO:75), B2_AAN52872_CAN 98-74 (SEQ ID NO:76), B2_AAN52873_CAN 98-75 (SEQ ID NO:77), B2_AAN52874_CAN 98-76 (SEQ ID NO:78), B2_AAN 52875_CAN 98-77 (SEQ ID NO:79), B2_AAN52876_CAN 98-78 (SEQ ID NO:80), B2_AAN52877_CAN 98-79 (SEQ ID NO:81), B2_AAN52882_CAN 00-13 (SEQ ID NO:82), B2_AAN52884_CAN 00-15 (SEQ ID NO:83), aMPV_C (SEQ ID NO:84), RSV_A, RSV_B (SEQ ID NO:85);

The amino acid sequences in FIG. 1D have been assigned sequence identifiers as follows: A1_NP690065_NDL 00-1 (SEQ ID NO:86), A1_AAS22100_JPS 03-180 (SEQ ID NO:87), A1_AAN52878_CAN 99-81 (SEQ ID NO:88), A1_AAN52883_CAN 00-14 (SEQ ID NO:89), A2_unsubmit_AUS -001 (SEQ ID NO:90), A2_AAN52880_CAN 97-83 (SEQ ID NO:91), A2_AAN52881_CAN 00-12 (SEQ ID NO:92), A2_AAN52885_CAN 00-16 (SEQ ID NO:93), A2_AAS22084_JPS 03-176 (SEQ ID NO:94), A2_AAS22092_JPS 03-178 (SEQ ID NO:95), A2_AAS22108_JPS 03-187 (SEQ ID NO:96), A2_AAS22124_JPS 03-240 (SEQ ID NO:97), B1_AAN52879_CAN 97-82 (SEQ ID NO:98), B1_AAS22076_JPS 02-76 (SEQ ID NO:99), B1_AAS22116_JPS 03-194 (SEQ ID NO:100), B2_AAN52871_CAN 98-73 (SEQ ID NO:101), B2_AAN52872_CAN 98-74 (SEQ ID NO:102), B2_AAN52873_CAN 98-75 (SEQ ID NO:103), B2_AAN52874_CAN 98-76 (SEQ ID NO:104), B2_AAN52875_CAN 98-77 (SEQ ID NO:105), B2_AAN52876_CAN 98-78 (SEQ ID NO:106), B2_AAN52877_CAN 98-79 (SEQ ID NO:107), B2_AAN52882_CAN 00-13 (SEQ ID NO:108), B2_AAN52884_CAN 00-15 (SEQ ID NO:109).

FIG. 2 T cell immunity to the G protein. T cell immunity of patients (#9, #13) was evaluated at various times (months) past hMPV diagnosis as indicated. (A); IFN- γ -secreting T cells were quantified by ELISPOT assay. PBMC (ex vivo) were incubated for 18 h with or without predictope peptide as shown. Results are expressed as IFN- γ -positive cells/ 10^6 PBMC. Cytotoxic T cells were measured by a

⁵¹Cr release assay. T cell lines (restimulated 2 or 5 times) were tested against autologous target cells with or without peptide as shown. Results are expressed as percent cytotoxicity (mean $\pm$ standard deviation) (SD was always <5%). Numbers represent peptide-specific cytotoxicity at an effector-to-target ratio of 10:1. (B); Protein sequence alignments for amino acids 1 to 60 of the G protein (236 amino acids) indicate that the identified CTL epitope is present in type A hMPV strains according to {van den Hoogen, 2002}. The transmembrane domain is boxed and the identified CTL epitope, H23 (G³², SLILIGITTL [SEQ ID NO: 9]/HLA-A*02) is shaded. The amino acid sequence in FIG. 2B have been assigned sequence identifiers as follows: A1_NP690070_NDL 00-1 (SEQ ID NO:110), A1_AAQ62715_NDL 01-10 (SEQ ID NO:111), A1_AAQ62716_NDL 02-2 (SEQ ID NO:112), A1_AAQ01368_AUS Q01-7182 (SEQ ID NO:113), A1_AAQ01369_AUS Q01-4199 (SEQ ID NO:114), A1_AAQ01370_AUS Q01-6410 (SEQ ID NO:115), A1_AAQ01371_AUS Q01-7262 (SEQ ID NO:116), A1_AAQ01372_AUS Q01-6346 (SEQ ID NO:117), A1_AAQ01373_AUS Q01-7292 (SEQ ID NO:118), A1_AAQ01374_AUS Q01-7152A (SEQ ID NO:119), A1_AAQ01376_AUS Q01-6297 (SEQ ID NO:120), A1_AAS22105_JPS 03-180 (SEQ ID NO:121), A2_unsubmit_AUS-001 (SEQ ID NO:122), A2_AAQ67699_CAN 97-83 (SEQ ID NO:123), A2_AAQ62717_NDL 00-17 (SEQ ID NO:124), A2_AAQ62718_NDL 81-1 (SEQ ID NO:125), A2_AAQ62722_NDL 95-1 (SEQ ID NO:126), A2_AAQ62724_NDL 96-3 (SEQ ID NO:127), A2_AAQ62725_NDL 01-22 (SEQ ID NO:128), A2_AAQ62729_NDL 02-3 (SEQ ID NO:129), A2_AAS22089_JPS 03-176 (SEQ ID NO:130), A2_AAS22097_JPS 03-178 (SEQ ID NO:131), A2_AAS22113_JPS 03-187 (SEQ ID NO:132), A2_AAS22129_JPS 03-240 (SEQ ID NO:133), B1_AAQ62730_NDL 99-1 (SEQ ID NO:134), B1_AAQ62731_NDL 00-11 (SEQ ID NO:135), B1_AAQ62733_NDL 01-5 (SEQ ID NO:136), B1_AAQ62735_NDL 01-21 (SEQ ID NO:137), B1_AAS22081_JPS 02-76 (SEQ ID NO:138), B1_AAS22121_JPS 03-0194 (SEQ ID NO:139), B2_AAQ62736_NDL 94-1 (SEQ ID NO:140), B2_AAQ62737_NDL 82-1 (SEQ ID NO:141), B2_AAQ62738_NDL 96-1 (SEQ ID NO:142), B2_AAQ62739_NDL 97-6 (SEQ ID NO:143), B2_AAQ62740_NDL 00-9 (SEQ ID NO:144), B2_AAQ62741_NDL 01-3

(SEQ ID NO:145), B2_patient #13 (SEQ ID NO:146), B2_AAQ67690_CAN 98-75 (SEQ ID NO:147).

FIG. 3 T cell immunity to the F protein. T cell immunity of patients (#2, #13, #16) was evaluated at various times (months) past hMPV diagnosis as indicated. (A, C); IFN- γ -secreting T cells were quantified by ELISPOT assay. PBMC (ex vivo) were incubated for 18 h with or without predictope peptide as shown. Results are expressed as IFN- γ -positive cells/ 10^6 PBMC. Cytotoxic T cells were measured by a ^{51}Cr release assay. T cell lines (restimulated 2 or 5 times) were tested against target cells with or without peptide as shown. Results are expressed as percent cytotoxicity (mean $\pm$ standard deviation) (SD was always $<5\%$). Numbers represent peptide-specific cytotoxicity at an effector-to-target ratio of 10:1. (B, D); Protein sequence alignments for amino acids 91 to 169 (B) and 421 to 491 (D) of the F protein (539 amino acids) indicate that the identified CTL epitopes are present in type A and B hMPV strains. The cleave site {Schowalter, 2006} and fusion domain {van den Hoogen, 2002} are boxed. Heptad repeat (HR-) regions 1 and 2 (••••) {van den Hoogen, 2002}, are also shown. The two identified CTL epitopes, H22 (F¹⁵⁷, VLATAVREL [SEQ ID NO:4]/HLA-A*02) and H15 (F⁴²⁹, KVEGEQHVIV [SEQ ID NO:5]/HLA-A*31 and A*11) are shaded. The amino acid sequences in FIG. 3B have been assigned sequence identifiers as follows: A1_NP690066_NDL 00-1 (SEQ ID NO:148), A1_AAN52908_CAN 99-81 (SEQ ID NO:149), A1_AAN52913_CAN 00-14 (SEQ ID NO:150), A1_AAS22101_JPS 03-180 (SEQ ID NO:151), A2_unsubmit_AUS -001 (SEQ ID NO:152), A2_AAN52910_CAN 97-83 (SEQ ID NO:153), A2_AAN52911_CAN 00-12 (SEQ ID NO:154), A2_AAN52915_CAN 00-16 (SEQ ID NO:155), A2_AAS22085_JPS 03-176 (SEQ ID NO:156), A2_AAS22093_JPS 03_178 (SEQ ID NO:157), A2_AAS22109_JPS 03-187 (SEQ ID NO:158), A2_AAS22125_JPS 03-240 (SEQ ID NO:159), B1_AAN52909_CAN 97-82 (SEQ ID NO:160), B1_AAS22077_JPS 02-76 (SEQ ID NO:161), B1_AAS22117_JPS 03-194 (SEQ ID NO:162), B2_AAN52901_CAN 98-73 (SEQ ID NO:163), B2_AAN52902_CAN 98-74 (SEQ ID NO:164), B2_AAN52903_CAN 98-75 (SEQ ID NO:165), B2_AAN52904_CAN 98-76 (SEQ ID NO:166), B2_AAN52905_CAN 98-77 (SEQ ID NO:167), B2_AAN52906_CAN 98-78 (SEQ

ID NO:168), B2_AAN52907_CAN 98-79 (SEQ ID NO:169), B2_AAN52912_CAN 00-13 (SEQ ID NO:170), B2_AAN52914_CAN 00-15 (SEQ ID NO:171).

The amino acid sequences in FIG. 3D have been assigned sequence identifiers as follows: A1_NP690066_NDL 00-01 (SEQ ID NO:172), A1_AAN52908_CAN 99-81 (SEQ ID NO:173), A1_AAN52913_CAN 00-14 (SEQ ID NO:174), A1_AAS22101_JPS 03-180 (SEQ ID NO:175), A2_unsubmit_AUS -001 (SEQ ID NO:176), A2_AAN52910_CAN 97-83 (SEQ ID NO:177), A2_AAN52911_CAN 00-12 (SEQ ID NO:178), A2_AAN52915_CAN 00-16 (SEQ ID NO:179), A2_AAS22085_JPS 03-176 (SEQ ID NO:180), A2_AAS22093_JPS 03-178 (SEQ ID NO:181), A2_AAS2109_JPS 03_187 (SEQ ID NO:182), A2_AAS22125_JPS 03-240 (SEQ ID NO:183), B1_AAN52909_CAN 97-82 (SEQ ID NO:184), B1_AAS22077_JPS 02-76 (SEQ ID NO:185), B1_AAS22117_JPS 03-194 (SEQ ID NO:186), B2_AAN52901_CAN 98-73 (SEQ ID NO:187), B2_AAN52902_CAN 98-74 (SEQ ID NO:188), B2_AAN52903_CAN 98-75 (SEQ ID NO:189), B2_AAN52904_CAN 98-76 (SEQ ID NO:190), B2_AAN52905_CAN 98-77 (SEQ ID NO:191), B2_AAN52906_CAN 98-78 (SEQ ID NO:192), B2_AAN52907_CAN 98-79 (SEQ ID NO:193), B2_AAN52912_CAN 00-13 (SEQ ID NO:194), B2_AAN52914_CAN 00-15 (SEQ ID NO:195).

FIG. 4 T cell immunity to the M2-1 protein. T cell immunity of patients (#13, #16) was evaluated at various times (months) past hMPV diagnosis as indicated. (A, B); IFN- γ -secreting T cells were quantified by ELISPOT assay. PBMC (ex vivo) were incubated for 18 h with or without predictope peptide as shown. Results are expressed as IFN- γ -positive cells/ 10^6 PBMC. Cytotoxic T cells were measured by a ^{51}Cr release assay. T cell lines (restimulated 2 or 5 times) were tested against target cells with or without peptide as shown. Results are expressed as percent cytotoxicity (mean $\pm$ standard deviation) (SD was always $<5\%$). Numbers represent peptide-specific cytotoxicity at an effector-to-target ratio of 10:1. (C); The identified CTL epitopes are present in type A and B hMPV strains according to protein sequence alignments for amino acids 141 to 171 of the M2-1 protein (187 amino acids) (C). The identified CTL epitopes, H14 (M2-1149, RLPREKLKK [SEQ ID NO:7]/HLA-A*11) and H24 (M2-1157, KLAKLIIDL [SEQ ID NO:6]/HLA-

A*02) are shaded. The amino acid sequences in FIG. 4C have been assigned sequence identifiers as follows: A1_NP690067_NDL 00-1 (SEQ ID NO:196), A1_AAS22102_JPS03-180 (SEQ ID NO:197), A2_unsubmit_AUS -001 (SEQ ID NO:198), AS_AAQ67696_CAN 97-83 (SEQ ID NO:199), A2_AAS22086_JPS03-176 (SEQ ID NO:200), A2_AAS22094_JPS03-178 (SEQ ID NO:201), A2_AAS22110_JPS03-187 (SEQ ID NO:202), A2_AAS22126_JPS03-240 (SEQ ID NO:203), B1_AAS22078_JPS02-76 (SEQ ID NO:204), B1_AAS22118_JPS03-194 (SEQ ID NO:205), B2_AAQ67687_CAN98-75 (SEQ ID NO:206).

FIG. 5 T cell immunity to the N and SH proteins. T cell immunity of patient (#13) was evaluated at various times (months) past hMPV diagnosis as indicated. (A, C); IFN- γ -secreting T cells were quantified by ELISPOT assay. PBMC (ex vivo) were incubated for 18 h with or without predictope peptide as shown. Results are expressed as IFN- γ -positive cells/ 10^6 PBMC. Cytotoxic T cells were measured by a ^{51}Cr release assay. T cell lines (restimulated 2 or 5 times) were tested against autologous target cells with or without peptide as shown. Results are expressed as percent cytotoxicity (mean $\pm$ standard deviation) (SD was always $<5\%$). Numbers represent peptide-specific cytotoxicity at an effector-to-target ratio of 10:1.(B,D); Protein sequence alignments for amino acids 271 to 331 of the N protein (394 amino acids) with similarity region {van den Hoogen, 2002} boxed (B) and amino acids 121 to 181 of the SH protein (183 amino acids) with second hydrophobic region {van den Hoogen, 2002} boxed (D), indicate that the identified CTL epitopes are present in type A and B (or subtype A1) hMPV strains. The identified CTL epitopes, H4 (N³⁰⁷, SPKAGLLSL [SEQ ID NO:8]/HLA-B*07) and H9 (SH¹⁵², KPAVGVIYHIV [SEQ ID NO:3]/HLA-B*07) are shaded. The hRSV epitope (N³⁰⁶, NPKASLLSL [SEQ ID NO:48]/HLA-B*07) {Heidema, 2004; Goulder, 2000} is shown in brackets (B). The amino acid sequences in FIG. 5B have been assigned sequence identifiers as follows: A1_NP690063_NDL 00-1 (SEQ ID NO:207), A1_AAN52900_CAN 99-81 (SEQ ID NO:208), A1_AAN52888_CAN 00-14 (SEQ ID NO:209), A1_AAS22098_JPS03-180 (SEQ ID NO:210), A2_unsubmit_AUS-001 (SEQ ID NO:211), A2_AAN52892_CAN 97-83 (SEQ ID NO:212), A2_AAN52886_CAN 00-12 (SEQ ID NO:213), A2_AAN52890_CAN 00-16 (SEQ ID NO:214),

A2_AAS22082_JPS03-176 (SEQ ID NO:215), A2_AAS22090_JPS03-178 (SEQ ID NO:216), A2_AAS22106_JPS03-187 (SEQ ID NO:217), A2_AAS22122_JPS03-240 (SEQ ID NO:218), B1_AAN52891_CAN 97-82 (SEQ ID NO:219), B1_AAS22074_JPS02-76 (SEQ ID NO:220), B1_AAS22114_JPS03-194 (SEQ ID NO:221), B2_AAN52893_CAN 98-73 (SEQ ID NO:222), B2_AAN52894_CAN 98-74 (SEQ ID NO:223), B2_AAN52895_CAN 98-75 (SEQ ID NO:224), B2_AAN52896_CAN 98-76 (SEQ ID NO:225), B2_AAN52897_CAN 98-77 (SEQ ID NO:226), B2_AAN52898_CAN 98-78 (SEQ ID NO:227), B2_AAN52899_CAN 98-79 (SEQ ID NO:228), B2_AAN52887_CAN 00-13 (SEQ ID NO:229), B2_AAN52889_CAN 00-15 (SEQ ID NO:230), aMPV_C (SEQ ID NO:231), hRSV_A (SEQ ID NO:232), hRSV_B (SEQ ID NO:233).

