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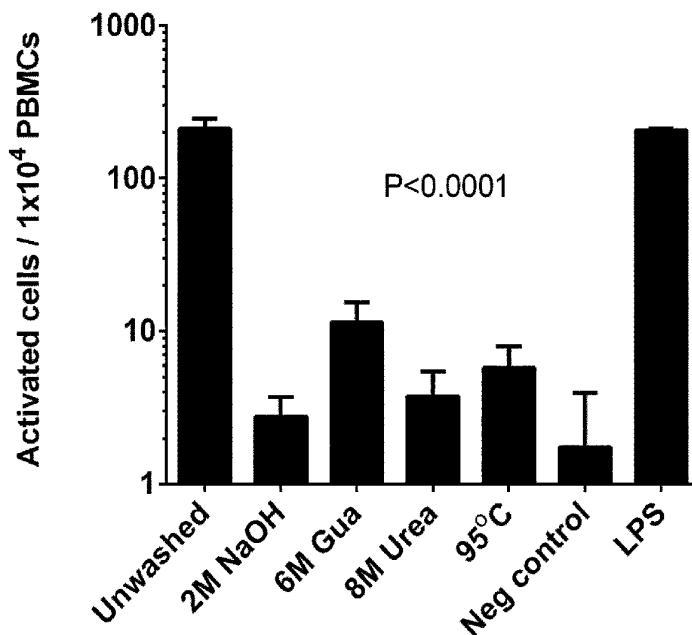
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(54) **T-CELL REACTIVITY PLATFORM**

(57) Methods for assaying antigen-specific T-cell activation, comprising the steps of (a) providing a phagocytatable particle, having a candidate antigen polypeptide tightly associated thereto, wherein the particle with the associated polypeptide has been subjected to a denaturing wash resulting in an endotoxin level low enough to not interfere with the subsequent steps; (b) providing a viable antigen-presenting cell; (c) contacting the washed particle with the antigen-presenting cell under conditions allowing phagocytosis of the particle by the antigen-presenting cell; (d) providing a T-cell sample to

be assayed comprising viable T-cells; (e) contacting the T-cell sample with the antigen-presenting cell contacted with the particle under conditions allowing specific activation of T-cells in response to an antigen presented by an antigen-presenting cell; and (f) determining the degree of T-cell activation in the T-cell sample. Methods for reducing endotoxin in a polypeptide sample. A collection of multiple different polypeptides tightly associated to phagocytatable particles, wherein the polypeptides have been subjected to denaturing wash subsequent to association to the particles.

Figure 1



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Description**TECHNICAL FIELD**

[0001] The present invention relates to the field of diagnostic and analytic methods involving determination of antigen-specific T-cell activation.

BACKGROUND TO THE INVENTION

[0002] The adaptive immune system constitutes the branch of the immune system that can specifically adapt and respond to different pathogens or cell-damaging challenges encountered by the organism, as opposed to the innate immune system that responds in a more generic way. The adaptive system includes both humoral immunity, i.e. antibodies secreted by B-cells, and T-cell-mediated immunity. The specificity of the adaptive immune system lies in the B- and T-cell receptors expressed on B- and T-cells. Through an intricate system of mixing of gene segments the body produces an almost infinite variety of B- and T-cells, each expressing a specific receptor for a certain protein or peptide.

[0003] T-cells are lymphocytes that form a major part of the adaptive immune system, where they play a central role in cell-mediated immunity. The defining T-cell receptor (TCR) is expressed on the cell surface, and each receptor recognises an antigen derived peptide presented in the context of an MHC (major histocompatibility complex) molecule. Several types of T-cells exist, each having a distinct function in the cellular immune response.

[0004] Briefly, there are two main types of T-cells with different functions. The CD8-positive cytotoxic T-cell will bind peptides presented on the MHC class I receptor (in humans, termed human leukocyte antigen (HLA) class I) on a cell. All nucleated cells express HLA class I. If the presented peptide is considered as foreign (most often a sign of viral infection), the cytotoxic T-cell will kill the cell either by the protein Granzyme B or Perforin. The helper T-cell (Th), expressing the surface marker CD4, does not kill, rather it orchestrates immune responses by secretion of cytokines, proteins that will augment both pro-inflammatory and sometimes inhibitory signals between cells. Another important function of T-helper cells is to induce class switching of B-cells, e.g. to turn an IgM-secreting B-cell into an IgG-secreting cell, which will increase the humoral immune response against an antigen.

