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(54) Title: ASSAYS, COMPOSITIONS, SYSTEMS, KITS, AND DEVICES FOR DIAGNOSING TYPHOID FEVER

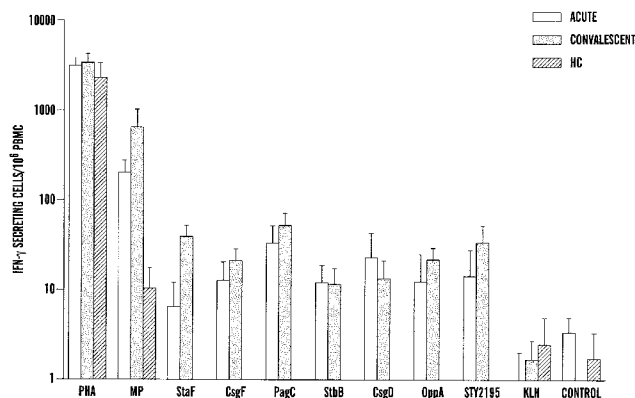


FIG. 1

(57) Abstract: Provided herein are methods for diagnosing typhoid fever and acute typhoid fever in a patient using isolated Salmonella enterica serotype Typhi antigens or proteins. Induction of a T-cell response upon contact with one or more of the isolated antigens or proteins indicates that the patient has typhoid fever.

ASSAYS, COMPOSITIONS, SYSTEMS, KITS, AND DEVICES FOR DIAGNOSING TYPHOID FEVER

SEQUENCE LISTING

[0001] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 30, 2012, is named 030258PC.txt and is 37,989 bytes in size.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] The present application claims benefit under 35 U.S.C. §119(e) of U.S. provisional application Serial No. 61/474,099 filed on April 11, 2011 and U.S. provisional application Serial No. 61/507,867 filed on July 14, 2011, the contents of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

[0003] Disclosed are assays, composition, systems, kits, and devices for diagnosis of typhoid fever in humans.

BACKGROUND OF THE INVENTION

[0004] *Salmonella enterica* serotype *Typhi* is a human-restricted intracellular pathogen and the cause of typhoid fever. Cellular immune responses are required to control and clear *Salmonella* infection. Despite this, there are limited data on cellular immune responses in humans infected with wild type *S. Typhi*.

[0005] Typhoid fever is a major cause of illness in resource-limited countries and a frequent cause of outbreaks in higher-income countries. Current typhoid diagnostic assays lack both sensitivity and specificity, especially in endemic zones, and are costly in both time and expense. For example, blood culture is only about 30-70% sensitive and requires a minimum of 48-72 hours from collection to reporting to the health provider. The serodiagnosis of typhoid infection using the Widal agglutination assay, relies on patients' antibodies to the O=9,12 lipopolysaccharide (LPS) proteins, H=d flagellar proteins, and the Vi capsular proteins. The Widal assay lacks sensitivity and specificity. In addition, to detect a four fold change in the amount of antibodies required for diagnosis using Widal assay, one needs both acute and convalescent blood samples. This essentially means that acute first time infections cannot be diagnosed using the method. Moreover, the Widal assay has as at best 85% specificity when analyzing both acute and convalescent phase responses in endemic zones.

[0006] Accordingly, diagnostic assays and methods for typhoid fever, also called enteric fever, that provide more accurate results, particularly at time of acute illness, are greatly needed.

SUMMARY OF THE INVENTION

[0007] The present invention provides proteins and methods for analyzing them that are useful in diagnosing typhoid fever including an acute typhoid fever. The methods, assays, kits, devices and systems described herein use one or more of the specific newly identified proteins, which have been discovered to induce a T-cell mediated cytokine response, including interferon-gamma (IFN- γ), IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a, response in blood cells from a patient with typhoid fever, including those patients in acute stage of typhoid fever, but not in a control. If the cytokine amount in the patient's sample in response to contact with one or more of these proteins is increased, the patient can be diagnosed as having typhoid fever or acute typhoid fever. If the amount is not increased, the condition in the patient is not typhoid fever or acute typhoid fever. Assays, kits, devices, and compositions for the diagnosis of typhoid fever and acute typhoid fever are thus provided.

[0008] The invention is based, at least in part, on the analysis of cellular immune responses or T cell responses to purified *S. Typhi* proteins in patients with typhoid fever. The analysis showed that affected patients generate significant CD4 and CD8 IFN- γ responses to specific *S. Typhi* proteins during typhoid fever, and that these responses are elevated at the time of clinical presentation. These data show that a cytokine based, such as an IFN- γ based, detection system can now be used to diagnose individuals with typhoid fever during the acute stage of illness.

[0009] Accordingly, in one embodiment, the invention provides an in vitro assay comprising the steps of: (a) contacting and incubating a biological sample comprising T-cells obtained from a human individual with at least one isolated and purified *S. Typhi* protein or a fragment thereof; and (b) detecting and quantifying the amount of *S. Typhi* protein responsive cytokine in the incubated sample.

[00010] In some aspects and all aspects of this embodiment, the at least one isolated and purified *S. Typhi* protein is selected from the group consisting of STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195, STY0206, STY0595, STY0909, STY1375, STY1522, STY2167 FliC, STY2381, STY3089, STY3090, STY3796 or any combination thereof.

[00011] In some aspects and all aspects of this embodiment, the at least one protein is selected from the group consisting of STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195 and any combination thereof.

[00012] In some aspects and all aspects of this embodiment, the assay further comprises a step of comparing the cytokine amount to a reference and diagnosing a typhoid fever in the human individual if the amount of cytokine amount is increased compared to a reference value.

[00013] In some embodiments, the typhoid fever is diagnosed if the cytokine amount is increased about 1.5 fold or more compared to the reference value. The increase in some embodiments can be more than 10%, more than 15%, more than 20%, more than 25%, more than 30%, more than 35%, more than 40%, more than 50%, more than 60%, more than 70%, more than 75%, more than 80%, more than 90%, or more than 100% compared to a reference. The increased amount can also be more than 2 fold of the reference.

[00014] In some aspects and all aspects of this embodiment, the typhoid fever is acute typhoid fever.

[00015] In some aspects and all aspects of this embodiment, the *S. Typhi* protein responsive cytokine is selected from the group consisting of IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a or any combination thereof. In some embodiments, the *S. Typhi* protein responsive cytokine is IFN- γ .

[00016] In some aspects and all aspects of this embodiment, the biological sample is a blood sample. In some embodiment, the blood sample is a fresh blood sample. In some embodiments, the biological sample is peripheral blood mononuclear cell sample.

[00017] In some aspects and all aspects of this embodiment, the step of detecting and quantifying the amount of *S. Typhi* protein responsive cytokine is performed using a stereomicroscope, an ELISA, an ELISPOT, a FLUOROSPOT, a FACS, an FP, an FLT or a FRET.

[00018] In some aspects and all aspects of this embodiment, the step of comparing the cytokine amount to a reference and/or diagnosing a typhoid fever is performed by a non-human machine.

[00019] In some aspects and all aspects of this embodiment, the non-human machine comprises a computer implemented software.

[00020] In another embodiment, the invention provides a computer program comprising computer program code means adapted to perform all the steps of any of the assays set forth herein when said program is run on a computer.

[00021] In yet another embodiment, the invention provides a composition comprising at least one isolated and purified protein selected from the group consisting of STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195, STY0206, STY0595, STY0909, STY1375, STY1522, STY2167 FliC, STY2381, STY3089, STY3090, and STY3796 or any combination thereof. The composition may be provided in a dry form or in a suitable solution, such as a storage buffer or a buffer suitable for incubating the protein with the biological sample.

[00022] In some aspects and all aspects of this embodiment, the at least one isolated and purified protein is selected from the group consisting of STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195 and any combination thereof.

[00023] The invention further provides a kit comprising the compositions comprising at least one isolated and purified protein selected from the group consisting of STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195, STY0206, STY0595, STY0909, STY1375, STY1522, STY2167 FliC, STY2381, STY3089, STY3090, and STY3796 or any combination thereof or at least one isolated and purified protein selected from the group consisting of STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195 and any combination thereof.

[00024] In some aspects and all aspects of this embodiment, the kit further comprises laboratory materials and instructions to implement an assay as described in any and all embodiments described above.

[00025] In some aspects and all aspects of this embodiment the kit further comprises a washing buffer.

[00026] In some aspects and all aspects of this embodiment the kit further comprises a solid support comprising an antibody against at least one of *S. Typhi* protein responsive cytokine against at least wherein the solid support is an ELISA plate and the marker is an enzyme.

[00027] In some aspects and all aspects of this embodiment the *S. Typhi* protein responsive cytokine in the kit is selected from the group consisting of IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a.

[00028] In some aspects and all aspects of this embodiment, the kit further comprises a secondary antibody directed against at least one *S. Typhi* protein responsive cytokine. Preferably the secondary antibody is against a different epitope than the first antibody used to bind the *S. Typhi* protein.

[00029] In some aspects and all aspects of this embodiment, the at least one *S. Typhi* protein responsive cytokine is selected from IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a.

[00030] In some aspects and all aspects of this embodiment, the secondary antibody is labeled with a detectable label.

[00031] In yet another embodiment, the invention provides a microfluidic device comprising at least one inlet for adding a blood sample; at least one chamber comprising at least one isolated and purified *S. Typhi* protein; and at least one chamber comprising at least one antibody against an *S. Typhi* protein responsive cytokine. In some aspects and all aspects of this embodiment, the microfluidic device may further comprise at least one chamber comprising at least one secondary antibody against the at least one *S. Typhi* protein responsive cytokine.

[00032] In some aspects of this embodiment, the at least one isolated and purified *S. Typhi* protein is selected from the group consisting of STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195, STY0206, STY0595, STY0909, STY1375, STY1522, STY2167 FliC, STY2381, STY3089, STY3090, STY3796 or any combination thereof.

[00033] In some aspects, the *S. Typhi* protein responsive cytokine is selected from the group consisting of IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a.

[00034] In some embodiments the device further comprises a chamber comprising at least one secondary antibody against the at least one *S. Typhi* protein responsive cytokine.

BRIEF DESCRIPTION OF DRAWINGS

[00035] Figure 1 shows interferon- γ ELISPOT responses to *S. Typhi* proteins including StaF (STY0202), CsgF (STY1177), PagC (STY1878), StbB (STY0372), CsgD (STY1179), OppA (STY1304), conserved hypothetical protein encoded by STY2195, *S. Typhi* membrane preparation (MP), control protein, and phytohaemagglutinin (PHA) and keyhole limpet hemocyanin (KLH) during acute and convalescent stage illness in *S. Typhi* bacteremic patients and in healthy controls (HC). Mean and standard error of the mean represented.

[00036] Figure 2 shows characterization of interferon- γ CD4 and CD8 responses to *S. Typhi* proteins including StaF (STY0202), PagC (STY1878), *S. Typhi* membrane preparation (MP), and PMA and keyhole limpet hemocyanin (KLH) during acute and convalescent stage illness in *S. Typhi* bacteremic patients. Mean and standard error of the mean represented.

[00037] Figure 3 shows cellular proliferation responses to *S. Typhi* proteins including StaF (STY0202), PagC (STY1878), conserved hypothetical protein encoded by STY2195, *S. Typhi* membrane preparation (MP), control protein, and phytohaemagglutinin (PHA) and keyhole limpet hemocyanin (KLH) during acute and convalescent stage illness in *S. Typhi* bacteremic patients and in healthy controls (HC). Stimulation index: net cpm with protein /net cpm without protein (media alone) for each individual on each day (acute and convalescent). Mean and standard error of the mean represented.

DETAILED DESCRIPTION OF THE INVENTION

[00038] Cellular immune responses, including CD4 and CD8-mediated interferon- γ responses, play a critical role in clearing and controlling systemic Salmonella infections [23, 40]. Despite this, there has been limited evaluation of cellular responses in humans to wild-type *S. Typhi*. No animal model fully replicates host-pathogen interactions and immunologic events that occur during this human-restricted infection. Evaluation in humans has largely focused on characterizing responses in recipients of attenuated vaccine strains of *S. Typhi* [23, 38, 41, 42, 43, 44].

[00039] The present invention provides novel assays, devices, compositions, and kits for diagnosing typhoid fever in humans.

[00040] There are about 4400 *S. Typhi* proteins. We have discovered a set of specific *S. Typhi* proteins that result in excess cytokine production in patients with typhoid fever, including those with acute typhoid fever but not in controls or patients whose symptoms are not caused by *S. Typhi*. The invention thus provides various assays for the detection of cytokine responses in patients to diagnose typhoid fever. In the assays, the patient sample comprising T cells is incubated with at least one of the isolated and purified *S. Typhi* proteins that were identified as causing excess cytokine secretion, and the amount of one or more secreted cytokines is measured. In some aspects, the sample is taken from a human in need to diagnosis for typhoid fever.

