(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 October 2015 (01.10.2015)

(10) International Publication Number
WO 2015/148960 A1

(51) International Patent Classification:
A61K 39/04 (2006.01)

(21) International Application Number:
PCT/US2015/023058

(22) International Filing Date:
27 March 2015 (27.03.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/972,202 28 March 2014 (28.03.2014) US

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,

[Continued on next page]

(54) Title: COMPOSITIONS COMPRISING SOLUBLE HLA/*M.* TUBERCULOSIS-SPECIFIC LIGAND COMPLEXES AND METHODS OF PRODUCTION AND USE THEREOF

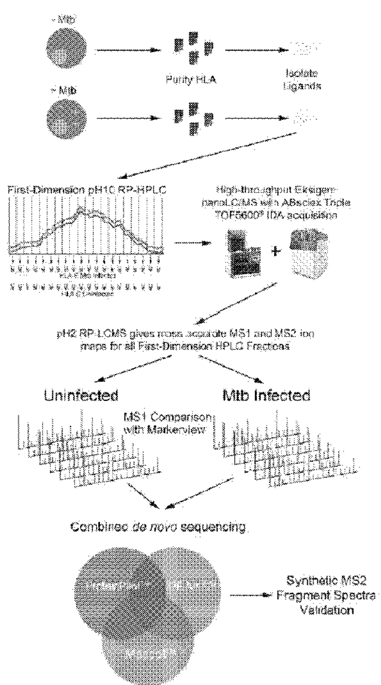

FIGURE 1

(57) **Abstract**: Compositions that include at least one isolated, class I HLA trimolecular complex that includes a peptide ligand unique to *M. tuberculosis*-infected cells are disclosed. Isolated compositions that include the three components of the trimolecular complex and/or a polynucleotide encoding one or more of the three components are also disclosed.

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LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

COMPOSITIONS COMPRISING SOLUBLE HLA/*M. TUBERCULOSIS*-SPECIFIC LIGAND COMPLEXES AND METHODS OF PRODUCTION AND USE THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS/ INCORPORATION BY REFERENCE STATEMENT

[0001] This application claims benefit under 35 USC 119(e) of US Serial No. 61/972,202, filed March 28, 2014. The entire contents of the above-referenced patent application are hereby expressly incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under Contract No. BAA-NIAID-DAIT-NIHAI2008032 awarded by the National Institutes of Health. The government has certain rights in this invention.

BACKGROUND

[0003] Class I major histocompatibility complex (MHC) molecules, designated class I HLA in humans, bind and display peptide antigen ligands upon the cell surface. The peptide antigen ligands presented by the class I MHC molecule are derived from either normal endogenous proteins ("self") or foreign proteins ("non-self") introduced into the cell. Non-self proteins may be products of malignant transformation or intracellular pathogens such as viruses. In this manner, class I MHC molecules convey information regarding the internal fitness of a cell to immune effector cells including but not limited to, $CD8^+$ cytotoxic T lymphocytes (CTLs), which are activated upon interaction with "non-self" peptides, thereby lysing or killing the cell presenting such "non-self" peptides.

[0004] Class I HLA molecules exhibit extensive polymorphism generated by systematic recombinatorial and point mutation events; as such, hundreds of different HLA types exist throughout the world's population, resulting in a large immunological diversity. Such extensive HLA diversity throughout the population results in tissue or organ transplant rejection between individuals as well as differing susceptibilities and/or resistances to

infectious diseases. HLA molecules also contribute significantly to autoimmunity and cancer. Because HLA molecules mediate most, if not all, adaptive immune responses, large quantities of pure isolated HLA proteins are required in order to effectively study transplantation, autoimmunity disorders, and for vaccine development.

[0005] Class I MHC molecules alert the immune response to disorders within host cells. Peptides, which are derived from viral- and tumor-specific proteins within the cell, are loaded into the class I MHC molecule's antigen binding groove in the endoplasmic reticulum of the cell and subsequently carried to the cell surface. Once the class I MHC molecule and its loaded peptide ligand are on the cell surface, the class I MHC molecule and its peptide ligand are accessible to cytotoxic T lymphocytes (CTL). CTL survey the peptides presented by the class I MHC molecule and destroy those cells harboring ligands derived from infectious or neoplastic agents within that cell.

[0006] Discerning disease-specific class I MHC ligands for CTL recognition is an important component of immunotherapy design. Ligands unique to infected cells can be tested and incorporated into vaccines designed to evoke a protective CTL response. Several methodologies are currently employed to identify potentially protective peptide ligands. One approach uses T cell lines or clones to screen for biologically active ligands among chromatographic fractions of eluted peptides (Cox et al. (1994) Science, 264:716-719, which is expressly incorporated herein by reference in its entirety). This approach has been employed to identify peptide ligands specific to cancerous or infected cells. A second technique utilizes predictive algorithms to identify peptides capable of binding to a particular class I MHC molecule based upon previously determined motif and/or individual ligand sequences (DeGroot et al. (2001) Emerging Infectious Diseases, 7:4, which is expressly incorporated herein by reference in its entirety). Peptides having high predicted probability of binding from a pathogen of interest can then be synthesized and tested for T cell reactivity in various assays, such as but not limited to, precursor, tetramer, and ELISpot assays.

[0007] *Mycobacterium tuberculosis* (Mtb) is a pathogenic bacterial species and the cause of most cases of tuberculosis (TB). There are close to two-million estimated deaths annually resulting from TB infection. Current TB vaccines are effective at protecting against

the disease during childhood, but there is no consistent protection against the contraction of pulmonary TB in adult. Thus, there exists a need to create a more effective vaccine against TB.

[0008] The non-classical HLA-E complex is distinct from classical HLA (such as HLA-A, HLA-B, and HLA-C) in that it is monomorphic in the human population, as compared with the classical HLA that are polymorphic. As such, all people in the population tend to have the same HLA-E on the surface of all their cells. Additionally, HLA-E provides a very specialized immune function that involves presentation of classical HLA leader sequences to inhibit NK cell function. Apart from this specialized function, HLA-E is thought to act like a classical class I HLA by presenting *M. tuberculosis*-derived peptide ligands to T-cells. However, this is unclear, and it is primarily known that HLA-E presents leader peptides from other proteins. Thus, identifying Mtb-derived peptide ligands that are made available by the HLA-E of Mtb-infected cells would be novel and of great value.

[0009] A number of potential strategies exist to identify Mtb peptide sequences that are presented by the HLA of Mtb-infected cells. In addition to those methods described herein above, HLA molecules isolated from cell lysates of pathogen-infected cells are also currently used to identify class I HLA-presented peptides; these methods, however, result in low peptide yields and/or relatively impure samples, and impurity and low concentrations lead to poor data. In addition, these current methods provide ambiguity as to which HLA allele is presenting said peptides; therefore, which HLA/peptide complex actually marks the surface of infected cells for targeting or diagnostics remains difficult to ascertain.

[0010] Many peptide ligands derived from Mtb have been reported in the context of classical HLA (i.e., HLA-A, HLA-B, and HLA-C). However, all Mtb-derived peptide ligands have been reported in the context of a non-classical HLA such as HLA-E. In addition, no previous reports of HLA-presented Mtb ligands have been reported in polymorphic HLA that differ from person to person, and thus the downstream diagnostics and therapies will only work on a subset of the population.