The amino acid sequences in FIG. 5D have been assigned sequence identifiers as follows: A1_NP690069_NDL 00-1 ((SEQ ID NO:234), A1_AAS22104_JPS03-180 (SEQ ID NO:235), A2_unsubmit_AUS-001 (SEQ ID NO:236), A2_AAQ67698_CAN 97-83 (SEQ ID NO:237), A2_AAS22088_JPS03-176 (SEQ ID NO:238), A2_AAS22096_JPS03-178 (SEQ ID NO:239), A2_AAS22112_JPS03_187 (SEQ ID NO:240), A2_AAS22128_JPS03-240 (SEQ ID NO:241), B1_AAS22080_JPS02-76 (SEQ ID NO:242), B1_AAS22120_JPS03-194 (SEQ ID NO:243), B2_AAQ67689_CAN 98-75 (SEQ ID NO:244), B2_patient #13 (SEQ ID NO:56).

FIG. 6 Peptide specific T cell lines recognize virus-infected targets. T cell lines specific for M¹⁹⁴ peptide (A) and G³² peptide (B) were tested against HLA-matched, hMPV-infected target cells or uninfected target cells as shown. Results are expressed as percent cytotoxicity (mean $\pm$ standard deviation) (SD was always <5%). Numbers represent peptide-specific cytotoxicity at an effector-to-target ratio of 10:1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, at least in part, on the discovery of effector and memory CTL responses to immunogenic epitopes from six hMPV proteins in patients with prior hMPV-associated respiratory disease, which were isolated from a bank of predictopes generated by bioinformatics. Characterisation of isolated hMPV immunogenic epitopes provides an opportunity for development of

immunotherapeutic compositions, and in particular vaccines, to combat hMPV-associated respiratory disease. Moreover, most of the identified epitopes are conserved among hMPV subtypes. Protection across various hMPV strains is desirable in a vaccine as hMPV strains co-circulate within the human population, typically with a different predominant strain from year to year.

Therefore in one particular form, the invention provides an isolated immunogenic peptide comprising or consisting essentially of eight to ten contiguous amino acids of a hMPV protein. Preferably, said isolated immunogenic peptides are useful as an immunotherapeutic composition and in particular, a vaccine.

In preferred embodiments, the hMPV protein is selected from the group consisting of an M protein, an F protein, an SH protein, an M2-1 protein and an N protein.

For the purposes of this invention, by “*isolated*” is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

By “*protein*” is meant an amino acid polymer. The amino acids may be natural or non-natural amino acids, D- or L- amino acids as are well understood in the art.

The term “*protein*” includes and encompasses “*peptide*”, which is typically used to describe a protein having no more than fifty (50) amino acids and “*polypeptide*”, which is typically used to describe a protein having more than fifty (50) amino acids.

In preferred embodiments, the isolated immunogenic peptide of the present invention is an isolated hMPV immunogenic epitope consisting essentially of an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and SEQ ID NO:8.

By “*immunogenic peptide*” is meant a sequence of amino acids encoded by a hMPV genome that is capable of eliciting an immune response, be it a cellular or humoral immune response. In a preferred form of the invention, the “immunogenic peptide” elicits a cellular immune response, and in preferred embodiments, elicits a cellular response by at least one T cell when the amino acid sequence is presented to the at least one T cell in the context of MHC class I *in vitro* or *in vivo*. In preferred embodiments, the isolated immunogenic peptide is an isolated hMPV immunogenic epitope. Typically, although not exclusively, the at least one T cell is a cytotoxic T lymphocyte (CTL).

In preferred embodiments, the isolated peptides and hMPV immunogenic epitopes of the present invention are CTL epitopes.

In the context of the present invention, by “*epitope*” is meant a peptide or amino acid sequence which is recognised by the immune system and in particular, antibodies, B cells or T cells.

In the context of the present invention, “*consisting essentially of*” is meant that the immunogenic peptide, and preferably the hMPV immunogenic epitope, has one, two or no more than three amino acid residues in addition to the immunogenic peptide sequence or hMPV immunogenic epitope sequence, at the N- and/or C-terminus of the epitope. The additional amino acid residues may occur at one or both termini of the immunogenic peptide or hMPV immunogenic epitope, but is not limited thereto.

Also contemplated are isolated immunogenic peptide variants.

Generally, as used herein, “*variants*” are isolated immunogenic peptides of the present invention in which one, two or three amino acid residues have been deleted or replaced by different amino acids without substantial alteration to immunogenicity. It is well understood in the art that some amino acids may be changed to others with broadly similar properties without changing the immunogenicity of the peptide, so called conservative substitutions.

Substantial changes in function are made by selecting substitutions that are less conservative and relatively fewer of these may be tolerated. Generally, the substitutions which are likely to produce the greatest changes in a protein's properties

are those in which (a) a hydrophilic residue (e.g., Ser or Thr) is substituted for, or by, a hydrophobic residue (e.g., Ala, Leu, Ile, Phe or Val); (b) a cysteine or proline is substituted for, or by, any other residue; (c) a residue having an electropositive side chain (e.g., Arg, His or Lys) is substituted for, or by, an electronegative residue (e.g., Glu or Asp) or (d) a residue having a bulky side chain (e.g., Phe or Trp) is substituted for, or by, one having a smaller side chain (e.g., Ala, Ser) or no side chain (e.g., Gly).

Non-limiting examples of naturally-occurring variant immunogenic peptides and in particular, hMPV immunogenic epitopes are provided in FIG. 4C and FIG. 5D and SEQ ID NOs:204-206 and SEQ ID NOs:234-244 and SEQ ID NO:56, which variants are derived from particular hMPV subtypes and strains thereof.

The invention also contemplates "*derivatives*" of isolated immunogenic peptides of the invention, such as created by chemical modification of amino acid residues, biotinylation, conjugation with fluorochromes, addition of epitope tags (for example c-*myc*, haemagglutinin and FLAG tags), and fusion partners that facilitate recombinant protein expression, detection and purification (such as glutathione-S-transferase, green fluorescent protein, hexahistidine and maltose-binding protein, although without limitation thereto).

With regard to chemical modification of amino acids, this includes but is not limited to, modification by acylation, amidination, pyridoxylation of lysine, reductive alkylation, trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS), amide modification of carboxyl groups and sulphydryl modification by performic acid oxidation of cysteine to cysteic acid, formation of mercurial derivatives, formation of mixed disulphides with other thiol compounds, reaction with maleimide, carboxymethylation with iodoacetic acid or iodoacetamide and carbamoylation with cyanate at alkaline pH, although without limitation thereto.

In this regard, the skilled person is referred to Chapter 15 of CURRENT PROTOCOLS IN PROTEIN SCIENCE, Eds. Coligan *et al.* (John Wiley & Sons NY 1995-2000) for more extensive methodology relating to chemical modification of proteins.

The present invention also provides an isolated protein comprising at least one isolated immunogenic peptide as hereinbefore described, wherein the isolated

protein is not an hMPV protein. Typically, the isolated protein of the present invention comprises one or more amino acid sequences of one or more proteins other than an hMPV protein.

In preferred embodiments, the isolated protein comprises at least one isolated hMPV immunogenic epitope consisting essentially of an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and SEQ ID NO:8, wherein the isolated protein is not a hMPV protein.

The invention further contemplates an isolated protein as a chimera comprising a fusion between one or a plurality of isolated immunogenic peptides of the present invention and an amino acid sequence derived from a heterologous protein.

In preferred embodiments, the heterologous protein is a protein capable of forming a virus-like particle such as, but not limited to, HBsAg from hepatitis B virus, VP1 protein from polyomavirus and L1 from papillomavirus. Preferably, the heterologous protein is HBsAg.

The present invention includes within its scope a chimera comprising a fusion between at least one isolated immunogenic peptide and one or a plurality of immunogenic epitopes derived from one or more pathogens other than hMPV. The invention is particularly well suited for generation of chimeras wherein the pathogen other than hMPV is a respiratory pathogen such as, but not limited to, respiratory syncytial virus and/or parainfluenza virus.

Exemplary immunogenic epitopes from RSV include RARRELPRF (SEQ ID NO:46), IAVGLLLYC (SEQ ID NO:47), NPKASLLSL (SEQ ID NO:48), QVMLRWGVLA (SEQ ID NO:49), RELPRFMNYT (SEQ ID NO:50), VELKESIYY (SEQ ID NO:51), RLPADVLKK (SEQ ID NO:52), AELDRTEEY (SEQ ID NO:53), LAKAVIHTI (SEQ ID NO:54), IPYSGLLLV (SEQ ID NO:55) and STYTAAVQY (SEQ ID NO:58).

A non-limiting example of an immunogenic epitope from parainfluenza virus is the region spanning amino acids 321 to 326 of the nucleoprotein. In this regard, reference is made to Dave *et al* (1994) *Virology* 199: 376-383, which describes CTL

determinants recognised by human parainfluenza virus type 1-specific cytotoxic T-cells.

In one embodiment, the invention also contemplates an isolated protein in the form of a "*polyepitope*" or "*polytope*" protein, and/or an isolated nucleic acid encoding the same.

Suitably, the polytope protein comprises a plurality of isolated immunogenic peptides of the present invention, preferably, hMPV immunogenic epitopes, and more preferably CTL epitopes of the invention. For example, said epitopes may be present singly or as repeats, which also includes tandemly repeated epitopes. Accordingly, the polytope protein may comprise a plurality of repeated same and/or different epitopes located either adjacent to each other and/or spaced apart by a spacer. Furthermore, one or more other CTL epitopes (eg parainfluenza and/or RSV) may be included. "Spacer" amino acids may also be included between one or more of the CTL epitopes present in said isolated protein. Such spacer amino acids may be amino acids that normally flank the CTL epitope or may be amino acids that do not normally flank the CTL epitope. Alternatively, the polytope protein may comprise two or more contiguous epitopes (*i.e* that lack intervening sequences between respective CTL epitopes). It will be appreciated that a particular advantage of a polytope protein is that a single protein may comprise CTL epitopes derived from a plurality of different pathogens and/or cover a plurality of different MHC specificities (*eg*. HLA types in humans).

The isolated immunogenic peptides, hMPV immunogenic epitopes, isolated proteins and chimeras of the present invention may be produced by any means known in the art, including but not limited to, chemical synthesis, recombinant DNA technology and proteolytic cleavage to produce peptide fragments.

In one embodiment, isolated immunogenic peptides and hMPV immunogenic epitopes may be in the form of peptides prepared by chemical synthesis, inclusive of solid phase and solution phase synthesis. Such methods are well known in the art, although reference is made to examples of chemical synthesis techniques as provided in Chapter 9 of SYNTHETIC VACCINES Ed. Nicholson (Blackwell Scientific Publications) and Chapter 15 of CURRENT PROTOCOLS IN PROTEIN SCIENCE

Eds. Coligan *et al.*, (John Wiley & Sons, Inc. NY USA 1995-2001). In this regard, reference is also made to International Publication WO 99/02550 and International Publication WO 97/45444.

In another embodiment, recombinant immunogenic peptides of the invention, or preferably, isolated proteins and chimeric proteins comprising isolated immunogenic peptides and hMPV immunogenic epitopes, may be conveniently prepared by a person skilled in the art using standard protocols as for example described in Sambrook *et al.*, MOLECULAR CLONING. A Laboratory Manual (Cold Spring Harbor Press, 1989), in particular Sections 16 and 17; CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.*, (John Wiley & Sons, Inc. NY USA 1995-2001), in particular Chapters 10 and 16; and CURRENT PROTOCOLS IN PROTEIN SCIENCE Eds. Coligan *et al.*, (John Wiley & Sons, Inc. NY USA 1995-2001, in particular Chapters 1, 5 and 6.

Nucleic acids and genetic constructs

The present invention provides isolated nucleic acids encoding isolated immunogenic peptides, variants thereof and isolated proteins as hereinbefore described.

Nucleotide sequences encoding hMPV immunogenic epitopes of the invention may be readily deduced from the complete genomic nucleic acid sequence of hMPV subtypes, published for example in van de Hoogen *et al.*, Nat Med. 2001 Jun;7(6):719-24 (hMPV subtype A1; NCBI Accession No. NC_004148.2), Biacchesi *et al.*, Virology, 2003, 315(1):1-9 (hMPV subtype A2; NCBI Accession No. AY297749), Herfst *et al.*, J Virol, 2004, 78(15):8264-8270 (subtype B1; NCBI Accession No. AY525843) and Biacchesi *et al.*, Virology, 2003, 315(1):1-9 (hMPV subtype B2; NCBI Accession No. AY297748).

The term “*nucleic acid*” as used herein designates single- or double-stranded mRNA, RNA, cRNA and DNA inclusive of cDNA, genomic DNA and DNA-RNA hybrids.

The invention also contemplates nucleic acids which have been modified such as by taking advantage of codon redundancy. By way of example only, codon usage may be modified to optimise expression of a nucleic acid in a particular

organism or cell type.

The present invention further contemplates use of modified purines (for example inosine, methlinosine and methyladenosine) and modified pyrimidines (for example, thiouridine and methylcytosine) in nucleic acids of the invention.

It will be well appreciated by a person of skill in the art that the isolated nucleic acids of the invention can be conveniently prepared by a person of skill in the art using standard protocols such as those described in Chapter 2 and Chapter 3 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Eds. Ausubel *et al.* John Wiley & Sons NY, 2000).

In one particular embodiment, an isolated nucleic acid of the present invention is operably linked to one or more regulatory nucleotide sequences in a genetic construct. A person skilled in the art will appreciate that a genetic construct is a nucleic acid comprising any one of a number of nucleotide sequence elements, the function of which depends upon the desired use of the construct. Uses range from vectors for the general manipulation and propagation of recombinant DNA to more complicated applications such as prokaryotic or eukaryotic expression of the isolated nucleic acid. Typically, although not exclusively, genetic constructs are designed for more than one application. By way of example only, a genetic construct whose intended end use is recombinant protein expression in a eukaryotic system may have incorporated nucleotide sequences for such functions as cloning and propagation in prokaryotes over and above sequences required for expression. An important consideration when designing and preparing such genetic constructs are the required nucleotide sequences for the intended application.

In view of the foregoing, it is evident to a person of skill in the art that genetic constructs are versatile tools that can be adapted for any one of a number of purposes.

Therefore in one particular aspect, the invention provides a genetic construct comprising an isolated nucleic acid of the invention operably linked to one or more regulatory nucleotide sequences.

In a preferred embodiment, the genetic construct is an expression construct which is suitable for recombinant expression. Preferably, the expression construct

comprises at least a promoter and in addition, one or more other regulatory nucleotide sequences which are required for manipulation, propagation and expression of recombinant DNA.

By "*operably linked*" is meant that said one or more other regulatory nucleotide sequence(s) is/are positioned relative to the nucleic acid(s) of the invention to initiate, regulate or otherwise control transcription thereof.

"*Regulatory nucleotide sequences*" present in the expression construct may include an enhancer, promoter, splice donor/acceptor signals, Kozak sequence, terminator and polyadenylation sequences, as are well known in the art and facilitate expression of the nucleotide sequence(s) to which they are operably linked, or facilitate expression of an encoded protein. Regulatory nucleotide sequences will generally be appropriate for the host cell or organism used for expression. Numerous types of appropriate expression constructs and suitable regulatory sequences are known in the art for a variety of host cells.

With regard to promoters, constitutive promoters (such as CMV, SV40, vaccinia, HTLV1 and human elongation factor promoters) and inducible/repressible promoters (such as *tet*-repressible promoters and IPTG-, metallothionine- or ecdysone-inducible promoters) are well known in the art and are contemplated by the invention. It will also be appreciated that promoters may be hybrid promoters that combine elements of more than one promoter.

Preferably, said expression construct also includes one or more selectable markers suitable for the purposes of selection of transformed bacteria (such as *bla*, *kanR* and *tetR*) or transformed mammalian cells (such as hygromycin, G418 and puromycin).

The expression construct may also include a fusion partner (typically provided by the expression vector) so that the recombinant protein of the invention is expressed as a fusion protein with said fusion partner. The main advantage of fusion partners is that they assist identification and/or purification of said fusion protein.

Examples of fusion partners have been hereinbefore described. Typically, fusion partners are particularly useful for isolation of a fusion protein by affinity chromatography. For the purposes of fusion polypeptide purification by affinity

chromatography, relevant matrices for affinity chromatography are antibody, protein A- or G-, glutathione-, amylose-, and nickel- or cobalt-conjugated resins respectively. Many such matrices are available in "kit" form, such as the QIAexpressTM system (Qiagen) useful with (HIS₆) fusion partners and the Pharmacia GST purification system.