[00041] Thus the present invention provides a method of assaying for selected *S. Typhi* protein-specific T-cells. The method comprises providing a biological fluid containing T-cells, adding an isolated and purified peptide to the fluid, incubating the fluid to cause cytokine release, and detecting the released cytokine. In some embodiments, the method

comprises contacting the fluid containing T-cells with a surface carrying an immobilized first antibody to the cytokine, adding the isolated and purified *S. Typhi* protein or a fragment thereof to the fluid, incubating the resulting fluid mixture under conditions to cause any peptide-specific T-cells that have been pre-sensitized *in vivo* to the peptide to secrete the cytokine, and detecting any secreted cytokine bound to the immobilized first antibody.

[00042] The assays described herein are generally based on a method of assaying for peptide-specific T-cells comprising adding peptide to a biological fluid sample comprising cells of fresh peripheral blood mononuclear cells, and detecting a cytokine produced by T-cells that have been pre-sensitized to the peptide. For example, U.S. Patent No. 7,575,870, incorporated herein by reference in its entirety, sets forth the general steps of the assay. However, the '870 patent does not set forth how the method could be used in diagnosis of typhoid fever including acute typhoid fever. Moreover, the '870 patent only describes the method for diagnosing viral diseases and not for bacterial diseases.

[00043] The biological sample used in the assays of the invention must comprise T cells. Thus, the biological sample can be, for example, blood, bone marrow or peripheral mononuclear cells (PMBC). In some embodiments the PMBC are isolated from blood. In some embodiments, fresh blood sample is used.

[00044] In some embodiments, the biological sample comprises PMBC or isolated PMBC. These cells may suitably be taken from a patient, such as a patient suspected of being infected or having symptoms of typhoid fever. In some embodiments, fresh cells are used, because cells cultured *in vitro* may develop altered characteristics thus reducing the diagnostic value of the assay. The purpose of the assay is to identify or quantitatively measure *S. Typhi* peptide- or protein-specific T-cells e.g. CD8+ or CD4+ cells that have been activated or pre-sensitized *in vivo* to a one or more of the particular *S. Typhi* peptides described herein. These are unrestimulated T-cells, i.e. cells capable of immediate effector function without the need to effect division/differentiation by *in vitro* culture. When the peptide in question is presented to such cells, the cells secrete various cytokines, herein referred to as *S. Typhi* protein specific cytokines, of which any one may be selected for the purposes of this assay.

[00045] The secreted cytokine can be detected by any of a variety of methods known in the literature. In some embodiments, the assay involves providing a surface carrying an immobilised first antibody to the cytokine. A biological fluid containing the PBMC or other fresh cells is placed in contact with that immobilised antibody. About 30% of the PBMC are CD8+ cells.

[00046] The method of the invention involves adding the isolated and purified or recombinant *S. Typhi* protein or peptide or fragment thereof to the biological fluid comprising T-cells. The resulting fluid mixture is incubated under conditions to stimulate any peptide-specific T-cells that may have been pre-sensitized to the *S. Typhi*-derived peptide *in vivo*. The peptide or peptide fragment typically needs to be of a length, e.g., about 6-15 amino acids, for example, 8-12 or 8-10, amino acid residues long, that is recognised by CD8+ cells. It has been proposed, that the generality of the CD8+ cells (and other PBMC) present the peptide to the small minority of CD8+ cells that may have been pre-sensitised to the *S. Typhi* peptide. If such activated or pre-sensitized peptide-specific T-cells are present in the test biological fluid, they respond by secreting a cytokine which then becomes bound to the, e.g., immobilized antibody.

[00047] The term "fragment" generally refers to any subject polypeptide having an amino acid residue sequence shorter than that of a polypeptide whose amino acid residue sequence is described herein. The fragments of the *S. Typhi* proteins useful according to the present invention are typically at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 amino acids long. For example, fragments of the length 6-20 or 8-12 consecutive amino acids from any of the disclosed *S. Typhi* proteins can be used.

[00048] In some aspects, the entire protein may be used, but it may be digested into smaller fragments. Thus, the invention provides compositions comprising fragments of any one or more of the *S. Typhi* proteins described herein. Such fragments may also be part of a kit or a device or an assay. The fragments typically comprise one or more epitopes.

[00049] An "epitope" is the collective features of a molecule, such as primary, secondary and tertiary peptide structure, and charge, that together form a site recognized by an immunoglobulin, T cell receptor or HLA molecule. Alternatively, an epitope can be defined as a set of amino acid residues which is involved in recognition by a particular immunoglobulin, or in the context of T cells, those residues necessary for recognition by T cell receptor proteins and/or Major Histocompatibility Complex (MHC) receptors.

[00050] Epitopes are present in nature, and can be isolated, purified or otherwise prepared/derived by human or non-human means. For example, epitopes can be prepared by isolating the *S. Typhi* peptides from a natural source, such as a bacterial culture, or they can be synthesized in accordance with standard protocols in the art.

[00051] The protein or polypeptide molecules that comprise one or more *S. Typhi* peptide epitopes of the invention as well as additional amino acid(s) are still within the

bounds of the invention. In certain embodiments, there is a limitation on the length of a polypeptide of the invention of, for example, not more than 120 amino acids, not more than 110 amino acids, not more than 100 amino acids, not more than 95 amino acids, not more than 90 amino acids, not more than 85 amino acids, not more than 80 amino acids, not more than 75 amino acids, not more than 70 amino acids, not more than 65 amino acids, not more than 60 amino acids, not more than 55 amino acids, not more than 50 amino acids, not more than 45 amino acids, not more than 40 amino acids, not more than 35 amino acids, not more than 30 amino acids, not more than 25 amino acids, 20 amino acids, 15 amino acids, or 14, 13, 12, 11, 10, 9 or 8 amino acids. In some instances, the embodiment that is length-limited occurs when the protein/polypeptide comprising an epitope of the invention comprises a region (i.e., a contiguous series of amino acids) having 100% identity with a native sequence.

[00052] In some embodiments, the *S. Typhi* peptide or protein is added in uncombined form to the fresh cells. While it is possible to add cultured cells that have been pulsed with the peptide, this is not necessary when using peptide epitopes. The peptides should be added in an amount sufficient to generate an observable signal; a preferred concentration range in the fluid is 0.01 up to 100 μM particularly 0.5-5.0 μM . In some embodiments, the peptide concentration in the incubation is about concentration 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 $\mu\text{g/ml}$. In some embodiments, concentration is about 0.7 $\mu\text{g/ml}$.

[00053] Incubation should be continued for a time sufficient to permit CD8+ cells that have been pre-sensitized *in vivo* to the particular peptide chosen to secrete the cytokine. The incubation should not continue for so long that quiescent CD8+ cells have time to differentiate and become activated by the peptide and start to secrete cytokines. This suggests an incubation time of about 4-24 hours, for example 20 hours. To speed up the assay, the incubation in some embodiments is performed within in a single working day or overnight, and without the use of sterile conditions required for cell culture *in vitro*.

[00054] During the incubation, any cytokine secreted by CD8+ cells becomes bound to the first antibody, which may be, immobilized on the surface. After incubation, the surface may be washed to remove unbound material. For detection, typically a labeled second antibody to the cytokine is used. When this is applied to the surface it becomes bound to any cytokine present. The second antibody should recognize a different epitope from the first antibody to allow effective binding to the cytokine. One or both of the first and second antibodies should preferably be monoclonal. The label may be any that is conventionally used in the field, including radioisotopes, enzymes to generate color or chemiluminescence,

fluorescent groups or groups for detection by mass spectrometry or refractive index (e.g. by surface plasmon resonance). It is convenient but not necessary to use a labeled antibody, any reagent that binds specifically to the cytokine could be labeled and used. Detection and perhaps quantification of the label is effected by means well known in the field and appropriate to the nature of the label used.

[00055] The assay may be conveniently carried out, for example, in a multiwell plate or on a microfluidic device. For example, each well of the plate has a surface carrying a bound first antibody. To each well is added a fluid containing an appropriate number, e.g. 10^3 - 10^6 of cells. Different peptides and/or controls can be added to individual wells of the plate or channels of the microfluidic device. Cells that secrete a cytokine during incubation show up as spots (spot forming cells or SFCs) and the number or density of these in each well can readily be determined.

[00056] Accordingly, the presently described assay comprises the steps of contacting, *in vitro*, biological sample comprising T-cells from a human patient with one or more *S. Typhi* proteins or fragments thereof, incubating the blood sample with the protein(s), and measuring the amount of one or more *S. Typhi* protein responsive cytokines, that is/are secreted by the cells in the blood sample in response to the contact with the *S. Typhi* protein(s).

[00057] The assay can be generally performed as described, e.g., in U.S. Patent No. 7,575,870, incorporated herein by reference in its entirety.

[00058] The assay can further comprise a step of diagnosing the patient as having typhoid fever if the amount of the cytokine is increased to at least about 1.5 fold or more compared to a reference amount. In some aspects of all the embodiments described in this application, the typhoid fever is acute typhoid fever. The step of diagnosing can be performed automatically, by a machine or a computer implemented software.

[00059] The *S. Typhi* proteins we have identified as eliciting this patient-specific and cytokine-specific response comprise STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195, STY0206, STY0595, STY0909, STY1375, STY1522, STY2167 FliC, STY2381, STY3089, STY3090, STY3796.

[00060] Any method well known from the skilled person may be used to obtain these proteins or fragments thereof, such as by isolation and purification, by chemical synthesis (solid phase method) or by molecular biology using nucleic acids encoding said proteins or fragments and expression vectors to make recombinant proteins or fragments thereof which may be further purified.

[00061] In the present invention, "isolated" refers to material removed from its native environment (e.g., the natural environment if it is naturally occurring), and thus is altered "by the hand of man" from its natural state.

[00062] The cytokines that show this specific and statistically significant relationship comprise *S. Typhi* responsive proteins, including, IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a. The group of cytokines consisting of IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a is herein also referred to as "specific *S. Typhi* protein responsive cytokines." In some aspects of all the embodiments of the invention, the cytokine is IFN- γ .

[00063] In some embodiments, a crude extract of *S. Typhi* can also be used to induce cytokine secretion to differentiate human patients who have acute typhoid fever from patients who do not have it.

[00064] Cytokine amount that is about 1.5 fold or higher than a reference, which is based on the normal or control cytokine level in a human, indicates that the human patient has a typhoid fever. In some aspects, the diagnosis of acute typhoid fever can be made when the cytokine amount is at least about 1.5 fold or more than the reference. The increase can also be measured in percentages compared to the reference, wherein at least 10% or more increase is indicative of increased cytokine amount to diagnose typhoid fever. In some aspects, an increase of 50% or more is used as a diagnostic threshold.

[00065] The amount of cytokines can be determined, measured or detected by well known methods to one skilled in the art. For example, one can measure the cytokines using antibodies that form immunocomplexes with the cytokines. The appropriate mediums, appropriate conditions, and protocols for the formation of the immunocomplexes are known for one skilled in the art working in the field of immunology. By way of example, various methods and protocols which can be used are described in "Current Protocols in Immunology" annually updated (4 volumes) and edited by the "National Institute of Health" by John E. Coligan, Ada M. Kruisbeek, David H. Margulies, Ethan M. Shevach, Warren Strober-Wiley Interscience.

[00066] The diagnosis can be done automatically by a computer implemented software executing a code that instructs it to set forth an output on a computer screen, via facsimile, to a file that can be e-mailed or posted to a secure server or printed out the amount detected in the patient sample and a reference value. The software can also execute instructions wherein if an amount of 1.5 or more times that of a reference value of the selected cytokines is detected, an output is generated either numerically, verbally, in color code, graph, or notation,

such as an asterisk, that indicates that the amount is abnormal or that the amount indicates a diagnosis typhoid fever or acute typhoid fever.

[00067] The blood sample can be whole blood or peripheral blood mononuclear cells (PBMC) isolated from blood.

[00068] The terms “patient”, “individual”, and “subject” used herein and throughout the specification all refer to human.

[00069] The term “reference value” means any reference range or maximum cut-off number of the amount of cytokines secreted by human cells that are taken from an individual not infected with *S. Typhi*. The reference value can be a number or a numeric range, a cut-off value or a graph or even sliding color scale showing normal cytokine levels or it can be a value obtained from a control sample that is run in a parallel reaction with the test sample.

[00070] In one embodiment, the amount is determined using a solid support, such as an ELISA plate and the method comprises: (a) incubating the blood sample or cells isolated from the blood sample with one or more *S. Typhi* protein on an ELISA plate coated with an antibody against the cytokine; (b) eliminating from the ELISA plate the cytokines not bound to the cytokine antibodies; (c) contacting the bound proteins with a secondary antibody conjugated with an enzyme, said secondary antibodies being capable of binding to the proteins of said sample, (d) eliminating from the ELISA plate the secondary antibodies not bound to the proteins of said serum or plasma sample, (e) adding the corresponding soluble substrate for the enzyme, and (f) reading the absorbance values of the wells of the ELISA plate in an ELISA reader at an appropriate wavelength, wherein the quantity of said cytokines is determined by means of the obtained absorbance values.