[0011] Therefore, there exists a need in the art for new and improved diagnostic and treatment methods for Mtb and identification of Mtb peptide ligands that are presented by the HLA of Mtb-infected cells, and particularly (but not limited to) the HLA-E of Mtb-

infected cells. It is to such methods, peptide sequences, and compositions containing such peptide sequences, that the presently disclosed and/or claimed inventive concept(s) is directed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] This patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[0013] Figure 1 graphically illustrates the deep ligand sequencing workflow utilized in accordance with the presently disclosed and/or claimed inventive concept(s), whereby sHLA (such as but not limited to, sHLA-E) was gathered from uninfected cells and cells infected with *M. tuberculosis*. The sHLA was purified, and the peptide pool was collected from the sHLA after acid elution. The directly eluted peptides were fractionated at high pH. The peptide rich fractions were then separated at low pH and directly injected into a mass spectrometer (such as, but not limited to, the TripleTOF® 5600+ mass spectrometer (AB Sciex Pte. Ltd., Framingham, MA)). MSl ion maps from Mtb-infected cells were compared to maps from the uninfected cells, and high throughput ion specific MS2 fragmentation was completed. A suite of software packages assigned sequences from the MS2 fragment spectra that were both unique to the infected cell and derived from Mtb. These Mtb sequences were validated with MS2 fragmentation of a corresponding synthetic peptide.

DETAILED DESCRIPTION

[0014] Before explaining at least one embodiment of the presently disclosed and/or claimed inventive concept(s) in detail by way of exemplary drawings, experimentation, results, and laboratory procedures, it is to be understood that the presently disclosed and/or claimed inventive concept(s) is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings, experimentation and/or results. The presently disclosed and/or claimed inventive concept(s) is capable of other embodiments or of being practiced or carried out in various ways. As such, the language used herein is intended to be given the

broadest possible scope and meaning; and the embodiments are meant to be exemplary - not exhaustive. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

[0015] Unless otherwise defined herein, scientific and technical terms used in connection with the presently disclosed and/or claimed inventive concept(s) shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. Generally, nomenclatures utilized in connection with, and techniques of, cell and tissue culture, molecular biology, and protein and oligo- or polynucleotide chemistry and hybridization described herein are those well known and commonly used in the art. Standard techniques are used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection). Enzymatic reactions and purification techniques are performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See e.g., Sambrook et al. Molecular Cloning: A Laboratory Manual (2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989) and Coligan et al. Current Protocols in Immunology (Current Protocols, Wiley Interscience (1994)), which are incorporated herein by reference. The nomenclatures utilized in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[0016] All patents, published patent applications, and non-patent publications mentioned in the specification are indicative of the level of skill of those of ordinary skill in the art to which this presently disclosed and/or claimed inventive concept(s) pertains. All patents, published patent applications, and non-patent publications referenced in any portion of this application are herein expressly incorporated by reference in their entirety

to the same extent as if each individual patent or publication was specifically and individually indicated to be incorporated by reference.

[0017] All of the compositions and/or methods disclosed and/or claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of the inventive concept(s) have been described in terms of particular embodiments, it will be apparent to those of ordinary skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the presently disclosed and/or claimed inventive concept(s). All such similar substitutes and modifications apparent to those of ordinary skill in the art are deemed to be within the spirit, scope and concept of the inventive concept(s) as defined by the appended claims.

[0018] As utilized in accordance with the present disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

[0019] The use of the word "a" or "an" when used in conjunction with the term "comprising" in the claims and/or the specification may mean "one," but it is also consistent with the meaning of "one or more," "at least one," and "one or more than one." The singular forms "a," "an," and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a compound" may refer to 1 or more, 2 or more, 3 or more, 4 or more or greater numbers of compounds. The term "plurality" refers to "two or more." The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure supports a definition that refers to only alternatives and "and/or." Throughout this application, the term "about" is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects. For example but not by way of limitation, when the term "about" is utilized, the designated value may vary by ± 20% or ± 10%, or ± 5%, or ± 1%, or ± 0.1% from the specified value, as such variations are appropriate to perform the disclosed methods and as understood by persons having ordinary skill in the art. The use of the term "at least one" will be understood to include

one as well as any quantity more than one, including but not limited to, 2, 3, 4, 5, 10, 15, 20, 30, 40, 50, 100, etc. The term "at least one" may extend up to 100 or 1000 or more, depending on the term to which it is attached; in addition, the quantities of 100/1000 are not to be considered limiting, as higher limits may also produce satisfactory results. In addition, the use of the term "at least one of X, Y and Z" will be understood to include X alone, Y alone, and Z alone, as well as any combination of X, Y and Z. The use of ordinal number terminology (i.e., "first," "second," "third," "fourth," etc.) is solely for the purpose of differentiating between two or more items and is not meant to imply any sequence or order or importance to one item over another or any order of addition, for example.

[0020] As used in this specification and claim(s), the terms "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

[0021] The term "or combinations thereof" as used herein refers to all permutations and combinations of the listed items preceding the term. For example, "A, B, C, or combinations thereof" is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AAB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The ordinary skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0022] As used herein, the term "substantially" means that the subsequently described event or circumstance completely occurs or that the subsequently described event or circumstance occurs to a great extent or degree. For example, the term "substantially" means that the subsequently described event or circumstance occurs at least 90% of the time, or at least 95% of the time, or at least 98% of the time.

[0023] The terms "*M. tuberculosis*-specific" and "Mtb-specific" are used interchangeably herein and refer to HLA peptide epitopes that have been identified as

being displayed in the context of HLA molecules on *Mycobacterium tuberculosis* (Mtb) - infected cells but not on cells which were not infected with Mtb. However, the terms "*M. tuberculosis*-specific" and "Mtb-specific" are not to be construed to mean that an HLA peptide epitope is limited to Mtb; indeed, multiple HLA peptide epitopes have been identified as being associated with other disease/infection states.

[0024] The presently disclosed and/or claimed inventive concept(s) relates generally to the identification of Mtb-derived peptide sequences that are presented by the HLA (such as, but not limited to, the HLA-E) of Mtb-infected cells. In the presently disclosed and/or claimed methods, milligram quantities of soluble HLA from cells that were infected with Mtb were harvested, and all the peptides ligands bound by the HLA of Mtb-infected cells were purified. Two-dimensional LCMS was then used to identify and sequence many of the peptide ligands presented by HLA. The use of soluble HLA in accordance with the presently disclosed and/or claimed inventive concept(s) provides a very pure preparation of peptides for HLA at a high concentration. This HLA ligand purity and concentration, in turn, allows the presently disclosed and/or claimed inventive concept(s) to superceed the sensitivity of other methods (biologic examples using a T-cell for example), and provides high-confidence HLA ligand sequences, thereby overcoming the defects and disadvantages of the prior art.

[0025] In particular (but not by way of limitation), Mtb peptide biomarkers presented by the HLA-E of Mtb-infected cells have been identified in accordance with the presently disclosed and/or claimed inventive concept(s). The HLA-E/Mtb ligands described and/or claimed herein were not previously known to be presented by the HLA-E of any infected cell. Because these HLA-E-presented Mtb peptides were purified and identified directly from infected cells, the presently disclosed and/or claimed inventive concept(s) has directly established that these HLA-E/Mtb complexes can be used as biomarkers of an infected cell, and targeted therapies can be developed based thereon. Additionally, since HLA-E is monomorphic, these targets can be applied to the entire human population. The HLA/ligand complexes discovered by direct comparison utilize soluble HLA molecules created *in vitro*. The soluble HLA molecules that enable direct comparative discovery of infected ligands do not exist in nature -- these soluble HLA have been created in the

laboratory. Therefore, the soluble HLA/ligand complexes distinct to Mtb-infected cells disclosed and/or claimed herein do not exist in nature.

[0026] The presently disclosed and/or claimed inventive concept(s) are based on a combination of methodologies for (1) the production of individual, soluble MHC molecules, and (2) epitope discovery and comparative ligand mapping methods (including methods of distinguishing infected cells from uninfected cells). The methods of production of individual, soluble MHC molecules have previously been described in detail in US Patent No. 7,521,202, issued April 21, 2009, to Hildebrand et al., while the methods of epitope discovery and comparative ligand mapping have previously been described in detail in US Patent No. 7,541,429, issued June 2, 2009, to Hildebrand et al. The entire contents of the above-referenced patents are expressly incorporated herein by reference. A brief description of each of these methodologies is included herein below for the purposes of exemplification and should not be considered as limiting.