[0005] A T-helper cell will by way of its TCR bind its corresponding peptide presented on MHC-II (in humans, termed HLA class II), a receptor specifically expressed on so-called antigen presenting cells (APCs) or endothelial cells. The T-cell activation is dependent on the micro-environment where the Th-APC interaction takes place and the type of so-called co-stimulatory molecules that are expressed on the APC. The T-helper cell can differentiate into different Th-subsets, e.g. pro-inflammatory Th1, Th2, Th17 cells or to an inhibitory T-helper cell type

called regulatory T-cells (Treg). The latter subset is of great importance to control immune responses, since an unrestrained immune system is harmful and can lead to tissue damage and autoimmunity.

5 [0006] Several methods suitable for determining antigen-specific T-cell activation exist, including ELISpot, Fluorospot, intracellular staining of cytokines with flow cytometry, FASCIA, proliferation assays (eg thymidine incorporation, CFSE or BrdU staining), specific TCR-detection with MHC-I or II tetramers, and ELISA- or Luminex
10 analysis of secreted cytokines.

[0007] With the ELISpot technique, one directly detects release of a specific cytokine by cells in response to a stimulus. The cells are seeded on a membrane and the number of cytokine secreting cells is measured. Depending on which cytokine is being measured, different variables such as macrophage or T-cell activation can be measured. FluoroSpot builds upon the ELISpot technique and allows for simultaneous readings of several
15 different secreted cytokines. This allows for a more exact and nuanced estimation of cell activation. Both of these methods offer a quick and easy way of measuring T-cell activation.

[0008] In recent years, large collections of recombinantly expressed proteins, i.e. proteins created with cloning techniques and bacterial or mammalian expression systems, have been produced by large-scale efforts such as the Human Atlas Project. The inventors have come to the realization that it would be of significant scientific and practical interest to be able to screen such polypeptide collections for antigens that are specifically T-cell activating. For instance, it would be of interest to screen T-cell samples from patients affected e.g. by an autoimmune disease, neoplastic disease, allergy or an infectious disease against a polypeptide library, to determine which epitopes the patient's T-cells are reacting to. Current methods are generally focused on determining which epitopes the patient's immune system is producing antibodies against (i.e. humoral response), failing to provide relevant information on a major part of the adaptive immune system, T-cell mediated immunity. Thus, being able to determine in large scale the antigens resulting in T-cell activation in a patient or a group of patients could tap into a new pool of novel biomarkers for the diseases, enable better diagnosis, support monitoring of disease progression in a patient, help identify therapeutic targets, etc.
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[0009] For practical reasons the large polypeptide collections that exist today are usually made up of *E. coli* expressed proteins, and therefore contain substantial amounts of endotoxins derived from the host cell outer membrane. Due to environmental contamination, even polypeptide collections prepared in eukaryotic hosts or prepared by non-biological peptide synthesis contain
30 practically significant levels of endotoxin. In the preparation of large collections, it is unpractical to establish a process that eliminates endotoxins to levels below detection, since endotoxins tend to bind to the proteins and
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are often difficult to remove once they contaminate a polypeptide sample.

[0010] Unfortunately, a common problem with assays determining T-cell activation is that even low levels of endotoxins that come into contact with the T-cells result in an activation masking the normally very low level of antigen-specific activation. Only a small fraction of the T-cell population being tested reacts in an antigen-specific manner to a given antigen (in the order of 1/10000 in blood from a subject that has recently encountered the antigen), whereas a large fraction of the cells will respond to endotoxins creating a high level of background. Given the ubiquitous endotoxin contamination it is not feasible to perform the screening outlined above with the current polypeptide collections.

[0011] Thus, there is still need in the art for methods for assaying antigen-specific T-cell activation able to tolerate endotoxin contamination in the candidate antigen preparation, enabling screening of large polypeptide collections. It is an object of the present invention to provide improved methods and means for assaying antigen-specific T-cell activation.