[00071] The detection can also be performed using an Enzyme-linked immunosorbent spot (ELISPOT) assay. ELISPOT assay allows visualization of the secretory product of individual activated or responding cells. ELISPOT assay is much more sensitive than ELISA assay and allows a convenient and better frequency analysis of rare cell populations, e.g., antigen-specific responses, such as *S. Typhi* protein or antigen. The exceptional sensitivity is in part because the product is rapidly captured around the secreting cell: before it is either diluted in the supernatant, captured by receptors of adjacent cells, or degraded. This makes ELISPOT assays much more sensitive than conventional ELISA measurements. Limits of detection are below 1/100,000 rendering enumerate the actively producing cells. This allows much of the analysis process to be automated, and permits a greater level of accuracy than what can be achieved using manual inspection.

[00072] Generally, the ELISPOT assays employ a technique very similar to the sandwich enzyme-linked immunosorbent assay (ELISA) technique. Either a monoclonal (preferred for greater specificity) or polyclonal capture antibody that is coated aseptically onto a PVDF (polyvinylidene fluoride) -backed microplate. These antibodies are chosen for their specificity for the analyte in question. The plate is blocked, usually with a serum protein that is non-reactive with any of the antibodies in the assay. After this, cells of interest are plated out at varying densities, along with antigen or mitogen, and then placed in a humidified 37°C CO₂ incubator for a specified period of time.

[00073] Cytokine secreted by activated cells is captured locally by the coated antibody on the high surface area PVDF membrane. After washing the wells to remove cells, debris, and media components, a biotinylated polyclonal antibody specific for the chosen analyte is added to the wells. This antibody is reactive with a distinct epitope of the target cytokine and thus is employed to detect the captured cytokine. Following a wash to remove any unbound biotinylated antibody, the detected cytokine is then visualized using an avidin-HRP, and a precipitating substrate (e.g., AEC, BCIP/NBT). The colored end product (a spot, usually a blackish blue) typically represents an individual cytokine-producing cell. The spots can be counted manually (e.g., with a dissecting microscope or a stereomicroscope) or using an automated reader to capture the microwell images and to analyze spot number and size.

[00074] The assay can also be a FluoroSpot assay, which is a modification of the ELISPOT assay. FluoroSpot can measure the secretion of several cytokines in each well by the use of fluorescent anti-cytokine antibodies. FluoroSpot assay is particularly useful if the methods of the invention are used to analyze two or more different cytokines as it can be applied to essentially any system where one wants to investigate dual or triple protein secretion at the single cell level or where one wants to benefit from the fact that several cytokines can be assessed simultaneously in the same well, for example when there is a limited supply of cells.

[00075] The principle and procedure for FluoroSpot is similar to ELISPOT. However, to detect cells secreting e.g. two different cytokines, a mixture of two capture antibodies, specific for the two cytokines, is coated in each well of a 96-well plate. Further on, after cellular stimulation, incubation and subsequent washing away of the cells, a mixture of two anti-cytokine antibodies is added to detect the secreted cytokines. To enable a separate detection of the two secreted cytokines, the two anti-cytokine antibodies are labelled with different tags, e.g. biotin and FITC. Following a wash to remove unbound antibody, the secreted cytokines are visualized by addition of a mixture of Streptavidin labelled with red

fluorochrome and antibody to FITC labelled with green fluorochrome. The membrane of a well will thus contain a mixture of green and red spots. A cell, which has secreted both cytokines, will thus give rise to a green and a red spot at the same position on the membrane.

[00076] To analyze the frequency of single and double-secreting cells, an automated FluoroSpot reader, equipped with separate filters for green and red fluorescence and software that detects single and co-localized green and red fluorescent spots, is preferred. Visually, spots representing dual secreting cells will appear as yellow in an overlay of green and red spot images (see Fig 2). By counting the number of spots in stimulated cultures and controls without stimulus the frequency of responding single and double secreting cells is determined.

[00077] Fluorescent activated cell sorting or FACS method can also be used to detect and quantify the cytokines. The antibodies are also in this method labeled with a fluorescent dye. Data analysis from FACS is typically performed with a computer.

[00078] Fluorescent polarization or FP analysis or a fluorescence polarization immunoassay (FPIA) can be used to detect and measure the cytokine response in the assays of the present invention.

[00079] In some aspects, a fluorescence resonance energy transfer (FRET) can be used to detect and measure the cytokine response in the assays of the present invention.

[00080] In certain aspects, a Fluorescence lifetime (FLT) assay can be used to detect and measure the cytokine response in the assays of the present invention.

[00081] The biological fluid can be peripheral blood mononuclear cells (PMBC). They may suitably isolated, e.g., from a patient's blood. In some embodiments, fresh cells are used, because cells cultured *in vitro* may develop altered characteristics thus reducing the diagnostic value of the assay. The purpose of the assay is to identify or quantitate peptide-specific T-cells e.g. CD8+ or CD4+ cells that have been activated or pre-sensitized *in vivo* to a particular peptide. These are unrestimulated T-cells, i.e. cells capable of immediate effector function without the need to effect division/differentiation by *in vitro* culture. When the peptide in question is presented to such cells, the cells secrete various cytokines, of which any one may be selected for the purposes of this assay.

[00082] In one embodiment, the method comprises use of coated 96-well nitrocellulose plates (Multiscreen HTS, Millipore). Plates can be coated, e.g., with e.g., about 100 μ l of 15 μ g/ml human monoclonal anti-interferon- γ antibody (1-D1K), e.g., about 8-24 hours, e.g., overnight at or at about 4°C. The plates can be washed and subsequently blocked with, e.g., about 10% FBS for about 2 h at room temperature.

[00083] For example, PBMCs from individual patients, and optionally controls, can be added at a concentration of 2×10^5 per well.

[00084] Individual *S. Typhi* proteins or control protein are added to wells at a concentration of, e.g., 140 ng/well of total preparation for each purified protein (in 200 μ l culture, final concentration 0.7 μ g/ml).

[00085] In separate wells, one can also optionally add *S. Typhi* membrane preparation at a final concentration of 10 μ g/ml in 200 μ l culture, phytohaemagglutinin (PHA; Murex Diagnostics Ltd, Temple Hill, UK) at a final concentration of 2.5 μ g/ml in 200 μ l culture, and keyhole limpet hemocyanin (KLH). One can further include additional control wells with media but lacking protein.

[00086] Following incubation of the plates at or at about 37°C in 5% CO₂ for 15-24, e.g., 20 hours, plates are washed, labeled, such as biotinylated monoclonal anti-cytokine, such as anti-interferon- γ antibody (7-B6-1-biotin; 1 500 dilution), is added and the plates are incubated at room temperature for an additional 1-4 hours, such as 2 hours. The wells are then washed, and streptavidin-HRP (1 500 dilution) added, and the wells are re-incubated for about 30 min-2 hours, such as 1 hour at room temperature.

[00087] If biotin label is used, the plates can be developed with aminoethylcarbazol plus H₂O₂, and the amount of interferon- γ secreted by the cells or the number of interferon- γ secreting cells can be counted. In some embodiments, the counting is performed using a stereomicroscope.

[00088] In some embodiments, the cytokine antibodies are coated on a solid support, such as a plate, a strip or a bead. The cytokine antibodies can be coated to different location (plate or a strip) or a different batch (beads) to allow measurement of one or more cytokines simultaneously.

[00089] The coating may be realized on various solid supports known from the skilled person, directly or indirectly using a spacer. The solid supports may include the glass, polystyrene, polypropylene, polyethylene, dextran, nylon or natural or modified celluloses. These supports may be soluble or insoluble. The support may also consist in microbeads, an enzyme linked immunosorbent assay (ELISA) strip or in a microtiter plate such as an (ELISA) plate.

[00090] Markers which may be used are well known from the skilled person and may be selected from the enzymes, the dyes, the luminescent agents such as the radioluminescent (such as ¹⁴C, ³⁶Cl, ⁵⁷Co, ⁵⁸Co, ⁵¹Cr, ¹⁵²Eu, ⁵⁹Fe, ³H, ¹²⁵I, ¹³¹I, ³²P, ³⁵S, ⁷⁵SE and ⁹⁹mTc which may be detected using for example gamma-ray counter or scintillation counter,

autoradiography), bioluminescent, chemiluminescent (luminol, dioxetane, luciferase, luciferin), fluorescent, and phosphorescent agents, the ligands such as Worm, avidin, streptavidin, digoxigenin, 5-bromo-deoxyuridine, radioactive isotopes. The secondary antibodies are conjugated for example with enzymes such as peroxidase, alkaline phosphatase, β -galactosidase, glucose oxydase, glucose amylase, anhydrase, acetylcholinesterase, lysozyme, la malate dehydrogenase or glucose-6 phosphate dehydrogenase. Fluorescent markers may be for example the fluorescein and its derived products, the fluorescein isothiocyanate (FITC), the allophycocyanin (APC), the phycoerythrin-cyanin 5 (PC5) and the phycoerythrine (PE), the calcein (AM), the red fluorescent tetramethyl-rhodamin or the rhodamin and its derived products, the GFP (Green Fluorescent Protein), the dansyl, the umbelliferone etc.

[00091] The enzyme and corresponding soluble substrate can be selected from the following combinations comprising: the alkaline phosphatase and the soluble substrate 4-nitrophenyl phosphate (PNPP); the peroxidase and the soluble substrate orthophenylene diamine (OPD); the β -galactosidase and the soluble substrate 2-nitrophenyl β -galactoside (ONPG); or the glucose-6-phosphate dehydrogenase and the soluble substrate glucose-6-phosphate (G6P).

Production of proteins

[00092] Preferably, the at least one recombinant *S. Typhi* protein is obtained by cloning the amplification product of the corresponding cDNA in an expression vector. The amplification product of the corresponding cDNA is obtained from an *S. Typhi* cDNA library using a couple of primers specific for said at least one *S. Typhi* protein.

[00093] Fragments can be produced either by enzymatic digestion of the proteins or by recombinant production using a nucleic acid encoding the desired fragment.

[00094] The skilled person has at his disposal the molecular and cellular biology tools to realize the cloning and recombinant expression of the STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195, STY0206, STY0595, STY0909, STY1375, STY1522, STY2167 FliC, STY2381, STY3089, STY3090, STY3796 proteins (see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989) and Ausubel et al., Current Protocols in Molecular Biology, J. Wiley & Sons, NY (1992)). A wide variety of expression host/vector combinations may be employed, such as bacterial hosts and corresponding known bacterial plasmids, yeast cells/yeast expression vectors, insect cells/insect expression vectors,

mammals cells/mammal expression vectors, etc.. In addition, any suitable expression control sequence may be used in these vectors.

[00095] For example, we selected *S. Typhi* proteins contained within operons identified during our previous application of IVIAT to *S. Typhi* [28]. IVIAT identifies proteins expressed *in vivo* during human infection and that generate an antibody response [28]. We obtained pDONR221 Gateway Based entry clones of the *S. Typhi* CT18 genes corresponding to selected proteins from the NIAID-sponsored Pathogen Functional Genomic Resource Center, J. Craig Venter Institute (JCVI, formerly The Institute for Genomic Research). We used LR clonase II enzyme reactions (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions to move inserts into pDEST17 (Invitrogen, Carlsbad, CA) to generate a fusion containing an amino terminal 6 × histidine (HIS) tag (SEQ ID NO: 19). We transformed DH5alpha-T1R competent cells with LR reactions and selected for ampicillin resistance. We confirmed insert presence by restriction digestion and PCR analysis and transformed purified plasmids into *E. coli* protein expression strain BL21 star (DE3) pLysS (Invitrogen).

[00096] We grew transformants harboring recombinant plasmids at 37°C as 1.5 ml cultures in 96-well blocks (Marsh Biomedical Products) to an OD600 of 0.6-0.8. We induced cultures with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) on a 96-well plate shaker (Multitron) (× 900 rpm). After 3 hours at 37°C, we harvested cells at 4°C and stored preparations at - 80°C for further use. We also induced BL21 star (DE3) pLysS containing pDEST17 but lacking an *S. Typhi* insert. This construct produced a truncated HIS-tagged protein MSYYHHHHHHLESTSLYKKAERERKMI (SEQ ID NO: 1) that we recovered and used as a control protein in immunological assays.