[0027] In the methods of production of individual, soluble class I MHC molecule-endogenous peptide complexes (sMHC), a construct encoding a truncated, soluble form of an individual class I MHC molecule is transfected into a cell line that is able to naturally process proteins into peptide ligands capable of being loaded into antigen binding grooves of class I MHC molecules. The cell line is then cultured under conditions which allow for expression of the individual soluble class I MHC molecules from the construct, and these conditions also allow for endogenous loading of a peptide ligand into the antigen binding groove of each individual soluble class I MHC molecule prior to secretion of the individual soluble class I MHC molecules from the cell. The secreted individual soluble class I MHC molecules having the endogenously loaded peptide ligands bound thereto are then isolated. The construct that encodes the individual soluble class I MHC molecule may further encode a tag, such as (but not limited to) a HIS tail, a FLAG tail, or a VLDL tail, which is attached to the individual soluble class I MHC molecule and aids in isolating the individual soluble class I MHC molecule.

[0028] The methods of epitope discovery and comparative ligand mapping may be utilized to identify a peptide of interest that is associated with an infected state; in the method, at least one endogenously loaded peptide ligand is identified that distinguishes an

infected cell from an uninfected cell, and said method utilizes the sMHC production technology described herein above. Any commercially available cell line (infected or uninfected) and/or lab created cell line (infected or uninfected) may be utilized, so long as the cell line is able to naturally process proteins into peptide ligands and is capable of loading these peptide ligands into antigen binding grooves of the soluble class I MHC molecules. For example (but not by way of limitation), (i) readily available, immortalized, uninfected cells line(s) and (ii) readily available infected cell line(s) from the same tissue/organ as the uninfected cell lines may be utilized; when commercially available cell lines are used, the two cell lines are infected with the construct encoding an individual soluble class I MHC molecule as described herein above with respect to the method of production of sMHC. Alternatively, the infected cell line may be produced from an uninfected cell line that already contains a construct that encodes an individual soluble class I MHC molecule. A portion of the uninfected cell line may become infected by any method known in the art or otherwise contemplated herein (such as, but not limited to, transfection with a construct encoding a gene).

[0029] The uninfected cell line and the infected cell line are each cultured under conditions which allow for expression of individual soluble class I MHC molecules from the construct. The culture conditions also allow for endogenous loading of a peptide ligand in the antigen binding groove of each individual soluble class I MHC molecule prior to secretion of the individual soluble class I MHC molecules from the cell. The secreted individual soluble class I MHC molecules having the endogenously loaded peptide ligands bound thereto are isolated from the uninfected cell line and the infected cell line, and the endogenously loaded peptide ligands are separated from the individual soluble class I MHC molecules from both the uninfected cell line and the infected cell line. The endogenously loaded peptide ligands are then isolated from both the uninfected cell line and the infected cell line, and the two sets of endogenously loaded peptide ligands are compared to identify at least one endogenously loaded peptide ligand presented by the individual soluble class I MHC molecule that distinguishes the infected cell line from the uninfected cell line.

[0030] The comparison of the two sets of peptide ligands may identify: (1) at least one peptide ligand presented on the infected cell line that is not presented on the uninfected

cell line; (2) at least one peptide ligand presented in a substantially greater amount on the infected cell line when compared to the uninfected cell line; (3) at least one peptide ligand presented on the uninfected cell line that is not presented on the infected cell line; and/or (4) at least one peptide ligand that is presented in a substantially greater amount on the uninfected cell line when compared to the infected cell line. The term "substantially greater amount" as used herein refers to an amount that is detectably greater than another amount; for example, the term "presented in a substantially greater amount" as used herein refers to an at least one-fold increase in a first amount of presentation when compared to a second amount of presentation, such as but not limited to, an at least two-fold increase, an at least three-fold increase, an at least four-fold increase, an at least five-fold increase, an at least six-fold increase, an at least seven-fold increase, an at least eight-fold increase, an at least nine-fold increase, an at least ten-fold increase, an at least 20-fold increase, an at least 30-fold increase, an at least 40-fold increase, an at least 50-fold increase, an at least 100-fold increase, and the like.

[0031] Following identification of the peptide ligand that distinguishes an infected cell from an uninfected cell, a source protein from which the endogenously loaded peptide ligand is obtained can be identified. Such source protein may be encoded by the microorganism and/or the gene from the microorganism with which the cell line was infected to form the infected cell line, or the source protein may be encoded by the uninfected cell line. When the source protein is encoded by the uninfected cell line, such protein may also demonstrate increased expression in an Mtb-infected cell line.

[0032] The presently disclosed and/or claimed inventive concept(s) includes at least one isolated peptide ligand for an individual class I MHC molecule that has been isolated by the methods described herein. In one embodiment, the isolated peptide ligand(s) distinguishes an Mtb-infected cell from an uninfected cell, and thus is designated as an Mtb-specific peptide ligand herein. The isolated peptide ligand(s) may be an endogenously loaded peptide ligand presented by an individual class I MHC molecule that is unique to Mtb-infected cells when compared to cells not infected with Mtb.

[0033] Certain particular, non-limiting embodiments of the presently disclosed and/or claimed inventive concept(s) are directed to an isolated Mtb-specific peptide ligand

comprising at least one sequence selected from the group consisting of SEQ ID NOS:1-29. In another embodiment, the isolated peptide ligand consists essentially of at least one sequence selected from the group consisting of SEQ ID NOS:1-29. The isolated Mtb-specific peptide ligand may consist essentially of a fragment of a source protein of one of SEQ ID NOS:30-44 (as outlined in Table 1) and comprise at least one sequence selected from the group consisting of SEQ ID NOS:1-29 (based on the relationship between peptide ligands and source proteins indicated in Table 1).

[0034] The length of the isolated Mtb-specific peptide ligand may be (for example, but not by way of limitation) from about 8 to about 20 amino acids. In addition, the isolated Mtb-specific peptide ligand may be for an HLA-E allele, such as but not limited to, HLA-E*01:01, HLA-E*01:03, HLA-E*01:04, HLA-E*01:05, HLA-E*01:06, and HLA-E*01:07. In particular, non-limiting embodiments, the isolated Mtb-specific peptide ligand is for HLA-E*01:01 or HLA-E*01:03.

[0035] In certain, non-limiting embodiments, the peptide ligands of the presently disclosed and/or claimed inventive concept(s) may be isolated by a method that includes providing a cell line containing a construct that encodes an individual soluble class I MHC molecule, wherein the cell line is able to naturally process proteins into peptide ligands capable of being loaded into antigen binding grooves of class I MHC molecules. The cell line is cultured under conditions which allow for expression of the individual soluble class I MHC molecules from the construct, and also allow for endogenous loading of a peptide ligand into the antigen binding groove of each individual soluble class I MHC molecule prior to secretion of the individual soluble class I MHC molecules from the cell. Secreted individual soluble class I MHC molecules having the endogenously loaded peptide ligands bound thereto are then isolated, and the peptide ligands are then separated from the individual soluble class I MHC molecules.

[0036] In another non-limiting embodiment, the isolated Mtb-specific peptide ligands of the presently disclosed and/or claimed inventive concept(s) may be identified by a method that includes providing uninfected and Mtb-infected cell lines, each containing the same construct that encodes an individual soluble class I MHC molecule, wherein the two cell lines are able to naturally process proteins into peptide ligands capable of being loaded

into antigen binding grooves of class I MHC molecules. The two cell lines are each cultured under conditions which allow for expression of the individual soluble class I MHC molecules from the construct and also allow for endogenous loading of a peptide ligand in the antigen binding groove of each individual soluble class I MHC molecule prior to secretion of the individual soluble class I MHC molecules from the cell. The secreted individual soluble class I MHC molecules having the endogenously loaded peptide ligands bound thereto are isolated separately from the two cell lines, and endogenously loaded peptide ligands are separated from the individual soluble class I MHC molecules for each of the two separate pools. The endogenously loaded peptide ligands obtained from the two cell lines are then isolated and compared to one another. Finally, at least one endogenously loaded peptide ligand presented by the individual soluble class I MHC molecule is identified that distinguishes between the two cell lines; the peptide ligand may be unique to one of the two cell lines, or the peptide ligand may be presented in a substantially greater amount on one cell line when compared to the other cell line.