Suitable host cells for expression may be prokaryotic or eukaryotic, such as *Escherichia coli* (BL-21 and derivatives for example), HuH7 cells, yeast cells, Sf9 cells utilized with a baculovirus expression system, mammalian cell lines such as lymphoblastoid cell lines and splenocytes isolated from transformed host organisms such as humans and mice, although without limitation thereto.

Expression constructs may be introduced into host cells or organisms by any of a number of well known techniques including, but not limited to, transformation by heat shock, electroporation, DEAE-Dextran transfection, microinjection, liposome-mediated transfection, calcium phosphate precipitation, protoplast fusion, microparticle bombardment, viral transformation and the like.

The invention contemplates that the genetic construct may comprise a nucleotide sequence encoding at least one isolated immunogenic peptide and/or at least one isolated protein according to any of the aforementioned aspects, wherein the peptide and/or protein is capable of forming a virus-like particle.

Therefore in a particular embodiment, the genetic or expression construct may be used for the production of VLPs.

Virus-like particles

The isolated immunogenic peptides, isolated hMPV immunogenic epitopes and isolated proteins of the present invention, inclusive of variants and derivatives thereof, are particularly well suited for inclusion into virus-like particles (VLPs) as a surface-exposed epitope (although without limitation thereto).

It is thought that the particulate nature of VLPs induces a more effective immune response than denatured or soluble proteins as immunogens. Hence, VLPs provide a convenient and efficient delivery vehicle for immunogenic epitopes. VLPs have a number of advantages over conventional vaccines: (i) VLP vaccines mimic the immunogenicity of live attenuated vaccines; (ii) they can induce a CTL and B cell

response in the absence of adjuvant; (iii) VLPs cannot replicate and are therefore non-infectious; (iv) they can be produced in large quantities; and (v) are easily enriched and purified. Viral structural proteins such as, but not limited to, capsid and envelope proteins have the ability to self-assemble into higher order molecular structures and ultimately, the macromolecular particulate structure of a VLP. Non-limiting examples of suitable viral structural proteins include, VP1 from polyomavirus, HBsAg from hepatitis B virus and L1 from human papillomavirus.

In preferred embodiments, the isolated immunogenic peptides, the isolated hMPV immunogenic epitopes and/or isolated proteins of the present invention are incorporated into a HBsAg-derived VLP.

HBsAg-derived VLPs are particularly advantageous due to the capacity to delete or excise one or more specific endogenous T cell epitopes of HBsAg and substitute with a foreign T cell epitope, i.e. an endogenous T cell epitope is replaced by a foreign T cell epitope. In addition, the endogenous T cell epitope is replaced with a foreign T cell epitope of similar structural characteristics, for example size and phobicity, i.e. replacing "*like with like*". This permits retention of tertiary conformation and retention of structural integrity within the HBsAg molecule. This strategy also exploits the powerful inherent immunogenicity of HBsAg which facilitates generation of immunogenic responses in an animal. Reference is made to International Publication Number WO 2006/108226, which describes protocols for like with like substitution of T cell epitopes in HBsAg and is incorporated herein by reference.

The present invention further contemplates co-delivery of foreign T and foreign B cell epitopes by means of insertion of one or more foreign B cell epitopes in HBsAg at particular insertion sites. In a preferred embodiment, the one or more foreign B cell epitopes are inserted within the a-determinant region of HBsAg.

A non-limiting example of a suitable B cell epitope is a mimotope from RSV with the sequence of HWSISKPQ (SEQ ID NO:59).

It will be appreciated that the invention contemplates HBsAg derived from a variety of viruses belonging to the family *Hepadnaviridae* such as but not limited to members of the genera *Avihepadnavirus* or *Orthohepadnavirus*. Non-limiting

examples of family members include hepatitis B virus, duck hepatitis B virus, woodchuck hepatitis B virus, ground squirrel hepatitis B virus, woolley monkey hepatitis B virus and snow goose hepatitis B virus.

A person of skill in the art will readily appreciate that the invention encompasses generation of a number of different VLPs. For example, the invention contemplates: (a) a single VLP with one or more immunogenic epitopes from a single pathogen or one or more immunogenic epitopes from a plurality of pathogens; or (b) a mixture of VLPs each having one or more epitopes from different pathogens. The single VLP of (a) may comprise, for example, (i) a plurality of a same structural protein encoding one or more immunogenic peptides or (ii) alternatively may comprise a plurality of different structural proteins. An example of (ii) includes combining a structural protein encoding one or more immunogenic peptides with another structural protein encoding different one or more immunogenic peptides. Alternatively, a VLP may comprise structural proteins encoding one or more immunogenic peptides and other structural proteins encoding one or more non-hMPV immunogenic epitopes.

Pharmaceutical compositions, immunotherapeutic compositions, vaccines and methods of treatment

One particular broad application of the present invention is provision of pharmaceutical compositions and methods of treating hMPV using the same.

Accordingly, the invention provides a method of immunizing an animal including the step of administering a pharmaceutical composition of the present invention to an animal to thereby induce immunity in said animal.

The invention also provides a method of treating an animal including the step of administering a pharmaceutical composition of the present invention to said animal to thereby modulate an immune response in said animal to prophylactically and/or therapeutically treat an hMPV-associated disease, disorder or condition.

In the context of the present invention, by "*hMPV-associated disease, disorder or condition*" is meant any clinical respiratory syndrome that is caused by hMPV virus infection of the upper and lower respiratory tract. Clinical respiratory syndromes range from mild to severe lower respiratory tract illness and may

exacerbate asthma and wheezing, particularly in young children. The causative agent of a hMPV-associated disease, disorder or condition may be any subtype or isolate of hMPV inclusive of subtypes A1, A2, B1 and B2, but without limitation thereto.

In a preferred form, the invention also provides a pharmaceutical composition as an immunogenic composition comprising at least one isolated immunogenic peptide of the invention, inclusive of variants and derivatives thereof, isolated proteins, isolated nucleic acids and/or VLPs. Preferably, the at least one isolated immunogenic peptide is at least one hMPV immunogenic epitope as hereinbefore described.

In a preferred embodiment, the pharmaceutical composition is an immunotherapeutic composition.

In a preferred embodiment, pharmaceutical compositions are immunotherapeutic compositions that provide a prophylactic or therapeutic treatment of hMPV-associated diseases, disorders or conditions.

In a particular preferred embodiment, the immunotherapeutic composition is a vaccine.

Such compositions may be delivered for the purposes of generating immunity, preferably protective immunity, to hMPV upon administration to a host, although without limitation thereto.

Suitable vaccines may be in the form of proteinaceous vaccines comprising at least one isolated immunogenic peptide of the invention, inclusive of peptide vaccines, whole protein vaccines, and VLP-based vaccines.

Alternatively, vaccine may be in the form of nucleic acid vaccine and in particular, a DNA vaccine. A useful reference describing DNA vaccinology is DNA Vaccines, Methods and Protocols, Second Edition (Volume 127 of Methods in Molecular Medicine series, Humana Press, 2006) and is incorporated herein by reference. A DNA vaccine may include a genetic construct encoding a VLP comprising one or more immunogenic peptides of the present invention, and preferably replacing "like for like" epitopes. In particular embodiments, the DNA vaccine comprises one or more immunogenic hMPV epitopes of the present invention. The DNA vaccine may also comprise other non-hMPV epitopes. Not

being bound by theory, such a DNA vaccine may have advantages in relation to CTL epitope processing within an antigen presenting cell, wherein an introduced epitope is processed efficiently due to its location at a native epitope.

The pharmaceutical compositions of the invention also contemplate use of the immunogenic peptides of the present invention together with other hMPV immunogenic epitopes. An example of another hMPV immunogenic epitope is SLILIGITTL (SEQ ID NO:9), although without limitation thereto.

The present invention is readily amenable to production of a multivalent vaccine designed to elicit immune responses to a plurality of pathogens. By way of example only, a mixed population of virus-like particles may be generated by means of introduction of one or more expression vectors into a cell. For example, the invention contemplates introduction of at least two separate expression vectors, one of which encodes hMPV immunogenic epitopes and the other vector encodes one or a plurality of immunogenic epitopes from a pathogen other than hMPV. Alternatively, an expression vector encoding a chimera as hereinbefore described may be introduced into a cell to thereby generate a single VLP that carries a plurality of foreign immunogenic epitopes.

In light of the foregoing, it will be appreciated that the invention is not limited to producing immune responses only to hMPV. In particular embodiments, the invention provides pharmaceutical compositions which induce immune responses to one or more other pathogens in addition to hMPV such as, but not limited to, respiratory syncytial virus and parainfluenza virus.

The invention further contemplates whole cell-based vaccines which use dendritic cells that have been loaded with exogenous antigen. Antigen may be in the form of synthetic peptides, whole proteins and nucleic acids (inclusive of DNA and RNA). Genetic and non-genetic methods may be employed for antigen loading of dendritic cells. Vari and Hart (2004, *Cytotherapy*, 6(2): 111-121) and Belz et al (2004, *Cytotherapy*, 6(2): 88-98) provides examples of techniques for loading dendritic cells with antigen and use of dendritic cells for vaccine design.

Any suitable procedure is contemplated for producing vaccine compositions. Exemplary procedures include, for example, those described in New Generation

Vaccines (1997, Levine *et al.*, Marcel Dekker, Inc. New York, Basel, Hong Kong), which is incorporated herein by reference.

The pharmaceutical composition may further comprise a pharmaceutically-acceptable carrier, diluent or excipient.

By “*pharmaceutically-acceptable carrier, diluent or excipient*” is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991) which is incorporated herein by reference.

It will be appreciated by the foregoing that the immunotherapeutic composition and/or vaccine of the invention may include an “*immunologically-acceptable carrier, diluent or excipient*”.

Useful carriers are well known in the art and include for example: thyroglobulin; albumins such as human serum albumin; toxins, toxoids or any mutant crossreactive material (CRM) of the toxin from tetanus, diphtheria, pertussis, *Pseudomonas*, *E. coli*, *Staphylococcus*, and *Streptococcus*; polyamino acids such as poly(lysine:glutamic acid); influenza; Rotavirus VP6, Parvovirus VP1 and VP2; hepatitis B virus core protein; hepatitis B virus recombinant vaccine and the like. Alternatively, a fragment or epitope of a carrier protein or other immunogenic protein may be used. For example, a T cell epitope of a bacterial toxin, toxoid or CRM may be used. In this regard, reference may be made to U.S. Patent No 5,785,973 which is incorporated herein by reference.

The “*immunologically-acceptable carrier, diluent or excipient*” includes within its scope water, bicarbonate buffer, phosphate buffered saline or saline and/or

an adjuvant as is well known in the art. As will be understood in the art, an "adjuvant" means a composition comprised of one or more substances that enhances the immunogenicity and efficacy of a vaccine composition. Non-limiting examples of suitable adjuvants include squalane and squalene (or other oils of animal origin); block copolymers; detergents such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; *Corynebacterium*-derived adjuvants such as *Corynebacterium parvum*; *Propionibacterium*-derived adjuvants such as *Propionibacterium acne*; *Mycobacterium bovis* (Bacille Calmette and Guerin or BCG); *Bordetella pertussis* antigens; tetanus toxoid; diptheria toxoid; surface active substances such as hexadecylamine, octadecylamine, octadecyl amino acid esters, lysolecithin, dimethyldioctadecylammonium bromide, *N,N*-dicoctadecyl-*N'*, *N'*-bis(2-hydroxyethyl-propanediamine), methoxyhexadecylglycerol, and pluronic polyols; polyamines such as pyran, dextran sulfate, poly IC carbopol; peptides such as muramyl dipeptide and derivatives, dimethylglycine, tuftsin; oil emulsions; and mineral gels such as aluminum phosphate, aluminum hydroxide or alum; interleukins such as interleukin 2 and interleukin 12; monokines such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon; combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide; liposomes; ISCOM® and ISCOMATRIX® adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as muramyl dipeptides or other derivatives; Avridine; Lipid A derivatives; dextran sulfate; DEAE-Dextran alone or with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer emulsions such as Neocryl A640 (e.g. U.S. Pat. No. 5,047,238); water in oil emulsifiers such as Montanide ISA 720; poliovirus, vaccinia or animal poxvirus proteins; or mixtures thereof.

With regard to subunit vaccines, an example of such a vaccine may be formulated with ISCOMs, such as described in International Publication WO97/45444.

An example of a vaccine in the form of a water-in-oil formulation includes Montanide ISA 720, such as described in International Publication WO97/45444.

Any safe route of administration may be employed for providing a patient

with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular and transdermal administration may be employed.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, nasal sprays, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be effected by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be effected by using other polymer matrices, liposomes and/or microspheres.

Pharmaceutical compositions of the present invention suitable for oral or parenteral administration may be presented as discrete units such as capsules, sachets or tablets each containing a pre-determined amount of one or more therapeutic agents of the invention, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the agents of the invention with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is pharmaceutically-effective. The dose administered to a patient, in the context of the present invention, should be sufficient to effect a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend on the subject to be

treated inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

Diagnostic methods

Broadly, the invention is directed towards diagnostic methods for metapneumovirus that utilise the hMPV immunogenic epitopes of the invention for detecting antigen-specific T cells from an individual infected with, or exposed to metapneumovirus.

In a particular embodiment, the invention provides a method of determining whether an animal harbors metapneumovirus by contacting one or more T cells isolated from the individual, with one or more immunogenic epitopes of present invention where a response to at least one of the one or more immunogenic epitopes by one or more T cells is indicative that the animal harbors, or has been exposed to, metapneumovirus. Non-limiting examples of suitable techniques to detect and quantify antigen-specific T cells include: cytolytic assays measuring chromium-51 release; antigen-specific induction of cytokines to detect specific T cells using ELISPOT and intracellular cytokine staining (reference is made to Letsch and Scheibenbogen, 2003, *Methods*, 31: 143-149); identification of specific T cells with fluorescently labelled tetrameric MHC-antigen complexes (reference is made to US Patent No. 5,635,363).

Antibodies

The present invention also contemplates antibodies against isolated immunogenic peptides of the invention. In a preferred embodiment, the antibody is raised against the hMPV immunogenic epitopes of the present invention.

Antibodies of the invention may be polyclonal or monoclonal. Well-known protocols applicable to antibody production, purification and use may be found, for example, in Chapter 2 of Coligan *et al.*, *CURRENT PROTOCOLS IN IMMUNOLOGY* (John Wiley & Sons NY, 1991-1994) and Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual*, Cold Spring Harbor, Cold Spring Harbor Laboratory, 1988, which are both herein incorporated by reference.

Generally, antibodies of the invention bind to or conjugate with a polypeptide, fragment, variant or derivative of the invention. For example, the antibodies may

comprise polyclonal antibodies. Such antibodies may be prepared for example by injecting a polypeptide, fragment, variant or derivative of the invention into a production species, which may include mice or rabbits, to obtain polyclonal antisera. Methods of producing polyclonal antibodies are well known to those skilled in the art. Exemplary protocols which may be used are described for example in Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY, *supra*, and in Harlow & Lane, 1988, *supra*.

In lieu of the polyclonal antisera obtained in the production species, monoclonal antibodies may be produced using the standard method as for example, described in an article by Köhler & Milstein, 1975, Nature **256**, 495, which is herein incorporated by reference, or by more recent modifications thereof as for example, described in Coligan *et al.*, CURRENT PROTOCOLS IN IMMUNOLOGY, *supra* by immortalizing spleen or other antibody producing cells derived from a production species which has been inoculated with one or more of the polypeptides, fragments, variants or derivatives of the invention.

The invention also includes within its scope antibodies which comprise Fc or Fab fragments of the polyclonal or monoclonal antibodies referred to above. Alternatively, the antibodies may comprise single chain Fv antibodies (scFvs) against the isolated proteins of the invention. Such scFvs may be prepared, for example, in accordance with the methods described respectively in United States Patent No 5,091,513, European Patent No 239,400 or the article by Winter & Milstein, 1991, Nature **349** 293, which are incorporated herein by reference. Labels may be associated with the antibody or antibody fragment of the invention.

The label may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorophore, a chemiluminescent molecule, a lanthanide ion such as Europium (Eu^{34}), a radioisotope and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.

A large number of enzymes useful as labels is disclosed in United States Patent Specifications U.S. 4,366,241, U.S. 4,843,000, and U.S. 4,849,338, all of

which are herein incorporated by reference. Enzyme labels useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, β -galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzyme label may be used alone or in combination with a second enzyme in solution.

By way of example, the fluorophore may be fluorescein isothiocyanate (FITC), oregon green, tetramethylrhodamine isothiocyanate (TRITL), allophycocyanin (APC) and R-Phycoerythrin (RPE), although without limitation thereto.

In other general embodiments, the invention also contemplates use of antibodies against isolated immunogenic peptides, and preferably isolated hMPV immunogenic epitopes, of the present invention as described herein to detect whether an individual has been exposed to hMPV. Applicable method is well known in the art such as ELISA, but is not limited thereto.