[00097] We performed protein purifications in an automated manner in 96-well plates using a BiomekFx (Beckman Coulter) robotic liquid handler as previously described [27]. For this 6xHIS (SEQ ID NO: 19) denaturing affinity purification, we thawed cell pellets at room temperature for 15 min, lysed them in the presence of protease inhibitors in 115 µl lysis buffer I (100 mM NaH₂PO₄, 10 mM Tris, pH 8.0), robotically resuspended product in a 96-well block and agitated at 900 rpm for 10 min (5 min in the clockwise direction and 5 min in the counterclockwise direction). We then added 10 µl of DNase mix (10 mg/ml DNase; Sigma Aldrich in 900 mM MgCl₂, 100 mM MnCl₂) to the lysate and agitated the preparation at 900 rpm for 10 min. Next, we added 115 µl of lysis buffer II (100 mM NaH₂PO₄, 10 mM Tris, 6 M guanidine hydrochloride, 10 mM, 2-mercaptoethanol, pH 8.0) to create denaturing conditions. We then allowed these cell lysates to bind to 30 µl of MagneHIS beads

(Promega) with shaking at 900 rpm for 20 min (10 min clockwise, 10 min counterclockwise), and separated beads using a magnabot (24-pin magnet; Promega). The robotic liquid handler then washed the MagneHIS beads with bound protein three times with wash buffer (100 mM NaH_2PO_4 , 10 mM Tris, 8M urea). We prevented bead adherence to the walls during washing by shaking the samples at 900 rpm for 2.5 min clockwise and then 2.5 min counterclockwise. We then washed the beads with bound protein using 100 μl of distilled water, and added 50 μl distilled water to make the final suspensions for analysis. We repeated this extraction cycle six times.

[00098] We analyzed proteins in an automated 96-well format using a capillary-based instrument, the LabChip90 (Caliper Sciences). We automated a system that resuspended 3 μl of protein sample in 7 μl analysis buffer (Caliper Sciences), heated these to 96°C for 5 min., cooled them to room temperature, and briefly centrifuged to collect the sample. We added distilled water (35 μl) to each sample prior to analysis. We primed the analysis chip (Caliper Sciences) according to the manufacturer's instructions. The automated protein analysis generated three different forms of output: a chromatogram that showed migration time; a virtual gel that mimicked a Coomassie stained gel; and a results table that included the estimated size, quality, and quantity of each peak. The LabChip90 analyzed 96 proteins at a time with analysis time of 40 seconds per sample. We parsed the output results and imported them into the Harvard Institute of Proteomics protein database. We assessed for presence of contaminating *E. coli* LPS using a HEK-Blue LPS Detection kit (InvivoGen, San Diego, CA).

[00099] Production and mass spectrometric analysis of *S. Typhi* crude membrane preparation. We prepared *S. Typhi* membrane preparation as previously described [29,30]. Briefly, we cultured *S. Typhi* Ty21a on sheep blood agar plates and harvested in Tris buffer (10 mM Tris, pH 8.0, 5 mM MgCl_2). We sonicated the mixture, and centrifuged at $1400 \times g$ for 10 minutes and transferred the supernatant to fresh tubes, centrifuging at $14900 \times g$ for 30 minutes. We suspended the pellet in 10 ml Tris buffer, and determined the protein content by the BioRad Protein Assay per the manufacturer's instructions.

[000100] We performed mass spectrometric analysis of the *S. Typhi* membrane preparation as previously described using a LTQ-Orbitrap XL (Thermo Fisher Scientific) instrument [19,31]. We identified peptides using SEQUEST (Thermo Fisher Scientific) through Bioworks Browser, version 3.3.1 SR1. MS/MS data were obtained using 10 ppm mass accuracy on precursor m/z and a 0.5 Da window on fragment ions. Fully enzymatic

tryptic searches with up to three missed cleavage sites were allowed. Oxidized methionines were searched as a variable modification and alkylated cysteines were searched as a fixed modification. Salmonella databases for CT18 were downloaded from EMBL-EBI and supplemented with common contaminants. We employed a reverse database strategy [32] using concatenating reversed protein sequences for each database entry in SEQUEST. We filtered peptides for each charge state to a false discovery rate (FDR) of 1%, and then grouped peptides into proteins using Occam's razor logic.

[000101] Based on the sequence information presented in Table 1, one skilled in the art can readily determine the appropriate polypeptide and nucleic acid sequences for any of the proteins or peptides described herein.

Compositions comprising novel proteins

[000102] The invention provides compositions comprising the novel isolated and purified or recombinantly or synthetically produced proteins, selected from the group consisting of STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195, STY0206, STY0595, STY0909, STY1375, STY1522, STY2167 FliC, STY2381, STY3089, STY3090, STY3796. The compositions can be used in assays guiding diagnosis for typhoid fever, including acute typhoid fever.

[000103] The compositions can contain one or more of the proteins. The compositions can further comprise reagents to facilitate contacting a patient sample with the compositions. Such reagent mixture can include, e.g., buffers, preservatives etc..

[000104] The protein compositions may be dried or in aqueous solution.

[000105] The proteins can also be provided in a combination of a solid support coated with an antibody against one or more of the cytokines selected from IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a. The composition may include buffers for storage of the protein in contact with the solid support, or the proteins may be dried on the plates.

Kits

[000106] The invention further provides kits for determining level of cytokines in a blood sample. The kit comprises (a) a composition consisting of at least one of the following isolated *S. Typhi* proteins: recombinant or synthesized or isolated STY0202, recombinant or synthesized or isolated STY0372, recombinant or synthesized or isolated STY1177, recombinant or synthesized or isolated STY1179, recombinant or synthesized or isolated STY1304, recombinant or synthesized or isolated STY1878, recombinant or synthesized or isolated STY2195, recombinant or synthesized or isolated STY0206, recombinant or

synthesized or isolated STY0595, recombinant or synthesized or isolated STY0909, recombinant or synthesized or isolated STY1375, recombinant or synthesized or isolated STY1522, recombinant or synthesized or isolated STY2167 FliC, recombinant or synthesized or isolated STY2381, recombinant or synthesized or isolated STY3089, recombinant or synthesized or isolated STY3090, and/or recombinant or synthesized or isolated STY3796 or any combination thereof.

[000107] In some embodiments, the kit further comprises a solid support in which one or more of antibodies against the *S. Typhi* protein responsive cytokines is attached directly or indirectly. The antibody can be selected from antibodies against IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a.

[000108] The kit may further comprise a secondary antibody against the cytokines for the detection of the amount of cytokines. The secondary antibody may be labeled or unlabeled.

[000109] The kit may further comprise buffers and washing solutions for facilitating incubation of the proteins with the blood sample and detection of the cytokines from the sample after incubation.

[000110] The kit may include reagents for detection of the label in the secondary antibody.

[000111] The kit may be a diagnostic kit for typhoid fever. In some embodiments the kit a kit for diagnosis of acute typhoid fever.

[000112] In some embodiments, the kit can include a solid support. In some embodiments, the solid support has bound to it an antibody that can bind one or more of the *S. Typhi* responsive cytokines. In some embodiments, the soli support is an ELISA plate. In some embodiments, the kit comprises a microfluidic device.

[000113] If the label on the secondary antibody is an enzymatic label, the kit may further comprise a solution containing a corresponding soluble substrate for the enzyme.

Antibodies against cytokines

[000114] Antibodies, both monoclonal and polyclonal, against the human cytokines useful according to the present invention are readily available to one skilled in the art from commercial sources. They can be readily picked according to the application, and can be purchased with or without specific labels already attached to them.

[000115] In addition, one of ordinary skill in the art having knowledge of the peptide sequences, can readily prepare additional antibodies that can be used in the assays and

devices of the invention. The human peptide and nucleic acid sequences of the cytokines useful according to the present invention are also readily available to one of ordinary skill in the art, e.g., in publicly available databases. Antibodies can be optionally produced by a cell line, a mixed cell line, an immortalized cell or clonal population of immortalized cells, as well known in the art. See, e.g., Ausubel, et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y. (1987-2001); Sambrook, et al., Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor, N.Y. (1989); Harlow and Lane, Antibodies, a Laboratory Manual, Cold Spring Harbor, N.Y. (1989); Colligan, et al., eds., Current Protocols in Immunology, John Wiley & Sons, Inc., NY (1994-2001); Colligan et al., Current Protocols in Protein Science, John Wiley & Sons, NY, N.Y., (1997-2001).

[000116] Antibodies can be attached to a solid surface with various well known techniques.

[000117] Similarly, the antibodies can be labeled using well known techniques.

[000118] The cytokines most useful according to the present invention include IFN- γ , which was discovered to be secreted in excess in response to the specific stimulation with the isolated and purifies proteins in patients with typhoid fever.

[000119] IL-1, particularly IL-1-beta, which is the secreted form of IL-1 was also discovered to be secreted in excess in response to the specific stimulation with the isolated and purifies proteins in patients with typhoid fever.

[000120] Further, IP-10, also referred to as CXCL10 was discovered to be secreted in excess in response to the specific stimulation with the isolated and purifies proteins in patients with typhoid fever.

[000121] IL-8, also referred to as CXCL8; GCP-1; GCP1; LECT; LUCT; LYNAP; MDNCF; MONAP; NAF; NAP-1; NAP1; C-X-C motif chemokine 8; OTTHUMP00000199825; T cell chemotactic factor; T-cell chemotactic factor; beta-thromboglobulin-like protein; chemokine (C-X-C motif) ligand 8; emoctakin; granulocyte chemotactic protein 1; interleukin-8; lymphocyte-derived neutrophil-activating factor; monocyte-derived neutrophil chemotactic factor; monocyte-derived neutrophil-activating peptide; neutrophil-activating peptide 1; small inducible cytokine subfamily B, member 8, was also discovered to be secreted in excess in response to the specific stimulation with the isolated and purifies proteins in patients with typhoid fever.

[000122] MCP-1, also referred to as GDCF-2; HC11; HSMCR30; MCAF; MCP-1; MCP1; MGC9434; SCYA2; SMC-CF; C-C motif chemokine 2; monocyte chemoattractant

protein 1; monocyte chemoattractant protein-1; monocyte chemotactic and activating factor; monocyte chemotactic protein 1; monocyte secretory protein JE; small inducible cytokine A2 (monocyte chemotactic protein 1, homologous to mouse Sig-je); small inducible cytokine subfamily A (Cys-Cys), member 2; small-inducible cytokine A2, was also discovered to be secreted in excess in response to the specific stimulation with the isolated and purifies proteins in patients with typhoid fever.

[000123] MDC, also referred to as CCL22 or macrophage derived cytokine, was also discovered to be secreted in excess in response to the specific stimulation with the isolated and purifies proteins in patients with typhoid fever.

[000124] MIP-1a, also referred to as chemokine (C-C motif) ligand 3, Macrophage inflammatory protein 1-alpha, G0S19-1, Tonsillar lymphocyte LD78 alpha protein, MIP-1-alpha, SIS-beta, MIP1A, G0/G1 switch regulatory protein 19-1, SCYA3, PAT 464.1, LD78ALPHA, C-C motif chemokine 3, small inducible cytokine A3 (homologous to mouse Mip-1a), Small-inducible cytokine A3, was also discovered to be secreted in excess in response to the specific stimulation with the isolated and purifies proteins in patients with typhoid fever.

[000125] Accordingly, one can use any of these cytokines to monitor the T-cell response in the biological sample from the patient that has been exposed to one or more of the *S. Typhi* proteins or antigens set forth in this specification.

Microfluidic devices

[000126] The invention further provides microfluidic devices for the diagnosis of typhoid fever. Specifically, the components of the assays described herein can be used in the format of a microfluidic device. Such devices have been well described in the art, see, e.g., U.S. Patent Nos. 6,444,461; 6,479,299; 7,041,509, incorporated herein by reference in their entirety.

[000127] The microfluidic devices can be designed to comprise a channel or chamber that contains one or more of the *S. Typhi* proteins either in dry form or in a suitable buffer so that a sample brought in contact with this chamber can be incubated in it for a suitable time. The device can further comprise a chamber or a portion of a channel, wherein antibodies against one or more cytokines are bound so that after the incubation, the secretion of cytokines can be conveniently measured by binding them to the antibody surfaces. The microfluidic device can be sold as a kit which further comprises incubation buffers, washing buffers and one or more secondary antibody which may be labeled to allow detection of the amount of cytokines.

Automation

[000128] As explained in the examples, the methods according to the present invention can be automated using robotics and computer directed systems. The entire assay can be automated from the point of introducing the biological fluid sample comprising T cells to an protein or a mixture thereof, incubating the reaction mixture, washing the reaction mixture, exposing the reaction mixture to a secondary antibody and detecting the amount of cytokine secreted by the cells. The step of comparing and displaying the results can also be automated and connected to the same system or in a remote system. Thus, the analysis can be performed in one location and the comparison and the analysis in another location, the only connection being, e.g., an internet connection in such way that the analysis result can be fed from the analysis module to the comparison module which can then either in the same location or by sending the result to a third location, which may or may not be the same location as the first location wherein the analysis was performed, to be displayed in a format suitable for either reading by a health professional or by a patient.

[000129] In one embodiment, the analysis, comparison and the result is performed in one location.

[000130] In one embodiment, the invention further provides a system to facilitate the diagnosis of typhoid fever in a human subject, comprising: a determination module configured to receive and output the amount of cytokines produced by the T-cells in the biological sample taken from a human and contacted with at least one *S. Typhi* protein or fragment thereof; a storage module configured to store output information from the determination module; a comparison module adapted to compare the data stored on the storage module with reference data and/or control data, and to provide a comparison content, and an output module for displaying the comparison content for the user, wherein if the cytokine amount is about 1.5 fold or more compared to the reference value, in the sample, then the subject will be indicated as having typhoid fever. The output may be in the form of a chart, color, text, numbers or a mere indication, such as an asterisk to indicate the diagnosis. The output may also be printing out the amount with a presentation of the normal cytokine range or cut-off value next to it.