[0037] The utility of the identified MHC/HLA-presented peptide epitopes which mark the Mtb-infected cell is three-fold. First, diagnostics designed to detect Mtb can use epitopes unique to Mtb-infected cells to ascertain the presence/absence of an infection. Second, peptides unique to Mtb-infected cells represent vaccine candidates. Third, the entire HLA trimolecular complex containing the Mtb-specific peptide epitope represents a possible therapeutic target. For example, but not by way of limitation, the presently disclosed and/or claimed inventive concept(s) describes and claims multiple peptide epitopes that are either unique to or presented in substantially greater amounts on Mtb-infected cells. Also, as demonstrated herein below, such epitopes are unlikely to be predicted without direct epitope discovery. These Mtb-specific peptides can be used for vaccine development, and the unique Mtb-specific peptide/HLA complex can be utilized for diagnostics and/or specific targeting.

[0038] Other particular, non-limiting embodiments of the presently disclosed and/or claimed inventive concept(s) are directed to compositions that comprise at least one isolated class I MHC/HLA trimolecular complex formed *in vitro*. The trimolecular complex includes a soluble, truncated HLA-E heavy chain that does not contain the transmembrane

and cytoplasmic domains of the native, full length HLA-E heavy chain. The trimolecular complex also includes beta-2-microglobulin and an Mtb-specific peptide ligand as described in detail herein above.

[0039] The soluble, truncated HLA-E heavy chain of the trimolecular complex may be a soluble, recombinantly produced HLA-E heavy chain. For example, the at least one isolated class I MHC/HLA trimolecular complex may be produced in a host cell made recombinant by a construct encoding the soluble, truncated HLA-E heavy chain, wherein the construct does not encode the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain. In certain embodiments, the peptide is an endogenous peptide that is produced by the host cell and loaded in the trimolecular complex by the host cell. Alternatively, the peptide may be a synthetic peptide; the synthetic peptide (or a gene encoding same) may be introduced into the host cell for *in vivo* loading into the trimolecular complex, or the synthetic peptide may be mixed *in vitro* with the soluble, truncated HLA-E heavy chain and beta-2-microglobulin to form the trimolecular complex. Such methods of producing trimolecular complexes are well known in the art, and thus no further description thereof is deemed necessary.

[0040] In certain non-limiting embodiments, the composition may include at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, or more isolated class I MHC/HLA trimolecular complexes as defined herein above. When multiple complexes are present, they may be unattached to one another, or they may be multimerized.

[0041] Thus, the compositions of the presently disclosed and/or claimed inventive concept(s) may include a multimer of isolated class I MHC/HLA trimolecular complexes. The term "multimer" as used herein will be understood to include two or more class I MHC/HLA-Mtb-specific epitope trimolecular complexes which are covalently or non-covalently attached together, either directly or indirectly. Each of the plurality of complexes present in the multimer may be multimerized by attachment to at least one

other complex by any covalent or non-covalent method known in the art or otherwise contemplated herein.

[0042] If necessary, one or more of the class I MHC/HLA-Mtb-specific epitope trimolecular complexes present in a multimer may be modified in some manner known in the art to enable attachment of the complexes to each other; alternatively, the multimer may be formed around a substrate to which each trimolecular complex is attached. A tail may be attached to a portion of one or more of the trimolecular complexes to aid in multimerization; examples of tails that may be utilized include, but are not limited to, a biotinylation signal peptide tail, an immunoglobulin heavy chain tail, a TNF tail, an IgM tail, a leucine zipper, a Fos/Jun tail, combinations thereof, and the like. The multimer can contain any desired number of trimolecular complexes and thus form any multimer desired, such as but not limited to, a dimer, a trimer, a tetramer, a pentamer, a hexamer, and the like. Streptavidin has four binding sites for biotin, so a BSP (biotinylation signal peptide) tail may be attached to a portion of the HLA molecule during production thereof, and a tetramer of the desired trimolecular complex(es) could be formed by combining the trimolecular complexes having the BSP tails with biotin added enzymatically *in vitro*. An immunoglobulin heavy chain tail may be utilized as a substrate for forming a dimer, while a TNF tail may be utilized as a substrate for forming a trimer. An IgM tail could be utilized as a substrate for forming various combinations, such as tetramers, hexamers, and pentamers. In addition, the trimolecular complexes may be multimerized through liposomal encapsulation or artificial antigen presenting cell technology (see US Patent Application Publication No. 2002/0122820, published to Hildebrand et al. on September 5, 2002, the contents of which are hereby expressly incorporated herein by reference). Further, the soluble HLA class II trimolecular complexes may be multimerized through the use of polymerized streptavidin and would produce what is termed a "STREPTAMER™" (IBA GmbH, Gottingen, Germany).

[0043] When the composition comprises a dimer of class I HLA trimolecular complexes, the peptides of the two trimolecular complexes may be the same or different. When the composition comprises a multimer of three or more class I HLA trimolecular complexes (such as but not limited to, a trimer, a tetramer, a pentamer, a hexamer, a heptamer, an

octamer, and the like), all of the peptides of the trimolecular complexes present in the multimer may be the same, at least one of the peptides of the trimolecular complexes present in the multimer may be different, or all of the peptides of the trimolecular complexes present in the multimer may be different.

[0044] The trimolecular complexes of the presently disclosed and/or claimed inventive concept(s) may further be modified for providing better performance and/or for aiding in stabilization of the monomer or multimer. Examples of modifications that may be utilized in accordance with the presently disclosed and/or claimed inventive concept(s) include, but are not limited to, modifying an anchor and/or tail attached to the trimolecular complex as described herein above, modifying one or more amino acids in the Mtb-specific peptide/HLA trimolecular complex, PEGylation, chemical cross-linking, changes in pH or salt (depending on the specific peptide of the trimolecular complex), addition of one or more chaperone proteins that stabilize certain trimolecular complexes, combinations thereof, and the like.

[0045] Particular, non-limiting embodiments of the compositions of the presently disclosed and/or claimed inventive concept(s) include at least one isolated class I HLA trimolecular complex formed *in vitro*, wherein the trimolecular complex comprises the soluble, truncated HLA-E heavy chain as described herein above, beta-2-microglobulin, and a peptide selected from the following: (a) a peptide comprising SEQ ID NO:1; (b) a peptide comprising SEQ ID NO:2 and/or 3; (c) a peptide comprising SEQ ID NO:4; (d) a peptide comprising SEQ ID NO:5; (e) a peptide comprising at least one of SEQ ID NOS:6-9; (f) a peptide comprising SEQ ID NO:10 and/or 11; (g) a peptide comprising SEQ ID NO:12 and/or 13; (h) a peptide comprising SEQ ID NO:14; (i) a peptide comprising SEQ ID NO:15; (j) a peptide comprising SEQ ID NO:16; (k) a peptide comprising at least one of SEQ ID NOS:17-20; (l) a peptide comprising SEQ ID NO:21; (m) a peptide comprising SEQ ID NO:22; (n) a peptide comprising SEQ ID NO:23 and/or 24; (o) a peptide comprising SEQ ID NO:25 and/or 26; (p) a peptide comprising SEQ ID NO:27; (q) a peptide comprising SEQ ID NO:28; and (r) a peptide comprising SEQ ID NO:29.