DEFINITIONS

[0012] *Endotoxins*, e.g. Lipopolysaccharide (LPS), comprise covalently linked lipid and polysaccharide subunits found on the outer cell wall of gram-negative bacteria, such as *Escherichia coli*.

[0013] *CD4⁺ T-cell or T-helper cells* are cells that orchestrate immune responses through cytokine secretion. They can both suppress or potentiate other immune cells such as stimulate antibody class switching of B-cells, expansion of cytotoxic T-cells or potentiate phagocytes. They get activated by antigen presentation via MHC class II on APCs and they express a T-cell receptor (TCR) specific for a stretch of approximately 15 amino acids (a so-called T-cell epitope) within a particular antigen.

[0014] *CD8⁺ T-cell or cytotoxic T-cells* are cells that kill tumour cells, infected cells or cells otherwise damaged. Unlike CD4⁺ T-cells they do not need APCs for activation. Their T-cell receptor recognizes antigen derived peptides (approximately 9-11 amino acids long) presented by MHC class I, a protein expressed on all nucleated cells.

[0015] *Antigen-specific T-cell activation* is a process requiring interaction between the TCR and a defined peptide presented on a MHC (HLA) molecule in combination with co-stimulation.

[0016] *Protein Epitope Signature Tag (PrEST)*: Recombinantly produced peptides, which are fragments of human proteins, representing unique peptide sequences of the protein they derive from. (See Lindskog M, Rockberg J, Uhlen M, Sterky F. Selection of protein epitopes for antibody production. *Biotechniques*. 2005;38(5):723-7)

[0017] *Antigen-presenting cells (APC)* are typically dendritic cells (DCs), B-cells or macrophages, cells that either phagocytose or internalise extra-cellular organisms

or proteins, i.e. antigens, and after processing present antigen-derived peptides on MHC class II to CD4⁺T-cells. In blood, monocytes are the most abundant antigen-presenting cells.

[0018] A *phagocytatable particle* is defined as a particle able to be phagocytosed by cells of the immune system, in particular monocytes.

[0019] *Peripheral blood mononuclear cells, PBMC*, is a fraction of human blood prepared by density gradient centrifugation of whole blood. The PBMC fraction mainly consists of lymphocytes (70-90%) and monocytes (10-30%), while red blood cells, granulocytes and plasma have been removed.

BRIEF DESCRIPTION OF THE FIGURES

[0020]

Figure 1. Comparison of different washes for endotoxin removal. Monocyte activity in response to protein coupled beads subjected to different washes was assessed, comparing the effectiveness of different denaturing washes to non-washed beads. Lower activation is desirable. P-value determined using one-way ANOVA. Staples denote SD.

Figure 2. Measurement of T-cell activation with a cell proliferation assay. Thymidine incorporation assay using splenocytes from ovalbumin sensitized mice. Comparison of ovalbumin coupled beads, bovine serum albumin coupled beads and medium. P-value determined with students T-test and indicated when $p < 0.05$ found. Staples denote SD.

Figure 3. IFN γ /IL-22/IL-17 Fluorospot assay comparing T-cell activation in multiple sclerosis (MS)-patients and healthy controls when stimulated with a suspected autoantigen in MS. P-values determined with Mann-Whitney-U test and written when found. Staples denote CI95% of mean.

Figure 4. Autoantigen screening in Multiple Sclerosis-patients against a library of 125 proteins. Comparison of the T-cell activation from MS-patients' PBMCs to that of PBMCs from healthy controls. Panel A, activation determination by IL22-FluoroSpot. Panel B, activation determination by IL17-FluoroSpot. Panel C, activation determination by IFN γ FluoroSpot. Patient's T-cells react significantly more to certain proteins in the library. Open squares and filled circles indicate mean activation in patients' and controls' PBMCs respectively, staples denote CI95% of mean. P-value determined using a two-way ANOVA. Asterisks denote P-value.

Figure 5. Effect of bead size on T-cell activation. Proliferation assay (with thymidine incorporation) with splenocytes from ovalbumin sensitized mice.