[000131] In one embodiment, the invention provides a computer readable storage medium comprising: a storing data module containing data from a sample obtained from a subject that represents a signal level from the cytokine assay after exposure of the human biological sample comprising T cells to at least one of the *S. Typhi* antigens/proteins or fragments thereof; a comparison module that compares the data stored on the storing data

module with a reference data and/or control data, and to provide a comparison content, and an output module displaying the comparison content for the user, wherein if the cytokine amount detected in the patient sample is greater than the reference value, the result shows that the subject has typhoid fever, and if the cytokine level is within the reference range, the subject does not have typhoid fever. In some embodiments, the cut off value is about 1.5 fold the reference value, which is or has been typically obtained from normal cytokine amount in a control human or a pool of control humans or an average of a set of control humans who is/are known not to be affected with typhoid fever.

Selection of patients

[000132] In some embodiments, the method further comprises selecting an individual having symptoms of typhoid fever, wherein the biological sample comprising T cells is taken from the individual.

[000133] Typhoid fever is characterized by a slowly progressive fever as high as 40 °C (104 °F), profuse sweating and gastroenteritis. Less commonly, a rash of flat, rose-colored spots may appear. Classically, the course of untreated typhoid fever is divided into four individual stages, each lasting approximately one week. In the first week, there is a slowly rising temperature with relative bradycardia, malaise, headache, and cough. A bloody nose (epistaxis) is seen in a quarter of cases and abdominal pain is also possible. There is leukopenia, a decrease in the number of circulating white blood cells, with eosinopenia and relative lymphocytosis, a positive reaction and blood cultures are positive for *Salmonella typhi* or *paratyphi*. The classic Widal test is negative in the first week.

[000134] In the second week of the infection, the patient lies prostrate with high fever in plateau around 40 °C (104 °F) and bradycardia (sphygmothermic dissociation), classically with a dicrotic pulse wave. Delirium is frequent, frequently calm, but sometimes agitated. This delirium gives to typhoid the nickname of "nervous fever". Rose spots appear on the lower chest and abdomen in around a third of patients. There are rhonchi in lung bases. The abdomen is distended and painful in the right lower quadrant where borborygmi can be heard. Diarrhea can occur in this stage: six to eight stools in a day, green with a characteristic smell, comparable to pea soup. However, constipation is also frequent. The spleen and liver are enlarged (hepatosplenomegaly) and tender, and there is elevation of liver transaminases. The Widal reaction is strongly positive with antiO and antiH antibodies. Blood cultures are sometimes still positive at this stage. (The major symptom of this fever is that the fever usually rises in the afternoon up to the first and second week.)

[000135] In the third week of typhoid fever, a number of complications can occur including intestinal hemorrhage due to bleeding in congested Peyer's patches (this can be very serious but is usually not fatal); intestinal perforation in the distal ileum: this is a very serious complication and is frequently fatal - it may occur without alarming symptoms until septicaemia or diffuse peritonitis sets in; encephalitis; neuropsychiatric symptoms (described as "muttering delirium" or "coma vigil"), with picking at bedclothes or imaginary objects; metastatic abscesses, cholecystitis, endocarditis and osteitis. The fever is still very high and oscillates very little over 24 hours. Dehydration ensues and the patient is delirious (typhoid state). By the end of third week the fever has started reducing this (defervescence). This carries on into the fourth and final week. A person may become an asymptomatic carrier of typhoid fever, suffering no symptoms, but capable of infecting others. According to the CDC approximately 5% of people who contract typhoid continue to carry the disease after they recover.

[000136] The assays of this invention can diagnose the typhoid fever both at the acute and later stages as well as in the latent stage.

[000137] The methods, assays, devices, kits and compositions described herein are based on our a screening approach that permitted us to evaluate interferon- γ and proliferation responses, as well as response to other cytokines, such as IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a to a number of bacterial proteins in *S. Typhi*-infected humans in Bangladesh.

[000138] We selected proteins that we had previously identified in immuno-affinity screening assays for humoral responses [28], and we recovered these selected proteins using an automated system and high throughput genomic and proteomic technologies. Although we were able to generate adequate samples for only approximately a third of our selected proteins for evaluation in humans, we believe that high throughput approaches such as the one we describe will assist in accelerating analysis of pathogens that express thousands of proteins.

[000139] For instance, *S. Typhi* contains approximately 4,400 open reading frames, and although protein microarrays can be used to screen for humoral responses across the immunoproteome, no comparable system has yet been developed to assess cellular immune responses in a high throughput manner, despite the critical role that cellular immune responses play against intracellular pathogens.

[000140] High throughput purification techniques may be compromised by issues of contamination, including with LPS when expression occurs in *E. coli* vectors. However, LPS contamination of all preparations was found to be less than the level of detection of our assay

kit (<300 fg/μl), we did not detect cellular immune responses to control protein expressed and purified from *E. coli* in the same manner as our *S. Typhi* proteins, and we detected cellular immune responses in patients but not healthy controls to purified *S. Typhi* proteins. All of these observations confirm that the responses we observed were protein-specific and not due to contaminating LPS.

[000141] In the *S. Typhimurium* mouse model, CD4 and CD8 cells are critical to the development of protective immunity, and control of Salmonella infection involves prominent expression of interferon-γ by both CD4 and CD8 cells [24,25,26]. Overall, only a relatively few defined class I and class II epitopes have been identified in the *S. Typhimurium* mouse model, including epitopes in FliC and SipC for CD4 cells, and OmpC and GroEL for CD8 cells [24,40,45,46,47,48]. A number of Salmonella proteins are also able to induce partially protective immunity when included in subunit-based vaccines in mice, including flagellin, MIG-14 and SseB (Salmonella proteins expressed *in vivo*), suggesting that immune responses against a number of Salmonella proteins could contribute to protective immunity [49,50,51].

[000142] In comparison to the murine data, evaluation of cellular responses to *S. Typhi* in humans have largely involved individuals who have received attenuated *S. Typhi* vaccine strains such as Ty21a and CVD908 [23,38,41,42,43,44]. In concordance with the mouse data, these studies have shown induction of interferon-γ-expressing CD4 and CD8 responses following vaccination [23,24,38,42,52]. Interestingly, CD8 responses may involve both classical (HLA-A, B and C in humans) and non-classical (HLA-E, F, and G) mediated T cell recognition [43,52]. Using an *ex vivo* model, Sztein and colleagues have also recently found that direct infection of protein-presenting dendritic cells with *S. Typhi* leads to expression of high levels of TNF-α, IL-6 and IL-8, and low levels of interferon-γ and IL-12 p70, but that dendritic cells can also ingest other infected human cells leading to high level expression of interferon-γ and IL-12 p70, with subsequent induction of a population of CD3+CD8+CD45RA-CD62L- effector/memory T cells in co-cultured lymphocytes [53].

[000143] We used recombinant proteins to assess and characterize IFN-γ and proliferation responses, as well as IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a responses, in infected humans in Bangladesh. We focused our initial efforts on a subset of proteins that we had previously identified as generating humoral immunity and being expressed *in vivo* during human infection [28]. These included a number involved in fimbrial attachment or adhesion such as StaF, StbB, CsgF, and CsgD, as well as OppA, a conserved hypothetical protein encoded by STY2195, and PagC, an outer membrane protein encoded by STY1878. We previously found that humans infected with *S. Typhi* develop a serum antibody response to

PagC and that this response increases at convalescence [28]. However, we did not at the time know that a similar response can be found at a cellular level with specific *S. Typhi* proteins. Here we report, for example, detection of a parallel cellular response against PagC during human infection, including both interferon- γ and proliferative responses, and show that responses in convalescence were higher than during acute stage illness although both were significantly increased compared to a control. Although the role of PagC during human infection is not fully understood, its expression is known to be controlled by the PhoP-regulon involved in intra-macrophage survival [19,54].

[000144] We also detected significant increases in cellular responses during convalescence against StaF, a fimbrial protein homologous to *E. coli* YadK that contains a Pfam motif believed to be involved in cellular adhesion [17], STY2195, a conserved hypothetical protein of unknown function, and a crude membrane preparation containing over 900 *S. Typhi* proteins, including GroEL, OmpC, OppA and PagC.

[000145] We found that *S. Typhi* proteins or antigens elicit both CD4+ and CD8+ IFN- γ expressing responses, with CD4 responses being more numerous than CD8 responses. We detected protein-specific IFN- γ responses in patients, including at the time that patients presented for clinical care, but similar responses were not seen on controls.

[000146] These data show that a protein-specific IFN- γ -based or other cytokine-based detection system can be used to diagnose individuals with typhoid fever during the acute stage of illness, similar to the approach used to diagnose infection with *Mycobacterium tuberculosis* [55,56,57]. Currently, all available diagnostic tests for typhoid fever lack either sensitivity and/or specificity, especially in areas of the world endemic for typhoid. For example, microbiological culturing of blood has approximately 30-70 % sensitivity, depending on the volume of blood obtained and whether previous antibiotics have been administered, and the Widal assay has at best 85% specificity when analyzing both acute and convalescent phase responses in endemic zones where typhoid exacts its highest burden [58,59,60].

Methodology

[000147] We used a novel automated approach to purify a subset of *S. Typhi* proteins identified in previous antibody-based immuno-affinity screens and proteins known to be expressed *in vivo*, including StaF-putative fimbrial protein-STY0202, StbB-fimbrial chaperone-STY0372, CsgF-involved in curli production-STY1177, CsgD- putative regulatory protein-STY1179, OppA-periplasmic oligopeptide binding protein precursor-STY1304, PagC-outer membrane invasion protein-STY1878, and conserved hypothetical

protein-STY2195. We also generated and analyzed a crude membrane preparation of *S. Typhi* (MP).

[000148] In comparison to samples collected from uninfected Bangladeshi and North American participants, we detected significant IFN- γ responses in PBMCs stimulated with MP, StaF, StbB, CsgF, CsgD, OppA, STY2195, and PagC in patients bacteremic with *S. Typhi* in Bangladesh. The majority of IFN- γ expressing T cells were CD4 cells, although CD8 responses also occurred. We also assessed cellular proliferation responses in bacteremic patients, and confirmed increased responses in infected individuals to MP, StaF, STY2195, and PagC in convalescent compared to acute phase samples and compared to controls. StaF is a fimbrial protein homologous to *E. coli* YadK, and contains a Pfam motif thought to be involved in cellular adhesion. PagC is expressed *in vivo* under the control of the virulence-associated PhoP-regulon required for intra-macrophage survival of *Salmonella*. STY2195 is a conserved hypothetical protein of unknown function.

[000149] Generation of expression clones. We selected 58 *S. Typhi* proteins contained within operons identified during our previous application of IVIAT to *S. Typhi* [28]. IVIAT identifies proteins expressed *in vivo* during human infection and that generate an antibody response [28]. We obtained pDONR221 Gateway Based entry clones of the *S. Typhi* CT18 genes corresponding to selected proteins from the NIAID-sponsored Pathogen Functional Genomic Resource Center, J. Craig Venter Institute (JCVI, formerly The Institute for Genomic Research). We used LR clonase II enzyme reactions (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions to move inserts into pDEST17 (Invitrogen, Carlsbad, CA) to generate a fusion containing an amino terminal 6 $\times$ histidine (HIS) tag (SEQ ID NO: 19). We transformed DH5 α -T1R competent cells with LR reactions and selected for ampicillin resistance. We confirmed insert presence by restriction digestion and PCR analysis and transformed purified plasmids into *E. coli* protein expression strain BL21 star (DE3) pLysS (Invitrogen).

[000150] Protein expression. We grew transformants harboring recombinant plasmids at 37°C as 1.5 ml cultures in 96-well blocks (Marsh Biomedical Products) to an OD600 of 0.6-0.8. We induced cultures with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) on a 96-well plate shaker (Multitron) ($\times$ 900 rpm). After 3 hours at 37°C, we harvested cells at 4°C and stored preparations at - 80°C for further use. We also induced BL21 star (DE3) pLysS containing pDEST17 but lacking an *S. Typhi* insert. This construct produced a truncated HIS-tagged protein MSYYHHHHHHLESTSLYKKAERERKMI (SEQ ID NO: 1) that we recovered and used as a control protein in immunological assays.