[0046] Other particular, non-limiting embodiments of the compositions of the presently disclosed and/or claimed inventive concept(s) include at least one isolated class I HLA

trimolecular complex formed *in vitro*, wherein the trimolecular complex comprises the soluble, truncated HLA-E heavy chain as described herein above, beta-2-microglobulin, and a peptide selected from the following: (a) a peptide consisting essentially of a fragment of SEQ ID NO:30 and comprising SEQ ID NO:1; (b) a peptide consisting essentially of a fragment of SEQ ID NO:31 and comprising SEQ ID NO:2 and/or 3; (c) a peptide consisting essentially of a fragment of SEQ ID NO:31 and comprising SEQ ID NO:4; (d) a peptide consisting essentially of a fragment of SEQ ID NO:31 and comprising SEQ ID NO:5; (e) a peptide consisting essentially of a fragment of SEQ ID NO:32 and comprising at least one of SEQ ID NOS:6-9; (f) a peptide consisting essentially of a fragment of SEQ ID NO:32 and comprising SEQ ID NO:10 and/or 11; (g) a peptide consisting essentially of a fragment of SEQ ID NO:33 and comprising SEQ ID NO:12 and/or 13; (h) a peptide consisting essentially of a fragment of SEQ ID NO:34 and comprising SEQ ID NO:14; (i) a peptide consisting essentially of a fragment of SEQ ID NO:35 and comprising SEQ ID NO:15; (j) a peptide consisting essentially of a fragment of SEQ ID NO:36 and comprising SEQ ID NO:16; (k) a peptide consisting essentially of a fragment of SEQ ID NO:37 and comprising at least one of SEQ ID NOS:17-20; (l) a peptide consisting essentially of a fragment of SEQ ID NO:38 and comprising SEQ ID NO:21; (m) a peptide consisting essentially of a fragment of SEQ ID NO:39 and comprising SEQ ID NO:22; (n) a peptide consisting essentially of a fragment of SEQ ID NO:40 and comprising SEQ ID NO:23 and/or 24; (o) a peptide consisting essentially of a fragment of SEQ ID NO:41 and comprising SEQ ID NO:25 and/or 26; (p) a peptide consisting essentially of a fragment of SEQ ID NO:42 and comprising SEQ ID NO:27; (q) a peptide consisting essentially of a fragment of SEQ ID NO:43 and comprising SEQ ID NO:28; and (r) a peptide consisting essentially of a fragment of SEQ ID NO:44 and comprising SEQ ID NO:29.

[0047] In addition, any of the components of the trimolecular complex may be provided in their peptide/polypeptide form or as a polynucleotide encoding the peptide/polypeptide form. Thus, another particular, non-limiting embodiment of the presently disclosed and/or claimed inventive concept(s) is directed to an isolated composition that includes (i) a soluble, truncated class I HLA-E heavy chain as described herein above or DNA encoding the soluble, truncated HLA-E heavy chain; (b) beta-2-microglobulin or DNA encoding beta-2-

microglobulin; and (c) at least one Mtb-specific peptide ligand as described herein above, or DNA encoding the at least one peptide.

EXAMPLES

[0048] Examples are provided hereinbelow. However, the presently disclosed and/or claimed inventive concept(s) is to be understood to not be limited in its application to the specific experimentation, results and laboratory procedures. Rather, the Examples are simply provided as one of various embodiments and are meant to be exemplary, not exhaustive.

Example 1: Identification of Mtb-Specific HLA-E Peptide Epitopes

[0049] The identification of novel, Mtb-specific epitopes is a critical step in the development of T cell receptor-mediated immunotherapeutics against TB. To convey intracellular health to the immune system, mammals utilize the major histocompatibility complex (MHC) class I molecule. Class I MHC molecules are nature's proteome scanning chip. The class I MHC molecules gather many small peptides of intracellular origin, including the products of proteasomal processing and of defective translation, and carry these intracellular peptides to the cell surface. Intracellular peptides derived from proteins found in multiple compartments within the cell, and derived from proteins of many cellular functions, are sampled and presented at the cell surface by class I MHC. Immune cells, including but not limited to $CD8^+$ cytotoxic T-lymphocytes (CTL), survey the peptides presented by class I MHC and target cells displaying Mtb-specific peptides. Therefore, class I MHC-presented peptides distinguish and promote the recognition of infected cells by the adaptive immune system.

[0050] Given that class I MHC molecules distinguish infected cells from healthy cells, a number of studies have aimed to identify class I MHC-presented Mtb antigens. Because class I MHC molecules can be difficult to produce and purify, immune-based studies using CTL raised to autologous tumors have been utilized to identify Mtb immune targets. Other immune-based methods have relied upon predictive algorithms and *in vitro* class I MHC peptide binding assays. Although these indirect approaches have identified putative tumor

antigens, a direct proteomics-based approach for identifying class I MHC Mtb-specific antigens is needed.

[0051] In addition, classic HLA (-A, -B, -C, and -G) are polymorphic; thus, in a diagnostic assay testing for the presence of Mtb, results based on peptides from classical HLA can lead to a large amount of variation in test results. Accordingly, a set of HLA-E binding peptides have been discovered by the methods described herein which are monomorphic in nature and would result in a superior assay or potential therapeutic.

[0052] Recognizing the protein production, isolation, and characterization challenges associated with the direct analysis of class I MHC proteome scanning chips, the presently disclosed and/or claimed inventive concept(s) utilized a method to obtain plentiful quantities of individual human class I MHC (HLA) from well-characterized Mtb-infected cell lines. Through expression of a secreted human class I MHC (sHLA) as described in US Patent No. 7,521,202 (incorporated *supra* and discussed in detail herein above), the cell's own class I HLA complexes remain on the cell surface, and only the transfected sHLA is harvested. Moreover, secretion of the human class I MHC molecule allows purification of the desired protein from tissue culture supernatants rather than requiring isolation of class I MHC from more complex detergent lysates.

[0053] The methodology of comparative ligand mapping as described in US Patent No. 7,541,429 (incorporated *supra* and discussed in detail herein above) and as illustrated in Figure 1 was utilized herein to identify HLA-E*01:03 epitopes that were unique to Mtb-infected cells. The uninfected cell line and the Mtb-infected cell line were each transfected with a construct encoding HLA-E*01:03, as described herein above and in US Patent No. 7,521,202 (incorporated *supra*). The class I HLA-E*01:03 allele was selected for its high frequency in the population.

[0054] The uninfected cell line and the Mtb-infected cell line were both cultured in hollow-fiber bioreactors under conditions as described herein above (and in detail in the '202 patent, incorporated *supra*) that allowed for expression of the construct encoding sHLA-E*01:03, loading of endogenously produced peptide therein, and secretion of the trimolecular complexes from the cells. The supernatant containing the sHLA-E*01:03/peptide trimolecular complexes was then harvested from the hollow-fiber

bioreactors, and the uninfected and Mtb-infected harvested supernatants were then treated in an identical manner post-removal from the hollow-fiber bioreactors.

[0055] Trimolecular sHLA-E*01:03/peptide complexes were affinity purified from the uninfected and Mtb-infected supernatants using an anti-HLA antibody (such as, but not limited to, W6/32). Following elution, peptides were isolated from the sHLA-E*01:03 molecules and separated by reverse phase HPLC fractionation. Separate but identical peptide purifications (including the same buffer preparations) were performed for each peptide-batch from both uninfected and infected cells.