Comparison of ovalbumin coupled to differently sized beads with a diameter of 5.6 μ m, 1 μ m and 0.2 μ m. P-values determined using students T-test and written indicated when $p < 0.05$ found. Staples denote SD.

SUMMARY OF THE INVENTION

[0021] The present invention relates to the following items. The subject matter disclosed in the items below should be regarded disclosed in the same manner as if the subject matter were disclosed in patent claims.

1. A method for assaying antigen-specific T-cell activation, comprising the steps of:

a. Providing a phagocytatable particle, having a candidate antigen polypeptide tightly associated thereto, wherein the particle with the associated polypeptide has been subjected to a denaturing wash resulting in an endotoxin level low enough to not interfere with the subsequent steps;

b. Providing a viable antigen-presenting cell;

c. Contacting the washed particle with the antigen-presenting cell under conditions allowing phagocytosis of the particle by the antigen-presenting cell;

d. Providing a T-cell sample to be assayed comprising viable T-cells;

e. Contacting the T-cell sample with the antigen-presenting cell contacted with the particle under conditions allowing specific activation of T-cells in response to an antigen presented by an antigen-presenting cell; and

f. Determining the degree of T-cell activation in the T-cell sample.

2. The method according to item 1, wherein the method further comprises the steps (a') tightly associating a candidate polypeptide to a phagocytatable particle and/or (a'') subjecting a candidate antigen associated with a particle to a denaturing wash.

3. A method for assaying antigen-specific T-cell activation, comprising the steps of:

a. Providing a phagocytatable particle;

b. Tightly associating a candidate antigen polypeptide to the particle;

c. Subjecting the particle with the associated

polypeptide to a denaturing wash resulting in an endotoxin level low enough to not interfere with the subsequent steps;

d. Providing a viable antigen-presenting cell;

e. Contacting the washed particle with the antigen-presenting cell under conditions allowing phagocytosis of the particle by the antigen-presenting cell;

f. Providing a T-cell sample to be assayed comprising viable T-cells;

g. Contacting the T-cell sample with the antigen-presenting cell contacted with the particle under conditions allowing specific activation of T-cells in response to an antigen presented by an antigen-presenting cell; and

h. Determining the degree of T-cell activation in the T-cell sample.

4. The method according to any of the preceding items, further comprising the step of comparing the degree of T-cell activation to a relevant reference, whereby a higher degree of T-cell activation in the sample compared to the reference is indicative of the conclusion that the candidate antigen results in antigen-specific T-cell activation in the sample.

5. The method according to any of the preceding items, wherein determining the degree of T-cell activation in the T-cell sample involves determining the fraction of activated T-cells in the sample.

6. The method according to any of the preceding items, wherein several candidate antigens are assayed against the same T-cell sample.

7. The method according to any of the preceding items, wherein at least 10 candidate antigens are assayed against the same T-cell sample.

8. The method according to any of the preceding items, wherein the particle has a largest dimension of less than 5.6 μ m, preferably less than 4 μ m, more preferably less than 3 μ m, even more preferably in the interval 0.5-2 μ m or most preferably about 1 μ m.

9. The method according to item 8, wherein the particle is substantially spherical.

10. The method according to any of the preceding items, wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a high pH, such as at least pH 13, more preferably at least pH 14, most preferably at least pH 14.3.

11. The method according to any of the preceding items, wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a low pH.

12. The method according to any of the preceding items, wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a high temperature, such as at least 90°C, more preferably at least 92°C, most preferably at least 95°C.

13. The method according to any of the preceding items, wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a denaturing agent, such as urea or guanidine hydrochloride at a sufficient concentration, such as at least 5M, 6M, 7M or 8M.

14. The method according to any of the preceding items, wherein the denaturing wash results in an endotoxin amount in the antigen being such that in the T-cell activation assay, the final concentration of endotoxin is less than 100 pg/ml, preferably less than 50 pg/ml, more preferably less than 25 pg/ml and most preferably less than 10 pg/ml.

15. The method according to any of the preceding items, wherein the particle has paramagnetic properties.

16. The method according to any of the preceding items, wherein the candidate antigen polypeptide is covalently linked to the particle.

17. The method according to any of the preceding items, wherein the candidate antigen polypeptide is linked to the particle via a metal chelate.