[000151] Automated 96-well protein purification. We performed protein purifications in 96-well plates using a BiomekFx (Beckman Coulter) robotic liquid handler as previously described [27]. For this 6xHIS (SEQ ID NO: 19) denaturing affinity purification, we thawed cell pellets at room temperature for 15 min, lysed them in the presence of protease inhibitors in 115 μ l lysis buffer I (100 mM NaH₂PO₄, 10 mM Tris, pH 8.0), robotically resuspended product in a 96-well block and agitated at 900 rpm for 10 min (5 min in the clockwise direction and 5 min in the counterclockwise direction). We then added 10 μ l of DNase mix (10 mg/ml DNase; Sigma Aldrich in 900 mM MgCl₂, 100 mM MnCl₂) to the lysate and agitated the preparation at 900 rpm for 10 min. Next, we added 115 μ l of lysis buffer II (100 mM NaH₂PO₄, 10 mM Tris, 6 M guanidine hydrochloride, 10 mM, 2-mercaptoethanol, pH 8.0) to create denaturing conditions. We then allowed these cell lysates to bind to 30 μ l of MagneHIS beads (Promega) with shaking at 900 rpm for 20 min (10 min clockwise, 10 min counterclockwise), and separated beads using a magnabot (24-pin magnet; Promega). The robotic liquid handler then washed the MagneHIS beads with bound protein three times with wash buffer (100 mM NaH₂PO₄, 10 mM Tris, 8M urea). We prevented bead adherence to the walls during washing by shaking the samples at 900 rpm for 2.5 min clockwise and then 2.5 min counterclockwise. We then washed the beads with bound protein using 100 μ l of distilled water, and added 50 μ l distilled water to make the final suspensions for analysis. We repeated this extraction cycle six times.

[000152] Automated 96-well protein analysis. We analyzed proteins in a 96-well format using a capillary-based instrument, the LabChip90 (Caliper Sciences). We automated a system that resuspended 3 μ l of protein sample in 7 μ l analysis buffer (Caliper Sciences), heated these to 96°C for 5 min., cooled them to room temperature, and briefly centrifuged to collect the sample. We added distilled water (35 μ l) to each sample prior to analysis. We primed the analysis chip (Caliper Sciences) according to the manufacturer's instructions. The automated protein analysis generated three different forms of output: a chromatogram that showed migration time; a virtual gel that mimicked a Coomassie stained gel; and a results table that included the estimated size, quality, and quantity of each peak. The LabChip90 analyzed 96 proteins at a time with analysis time of 40 seconds per sample. We parsed the output results and imported them into the Harvard Institute of Proteomics protein database. We assessed for presence of contaminating *E. coli* LPS using a HEK-Blue LPS Detection kit (InvivoGen, San Diego, CA).

[000153] Production and mass spectrometric analysis of *S. Typhi* crude membrane preparation. We prepared *S. Typhi* membrane preparation as previously described [29,30]. Briefly, we cultured *S. Typhi* Ty21a on sheep blood agar plates and harvested in Tris buffer (10 mM Tris, pH 8.0, 5 mM MgCl₂). We sonicated the mixture, and centrifuged at 1400 × g for 10 minutes and transferred the supernatant to fresh tubes, centrifuging at 14900 × g for 30 minutes. We suspended the pellet in 10 ml Tris buffer, and determined the protein content by the BioRad Protein Assay per the manufacturer's instructions.

[000154] We performed mass spectrometric analysis of the *S. Typhi* membrane preparation as previously described using a LTQ-Orbitrap XL (Thermo Fisher Scientific) instrument [19,31]. We identified peptides using SEQUEST (Thermo Fisher Scientific) through Bioworks Browser, version 3.3.1 SR1. MS/MS data were obtained using 10 ppm mass accuracy on precursor m/z and a 0.5 Da window on fragment ions. Fully enzymatic tryptic searches with up to three missed cleavage sites were allowed. Oxidized methionines were searched as a variable modification and alkylated cysteines were searched as a fixed modification. Salmonella databases for CT18 were downloaded from EMBL-EBI and supplemented with common contaminants. We employed a reverse database strategy [32] using concatenating reversed protein sequences for each database entry in SEQUEST. We filtered peptides for each charge state to a false discovery rate (FDR) of 1%, and then grouped peptides into proteins using Occam's razor logic.

[000155] Collection of specimens from study subjects. Individuals (1-59 years of age) with fever of 3-7 days duration ($\geq 39^{\circ}\text{C}$) having clinical symptoms and signs suggestive of typhoid fever and lacking an alternate diagnosis who presented to the Kamalapur field site of the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) Dhaka hospital were eligible for enrollment. We collected venous blood (for children <5 years of age, 3 ml of blood; for older individuals, 5 ml of blood) for culture (n = 69). We used the BacT/Alert automated system and identified *S. Typhi* organisms using standard biochemical methods and by reaction with Salmonella-specific antisera [30,33]. Following informed consent from patients or guardians in the case of children, we collected an additional 5 ml of blood from bacteremic individuals within 72 hours of the patient presenting for medical care, and a follow-up sample 21-28 days later (n = 16; ages 2-22 years). All patients with 3 days or longer of fever were treated initially with amoxicillin or cefixime at the discretion of the attending physician until scheduled follow-up 48-72 hours later, or sooner as clinically indicated. Individuals with documented *S. Typhi* bacteremia were continued on amoxicillin if they showed signs of improvement and their blood isolates showed sensitivity to first line

treatment; or were switched to parenteral ceftriaxone or oral ciprofloxacin, if their isolates were not sensitive and/or they failed to improve by 72 hours; therapy was continued for up to 14 days, or up to 7 days beyond defervescence, whichever occurred first. All patients recovered. We also collected 5 ml of blood from North American volunteers (n = 3) without a history of international travel who had never received typhoid vaccination and who did not have previous known *Salmonella* infection, and we collected 5 ml of blood from healthy Bangladeshi volunteers (n = 4) who did not have illness, fever or diarrhea in the preceding three months [34]. Studies were approved by the Institutional Review Boards of the ICDDR, B and Massachusetts General Hospital.

[000156] PBMC isolation. We diluted heparinized blood in phosphate buffered saline (PBS; 10 mM, pH 7.2) and isolated peripheral blood mononuclear cell (PBMC) by gradient centrifugation on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden). We re-suspended isolated PBMCs to a concentration of 1×10^6 cells/ml in RPMI complete medium RPMI-1640 (Gibco, Gaithersburg, Md) with 10% heat-inactivated fetal bovine serum (Hyclone-Thermo Scientific, Waltham, MA, USA), 100 units/ml penicillin, 100 μ g/ml streptomycin, 100 mM pyruvate, and 200 mM L-glutamine (Gibco) [35].

[000157] Interferon gamma ELISPOT assay. We used PBMCs to measure human interferon- γ expression using an ELISPOT format with MabTech antibodies, according to the manufacturers' instructions (Mabtech Inc, Cincinnati, OH, USA). In brief, we coated 96-well nitrocellulose plates (Multiscreen HTS, Millipore) with 100 μ l of 15 μ g/ml human monoclonal anti-interferon- γ antibody (1-D1K) overnight at 4°C. Following washing the plates and subsequent blocking with 10% FBS for 2h at room temperature, we added PBMCs from individual patients or controls at a concentration of 2×10^5 per well for each experimental condition. We added individual *S. Typhi* proteins or control protein to wells at a concentration of 140 ng/well of total preparation for each purified protein (in 200 μ l culture, final concentration 0.7 μ g/ml). In separate wells, we also added *S. Typhi* membrane preparation at a final concentration of 10 μ g/ml in 200 μ l culture, phytohaemagglutinin (PHA; Murex Diagnostics Ltd, Temple Hill, UK) at a final concentration of 2.5 μ g/ml in 200 μ l culture, and keyhole limpet hemocyanin (KLH). We included additional control wells with media but lacking protein. Following incubation of plates at 37°C in 5% CO₂ for 20 hours, we washed plates, added biotinylated monoclonal anti-interferon- γ antibody (7-B6-1-biotin; 1:500 dilution), incubated plates at room temperature for an additional 2 hours, washed them, added streptavidin-HRP (1:500 dilution), and re-incubated for 1 hour at room temperature. We developed plates with aminoethylcarbazol plus H₂O₂, and counted IFN- γ

secreting cells using a stereomicroscope. We subtracted results for wells containing media only and expressed results as the number of spots/106 PBMC in each experimental condition [36].

[000158] We also measured other cytokines and our data show that in addition to IFN- γ , also IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1 α can distinguish patients with typhoid fever from individuals with other febrile illnesses as shown in Table 1.

Table 1: Cytokine/chemokine patterns in the serum of typhoid fever patients in Bangladesh

	Cytokine/ Chemokine	Acute typhoid (N=10)	Convalescent (N=10)	P value*	Non- typhoid (N=8)	P value**
Elevated in typhoid fever compared to other febrile patients	IFN- γ	36 pg/mL	6 pg/mL	0.008	16 pg/mL	0.05
	IL-1	145 pg/mL	35 pg/mL	0.003	32 pg/mL	0.02
	IP-10	1450 pg/mL	350 pg/mL	0.0008	749 pg/mL	0.03
Less elevated in typhoid	IL-6	9 pg/mL	2 pg/mL	0.03	36 pg/mL	0.002
Unchanged or decreased in typhoid fever, but increased in other febrile patients	IL-8	13 pg/mL	18 pg/mL	0.2	25 pg/mL	0.07
	MCP-1	127 pg/mL	187 pg/mL	0.009	656 pg/mL	0.01
	MDC	263 pg/mL	282 pg/mL	0.5	831 pg/mL	0.00007
	MIP-1 α	14 pg/mL	18 pg/mL	0.5	86 pg/mL	0.003
*First P value denotes paired comparison between acute and convalescent samples						
**Second P value denotes comparison between typhoid fever and febrile patients with negative blood culture and negative <i>S. Typhi</i> serologies at presentation and in convalescence.						

[000159] Intracellular cytokine staining. To characterize the IFN- γ T cell response further, we resuspended PBMCs at a concentration of 1×10^6 cells/mL in RPMI medium (Gibco, Carlsbad, CA) and supplemented with 10% fetal calf serum (FCS, Gibco). We cultured PBMCs in U-bottom tissue culture plates (Nunc, Denmark) in the presence of Salmonella membrane preparation (MP; 10 μ g/ml), StaF (7 μ g/ml), PagC (7 μ g/ml), KLH (2.5 μ g/ml as a negative control) or PMA (5.0 ng/ml as a positive control; Phorbol 12-myristate 13-acetate) with ionomycin (1.0 μ g/ml). Samples containing only unstimulated cells were included to assess *in vivo* stimulation. We used 1.0 μ g/ml of anti-CD28 (clone 28.2; BD Pharmingen) and anti-CD49d (clone 9F10; BD Pharmingen) for co-stimulation. We incubated PBMCs and proteins for 2 hours at 37° C in 5% CO₂. After 2 hours, we added 10 μ g/mL of brefeldin A (BFA, Sigma) and continued incubating the plates for an additional

4 hours [37]. Following stimulation, we washed cells with PBS and 2% FCS. We then stained cells for 30 min at 4°C with the following surface monoclonal antibodies: anti-CD3-APC, anti-CD4-perCP, and anti-CD8-FITC (Becton Dickinson, San Jose, USA). Following surface staining, we washed the cells and incubated the preparations with FACS Lysing Solution (BD Bioscience) for 10 minutes, and then re-washed and permeabilized the preparations with FACS permeabilizing solution (BD Bioscience) for 10 min at room temperature. We washed the permeabilized cells and stained them for 30 min at 4°C with fluorochrome-conjugated anti-IFN- γ -PE (BD Bioscience). Following staining, we re-washed the cells, and fixed them in formaldehyde before performing flow cytometry using a FACS Calibur (BD, San Jose, CA) [37]. We identified the lymphocyte population on forward versus side scatter plot, then gated CD3+CD4+ and CD3+CD8+ subpopulations, and identified CD4+IFN- γ + and CD8+IFN- γ + subpopulations. We subtracted unstimulated responses, and expressed results as IFN- γ + T cells per 10 $\times$ 6 PBMC.

[000160] T-cell proliferation assay. To evaluate proliferative responses to proteins, we cultured PBMCs (105 cell per well) in DMEM/F12 medium (Gibco, GlutaMAX) supplemented with 1% gentamicin and 5% human AB+ serum in triplicate wells in round-bottomed 96-well plates. We added *S. Typhi* proteins and controls to wells at the same concentrations used in the interferon- γ ELISPOT assay and with a final culture volume of 200 μ l. We incubated plates at 37°C in 5% CO₂ for 5 days. After 48 h incubation, we replaced 100 μ l of the medium per well with fresh medium. After 5 days of incubation, we added 3H-thymidine (1 μ Ci) to each well under sterile conditions, incubated plates for an additional 8 hours, harvested cells in Bray's scintillation fluid (Ultimagold, PerkinElmer, Boston, MA) using a cell harvester (Skatron instruments, Norway), and assessed [3H] thymidine incorporation using a liquid scintillation β -counter (Beckman LS6500 multipurpose scintillation counter, USA) as previously described [22,38]. We expressed results as counts per minute (cpm), and calculated stimulation indices for each protein according to the formula: net cpm with protein /net cpm without protein (media alone) for each individual on each day (day 5 and day 20) [39].

[000161] Statistical analysis. We used Prism4 (version 4.03, GraphPad Software, Inc.) for data management, analysis and graphical presentation. We used unpaired T tests to compare differences between groups, and paired T tests to evaluate differences between study days within groups.