So that the invention may be readily understood and put into practical effect, the following non-limiting Examples are provided.

EXAMPLES

Example 1

MATERIALS and METHODS

Epitope prediction and peptide synthesis

T cell (MHC class I) epitopes of human metapneumovirus (hMPV) were predicted and confirmed by immunoinformatics, essentially as described previously {Herd, 2006}. In brief, sequences for seven of nine hMPV proteins (N, M, F, M2-1, M2-2, SH and G) were obtained from the genome record of type A hMPV /NDL 00-1 (NCBI accession no. NC_004148.1, derived from AF371337){van den Hoogen, 2001}. These proteins were chosen by analogy to human respiratory syncytial virus (hRSV), the most closely related human pathogen, in which the corresponding hRSV proteins elicit CTL responses during infection {Cherrie, 1992}. The large (L) polymerase protein was not included in epitope predictions due to its size (2000 amino acids).

Two independent online algorithms, SYFPEITHI with PAMProC {Rammensee, 1999; Kuttler, 2000} and ProPred1 {Singh, 2003} were used to predict proteasomal

cleavage and MHC binding for class I alleles, representing 9 MHC supertypes ('A1', 'A2', 'A3', 'A24', 'B7', 'B27', 'B44', 'B58', 'B62') {Sette, 1999}. Ultimately, the results from each algorithm were combined in a consensus prediction of 74 predicted epitopes or 'predictopes' (8-16 predictopes per supertype).

The predictopes for the most common MHC supertypes (>40% frequency in population), 'B7' (represented by HLA-B*0702, *3501, *5101, *5102 and *5103 and including B*08), 'A3' (HLA-A*03, *1101, *3101, *3302, *6801) and 'A2' (HLA-A*0201, *0203, *0205) are shown in Table 1. Predictopes were also selected for the less common MHC supertypes (with mean population frequencies of $\leq 40\%$) 'A24' (HLA-A*2402), 'B44' (HLA-B*3701, *40, *4001=B60, *4006=B61, *4402, *4403), 'A1' (HLA-A*01, *26), 'B27' (HLA-B*14, *1510, *2702, *2705, *2709, *3801, *3901, *3902), 'B62' (HLA-B*1501=B62, *5201) and 'B58' (HLA-B*5801) (not shown).

MHC predictopes were synthesized as a PepSet (>1 μ mol scale; Mimotopes, Clayton, Victoria, Australia). The peptides were dissolved in dimethyl sulfoxide at 10 mg/ml (or dimethyl formamide if the sequence included Cys, Met, or Trp).

MHC supertype predictopes corresponding to the MHC class I alleles of each individual patient were used to evaluate T-cell immunity in the patients with a history of hMPV associated disease.

Patients and samples

Study approval was obtained from the Ethics Committee of the Royal Children's Hospital and Health Service District, Queensland, Australia. Patients with symptoms of respiratory tract infection and confirmed hMPV diagnosis were included in this study. Symptoms at the time of hMPV diagnosis included upper and lower respiratory tract infection (bronchitis or viral pneumonia). Patient demographics and clinical data are summarized in Table 2. All seven patients tested were lung transplant recipients, in whom hMPV-associated respiratory disease occurred 1-7 years or 2 months (patient #16) after transplant. Patients had received varying degrees of immunosuppression. All patients fully recovered from the acute hMPV infection, either with (#1, #2, #3, #15, #16) or without (#9, #13) anti-viral therapy. Nasopharyngeal aspirates and/or bronchoalveolar lavages samples were

tested for the presence of hMPV RNA by real-time reverse-transcription PCR targeting the nucleoprotein gene {Maertzdorf, 2004; Mackay, 2003} . These samples had earlier been tested as described {Syrmis, 2004} and were negative for common respiratory pathogens including hRSV, hAdV, hPIV-1,-2 and -3. One patient (#2) had co-infection with Influenza-A virus. Blood samples were collected with informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, and either used immediately for assay and the generation of cell lines, or stored frozen. Patients were HLA typed by serology.

Cell Culture

Cells were cultured in RPMI (Invitrogen, Mt Waverley, Victoria, Australia) with 20 mM HEPES, 2 mM L-glutamine (GlutaMax I; Invitrogen), 1mM sodium pyruvate, 100 IU/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol and 10% fetal bovine serum. T-cell growth medium contained in addition purified human recombinant interleukin-2 (rIL2) (10-20 U/mL; Sigma, Castle Hill, New South Wales, Australia) and supernatant (30%v/v) from the T-cell line, MLA 144 (Gibbon ape lymphoma; American Type Culture Collection, Bethesda, Md.).

Target- and stimulator cell lines were generated as described elsewhere {Elkington, 2003}. In brief, phytohemagglutinin (PHA)-blasts were generated by stimulation of PBMCs with PHA (20 µg/mL, Sigma) for 3 days, and then maintained in T-cell growth medium (without further addition of PHA) for up to 6 weeks. PHA-blasts were used as target cells in cytotoxicity assays. EBV-transformed lymphoblastoid cell lines (LCLs) were established by exogenous virus transformation of peripheral B cells using B95-8 virus isolates. Autologous LCLs, pulsed with peptide, were used to stimulate T-cell lines on a weekly basis. HLA-matched LCLs were used as target cells in cytotoxicity assays. In some assays LCL target cells were infected with hMPV at multiplicity of infection of ca n:1. Expression of hMPV was determined in ca. 10% of cells by indirect immunofluorescence using mouse monoclonal antibody to the N protein and anti-mouse Ig/FITC.

T-cell lines were established essentially as described {Elkington, 2003}. In brief, PBMC (2×10^6) from patients previously infected with hMPV were restimulated in vitro with peptide-sensitized autologous PBMCs (1×10^6), then

expanded after 3 days by adding T-cell growth medium. T-cell lines were restimulated weekly with peptide-sensitized, γ -irradiated (8000 rad) autologous LCLs and were maintained in T-cell growth medium. After two or five restimulations, T-cell lines were tested for cytotoxic activity.

T cell assays

Effector T-cell responses were tested by reacting ex vivo PBMCs with MHC-appropriate predictope peptides and IFN- γ ELISPOT assay essentially as described {Elkington, 2003}. In brief, capture antibody (anti-human IFN- γ ; clone 1-D1K; Mabtech, Nacka, Sweden) was used at 5 μ g/mL to coat membrane-based microwell plates (MultiScreen-HA; Millipore, North Ryde, New South Wales, Australia). Plates were blocked with culture medium containing 10% fetal bovine serum. PBMCs were added ($2.0\text{--}2.5 \times 10^5$ / well) and incubated with peptide (10 μ g/mL) or without, for 16-20 h at 37°C, 5%CO₂. Plates were washed with PBS containing 0.02% Tween 20. Detection antibody (biotinylated anti-human IFN- γ , clone 7-B6-1; Mabtech) was used at 2 μ g/mL. Plates were developed using streptavidin-alkaline phosphatase (BD PharMingen, San Diego, CA) and BCIP-NBT substrate (5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium; Sigma, Castle Hill, New South Wales, Australia) and spots were counted manually with a light microscope. Results were expressed as IFN- γ positive cells / 10^6 PBMC. Frequency (f) was defined as (number of positive cells with peptide) minus (number of positive cells without peptide) / 10^6 PBMCs. Activation Index (AI) was defined as (number of positive cells with peptide) divided by (number of positive cells without peptide). Predictope peptides giving a positive result ($>\text{mean}+3$ standard deviations of negative control) were considered to be epitopes.

Memory T-cell responses were evaluated by ⁵¹Chromium-release assays, using restimulated T-cell lines and epitope peptides, essentially as described {Elkington, 2003}. In brief, target cells (3×10^6) were labelled with 100 μ Ci of ⁵¹Cr (as sodium chromate; MP Biomedicals, Seven Hills, New South Wales, Australia) for 90 min, then washed and incubated at 10^4 cells/microwell, with peptide (10 μ g/mL) or without, for 2 h at 37°C. T-cell lines were added at the specified E:T ratios, assay plates were incubated for 4 to 6 h at 37°C, then radioactivity (counts

per minute, cpm) in cell-free supernatant was determined using a MicroBeta counter (Perkin Elmer). Results were calculated as % cytotoxicity using the formula $[(E-S)/(T-S)] \times 100$, where E represents cpm released in the presence of effector cells, S represents spontaneous cpm with medium only and T represents total cpm in the presence of 5% sodium dodecyl sulfate [S values were always less than 25% of T]. Peptide specific cytotoxicity was defined as (% cytotoxicity for targets with peptide) minus (% cytotoxicity for targets without peptide).

Protein sequence alignment

Protein sequences for various hMPV strains were obtained from NCBI, and alignments were performed with CLUSTAL W {Thompson, 1994}. Representative hMPV strains were NDL 00-1, CAN 97-83, CAN 97-82, and CAN 98-75 for A1, A2, B1, and B2 subtypes, respectively. When available, protein sequences for other hMPV strains that have been detected in Australia, Canada, and Japan were also included in sequence alignments.

RESULTS

Prediction of MHC-I-restricted T-cell epitopes and patient evaluation.

Epitopes were predicted using two computer-based algorithms, SYFPEITHI (with PAMProC) {Kuttler, 2000; Rammensee, 1999} and ProPred1 {Singh, 2003}. We applied these algorithms to seven proteins (M, F, N, M2-1, M2-2, G and SH) of type A hMPV for HLA alleles representing nine MHC supertypes. Seventy four predicted epitopes (predictopes) were selected across the range of hMPV proteins and MHC supertypes. Twenty four predictopes were selected for the F protein 18 for the N protein, 11 for the M2-1 protein, 9 for the M protein, 6 for the G protein, 4 for the SH protein, and 2 for the M2-2 protein. Predictopes for the most common MHC supertypes 'B7' (13 predictopes), 'A3' (7 predictopes), and 'A2' (16 predictopes) are shown in Table 1. For the less common supertypes 'A24', 'B44', 'A1', 'B27', 'B62' and 'B58', a further 38 predictopes were selected (not shown).

The predictope set was used to evaluate hMPV-specific T-cell immunity in 7 patients (Table 2) 3-20 months after diagnosis of hMPV infection (except patient

#3 evaluated at 2 weeks after diagnosis), as patient availability allowed. To evaluate effector T-cell responses, ex vivo PBMC were tested in IFN- γ ELISPOT assays with or without MHC supertype appropriate predictope peptides. To evaluate memory T-cell responses, peptide-restimulated T cell lines derived from patients with hMPV-directed effector responses were tested in ^{51}Cr release cytotoxicity assays. hMPV-directed T cell effector and memory responses were demonstrated in four patients (below).

Effector and Memory CTL responses to hMPV proteins

The M protein contains two predictopes (H6, H7) for alleles of the 'B7' supertype (Table 1). Four out of five patients (#1, #2, #9, #13, #16) (80%) with alleles representing the 'B7' supertype displayed ex vivo IFN- γ secretion (effector) and post-restimulation cytotoxic T-cell (memory) responses to the H7 predictope (Fig 1C). One patient (#13) displayed IFN- γ secretion response ex vivo and post-restimulation cytotoxic T-cell responses to the H6 predictope (Fig 1A).

The G protein contains two predictopes (H23, H31) for alleles of the 'A2' supertype (Table 1). Two of five patients (#1, #3, #9, #13, #16), with alleles representing the 'A2' supertype displayed ex vivo IFN- γ secretion and post-restimulation cytotoxic T-cell responses to the H23 predictope (Fig 2A). One further patient (#16) did not display an ex vivo IFN- γ secretion response, but did display a post-restimulation cytotoxic CTL response to the H23 predictope (not shown).

The F protein contains three predictopes (H15, H16, and H17) for alleles of the 'A3' supertype (Table 1). Two of three patients (#2, #15, #16), with alleles representing the 'A3' supertype displayed ex vivo IFN- γ secretion and post-restimulation cytotoxic T-cell responses to the H15 predictope (Fig 3C). The F protein also contains five predictopes (H22, H26, H28, H30 and H33) for alleles of the 'A2' supertype (Table 1). One of five patients (#1, #3, #9, #13, #16), with alleles representing the 'A2' supertype displayed ex vivo IFN- γ secretion and post-restimulation cytotoxic T-cell responses to the H22 predictope (Fig 3A).

The M2-1 protein contains three predictopes (H15, H16, and H17) for alleles of the 'A3' supertype (Table 1). One of three patients (#2, #15, #16), with alleles

representing the 'A3' supertype displayed ex vivo IFN- γ secretion and post-restimulation cytotoxic T-cell responses to the H14 predictope (Fig 4B). The M2-1 protein also contains three predictopes (H24, H29, and H34) for alleles of the 'A2' supertype (Table 1). One of five patients (#1 #3, #9, #13, #16), with alleles representing the 'A2' supertype displayed ex vivo IFN- γ secretion and post-restimulation cytotoxic T-cell responses to the H24 predictope (Fig 4A).

The N protein contains three predictopes (H2, H4, and H10) for alleles of the 'B7' supertype (Table 1). One of three patients (#2, #15 #16), with alleles representing the 'B7' supertype displayed ex vivo IFN- γ secretion and post-restimulation cytotoxic T-cell responses to the H4 predictope (Fig 5A).

The SH protein contains one predictope (H9) for alleles of the 'B7' supertype (Table 1). One of five patients (#1, #2, #9, #13, #16), with alleles representing the 'B7' supertype displayed ex vivo IFN- γ secretion and post-restimulation cytotoxic T-cell responses to the H9 predictope (FIG. 5C).

The frequency of recognition per protein differed; responses to proteins M and G were recorded significantly more frequently than to proteins F, M-1, N and SH (Chi-square =40.1 *df*5 *p*<0.001; normalized for number of predictopes per protein) .

Together these data indicate that (at least) six hMPV proteins generate CTL responses in patients who have experienced hMPV-associated respiratory disease. Furthermore, the data define nine CTL epitopes (Table 4) restricted through six HLA class-I alleles, representing the commonest three MHC supertypes ('A2', 'A3', 'B7') in the human population. The CTL epitopes are generated by processing from hMPV whole proteins; we further demonstrated the relevance of response to epitope by showing that a M¹⁹⁴ epitope specific T cell line (from patient #2) specifically lysed MHC matched LCL target cells infected with hMPV (specific lysis 6% at E:T ratio 10:1).

Frequency and magnitude of CTL responses

IFN- γ secreting effector T cell responses were detected in ex vivo PBMC from four of seven patients who had experienced clinical hMPV infection. Memory CTLs could be recalled in all four patients by restimulation of T cell lines with

hMPV epitope. Establishment and restimulation of T cell lines in the absence of an ex vivo effector response was not attempted.

hMPV specific IFN γ -producing effector cells were detected in ex vivo PBMC at 5-6 mo (up to 10 mo) past diagnosis at a frequency of up to 35-275 /10⁶ PBMC (Figs 1-5).

hMPV specific memory CTLs demonstrated 5-45% cytotoxicity against hMPV epitope-pulsed targets and were generated in vitro from PBMC at 11-12 mo (up to 21 mo) past diagnosis (Figs 1-5).

T cell responses per haplotype

Four of five patients (#2, #9, #13, #16, but not #1) with alleles from the 'B7' supertype showed T-cell immunity, with 4 CTL epitopes identified. We confirmed MHC restriction in one incidence by demonstrating that a M¹⁹⁴ epitope specific T cell line derived from patient #2 specifically lysed M¹⁹⁴ pulsed LCL target cells (33% specific lysis at E:T ratio 10:1) derived from one of the patients in the study, where the only common allele shared between the two patients was B*07.

Two of five patients (#9, #13, but not #1, #3, #16) with alleles from the 'A2' supertype showed T-cell immunity, with 3 CTL epitopes identified. Two of three patients (#2, #16, but not #15) with an allele from the 'A3' supertype showed T-cell immunity, with 2 CTL epitopes identified. There was no significant difference in frequency of response among 'A2' 'A3' and 'B7' supertypes (Chi-square=0.01 *df*2 *p*> 0.05).

T cell responses of individual patients.

The four patients with evidence of T cell immunity all had responses to 2 or more hMPV proteins.

Patient #2 responded to 2 identified epitopes from M and F proteins (presented by 'B7' and 'A3' alleles respectively), with IFN- γ release effector response by ex vivo PBMC (at 6 months past diagnosis) and memory cytotoxic activity of restimulated T cell lines (at 11 mo past diagnosis).

Patient #9 responded to 2 identified epitopes from M and G proteins (presented by 'B7' and 'A2' alleles respectively) with IFN- γ release effector response by ex vivo PBMC (at 5 months past diagnosis) and memory cytotoxic

activity of restimulated T cell line (at 19 months past diagnosis).