[0056] Fractionated peptides were then mapped by mass spectrometry to generate fraction-based ion maps. The MS ion maps so produced were assessed for the presence of differences in the ions represented by the spectra. Ions corresponding to the following categories were selected for MS/MS sequencing: (1) upregulation in Mtb-infected cells (at least 1.5-fold over the same ion in uninfected cells), (2) down regulation in Mtb-infected cells (at least 1.5-fold over the same ion in the uninfected cells), (3) presence of the ion only in Mtb-infected cells, or (4) absence of ion in Mtb-infected cells that is present in uninfected cells. In addition, multiple parameters were established before peptides were assigned to one of the above categories, including checking the peptide fractions preceding and following the peptide fraction by MS/MS (to ensure that the peptide of interest was not present in an earlier or later fraction) as well as generation of synthetic peptides and subjection to MS/MS to check for an exact match.

[0057] Table I lists peptide ligands that have been identified as being presented by the HLA-E*01:03 molecule on the Mtb-infected cell line but not on the uninfected cell line, or presented in a substantially greater amount on the Mtb-infected cell line when compared to the uninfected cell line. One of ordinary skill in the art can appreciate the novelty and usefulness of these peptide ligands and the importance such identification has for numerous therapeutic (i.e., vaccine development, drug targeting, and the like) and diagnostic tools.

[0058] The sequences in Table I represent single-letter abbreviations of amino acids within a peptide. A peptide is defined as a short polymer of amino acids. The order of these

amino acids is written from N-terminal end of the peptide to the C-terminal end of the peptide.

[0059] The presently disclosed and/or claimed inventive concept(s) includes improved methodology for ligand screening titled Deep Ligand Sequencing (DLS), performed with a combination of next-generation, high sensitivity, high mass accurate quadruple time of flight (QTOF; TripleTOF® 5600+ mass spectrometer (AB Sciex Pte. Ltd., Framingham, MA)) with two-dimensional nano-LC/MS. This method is summarized in Figure 1.

TABLE 1: HLA-E Peptide Epitopes Unique to Mtb-infected Cells

Peptide SEQ ID NO:	Peptide Sequence	Gene	Source Protein	Source Protein SEQ ID NO:
1	AERAPVEADAGGGQKVLVRN	epsA	ESX-1 secretion-associated protein A, EspA	30
2	STEGNVTGMFA	esxA	6 kDa early secretory antigenic target EsxA (ESAT-6)	31
3	STEGNVTGM(+15.99)FA	esxA	6 kDa early secretory antigenic target EsxA (ESAT-6)	31
4	LLDEGKQSL	esxA	6 kDa early secretory antigenic target EsxA (ESAT-6)	31
5	SLLDEGKQSL	esxA	6 kDa early secretory antigenic target EsxA (ESAT-6)	31
6	SLLDAHIPQ	esxG	ESAT-6 like protein EsxG	32
7	SLLDAHIPQL	esxG	ESAT-6 like protein EsxG	32
8	LLDAHIPQL	esxG	ESAT-6 like protein EsxG	32
9	LLDAHIPQ	esxG	ESAT-6 like protein EsxG	32
10	TLLDVAQANLGEAAGTYV	esxG	ESAT-6 like protein EsxG	32
11	NLGEAAGTYV	esxG	ESAT-6 like protein EsxG	32
12	IMYNYPAML	esxH	Low molecular weight protein antigen 7 EsxH	33
13	IM(+15.99)YNYPAM(+15.99)L	esxH	Low molecular weight protein antigen 7 EsxH	33
14	PALPPAPPSP	GI:5305335	Proline-rich muscin homolog	34
15	LPPAPPAPPS	GI:544648605	Proline-rich muscin-like protein	35
16	GLIDIAPHQISSVAA	iniB	Isoniazid inductible gene protein IniB	36
17	GGILIGSDTDLT	lpqI	Probable conserved lipoprotein LpqI	37
18	HVGGILIGSDTDLT	lpqI	Probable conserved lipoprotein LpqI	37
19	VGGILIGSDTDLT	lpqI	Probable conserved lipoprotein LpqI	37
20	AEIVAGGGPLPL	lpqI	Probable conserved lipoprotein LpqI	37
21	PAILRPGRLD	mpa	Mycobacterial proteasome ATPase Mpa	38
22	RVVPEGLAAA	PE5	PE family protein PE5	39
23	ADVVGSDDLIE	rplA	50S ribosomal protein L1 RplA	40
24	AGADVVGSDDLIE	rplA	50S ribosomal protein L1 RplA	40
25	ATNRPDLID	Rv0435c/ftsH	Putative conserved ATPase/ Membrane-bound protease FtsH	41
26	TNRPDLID	Rv0435c/ftsH	Putative conserved ATPase/ Membrane-bound protease FtsH	41
27	EIEVDDDLIQK	Rv0634A	Hypothetical protein	42
28	S(+42.01)TIIAGALLLVL	Rv3479	Possible transmembrane protein	43
29	LIGPPPSP	Rv3491	Hypothetical protein	44

+15.99 refers to an oxidized methionine; +42.01 refers to an N-terminal acetylation

[0060] Directly eluted HLA peptides are very complex and contain a mixture of tens of thousands of biochemically similar peptides of comparable mass. Thus, multiple dimensions of biochemical separation are needed for comparative analysis of individual peptide ligands. Fortunately, modern mass spectrometers are equipped with two-dimensional HPLC that precedes mass spectrometric separation by mass and charge; thus, multiple dimensions of candidate epitope separation are incorporated into the latest equipment. Here, a two-dimensional LC-MS approach followed by MS1 and MS2 enabled efficient separation and sequencing of directly eluted ligands (Figure 1). A precise assignment of mass eliminated false positive hits in the MASCOT™ (Matrix Science, Boston, MA), PEAKS® (Bioinformatic Solutions Inc., Waterloo, ON, Canada), and ProteinPilot™ (AB Sciex Pte. Ltd., Framingham, MA) algorithms. The TripleTOF® 5600+ mass spectrometer (AB Sciex Pte. Ltd., Framingham, MA) has been applied herein for accurate and sensitive identification of ligand sequences from MS2 fragment spectra. Using this deep ligand sequencing method, it was possible to reach T-cell sensitivities while still allowing for the *de novo* identification of greater than 10,000 high-confidence ligand sequences.

[0061] For the first dimension, the directly eluted peptide pool was loaded on a reverse phase JUPITER® 4 µm Proteo 90 Å, 2 mm id x 150 mm long column (Phenomonex, Inc., Torrance, CA) with a Paradigm MG4 HPLC (Michrom Biosciences Inc., Auburn, CA). Peptides were eluted from the column in high pH conditions (pH 10) with a gradient of 2-10% acetonitrile in water in 2 minutes, then 10-60% in 60 minutes. Forty peptide-rich fractions were collected along the gradient. All 40 fractions from the first-dimension were dried by vacuum centrifugation. Each fraction was solubilized in a 1:1 second dimension solvent A/dimethylformamide and placed into the 96-well high-throughput autosampler of an Eksigent NanoLC 400 U-HPLC system (AB Sciex Pte. Ltd., Framingham, MA). One-tenth of each sample was sequentially run in triplicate on a reversed-phase nano-HPLC column equilibrated at pH 2. The second-dimension nano-HPLC setup included a 350 micron id x 0.5 mm long ChromXP C18 trap column (AB Sciex Pte. Ltd., Framingham, MA) with 3 micron particles and 120 Angstrom pores and a ChromXP C18 separation column (AB Sciex Pte. Ltd., Framingham, MA) of dimensions 75 micron id x 15 cm length packed with the same media. The solvent system was comprised of two solvents, A and B; solvent A contained

0.1% formic acid in 98% water and 2% acetonitrile, while solvent B contained 0.1% formic acid in 5% water and 95% acetonitrile. Samples were loaded on the trap column at a flow rate of 10 μl/minute.

[0062] Next, the trap column was placed in-line with the separation column running at a flow rate of 300 nL/minute. Peptides were then eluted utilizing a program with two linear gradients. The bulk of the peptides were eluted in the initial gradient of 5% to 40% solvent B over 70 minutes. Any remaining peptides were eluted in the second gradient of 40% to 80% solvent B over ten minutes. The second-dimension HPLC column effluent was connected to the nanospray III ion source of a quadrupole-TOF mass spectrometer (TripleTOF® 5600+ mass spectrometer (AB Sciex Pte. Ltd., Framingham, MA)) to generate LC/MS ion maps and parent ion MS/MS fragmentation spectra.