Results

[000162] Automated production of *S. Typhi* proteins. We estimated that we required at least 20 µg of a specific protein for use in our planned immunological assays. Our six production runs resulted in the production of 20 µg or more for 25 of our selected 58 proteins; nine of these samples had purity by LC90 Caliper analysis of >90%, and 17 had purity greater than >80%. Purity was defined as the quantity of protein matching the molecular size of the desired product. The LPS contamination of all preparations was found to be less than the level of detection of our assay kit (<300 fg/µl). Of these 17 proteins with sufficient quantity and purity, we selected 7 proteins for our initial analysis (Table 1). These proteins represented a range of cellular location and function, including a number involved in fimbrial attachment or adhesion such as StaF (putative fimbrial protein encoded by STY0202), StbB (fimbrial chaperone encoded by STY0372), CsgF (involved in curli production encoded by STY1177), and CsgD (a putative regulatory protein encoded by STY1179), as well as OppA (a periplasmic oligopeptide binding protein precursor involved in peptide transport encoded by STY1304), a conserved hypothetical protein encoded by STY2195, and PagC, an outer membrane protein encoded by STY1878 whose expression is regulated by the PhoP regulon involved in intra-macrophage survival [19,28].

[000163] Mass spectrometric analysis of the *S. Typhi* membrane preparation. Our mass spectrometric analysis of *S. Typhi* membrane preparation identified 934 *S. Typhi* proteins (636 with three or more spectral counts), including many involved in energy metabolism, protein synthesis and fate, cell envelope or peptidoglycan synthesis or maintenance, cellular processes, proteins involved in transport, proteins involved in regulatory functions, and proteins involved in virulence and pathogenesis (Table 2 and 3 and Supplemental Table 1). We also identified two of our 7 selected proteins (OppA and PagC) in the *S. Typhi* membrane preparation.

[000164] IFN-γ ELISPOT responses and T cell characterization We found that patients with *S. Typhi* bacteremia had elevated interferon-γ ELISPOT responses at both acute and convalescent stages of infection compared to healthy controls for all seven of the purified *S. Typhi* proteins, as well as against *S. Typhi* crude membrane preparation ($P < 0.05$) (Figure 1). In contrast, responses to PHA did not differ significantly between patients and healthy controls, and minimal responses were detected against control protein and KLH in both patients and healthy controls. To assess whether interferon-γ responses were CD4 or CD8-derived, we used intracellular cytokine staining following stimulation with a subset of

proteins, and found that the majority of inteferon- γ expressing cells were CD4-positive, although a CD8 positive response was also detected (Figure 2).

[000165] Proliferation responses. To further evaluate responses, we selected the three proteins associated with the highest interferon- γ expression levels in convalescent phase samples, as well as membrane preparation, for inclusion in cellular proliferation assays. In comparison to healthy Bangladeshi controls residing within the same *S. Typhi* endemic area, individuals with documented *S. Typhi* bacteremia had significantly elevated proliferation indices at the acute stage of illness to StaF and PagC ($P < 0.01$ - 0.0008), but not to STY2195, or crude membrane preparation, and these acute stage responses further significantly increased within bacteremic individuals by the convalescent period compared to the acute stage responses ($P < 0.02$ - 0.001) (Figure 3). We also detected a significantly increased proliferation response to STY2195 and *S. Typhi* membrane preparation in bacteremic patients at convalescence compared to acute phase samples and compared to control patients ($p < 0.01$).

[000166] In summary, we have used a screening format to characterize *S. Typhi* protein-specific IFN- γ responses in patients with typhoid fever. We believe that this is the first characterization of such responses in humans. Our detection of protein-specific IFN- γ and other cytokine responses provide novel cytokine-based diagnostic assays and kits for typhoid fever.

[000167] Table 2 sets forth the *S. Typhi* protein sequences useful in the methods of the present invention.

[000168] STY0202, STY0372, STY1177, STY1179, STY1304, STY1878, STY2195, STY0206, STY0595, STY0909, STY1375, STY1522, STY2167 FliC, STY2381, STY3089, STY3090, STY3796

Table 2		
STY-Numbers	Accession Numbers	SEQ ID NOs
STY0202	NP_804068.1	SEQ IS NO: 2
STY0372	NP_806248.1 GI: 29142906	SEQ IS NO: 3
STY1177	NP_455634.1	SEQ IS NO: 4
STY1179	NP_455636.1	SEQ IS NO: 5
STY1304	NP_455753.1	SEQ IS NO: 6
STY1878	NP_456268.1	SEQ IS NO: 7
STY2195	NP_456548.1	SEQ IS NO: 8
STY0206	NP_454797.1	SEQ IS NO: 9

Table 2		
STY-Numbers	Accession Numbers	SEQ ID NOs
STY0595	NP_455137.1	SEQ IS NO: 10
STY0909	NP_455403.1	SEQ IS NO: 11
STY1375	NP_455817.1	SEQ IS NO: 12
STY1522	NP_455945.1	SEQ IS NO: 13
STY2167 FliC	NP_456520.1	SEQ IS NO: 14
STY2381	NP_456711.1	SEQ IS NO: 15
STY3089	NP_457347.1	SEQ IS NO: 16
STY3090	NP_457348.1	SEQ IS NO: 17
STY3796	NP_457978.1	SEQ IS NO: 18

REFERENCES

[000169] The references cited below and throughout the specification are herein incorporated by reference in their entirety.

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CLAIMS

We claim

1. An *in vitro* assay comprising the steps of:
 - a. contacting and incubating a biological sample comprising T cells obtained from a human individual with at least one isolated and purified *S. Typhi* protein; and
 - b. detecting and quantifying the amount of at least one *S. Typhi* protein responsive cytokine in the incubated sample.
2. The *in vitro* assay of claim 1, wherein the at least one isolated and purified *S. Typhi* protein is selected from the group consisting of STY0202 (SEQ ID NO: 2), STY0372 (SEQ ID NO: 3), STY1177 (SEQ ID NO: 4), STY1179 (SEQ ID NO: 5), STY1304 (SEQ ID NO: 6), STY1878 (SEQ ID NO: 7), STY2195 (SEQ ID NO: 8), STY0206 (SEQ ID NO: 9), STY0595 (SEQ ID NO: 10), STY0909 (SEQ ID NO: 11), STY1375 (SEQ ID NO: 12), STY1522 (SEQ ID NO: 13), STY2167 FliC (SEQ ID NO: 14), STY2381 (SEQ ID NO: 15), STY3089 (SEQ ID NO: 16), STY3090 (SEQ ID NO: 17), STY3796 (SEQ ID NO: 18) or any combination thereof.
3. The *in vitro* assay of any one of the preceding claims, wherein the at least one protein is selected from the group consisting of STY0202 (SEQ ID NO: 2), STY0372 (SEQ ID NO: 3), STY1177 (SEQ ID NO: 4), STY1179 (SEQ ID NO: 5), STY1304 (SEQ ID NO: 6), STY1878 (SEQ ID NO: 7), STY2195 (SEQ ID NO: 8) and any combination thereof.
4. The *in vitro* assay of any one of the preceding claims further comprising a step of comparing the cytokine amount to a reference and diagnosing a typhoid fever in the human individual if the amount of cytokine amount is increased compared to a reference value.
5. The *in vitro* assay of claim 4, wherein the typhoid fever is diagnosed if the cytokine amount is increased about 1.5 fold or more compared to the reference value.
6. The *in vitro* assay of any one of the preceding claims wherein the typhoid fever is acute typhoid fever.
7. The *in vitro* assay of any one of the preceding claims, wherein the at least one *S. Typhi* protein responsive cytokine is selected from the group consisting of IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a or any combination thereof.

8. The *in vitro* assay of claim 7, wherein the at least one *S. Typhi* protein responsive cytokine is IFN- γ .
9. The *in vitro* assay of any one of the preceding claims, wherein the biological sample is a blood sample.
10. The *in vitro* assay of claim 9, wherein the blood sample is a fresh blood sample.
11. The *in vitro* assay of any one of the preceding claims, wherein the biological sample is isolated peripheral blood mononuclear cells.
12. The *in vitro* assay of any one of the preceding claims wherein the step of detecting and quantifying the amount of at least one *S. Typhi* protein responsive cytokine is performed using stereomicroscope, ELISA, ELISPOT, FLUOROSPOT, FACS, FP, FLT or FRET.
13. The *in vitro* assay of any of the preceding claims, wherein the step of comparing the cytokine amount to a reference and diagnosing a typhoid fever is performed by a non-human machine.
14. The *in vitro* assay of claim 13, wherein the non-human machine comprises a computer implemented software.
15. A computer program comprising computer program code means adapted to perform all the steps of any of the assays of any of the preceding claims when said program is run on a computer.
16. A composition comprising at least one isolated and purified protein selected from the group consisting of STY0202 (SEQ ID NO: 2), STY0372 (SEQ ID NO: 3), STY1177 (SEQ ID NO: 4), STY1179 (SEQ ID NO: 5), STY1304 (SEQ ID NO: 6), STY1878 (SEQ ID NO: 7), STY2195 (SEQ ID NO: 8), STY0206 (SEQ ID NO: 9), STY0595 (SEQ ID NO: 10), STY0909 (SEQ ID NO: 11), STY1375 (SEQ ID NO: 12), STY1522 (SEQ ID NO: 13), STY2167 FliC (SEQ ID NO: 14), STY2381 (SEQ ID NO: 15), STY3089 (SEQ ID NO: 16), STY3090 (SEQ ID NO: 17), STY3796 (SEQ ID NO: 18) and any combination thereof.
17. The composition of claim 16, wherein the at least one isolated and purified protein selected from the group consisting of STY0202 (SEQ ID NO: 2), STY0372 (SEQ ID NO: 3), STY1177 (SEQ ID NO: 4), STY1179 (SEQ ID NO: 5), STY1304 (SEQ ID NO: 6), STY1878 (SEQ ID NO: 7), STY2195 (SEQ ID NO: 8) and any combination thereof.
18. A kit comprising a composition of any one of claims 16-17.

19. The kit of claim 18 further comprising the composition of claims 16-17, and laboratory materials and instructions to implement an assay of any one of the claims 1-12.
20. The kit of any one of the claims 16-19, which further comprises a washing buffer.
21. The kit of any one of claims 16-19, further comprising a solid support comprising an antibody against at least one *S. Typhi* protein responsive cytokine against at least wherein the solid support is an ELISA plate and the marker is an enzyme.
22. The kit of claim 21, wherein the *S. Typhi* protein responsive cytokine is selected from the group consisting of IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a.
23. The kit of any one of the claims 15-17, further comprising a secondary antibody directed to at least one *S. Typhi* protein responsive cytokine.
24. The kit of claim 18, wherein the at least one *S. Typhi* protein responsive cytokine is selected from IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a.
25. The kit of claims 17-22, wherein the secondary antibody is labeled with a detectable label.
26. A microfluidic device comprising
 - a. an inlet for adding a blood sample;
 - b. a chamber comprising at least one isolated and purified *S. Typhi* protein; and
 - c. a chamber comprising at least one antibody against an *S. Typhi* protein responsive cytokine.
27. The microfluidic device of claim 25 further comprising a chamber comprising at least one secondary antibody against the at least one *S. Typhi* protein responsive cytokine.
28. The microfluidic device of any one of claims 25-26, wherein the at least one isolated and purified *S. Typhi* protein is selected from the group consisting of STY0202 (SEQ ID NO: 2), STY0372 (SEQ ID NO: 3), STY1177 (SEQ ID NO: 4), STY1179 (SEQ ID NO: 5), STY1304 (SEQ ID NO: 6), STY1878 (SEQ ID NO: 7), STY2195 (SEQ ID NO: 8), STY0206 (SEQ ID NO: 9), STY0595 (SEQ ID NO: 10), STY0909 (SEQ ID NO: 11), STY1375 (SEQ ID NO: 12), STY1522 (SEQ ID NO: 13), STY2167 FlIC (SEQ ID NO: 14), STY2381 (SEQ ID NO: 15), STY3089 (SEQ ID NO: 16), STY3090 (SEQ ID NO: 17), STY3796 (SEQ ID NO: 18) or any combination thereof.
29. The microfluidic device of any one of claims 25-27, wherein the *S. Typhi* protein responsive cytokine is selected from the group consisting of IFN- γ , IL-1, IP-10, IL-8, MCP-1, MDC, and MIP-1a.