Patient #16 responded to 4 identified epitopes from M, F, M2-1 and G proteins (two presented by 'A3', one by 'B7', and one by 'A2' alleles respectively) with IFN- γ release effector response by ex vivo PBMC (at 10 months past diagnosis) and memory cytotoxic activity of T cell lines (at 12 months past diagnosis).

Patient #13 responded to 7 identified epitopes from M, G, F, N, SH and M2-1 proteins (four presented by 'B7', three by 'A2' alleles) with IFN- γ release effector response by ex vivo PBMC (at 5 months past diagnosis) and memory cytotoxic activity of T cell lines (at 20 months past diagnosis)

Conservation of identified epitopes the hMPV proteome

The present study has identified nine (9) new CTL epitopes from 6 proteins of hMPV restricted through alleles of the commonest MHC class 1 supertypes in the human population. These epitopes are candidates for inclusion in a putative vaccine for hMPV designed to elicit a cellular immune response. Type A hMPV protein sequences has been used for epitope prediction. Since the prevalent circulating hMPV types vary both temporally and geographically or may co-circulate, a vaccine would ideally be protective against strains of both type A and type B hMPV. It is relevant to ask whether the CTL epitopes that have been identified are present in both virus types. Protein sequence alignments were performed for hMPV strains representing subtypes A1, A2, B1, and B2.

The M protein (254 amino acids) is highly conserved (>95% aa sequence identity between viral types). The H7 epitope (M¹⁹⁴, IAPYAGLIMI [SEQ ID NO:2] /HLA-B*07 and -B*51), is located at the C-terminal, and is present in all ($n=24$) strains analyzed (Figure 1D) The H6 epitope (M¹², IPYTAAVQV [SEQ ID NO:1] /HLA-B*07) is located at the N-terminal and is present in all ($n=23$) strains analyzed (Figure 1B).

The G protein (236 amino acids) is highly variable (~35% aa sequence identity between viral types). The H23 epitope (G³², SLILIGITTL [SEQ ID NO:9] /HLA-A*02) is located in the trans-membrane region which exhibits minimal variability in this otherwise variable surface glycoprotein. It is present in all ($n=24$)

type A strains analyzed (Figure 2B). Representative type B strains have amino acid (aa) differences at non-anchor positions TLILIGLTAL (SEQ ID NO: 57) which may still allow MHC binding (except CAN 98-75 which has L→P at position 10, so MHC binding is less likely) (Figure 2B).

The F protein (539 amino acids) is highly conserved (>95% aa sequence identity between viral types). The H15 epitope (F⁴²⁹, KVEGEQHVIV [SEQ ID NO:5] /HLA-A*31, A*11) is located in the C-terminal (F1) subunit, and is present in all (*n*=24) strains analyzed (Fig 3D). The H22 epitope (F¹⁵⁷, VLATAVREL [SEQ ID NO:4]/HLA-A*02), is also located in the C-terminal (F1) subunit, and is present in all (*n*=24) strains analyzed (Figure 3B).

The M2-1 protein (187 amino acids) is highly conserved (~95% aa sequence identity between viral types). The H14 epitope (M2-1¹⁴⁹, RLPREKLKK [SEQ ID NO:7]/HLA-A*11) is located at the C-terminal and is present in almost all (*n*=11) strains analyzed (Fig 4C). The B type has 1aa difference at a non-anchor position which may allow MHC binding. The H24 epitope (M2-1¹⁵⁷, KLAKLIIDL [SEQ ID NO:6]/HLA-A*02) is also located at the C-terminal and is present in almost all (*n*=11) strains analyzed. Again, the B type has 1aa difference at a non-anchor position (FIG. 4C).

The N protein (394 amino acids) is highly conserved (>95% aa sequence identity between viral types). The H4 epitope (N³⁰⁷, SPKAGLLSL [SEQ ID NO:8]/HLA-B*07) is located in a 'similarity region' for mononegaviruses. It is present in all (*n*=24) strains analyzed (FIG. 5B).

The SH protein (183 amino acids) is highly variable (~58% aa sequence identity between viral types). The H9 epitope (SH¹⁵², KPAVGVIYHIV [SEQ ID NO:3] /HLA-B*07) is located in the second hydrophobic domain (aa 155-170). It is present in only one of A1 subtype strains analyzed. The remaining subtypes have amino acid differences at anchor positions and MHC binding is unlikely (FIG. 5D). They do not fit the MHC binding motif for B*07 which consists of proline at position 2 and a hydrophobic C-terminal residue. Furthermore, representative B type strains have very little amino acid sequence identity (only 1 or 2/10aa).

In summary, five of the nine epitopes in the M, F and N proteins are totally

conserved among all hMPV strains for which sequence data is available. Two epitopes in the M2-1 protein are conserved among almost all hMPV strains. One epitope in the G protein is conserved among all type A strains, and may be recognized in type B strains. One epitope in the SH protein is present only in the type A hMPV strain tested.

DISCUSSION

hMPV is a recently discovered pan-global respiratory pathogen, ranking in severity between hPSV and parainfluenza type 3 (PIV3) {Macphail, 2004}. The morbidity associated with infection in the young {van den Hoogen, 2001}, the immunocompromised {Larcher, 2005; Williams, 2005} and the elderly {Falsey, 2003}, and the association of hMPV infection with childhood asthma {Nissen 2002}, underscore the need for an understanding of the immunobiology of hMPV infection. hMPV infects the upper and lower respiratory tract, where it establishes productive infection in mucosal epithelial cells. As with other viral infections (eg human papillomavirus, hRSV) displaying similar tropism, hMPV proteins expressed during infection are likely to be targets of CD8⁺ T cells responses, which may be associated with reduced viral titres and disease. CD8⁺ T cells are thought to contribute to recovery from infection with closely-related RSV {Chiba, 1989; Tripp, 2002}, and CTL protection against infection has been described in murine models of hRSV infection {Connors, 1991; Simmons, 2001} and hMPV infection {Herd, 2006}.

Very little is known of the T cell response to hMPV infection in humans. In the present study of patients with prior hMPV-associated respiratory disease, it was demonstrated that at least six hMPV proteins are immunogenic for the generation of hMPV-directed IFN- γ secreting- and cytotoxic T cell responses. We show that responses generated through alleles representing the three commonest MHC supertypes, together encompassing the majority of human population, and the first MHC class-I CTL epitopes reported for hMPV have been defined.

Furthermore, the present study found that hMPV epitope peptides elicit IFN- γ secretion within 18 hours in ex vivo PBMC from patients with prior hMPV-associated respiratory disease up, indicating an effector T cells response. The present study also generated 'memory' T cell lines cytotoxic for target cells presenting

hMPV epitope by repeatedly restimulating PBMC from patients making effector responses. While effector responses in four of the seven patients tested were recorded, it cannot be excluded that the patients not making effector responses nonetheless had HMPV-directed T cell memory. Indeed from the one patient who had no effector response, a memory T cell line was generated which killed hMPV epitope expressing target cells (not shown). Furthermore, two of the response-negative patients carried the A*29 allele which is not assigned to any supertype thereby precluding testing with predicted epitopes.

Whilst there is no data on longevity of hMPV CTL responses induced by natural infection, the present results indicate that an effector response may be detected for a number of months after infection (up to 10 months, patient #16). This indicates either that the effector response is long-lived, that immunogenic hMPV persists sub-clinically after resolution of disease, or that patients acquired sub-clinical re-infection(s). The latter scenario is likely in view of the annual winter 'peaks' of hMPV incidence in the general population {Sloots, 2006}. The present inventors were able to recall memory cytotoxic T cell responses at least up to 20 months post-disease.

Responses to more than one hMPV protein were made in all patients in which cellular immunity to hMPV was detected. Two patients (#9, #2) made responses to two proteins, one patient (#16) made responses to four proteins, and one patient (#13) made responses to six proteins. Intriguingly, patient #13 with the broadest immune response resolved her respiratory disease without anti-viral therapy (Table 2). It is interesting to speculate that recovery may have been immune response mediated.

All of these patients responded to the M protein and three of the four to the G protein. Responses to the M protein and G protein were seen significantly more frequently than responses to the other five proteins evaluated. Similarly, responses to hRSV M and G were among the commonest recalled in RSV infection {Cherrie, 1992}. More than one epitope in the M protein was recognised by patient #13 through more than one allele. The F protein was recognized by different patients (eg. #2 and #13) through different epitopes (F157/ A*02 and F429 /HL A*31 respectively). The responses to the nine newly identified epitopes in the six hMPV proteins were

restricted through 5 class-I alleles. Responses to epitope M¹⁹⁴ epitope were restricted through two different alleles (B*07 and B*51) of the 'B7' supertype in different patients. Similarly, responses to the F⁴²⁹ epitope were restricted through alleles A*31 and A*11 of the 'A3' supertype in different patients. The A*02-restricted G32 epitope recognized by two patients (# 9, #13,), was first recorded in HLA A2 transgenic mice the present inventors {Herd, 2006} .

The M, F and N proteins of hMPV are highly conserved among all hMPV strains for which sequence data is available, and epitopes we have identified within these proteins are totally conserved (FIG. 1B, D; FIG. 3B,D; FIG. 5B). This allows the prediction that infection with any of the hMPV A1, A2, B1, and B2 sub-types might elicit CTL responses to these epitopes in individuals expressing the appropriate class I alleles. Intriguingly, the two patients (#9 and #13) recognizing the G³² epitope (SLILIGITTL; SEQ ID NO:9) derived from hMPV type A, were diagnosed as having hMPV type B viral infection , in which the corresponding sequence of the G protein (in a majority of type B strains) is TLILIGLTAL (SEQ ID NO: 57). Since binding anchor residues for the A*02 restricting allele (underlined) are conserved in these two sequences, the possibility is raised that that the type B sequence is a cross-reactive epitope. A response to the SH¹⁵² epitope (Table 3) also recognized by patient #13, would not be predicted by type B virus infection. Thus patients #9 and/or #13 may have had a previous sub-clinical infection with type A hMPV.

Interestingly, two of the newly identified hMPV epitopes occur at near-identical locations to RSV CTL epitopes in the corresponding RSV proteins. Thus, the N³⁰⁷ epitope (³⁰⁷SPKAGLLSL³¹⁶ [SEQ ID NO: 8]/B*07) and the M¹⁹⁴ epitope (¹⁹⁴IAPYAGLIM²⁰⁴ [SEQ ID NO:2]/B*07, B*51) of hMPV are near- identically positioned to N³⁰⁶ (³⁰⁶NPKASLLSL³¹⁴ [SEQ ID NO:48]/B*07) epitope {Goulder, 2000} and the ¹⁹⁵IPYSGLLLV²⁰³ [SEQ ID NO:55]/B*51 epitope {Heidema, 2004} within the corresponding N and M proteins of RSV, respectively (note the identical MHC restricting alleles between corresponding hMPV and RSV epitopes; anchor residues are underlined). Also there is a conservation between the M¹² epitope (¹²IPYTAAVQV²¹; SEQ ID NO:1) of hMPV and the corresponding location in the M

protein of RSV (STYTAAVQY; SEQ ID NO:58). These homologies likely reflect the evolutionary relatedness of hMPV and hRSV, and the susceptibility of these locations within the respective proteins to appropriate antigen processing. The degree of amino acid homology/conservation between hMPV and RSV epitopes, (particularly at anchor residues), opens up the intriguing possibility of CTL-mediated cross protection between these viruses brought about by natural infection or by epitope vaccination.

An IL-6 associated pattern of innate immunity develops in pneumovirus infection, particularly hMPV, but also RSV {Douville, 2006}. IL-6 has been shown to suppress IFN- γ by primed T cells {Diehl, 2002}. This is likely to be relevant to the limited expansion of cognate T cells in natural RSV infection, and in mouse models {Chang, 2002}. A similar circumscribed cellular immune response to hMPV may underlie the life-long susceptibility to hMPV re-infection {Boivin, 2002; Falsey, 2003}. It might also explain the somewhat lower IFN- γ recall responses we observe to hMPV epitopes in comparison to those observed in some other viral infections eg. CMV {Elkington, 2003}.

There is evidence that a T-cell response to hMPV is protective against infection in a mouse model of hMPV infection. Alvarez and Tripp have demonstrated that primary hMPV infection elicits a delayed CTL response associated with decreased hMPV titres in the lung, suggesting that CTL might contribute to control of hMPV infection {Alvarez, 2005}. Lungs of infected mice exhibit mononuclear cell infiltration and an inflammatory environment conducive to CTL response induction. A CTL response can be demonstrated at the time when viral load starts to diminish following viral challenge {Alvarez, 2005}. Furthermore, depletion of T cells or NK cells results in increased viral titers in the lungs of infected mice, indicating at least some T cell mediated immune control of viral persistence {Alvarez, 2004}. Nonetheless, virus persists at a low level in spite of the T cell response, re-inforcing a view that immunity acquired by natural infection or viral challenge with hMPV is insufficient to eliminate infection either in the murine model {Alvarez, 2004} or in humans {van den Hoogen, 2001}.

As for hRSV, a vaccine to protect against hMPV is desirable. From our data

it might be surmised that immunization with an appropriately formatted whole- or subunit- hMPV vaccine would elicit epitope-specific CTL responses which might protect against infection and disease mediated by any of the hMPV sub-types types {Herd, 2006}. Indeed we have previously demonstrated that anhMPV epitope vaccine affords CTL-associated protection across hMPV strains in a murine model {Herd, 2006}. Protection across strains will be important in a human vaccine since not only do hMPV strains co-circulate within the human population, but the predominant strain can differ from year to year {Sloots, 2006}. An immune system vaccine-primed for hMPV CTL specific for non-structural proteins would kill virally infected cells expressing hMPV epitopes in conjunction with MHC class I on the cell surface before complete infectious virus could be produced. So while not preventing infection, this mechanism could stop viral replication and halt viral spread at an early stage. Used in conjunction with measures to neutralize virus and thereby minimize infection (ie. vaccination for antibody or passive administration of antibody) a CTL-inducing vaccine might afford maximum protection. This approach should be feasible since the MHC supertypes (A2, A3, B7) restricting the epitopes we describe together cover a majority of the human population (generalized across ethnicities) {Dawson, 2001}. Furthermore a proportion of the population carry two or more supertypes, which would predispose to a diverse immune response to multiple epitopes restricted through alleles of more than one I supertype within a given individual.

In summary, the present study demonstrate effector and memory CTL responses spread over six hMPV proteins in patients with prior hMPV-associated respiratory disease. Some patients made responses to multiple epitopes, and some epitopes elicited responses in multiple patients sharing the corresponding MHC class I supertype. The nine newly defined CTL epitopes are restricted over a range of common supertypes covering a majority human HLA class I polymorphism. It was confirmed that an hMPV epitope-specific T cell line killed hMPV-infected target cells. Most of the identified epitopes are conserved among hMPV sub-types. These observations clearly have implications for understanding the immune response to, and immunoregulation of, hMPV infection, and for future vaccines designed to

alleviate hMPV pathogenesis.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can be made in the particular embodiments exemplified without departing from the scope of the present invention.

All computer programs, algorithms, patent and scientific literature referred to herein is incorporated herein by reference.