18. The method according to any of the preceding items, wherein the antigen-presenting cell and the T-cell sample are derived from the same individual.

19. The method according to any of the preceding items, wherein the antigen-presenting cell and the T-cell sample are derived from the same blood sample.

20. The method according to any of the preceding items, wherein the antigen-presenting cell and the T-cell sample are derived from a PBMC-sample from the same individual.

21. The method according to item 20, wherein the PBMC sample is fresh or has been subjected to freezing.

22. The method according to any of items 1-18,

wherein the T-cell sample is derived from a tumour, preferably a lymphatic vessel in a tumour.

23. The method according to any of items 1-18, wherein the T-cell sample is derived from ascites.

24. The method according to any of the preceding items, wherein the T-cell sample comprises CD4+ and/or CD8+ T-cells.

25. The method according to any of the preceding items, wherein the washed particle, the antigen presenting cell and the T-cell sample are contacted concurrently.

26. The method according to any of the preceding items, wherein determining the degree of T-cell activation in the T-cell sample comprises determining the fraction of T-cells contacted with the antigen-presenting cell responding by secretion of IFN- γ , IL-17, IL-22 or a combination thereof.

27. The method according to any of the preceding items, wherein the method is for diagnosing multiple sclerosis, or following the course of multiple sclerosis, and involves determining the degree of T-cell activation in the T-cell sample comprises determining the fraction of T-cells contacted with the antigen-presenting cell responding by secretion of IL-17 and/or IL-22.

28. The method according to any of the preceding items, wherein determining the degree of T-cell activation in the T-cell sample is performed using an ELISpot or a FluoroSpot-technique.

29. The method according to any of the preceding items, wherein the candidate antigen polypeptide comprises at least 50 amino-acids, preferably at least 75 amino-acids, most preferably at least 100 amino-acids.

30. The method according to any of the preceding items, wherein the candidate antigen polypeptide is in form of a Protein Epitope Signature Tag (PrEST).

31. The method according to any of the preceding items, wherein the T-cell sample is from a human and the candidate antigen polypeptide sequence is derived from a human.

32. The method according to any of the preceding items, wherein the candidate antigen polypeptide is derived from an antigen known or suspected to be associated with disease.

33. The method according to any of the preceding items, wherein the candidate antigen polypeptide is

derived from a polypeptide being:

- a. known to be highly expressed in a tissue or cell affected in an autoimmune disease;
- b. known to be associated with a neoplastic disease;
- c. known to be associated with an autoimmune disease;
- d. known to be associated with an infectious disease; or
- e. known to be associated with allergy or similar hypersensitivity.

34. A method for according to any of the preceding items, for diagnosing autoreactivity in a subject comprising the steps of:

- a. Providing a phagocytatable particle having a candidate auto-antigen peptide tightly associated thereto, wherein the candidate auto-antigen has been subjected to a denaturing wash subsequent to association to the particle resulting in an endotoxin level low enough to not interfere with the subsequent steps;
- b. Providing a peripheral blood mononuclear cell (PBMC) sample from the subject comprising viable T-cells and viable antigen-presenting cells;
- c. Contacting the PBMC sample with the particles under conditions allowing phagocytosis of the particles by antigen-presenting cells and allowing specific activation of T-cells in response to an antigen presented by an antigen-presenting cell;
- d. Quantitating the fraction of activated T-cells in the contacted PBMC sample; and
- e. Comparing the quantitated fraction to comparably quantitated fraction from a healthy subject, whereby higher fraction of activated T-cells in the subject is indicative of autoreactivity against the auto-antigen in the subject.

35. A method for reducing endotoxin in a polypeptide sample, comprising the steps of:

- a. Tightly associating the polypeptide of the sample to a particle; and
- b. Subjecting the particle with the associated polypeptide to a denaturing wash to reduce endotoxins in the polypeptide sample, wherein the

denaturing wash results in an endotoxin level low enough to not interfere with a method for assaying antigen-specific T-cell activation in response to the polypeptide sample.