[0063] Forty second dimension LC/MS1 spectra were comparatively analyzed, and candidate ligand sequences from Mtb-infected cells were identified (Figure 1). Briefly, corresponding LC/MS1 spectra from each fraction were aligned; after alignment, corresponding LC/MS1 ion maps from Mtb-infected and uninfected cells were compared using MarkerView™ software (AB Sciex Pte. Ltd., Framingham, MA). Unique ions corresponding to the Mtb cell line fraction were identified, placed on an inclusion list for Information Dependant Acquisition (AB Sciex Pte. Ltd., Framingham, MA), and subjected to LC/MS2 fragmentation (if not completed in the first round of MS acquisition) in order to identify amino acid sequences.

[0064] MS2 fragment spectra for directly eluted HLA peptides can be challenging to interpret, because HLA-presented peptides are not tryptic peptides: the C-terminus thereof often lacks a charge. Because of the unique nature of HLA peptides, a multi-layered complementary approach was taken to resolve MS2 fragmentation data, utilizing a combined application of the current algorithms MASCOT™ (Matrix Science, Boston, MA), PEAKS® (Bioinformatic Solutions Inc., Waterloo, ON, Canada), and ProteinPilot™ (AB Sciex Pte. Ltd., Framingham, MA). Using this approach, over 10,000 peptides were sequenced with high confidence, including any peptides that were post-translationally modified (which includes approximately greater than 20% of the ligands). To avoid false positive sequences, the corresponding synthetic peptide of any assigned sequence was generated and

subjected to the same LC/MS/MS method. The assigned sequence would only be considered correct when the MS/MS spectra and LC retention time from the synthetic peptide matched the native peptide.

[0065] Thus, in accordance with the presently disclosed and/or claimed inventive concept(s), there has been provided compositions that include HLA-E binding epitopes identified by a method of epitope discovery, comparative ligand mapping, and deep ligand sequencing, as well as compositions including same and methods of identification, production, and use thereof, that fully satisfy the objectives and advantages set forth herein above. Although the inventive concept(s) has been described in conjunction with the specific drawings, experimentation, results and language set forth herein above, it is evident that many alternatives, modifications, and variations will be apparent to those of ordinary skill in the art. Accordingly, it is intended to embrace all such alternatives, modifications, and variations that fall within the spirit and broad scope of the inventive concept(s).

<u>What is claimed is</u>:

1. A composition, comprising at least one of:

   (a) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:1, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

   (b) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:2 and/or 3, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

   (c) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:4, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

   (d) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:5, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

   (e) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising at least one of SEQ ID NOS:6-9, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

   (f) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular

complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:10 and/or 11, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(g) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:12 and/or 13, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(h) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:14, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(i) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:15, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(j) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:16, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(k) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising at least one of SEQ ID NOS:17-20, and wherein the soluble, truncated HLA-E heavy chain does not contain the

transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(l) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:21, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(m) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:22, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(n) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:23 and/or 24, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(o) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:25 and/or 26, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(p) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:27, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(q) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular

complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:28, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain; and

(r) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:29, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain.

2. The composition of claim 1, wherein the HLA-E of (a)-(r) is HLA-E*01:01 or HLA-E*01:03.

3. The composition of claim 1, wherein the HLA-E heavy chain of at least one of (a)-(r) is further defined as a soluble, recombinantly produced HLA-E heavy chain.

4. The composition of claim 3, wherein the class I HLA trimolecular complex of at least one of (a)-(r) is produced in a host cell made recombinant by a construct encoding the soluble, truncated HLA-E heavy chain, and wherein the construct does not encode the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain, and wherein the peptide is further defined as an endogenous peptide that is produced by the host cell and loaded in the trimolecular complex by the host cell.

5. The composition of claim 1, wherein the peptide of at least one of (a)-(r) is a synthetic peptide.

6. The composition of claim 1, wherein the peptide of at least one of (a)-(r) is further defined as a peptide having a length of from 8 to 20 amino acids.

7. The composition of claim 1, further defined as comprising at least two of (a)-(r).

8. The composition of claim 1, further defined as comprising at least three of (a)-(r).

9. The composition of claim 1, further defined as comprising at least four of (a)-(r).

10. The composition of claim 1, further defined as comprising at least five of (a)-(r).

11. A composition, comprising at least one of:
   (a) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:30 and comprising SEQ ID NO:1, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;
   (b) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:31 and comprising SEQ ID NO:2 and/or 3, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;
   (c) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:31 and comprising SEQ ID NO:4, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;
   (d) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:31 and comprising SEQ ID NO:5, and wherein the soluble, truncated HLA-

E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(e) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:32 and comprising at least one of SEQ ID NOS:6-9, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(f) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:32 and comprising SEQ ID NO:10 and/or 11, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(g) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:33 and comprising SEQ ID NO:12 and/or 13, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(h) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:34 and comprising SEQ ID NO:14, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(i) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:35 and comprising SEQ ID NO:15, and wherein the soluble, truncated

HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(j) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:36 and comprising SEQ ID NO:16, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(k) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:37 and comprising at least one of SEQ ID NOS:17-20, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(l) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:38 and comprising SEQ ID NO:21, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(m) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:39 and comprising SEQ ID NO:22, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(n) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:40 and comprising SEQ ID NO:23 and/or 24, and wherein the soluble,

truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(o) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:41 and comprising SEQ ID NO:25 and/or 26, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(p) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:42 and comprising SEQ ID NO:27, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

(q) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:43 and comprising SEQ ID NO:28, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain; and

(r) an isolated class I HLA trimolecular complex formed *in vitro*, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide consisting essentially of a fragment of SEQ ID NO:44 and comprising SEQ ID NO:29, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain.

12. The composition of claim 11, wherein the HLA-E of (a)-(r) is HLA-E*01:01 or HLA-E*01:03.

13. The composition of claim 11, wherein the HLA-E heavy chain of at least one of (a)-(r) is further defined as a soluble, recombinantly produced HLA-E heavy chain.

14. The composition of claim 13, wherein the class I HLA trimolecular complex of at least one of (a)-(r) is produced in a host cell made recombinant by a construct encoding the soluble, truncated HLA-E heavy chain, and wherein the construct does not encode the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain, and wherein the peptide is further defined as an endogenous peptide that is produced by the host cell and loaded in the trimolecular complex by the host cell.

15. The composition of claim 11, wherein the peptide of at least one of (a)-(r) is a synthetic peptide.

16. The composition of claim 11, wherein the peptide of at least one of (a)-(r) is further defined as a peptide having a length of from 8 to 20 amino acids.

17. The composition of claim 11, further defined as comprising at least two of (a)-(r).

18. The composition of claim 11, further defined as comprising at least three of (a)-(r).

19. The composition of claim 11, further defined as comprising at least four of (a)-(r).

20. The composition of claim 11, further defined as comprising at least five of (a)-(r).

21. An isolated composition, comprising:

a soluble, truncated class I HLA-E heavy chain or DNA encoding a soluble, truncated HLA-E heavy chain, wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain;

beta-2-microglobulin or DNA encoding beta-2-microglobulin; and

at least one peptide comprising at least one of SEQ ID NOS:1-29, or DNA encoding the at least one peptide.