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TABLES

Table 1 Predicted epitopes for hMPV for 'A2', 'A3' and 'B7' MHC class-I supertypes

MHC SUPERTYPE	PEPTIDE CODE	PROTEIN	SEQUENCE	MHC alleles	Sequence Identifier
'B7'	H1	F ⁹⁷	N P R Q S R F V L	B*07/0702, 3501	10
	H2	N ¹⁵⁴	A P D T P I I L L	B*07/0702	11
	H3	M2-1 ¹⁵⁰	L P R E K L K K L	B*07/0702, 3501, 5101, [08]	12
	H4	N ³⁰⁷	S P K A G L L S L	B*07/0702, 3501, [08]	13
	H5	M2-1 ²⁰⁸	N P K G I F K K L	B*07/0702, 3501, [08]	14
	H6	M ¹²	I P Y T A A V Q V	B*5101, 5102, 5103	15
	H7	M ¹⁹⁴	I A P Y A G L I M I	B*5101	16
	H8	G ⁴³	I A L N I Y L I I	B*5101	17
	H9	SH ¹⁵²	K P A V G V Y H I V	B*5101	18
	H10	N ¹⁵¹	R P S A P D T P I I	B*07/0702, 5101	19
	H11	F ¹¹⁶	A A V T A G V A I	B*5101, 5102, 5103	20
	H12	F ¹¹⁴	T A A A V T A G V	B*5101	21
	H13	F ¹²²	V A I A K T I R L	B*5102, [08]	22
'A3'	H14	M2-1 ¹⁴⁹	R L P R E K L K K	A*03	23
	H15	F ⁴²⁹	K V E G E Q H V I K	A*03	24
	H16	F ³⁹⁶	R V G I I K Q L N K	A*03	25
	H17	F ¹⁴⁸	V S T L G N G V R	A*3302, 6801	26
	H18	N ¹⁶³	C V G A L I F T K	A*03	27
	H19	N ¹⁸⁹	R V L S D A L K R	A*03	28
	H20	M2-1 ¹⁴⁶	N L K R L P R E K	A*03	29
'A2'	H21	M ⁴⁷	V L L D Q L K T L	A*0201, 0205	30
	H22	F ¹⁵⁷	V L A T A V R E L	A*0201	31
	H23	G ³²	S L I L I G I T T L	A*0201, 0205	32
	H24	M2-1 ¹⁵⁷	K L A K L I I D L	A*0201	33
	H25	N ²	S L Q G I H L S D L	A*0201	34
	H26	F ²⁵⁸	I L I G V Y G S S V	A*0201	35
	H27	SH ³⁵	K L I L A L L T F L	A*0201, 0205	36
	H28	F ¹⁰	S L L I T P Q H G L	A*0201	37
	H29	M2-1 ¹⁴²	S L I N N L K R L	A*0201	38
	H30	F ³⁷⁴	A L S P L G A L V	A*0201	39
	H31	G ³⁴	I L I G I T T L S I	A*0201	40
	H32	N ³⁹	S L Q Q E I T L L	A*0201	41
	H33	F ¹⁵⁰	T L G N G V R V L	A*0201	42
	H34	M2-1 ¹⁰⁰	N I I K Q L Q E V	A*0201	43
	H35	N ¹⁷¹	K L A S T I E V G L	A*0201	44
	H36	SH ³²	I V L K L I L A L L	A*0201	45

anchor positions are bolded

Table 2 A. patient demographics

patient #	age/se x	sample type /hMPV diagnosis		clinical data and HMPV disease outcome	time past diagnosis IFNg / T cell assay
		viral type	time post Tx		
1 MaOs	57/F	BAL+NPA A2 4yr		URTI progressing to LRTI standard therapy +anti-viral full recovery	3mo / 3mo
2 BeBe	37/F	NPA+BAL A2 7yr		URTI + LRTI (bronchitis) standard therapy +anti-viral full recovery	6mo / 11mo
3 RoFa	60/F	NPA B2 1yr		URTI standard therapy +anti-viral full recovery	2wk / nt
9 MIDW	42/M	NPA B1 7yr		URTI standard therapy, no anti-viral full recovery	5mo / 19mo
13 JoWa	66/F	NPA B2 4yr		URTI outpatient no therapy, no anti-viral full recovery	5mo / 20mo
15 AnCo	64/M	BAL B2 2yr		URTI + LRTI (bronchitis) standard therapy +anti-viral full recovery	12mo / nt
16 GaMc	54/M	BAL A1 2mo		URTI + LRTI (viral pneumonia) standard therapy +anti-viral full recovery	10mo / 12mo

sample type: NPA= nasopharangeal aspirate; BAL= bronchoalveolar lavage

clinical data and outcome : URTI=upper respiratory tract infection; LRTI=lower respiratory tract infection

standard therapy= IV pulse methylprednisolone, IV broad spectrum anti-bacterial and nebulized bronchodilators. Anti-viral= ribavirin

Table 2 *cont.*

B. Patient MHC class-I haplotype

patient #	HLA alleles				MHC 'SUPERTYPE'				# predictopes (MHC-appropriate)
1 MaOs	A1	A2	B8	B61	'A1'	'A2'	'B7'	'B44'	42
2 BeBe	A25	A31	B7	B51	'A1'	'A3'	'B7'	'B7'	25
3 RoFa	A2	A29	B44 ^(a)	B44 ^(a)	'A2'	'A?'	'B44'	'B44'	24
9 MiDw	A2 ^(a)	A2 ^(a)	B44	B51	'A2'	'A2'	'B44'	'B7'	37
13 JoWa	A2 ^(a)	A2 ^(a)	B7	B62	'A2'	'A2'	'B7'	'B62'	33
15 AnCo	A29	A31	B37	B57	'A?'	'A3'	'B44'	'B58'	17
16 GaMc	A2	A11	B7	B55	'A2'	'A3'	'B7'	'B7'	36

(a) homozygous

Table 3. hMPV CTL epitopes

Epitope	hMPV protein	'supertype'	HLA restriction	No. reactive patients/ No. patients tested ³	Sequence identifier
IPYTAAVQV	M ¹²⁻²¹	'B7'	B*07	1/5	SEQ ID NO:1
IAPYAGLIMI	M ¹⁹⁴⁻²⁰⁴	'B7'	B*07,*51 ¹	4/5	SEQ ID NO:2
KPAVGVYHIV	SH ¹⁵²⁻¹⁶¹	'B7'	B*07	1/5	SEQ ID NO:3
VLATAVREL	F ¹⁵⁷⁻¹⁶⁶	'A2'	A*02	1/3	SEQ ID NO:4
KVEGEQHVIV	F ⁴²⁹⁻⁴³⁹	'A3'	A*31,*11 ²	2/3	SEQ ID NO:5
KLAKLIIDL	M2-1 ¹⁵⁷⁻¹⁶⁶	'A2'	A*02	1/3	SEQ ID NO:6
RLPREKLKK	M2-1 ¹⁴⁹⁻¹⁵⁷	'A3'	A*11	1/3	SEQ ID NO:7
SPKAGLLSL	N ³⁰⁷⁻³¹⁶	'B7'	B*07	1/5	SEQ ID NO:8
SLILIGITTL	G ³²⁻⁴²	'A2'	A*02	3/5	SEQ ID NO:9

¹ Of the four patients recognising M¹⁹⁴⁻²⁰⁴, #2 carries both B*07 and B*51 alleles, #9 and #16 the carry B*51 allele alone, # 13 carries the B*07 allele alone.

² The two patients (#2 and #16) recognising F⁴²⁹⁻⁴³⁹ carry separate alleles within the 'A3'supertype (A*31 and A*11 respectively).

³ Patients with appropriate MHC class-I supertype

CLAIMS

1. An isolated immunogenic peptide consisting essentially of eight to ten contiguous amino acids of a hMPV protein.
2. The isolated immunogenic peptide of claim 1, wherein the hMPV protein is selected from the group consisting of an M protein, an F protein, an SH protein, an M2-1 protein and an N protein.
3. The isolated immunogenic peptide according to any one of the preceding claims, wherein the isolated immunogenic peptide is an isolated hMPV immunogenic epitope consisting essentially of an amino acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO:6, SEQ ID NO:7 and SEQ ID NO:8.
4. The isolated immunogenic peptide according to any one of the preceding claims, wherein the isolated immunogenic peptide is a T cell epitope.
5. The isolated immunogenic peptide according of claim 4, wherein the T cell epitope is a CTL epitope.
6. An isolated immunogenic peptide which is a variant of the isolated immunogenic peptide according to any of the preceding claims.
7. The isolated immunogenic peptide according to claim 6, wherein the variant has an amino acid sequence according to any one of SEQ ID NOs:204-206, SEQ ID NOs:234-244 and SEQ ID NO:56.
8. An isolated protein comprising at least one isolated immunogenic peptide according to any of the preceding claims, wherein the isolated protein is not an hMPV protein.
9. The isolated protein according to claim 8, wherein the isolated protein is a

chimera comprising an amino acid sequence of at least one isolated immunogenic peptide according to any one of claims 1 to 7 and an amino acid sequence derived from an heterologous protein.

10. The isolated protein according to claim 8 or claim 9, wherein the isolated protein further comprises one or a plurality of immunogenic epitopes of one or more pathogens other than hMPV.

11. The isolated protein according to any one of claims 8 to 10, wherein the one or more pathogens other than hMPV is selected from the group consisting of respiratory syncytial virus and parainfluenza virus.

12. The isolated protein according to any one of claims 8 to 11, wherein the one or a plurality of immunogenic epitopes of respiratory syncytial virus are selected from the group consisting of SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:58 and SEQ ID NO:248.

13. The isolated protein according to any one of claims 8 to 12, wherein the immunogenic epitope from parainfluenza virus is the region spanning amino acids 321 to 326 of the nucleoprotein from parainfluenza virus.

14. The isolated protein according to any one of claims 8 to 13, wherein the heterologous protein is a HBsAg.

15. An isolated nucleic acid encoding the isolated immunogenic peptide or variant thereof, of any one of claims 1 to 7.

16. An isolated nucleic acid encoding the isolated protein of any one of claims 8 to 14.

17. The isolated nucleic acid according to any one of claims 15 to 16, wherein the

isolated nucleic acid is DNA.

18. A genetic construct comprising the isolated nucleic acid of any one of claims 15 to 17 operably linked to one or more regulatory nucleotide sequences.

19. The genetic construct according to claim 18, wherein the genetic construct is an expression construct for expression.

20. A host cell comprising the genetic construct of any one of claims 18 to 19.

21. The host cell of claim 20, wherein the host cell is of eukaryotic origin.

22. The host cell of any one of claims 20 to 21, wherein the host cell is a mammalian cell.

23. A VLP comprising an immunogenic agent selected from the group consisting of the isolated immunogenic peptide of any one of claims 1 to 7 and the isolated protein of any one of claims 8 to 14.

24. The VLP of claim 23, wherein the virus-like particle is a HBsAg-derived VLP.

25. The VLP of any one of claims 22 to 24 comprising the isolated nucleic acid of any one of claims 15 to 17.

26. A pharmaceutical composition comprising an immunogenic agent selected from the group consisting of the isolated immunogenic peptide of any one of claims 1 to 7, the isolated protein of any one of claims 8 to 14, the isolated nucleic acid of any one of claims 15 to 17, the genetic construct of any one of claims 18 to 19 and the VLP of any one of claims 23 to 25, together with a pharmaceutically acceptable carrier, diluent or excipient.

27. The pharmaceutical composition of claim 26, wherein the pharmaceutical composition is an immunotherapeutic composition.

28. The pharmaceutical composition of any one of claims 26 to 27, which is a vaccine.

29. A method of immunising an animal, said method including the step of administering the pharmaceutical composition of any one of claims 26 to 28 to said animal to thereby induce an immune response in said animal.

30. The method of claim 29, wherein the immune response is selected from the group consisting of a CTL-mediated response and a Th1 response.

31. The method of any one of claims 29 to 30, wherein the animal is a human.

32. A method of treating an animal, said method including the step of administering the pharmaceutical composition of any one of claims 26 to 28 to said animal to thereby modulate an immune response in said animal to prophylactically and/or therapeutically treat an hMPV-associated disease, disorder or condition.

33. The method of claim 32, wherein the immune response is a T cell mediated response.

34. The method of any one of claims 32 to 33, wherein the T cell mediated response is a protective immune response.

35. The method of any one of claims 32 to 34, wherein the animal is a human.

36. A method of determining whether an animal harbours, or has been exposed to, metapneumovirus, said method including the step of contacting one or more T cells isolated from said individual with one or more isolated immunogenic peptides of any one of claims 1 to 7, whereby a response to at least one of the one or more isolated immunogenic peptides by said one or more T cells indicates that the animal harbours, or has been exposed to, metapneumovirus.

37. The method of claim 36, wherein the animal is a human.

38. An isolated antibody which binds one or more isolated immunogenic peptides of any of claims 1 to 7.
39. An isolated antibody raised against one or more isolated immunogenic peptides of any of claims 1 to 7.
40. A method of determining whether an animal harbours, or has been exposed to, metapneumovirus, said method including the step of contacting at least one isolated antibody of claim 38 or claim 39 with a sera of said animal, whereby a response to at least one isolated antibody indicates that the animal harbours, or has been exposed to, metapneumovirus.
41. The method of claim 40, wherein the animal is a human.

(A) H6 (M¹², IPYTAADVQV)

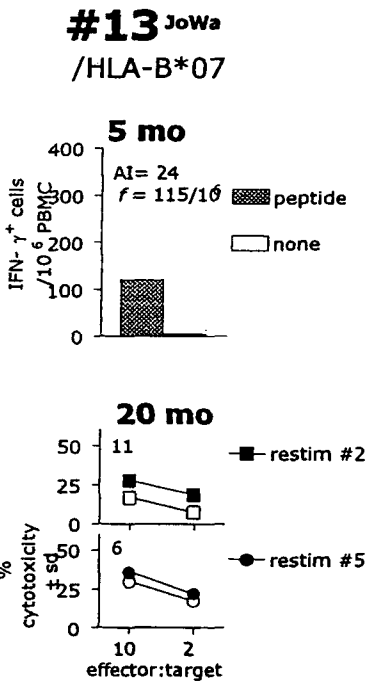


FIG. 1

2/16

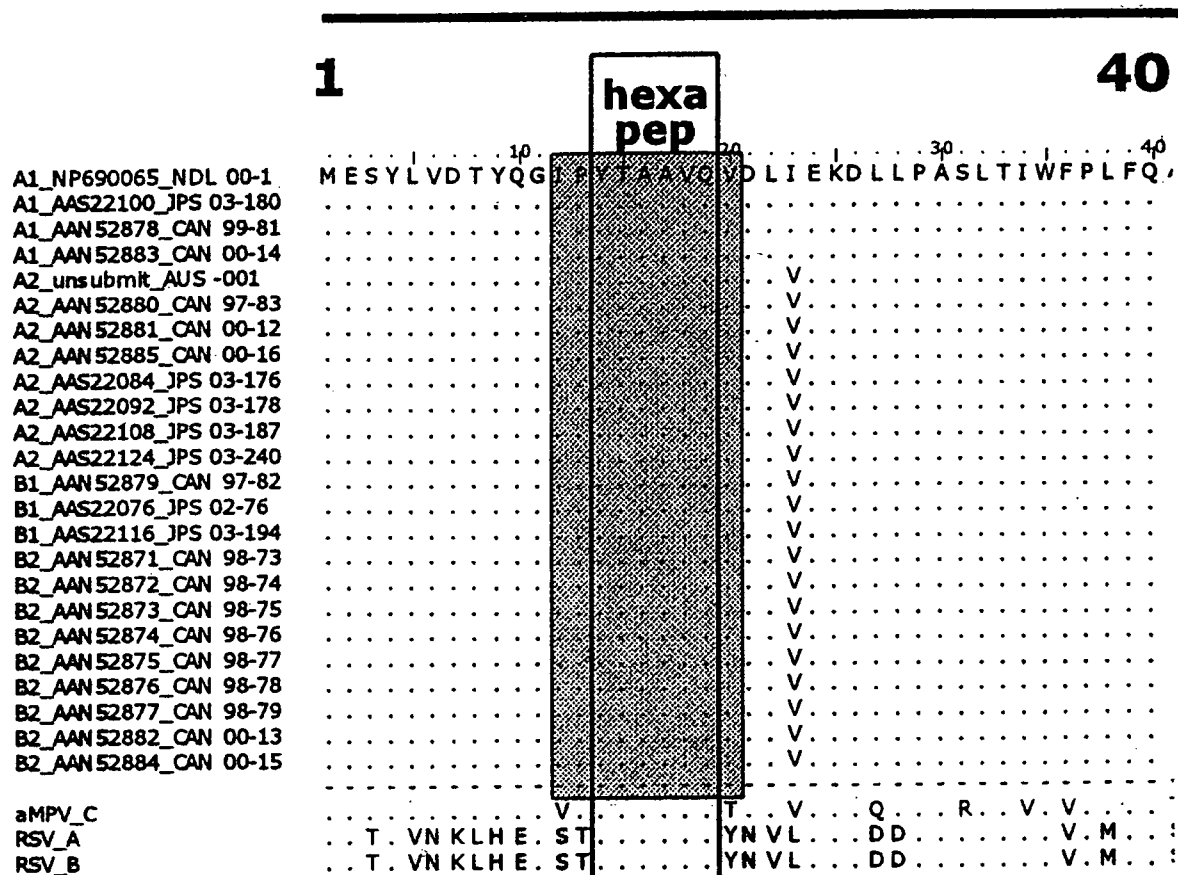
(B)**M protein**

FIG. 1 CONTINUED

3/16

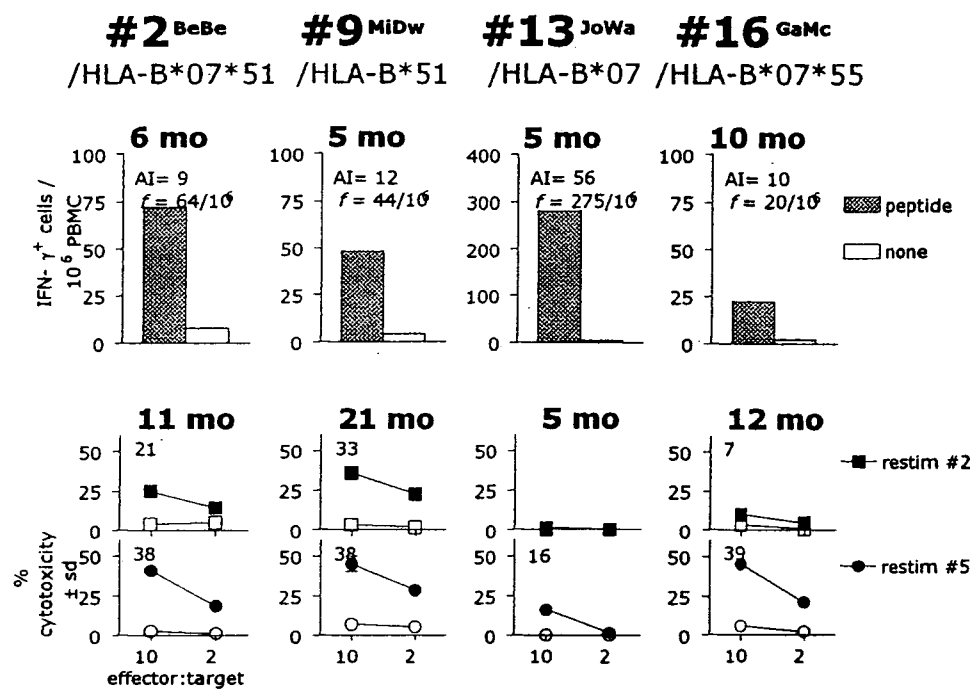
(C) H7 (M¹⁹⁴, IAPYAGLIMI)