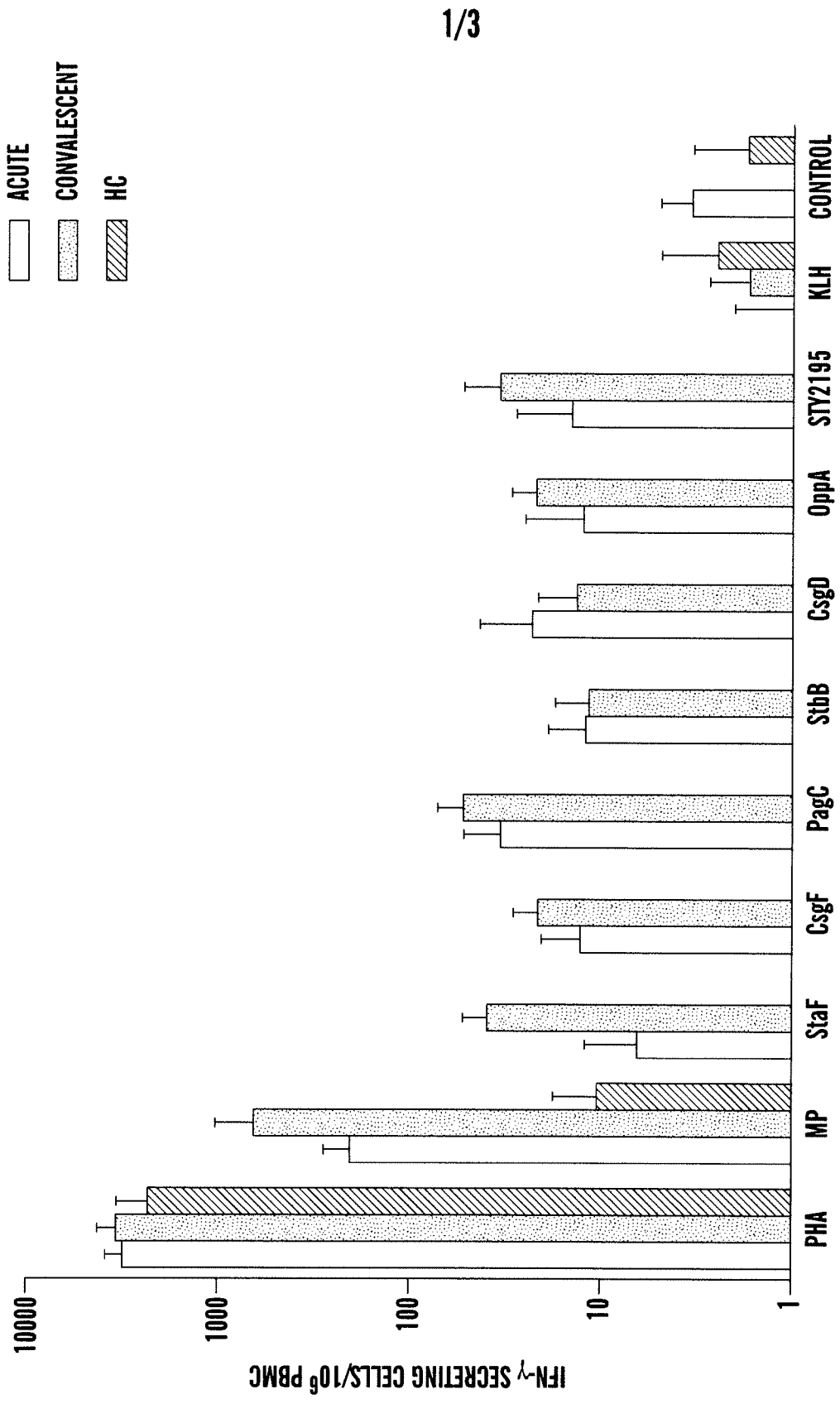


FIG. 1

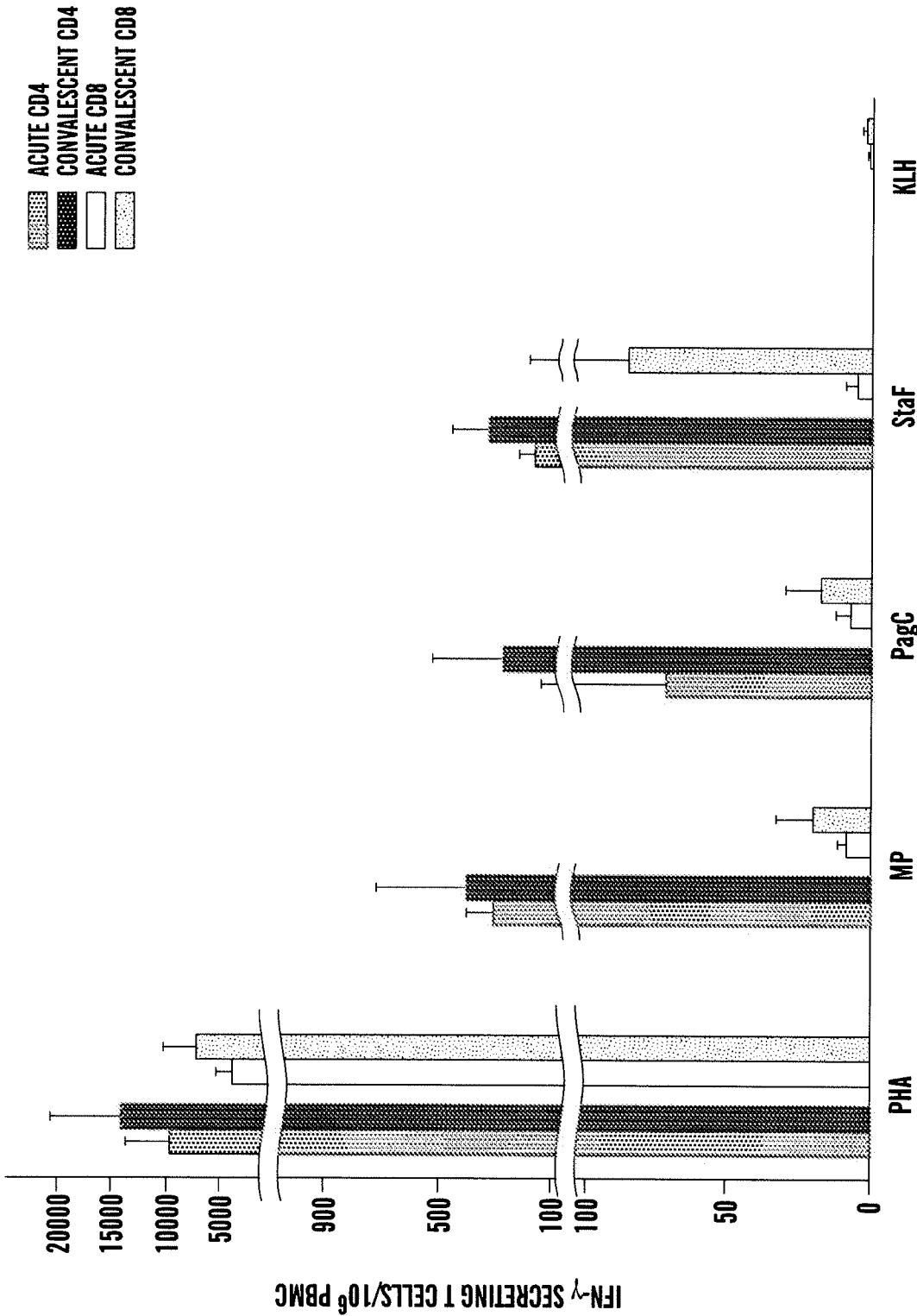


FIG. 2

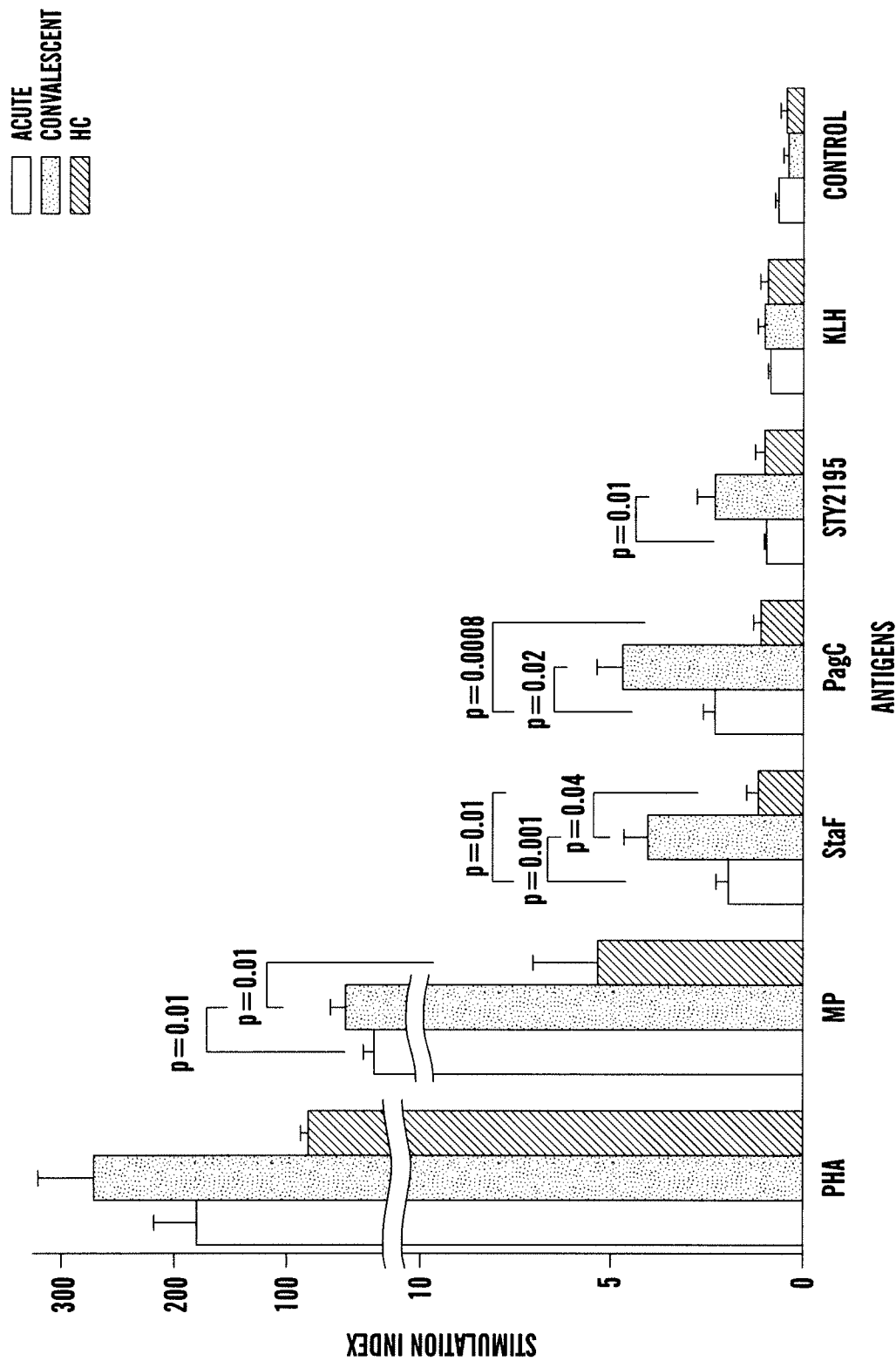


FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/032874

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/68 (2006.01)
 C07K 14/52 (2006.01)
 C07K 14/255 (2006.01)
 G06F 19/00 (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)

G01N 33/68, C07K 14/52, 14/255, G06F 19/00, G01N 33/96

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)

GoogleScholar, Esp@cenet, PCT Online, USPTO DB, WIPO, RUPTO, EAPATIS, PAJ, NCBI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PAI M. et al., "Interferon- γ assays in the immunodiagnosis of tuberculosis: a systematic review", Lancet Infectious Diseases 2004; 4: 761-763	1-29
Y	WYANT T.L. et al., "Salmonella typhi Flagella Are Potent Inducers of Proinflammatory Cytokine Secretion by Human Monocytes", Infection and Immunity, 1999, 67(7):3619-3624	1-29
Y	HARRIS J.B. et al., "Identification of In Vivo-Induced Bacterial Protein Antigens during Human Infection with Salmonella enterica Serovar Typhi", Infection Immunity, 2006; 74(9): 5161-5168	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AB0525, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AB0525 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AE0544, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AE0544 >	2, 3, 16, 17, 28

☒ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier document but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

20 July 2012 (20.07.2012)

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2012/032874

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE, GenPept, AE0635, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AE0635 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AG0635, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AG0635 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AG0650, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AG0650 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AG0717, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AG0717 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AD0754, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AD0754 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AF0525, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AF0525 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AD0570, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AD0570 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AI0605, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AI0605 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AB0659, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AB0659 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AG0675, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AG0675 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AH0750, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AH0750 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AE0776, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AE0776 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AB0860, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AB0860 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AC0860, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AC0860 >	2, 3, 16, 17, 28
Y	DATABASE, GenPept, AC0941, 18.11.2002 [retrieved on 19.07.2012]. Retrieved from the Internet:<URL: http://www.ncbi.nlm.nih.gov/protein/AC0941 >	2, 3, 16, 17, 28
Y	US 2007/0009884 A1 (GHC TECHNOLOGIES INC) 11.01.2007, paragraphs [0034], [0081]-[0082], [0210]-[0226], [0244]-[0246]	13-15, 18-29