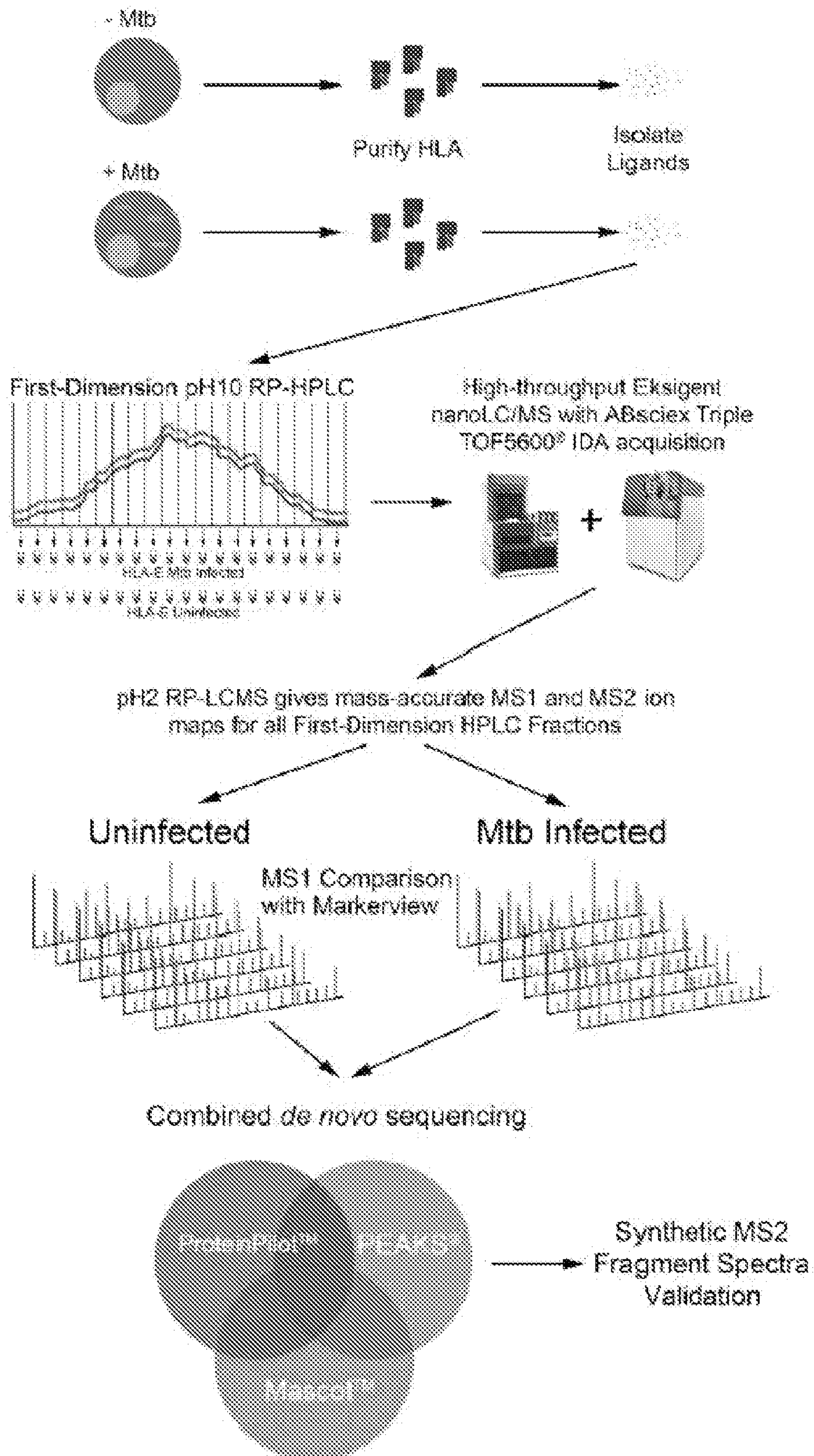

FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/023058

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/04 (2015.01)
CPC - A61K 39/04 (2015.07)
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)
IPC(8) - A61K 39/00, 39/04, 39/08, 39/085, 39/09, 39/145 (2015.01)
CPC - A61K 39/00, 39/0007, 39/0008, 39/04, 39/08, 39/085 (2015.07)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - A61K 39/00, 39/0007, 39/0008, 39/04, 39/08, 39/085 (2015.07) (keyword delimited)
US Classes - 424/184.1, 185.1, 190.1, 193.1, 194.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatBase, Google Patents, Google, PubMed

Search terms used: trimolecular, HLA, HLA-E, heavy chain, B2 microglobulin, EspA, tuberculosis, mycobacteria, fusion protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2008/0274475 A1 (BRAUD et al) 06 November 2008 (06.11.2008) entire document	1-21
Y	WO 2011/092253 A1 (GLAXOSMITHKLINE BIOLOGICALS S.A. et al) 04 August 2011 (04.08.2011) entire document	1-21
A	US 2013/0143235 A1 (HILDEBRAND et al) 06 June 2013 (06.06.2013) entire document	1-21
A	JOOSTEN et al. "Mycobacterium tuberculosis Peptides Presented by HLA-E Molecules Are Targets for Human CD8+ T-Cells with Cytotoxic as well as Regulatory Activity," 26 February 2010 (26.02.2010), PLoS Pathogens, Vol. 6, Iss. 2, Pgs. 1-15. entire document	1-21
A	GARCES et al. "EspA Acts as a Critical Mediator of ESX1-Dependent Virulence in Mycobacterium tuberculosis by Affecting Bacterial Cell Wall Integrity," 24 June 2010 (24.06.2010), PLoS Pathogens, Vol. 6, Iss. 6, Pgs. 1-11. entire document	1-21

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
17 August 2015

Date of mailing of the international search report
04 SEP 2015

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PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2015/023058

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see Extra Sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1, 3-6, 11, 13-16, and 21 will be searched to the extent that they read on the trimolecular complex containing a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide of SEQ ID NO:1.

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-21 are drawn to a composition comprising an isolated class I HLA trimolecular complex.

The first invention of Group I+ is restricted to a composition comprising an isolated class I HLA trimolecular complex, wherein the trimolecular complex is selected to be a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:1, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain. It is believed that claims 1, 3-6, 11, 13-16, and 21 read on this first named invention and thus these claims will be searched without fee to the extent that they read on the trimolecular complex containing a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide of SEQ ID NO:1.

Applicant is invited to elect additional peptides and their corresponding SEQ ID NOs to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be a composition comprising an isolated class I HLA trimolecular complex formed in vitro, the trimolecular complex containing a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide selected to be SEQ ID NO:2, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain. Additional peptides and their corresponding SEQ ID NOs will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element, requiring the selection of alternatives for the peptide of the trimolecular complex where "at least one peptide comprising at least one of SEQ ID NOS:1-29, or DNA encoding the at least one peptide."

The Groups I+ share the technical features of an isolated composition, comprising an isolated class I HLA trimolecular complex formed in vitro, the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide comprising SEQ ID NO:1, and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain. However, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2008/0274475 A1 to Braud et al. discloses an isolated composition (a compound, Para. [0024]; a recombinant HLA-E, Para. [0031]), comprising an isolated class I HLA trimolecular complex formed in vitro (the invention provides a multimer of HLA-E, Para. [0030]; in vitro, Para. [0058]), the trimolecular complex comprising a soluble, truncated HLA-E heavy chain, beta-2-microglobulin, and a peptide (constructing complexes by refolding the HLA-E heavy chains in vitro with β2m molecules and with a synthetic leader peptide from HLA-B, Para. [0058]; the invention provides a multimer of HLA-E comprising...alpha 1, alpha 2 and alpha 3 HLA-E domains, together with beta-2-microglobulin, and a suitable peptide in the peptide binding groove, Para. [0030]; a soluble HLA-E molecule, Para. [0063]), and wherein the soluble, truncated HLA-E heavy chain does not contain the transmembrane and cytoplasmic domains of the native, full length HLA-E heavy chain (a soluble HLA-E molecule; the protein may be truncated by removing both the cytoplasmic and the transmembrane domains, Para. [0063]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.