FIG. 1 CONTINUED

4/16

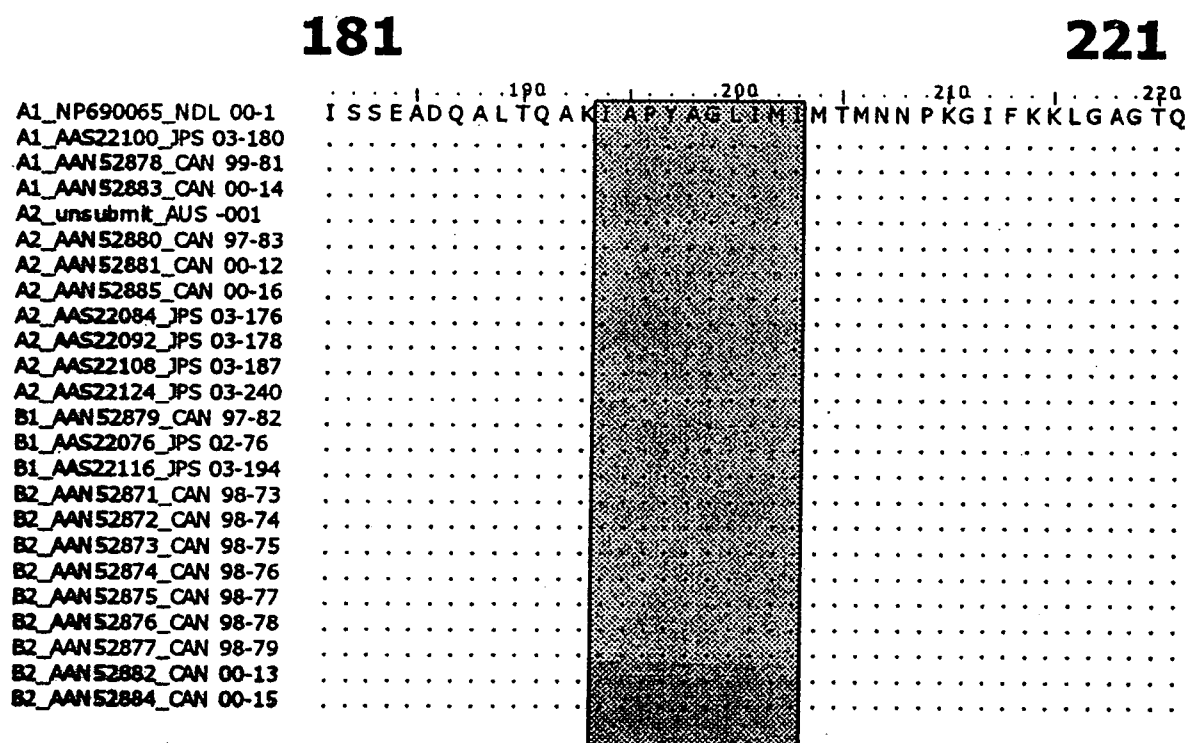
(D)**M protein**

FIG. 1 CONTINUED

5/16

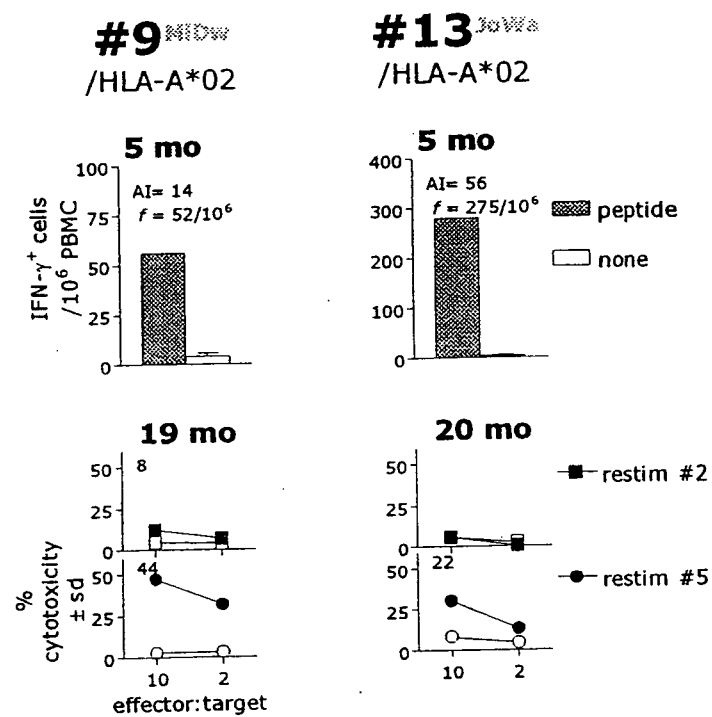
(A) H23 (G³², SLILIGITTL)

FIG. 2

6/16

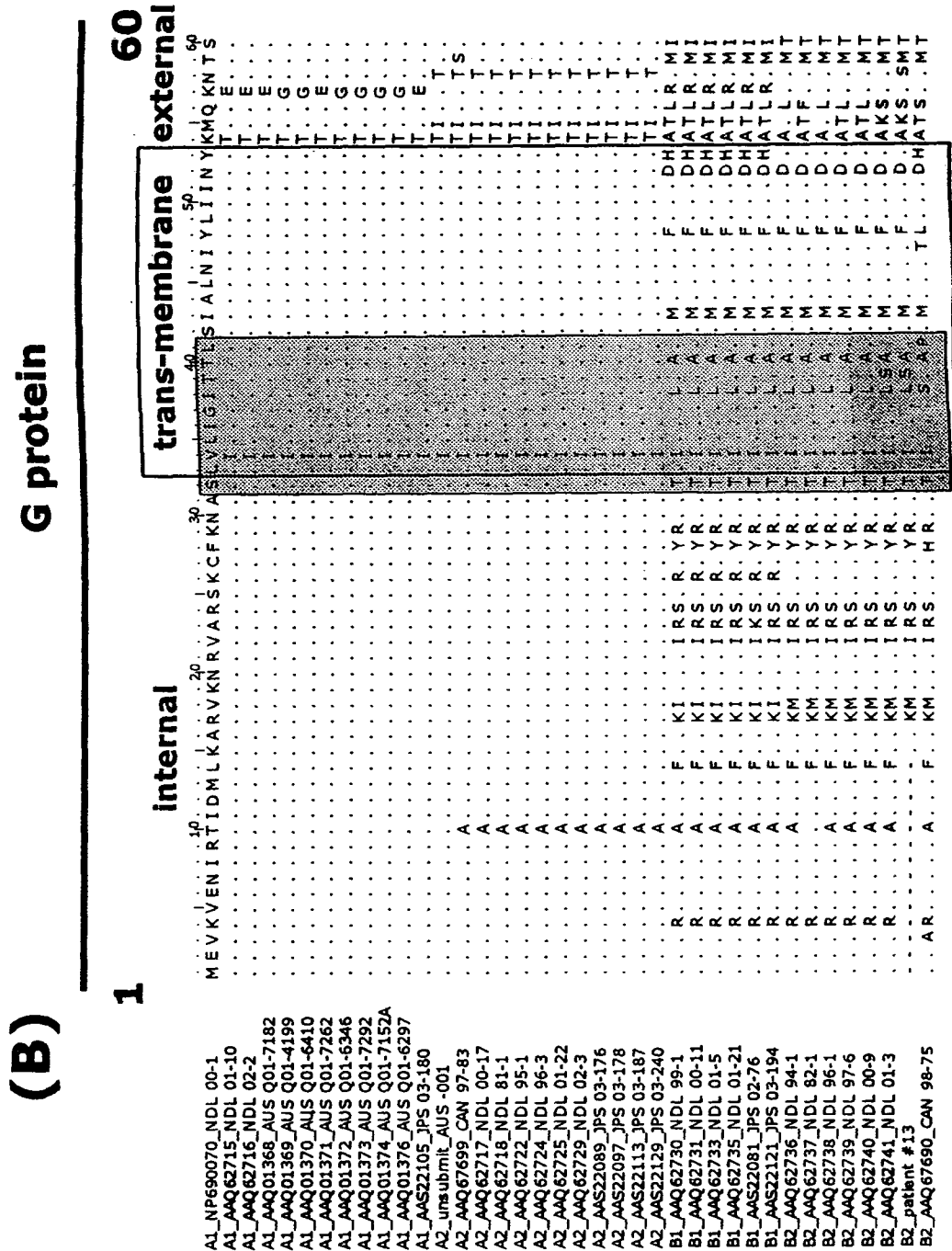


FIG. 2 CONTINUED

7/16

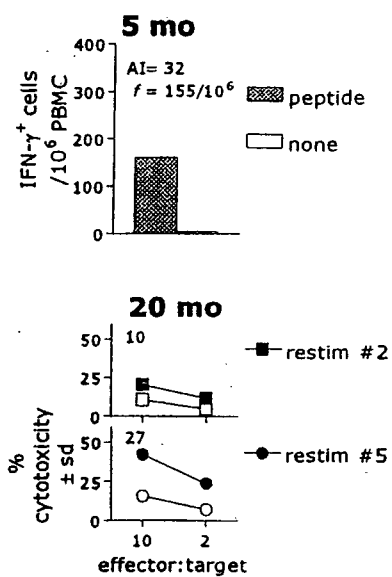
(A) H22 (F¹⁵⁷, VLATAVREL)**#13**^{30W2}
/HLA-A*02

FIG. 3

(B) F protein

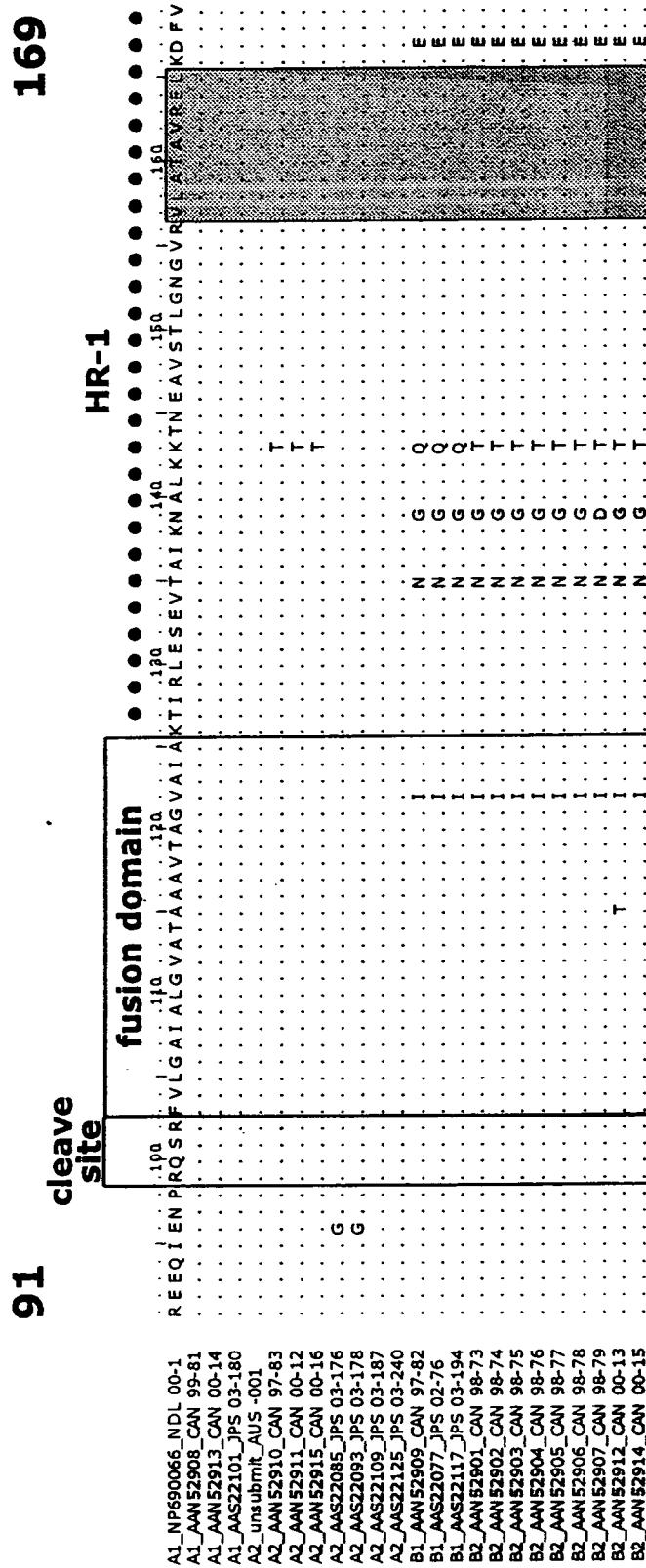


FIG. 3 CONTINUED

9/16

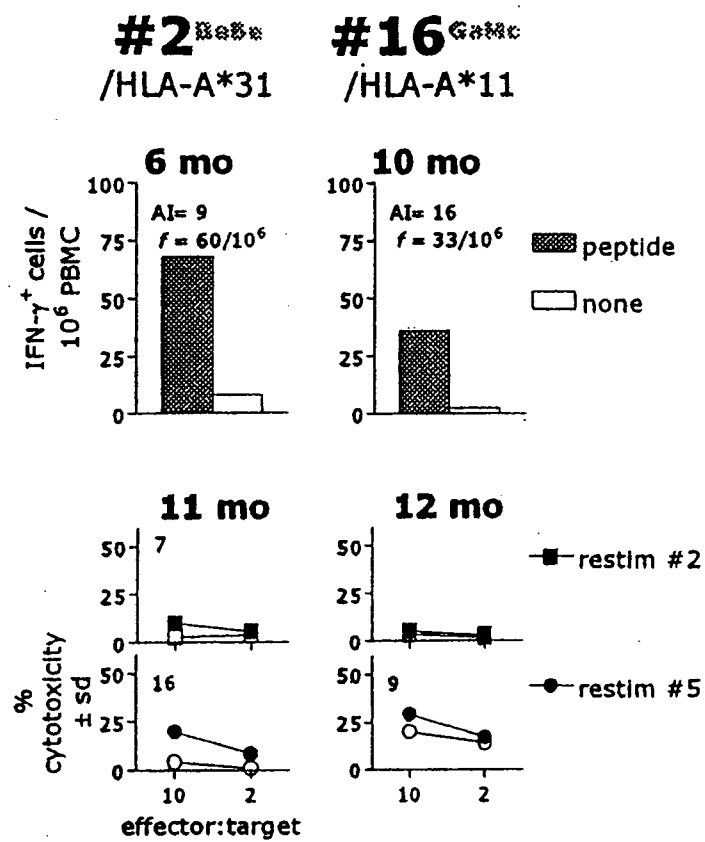
(C) H15 (F⁴²⁹, KVEGEQHVIVK)

FIG. 3 CONTINUED

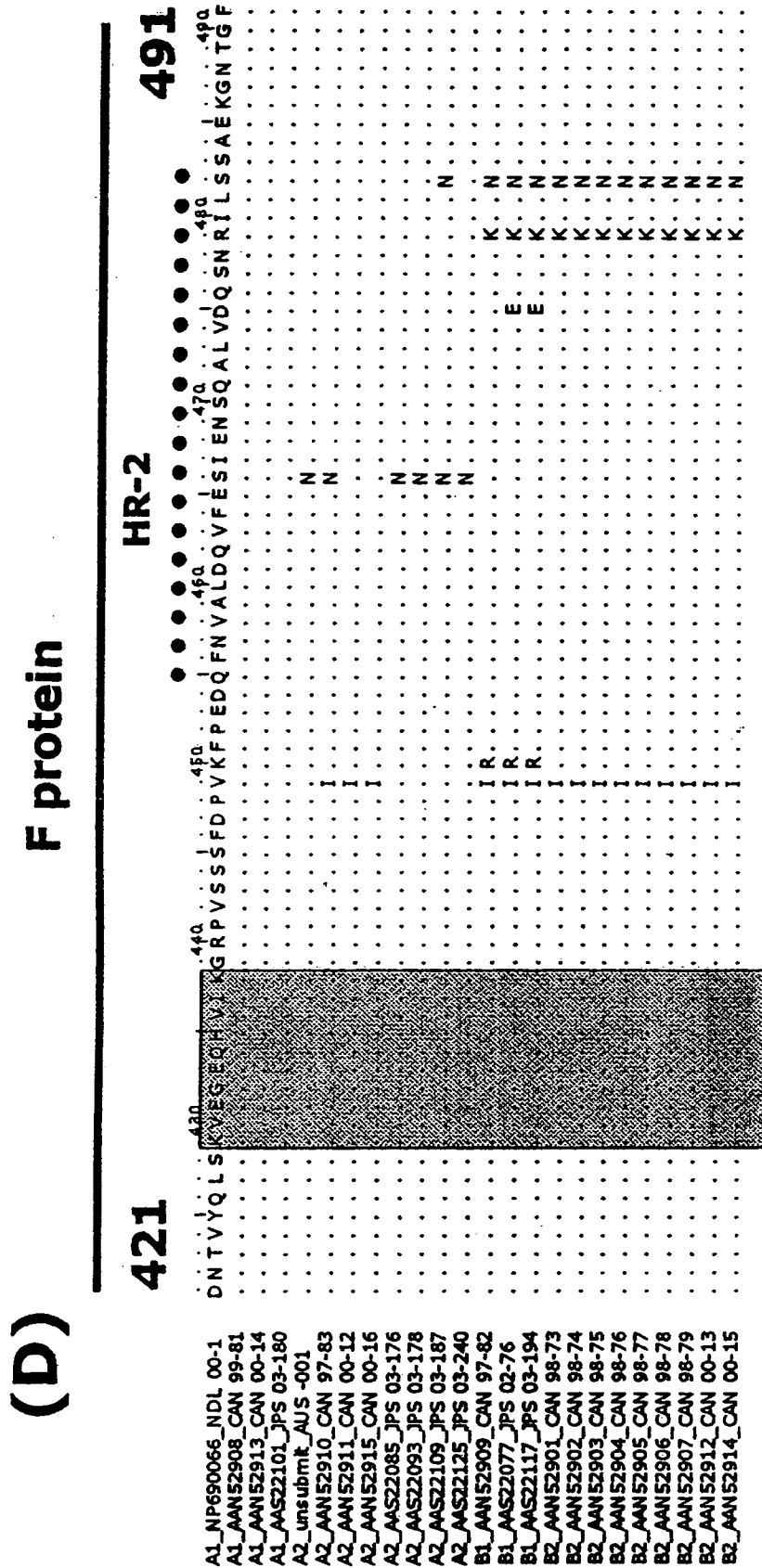


FIG. 3 CONTINUED

11/16

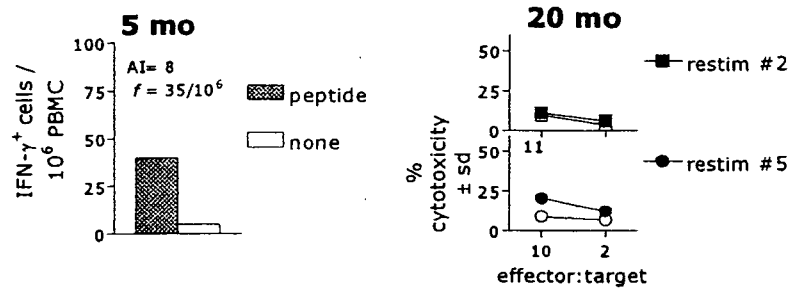
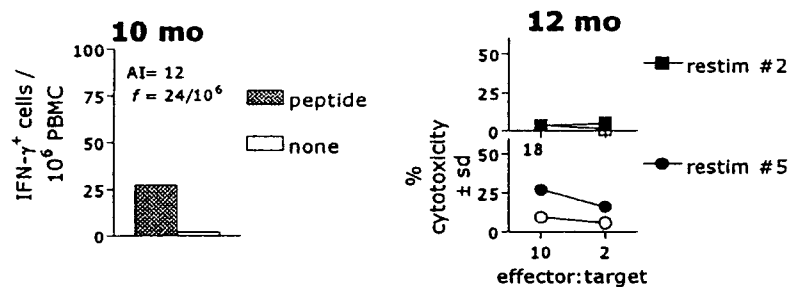
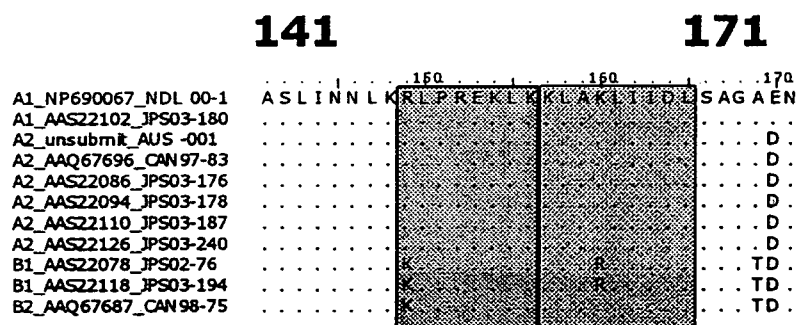
(A) H24 (M2-1¹⁵⁷, KLAKLIIDL)**#13**^{30W*}
/HLA-A*02**(B) H14 (M2-1¹⁴⁹, RLPREKLK)****#16**^{G28*}
/HLA-A*11**(C) M2-1 protein**

FIG. 4

12/16

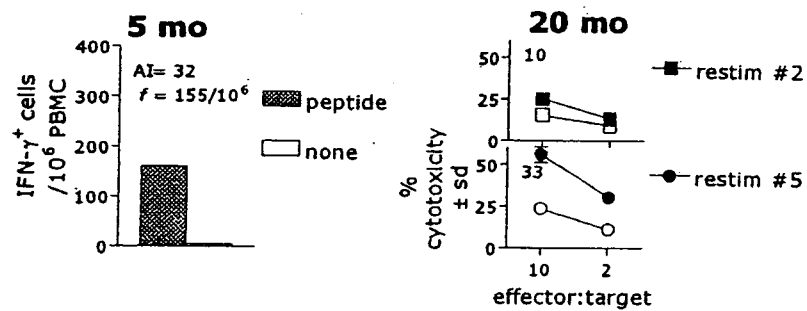
(A) H4 (N³⁰⁷, SPKAGLLSL)**#13**^{JoWz}
/HLA-B*07

FIG. 5

13/16

(B)

N protein

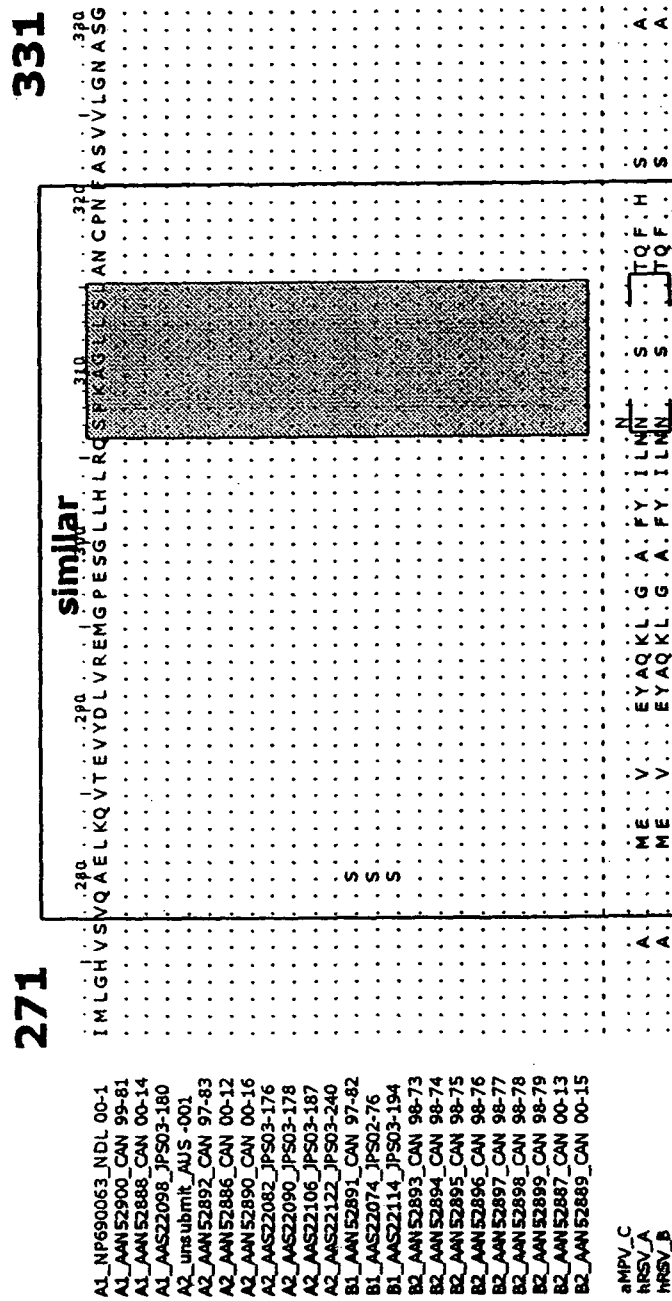


FIG. 5 CONTINUED

14/16

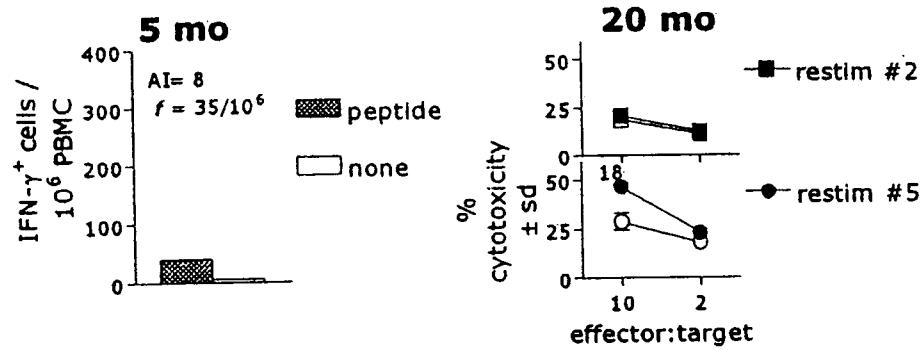
(C) H9 (SH¹⁵², KPAVGVYHIV)**#13** JxWx
/HLA-B*07

FIG. 5 CONTINUED

16/16

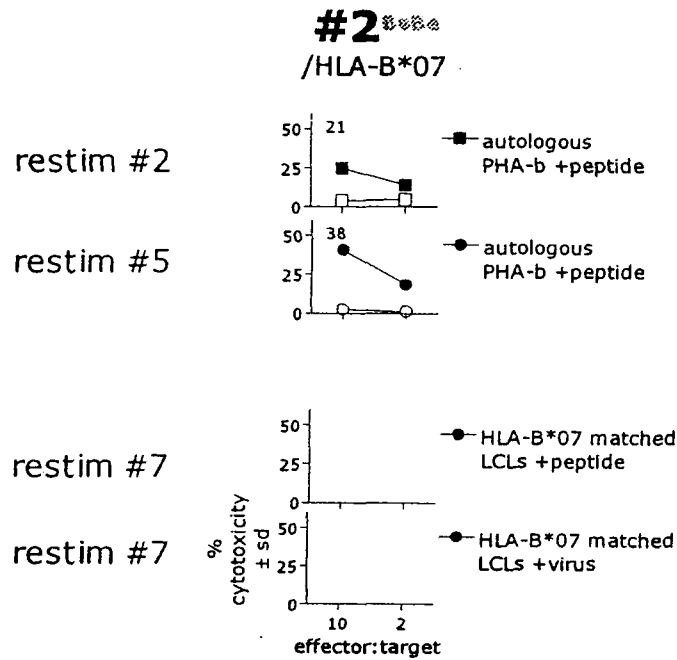
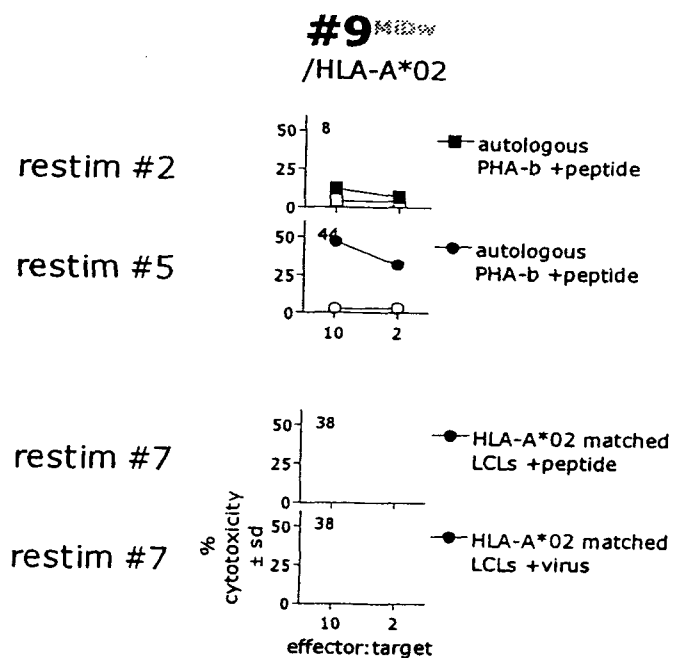
(A) TC-H7 (M¹⁹⁴, IAPYAGLIMI)**(B) TC-H23 (G³², SLILIGITTL)**

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2008/000821

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C07K 4/02 (2006.01) *C07K 7/06* (2006.01) *C12N 15/45* (2006.01) *C12N 15/62* (2006.01)
A61K 38/08 (2006.01) *A61K 38/16* (2006.01) *A61K 39/155* (2006.01) *A61K 39/295* (2006.01)
A61P 31/14 (2006.01) *C07K 16/10* (2006.01)

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)

See electronic databases below

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)

STN: Medline, CA - keyword search (hMPV, epitope)

Genomequest: Blast search based on SEQ ID NOs:1-8

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/108226 A1 (THE UNIVERSITY OF QUEENSLAND et al.) 19 October 2006 See abstract; p4, lines 11-13; figures 11-13 & 21; claims 17-35, 37-40 & 42-45.	1, 2, 4-6, 8-12, 14-41
Y	See claim 13.	13
X	HERD et al. "Cytotoxic T-Lymphocyte Epitope Vaccination Protects against Human Metapneumovirus Infection and Disease in Mice" <i>J. Virol.</i> 2006 , 80(4), 2034-2044 Also cited in the application See abstract; table 1; figure 6; pp 2035-6.	1, 2, 4-6, 15, 17, 26-30, 32-34, 38-41



Further documents are listed in the continuation of Box C



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	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"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
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 July 2008

Date of mailing of the international search report

08 JUL 2008

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2008/000821

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Cat.*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MELENDI et al. "Mapping and Characterization of the Primary and Anamnestic H-2 ^d -Restricted Cytotoxic T-Lymphocyte Response in Mice against Human Metapneumovirus" <i>J. Virol.</i> 2007 , 81(20), 11461-11467 See abstract; table 1; p11462-11464; figures 1 & 2.	1-7, 15, 17, 26-41
P,X	HERD et al. "Major Histocompatibility Complex Class I Cytotoxic T Lymphocyte Immunity to Human Metapneumovirus (hMPV) in Individuals with Previous hMPV Infection and Respiratory Disease" <i>J. Infect. Dis.</i> 2008 , 197(4), 584-592 See abstract; table 1; figures 3-6.	1-7, 15, 17, 26-30, 38-41
Y	DAVE et al. "Viral cross-reactivity and antigenic determinants recognized by human parainfluenza virus type 1-specific cytotoxic T-cells" <i>Virology</i> 1994 , 199(2), 376-383 Also cited in the application. See abstract.	13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2008/000821

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member
WO 2006/108226	NONE
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.	
END OF ANNEX	