36. The method according to item 35 wherein the particle is a phagocytatable particle.

37. The method according to item 35 or 36 wherein the particle has a largest dimension of less than 5.6 μm , preferably less than 4 μm , more preferably less than 3 μm , even more preferably in the interval 0.5-2 μm or most preferably about 1 μm .

38. The method according to any of items 35-37 wherein the particle has paramagnetic properties.

39. The method according to any of items 35-38 wherein the association is covalent.

40. The method according to any of items 35-39 wherein the association is by metal chelation.

41. The method according to any of items 35-40 wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a high pH, such as at least pH 13, more preferably at least pH 14, most preferably at least pH 14.3.

42. The method according to any of items 35-41 wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a low pH.

43. The method according to any of items 35-42, wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a high temperature, such as at least 90°C, more preferably at least 92°C, most preferably at least 95°C.

44. The method according to any of items 35-43, wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a denaturing agent, such as urea or guanidine hydrochloride at a sufficient concentration, such as at least 5M, 6M, 7M or 8M.

45. The method according to any of items 35-44, wherein the denaturing wash results in an endotoxin amount in the polypeptide sample being such that in a T-cell activation assay performed on the polypeptide sample, the final concentration of endotoxin is less than 100 pg/ml, preferably less than 50 pg/ml, more preferably less than 25 pg/ml and most preferably less than 10 pg/ml.

46. A collection of multiple different polypeptides tightly associated to phagocytatable particles, wherein the polypeptides have been subjected to denaturing

wash subsequent to association to the particles resulting in an endotoxin level low enough to not interfere with a method for assaying antigen-specific T-cell activation in response to a polypeptide of the collection.

47. The collection according to item 46 comprising at least 100 different polypeptides.

48. The collection according to item 46 or 47, wherein at least one of the polypeptides is derived from a bacterial expression system.

49. The collection according to any of items 46-48, wherein the association is covalent.

50. The collection according to any of items 46-48, wherein the association is by metal chelation.

51. The collection according to any of items 46-50, wherein the particles have paramagnetic properties.

52. The collection according to any of items 46-51, the particles having a largest dimension of less than 5.6 μm , preferably less than 4 μm , more preferably less than 3 μm , even more preferably in the interval 0.5-2 μm or most preferably about 1 μm .

53. The collection according to any of items 46-52 wherein the sets of particles with a particular polypeptide are in separate containers.

54. The collection according to any of items 46-53, prepared by a method according to any of items 35-45

55. The collection according to any of items 46-54, wherein the endotoxin amount in the collection is such that in a T-cell activation assay performed on a polypeptide of the collection, the final concentration of endotoxin is less than 100 pg/ml, preferably less than 50 pg/ml, more preferably less than 25 pg/ml and most preferably less than 10 pg/ml.

DETAILED DESCRIPTION

[0022] The present invention provides methods and means for assaying antigen-specific T-cell activation as disclosed in more detail below. By solving the problem associated with using endotoxin-contaminated polypeptides in an assay to antigen-specific T-cell activation, the methods and means enable and facilitate large-scale screening of new and existing polypeptide collections for biomarker discovery, diagnosis, monitoring of disease progression in patients, identification of therapeutic targets, etc.

Methods for assaying antigen-specific T-cell activation

[0023] In a first aspect, the present invention provides a method for assaying antigen-specific T-cell activation, comprising the steps of:

a. Providing a phagocytatable particle, having a candidate antigen polypeptide tightly associated thereto, wherein the particle with the associated polypeptide has been subjected to a denaturing wash resulting in an endotoxin level low enough to not interfere with any of the subsequent steps (b-f) of the method;

b. Providing a viable antigen-presenting cell (APC);

c. Contacting the washed particle with the antigen-presenting cell contacted with the particle under conditions allowing phagocytosis of the particle by the antigen-presenting cell;

d. Providing a T-cell sample to be assayed comprising viable T-cells;

e. Contacting the T-cell sample with the antigen-presenting cell under conditions allowing specific activation of T-cells in response to an antigen presented by an antigen-presenting cell; and

f. Determining the degree of T-cell activation in the T-cell sample.

[0024] The method further may comprise the steps of (a') tightly associating a candidate polypeptide to a phagocytatable particle and/or (a'') subjecting a candidate antigen associated with a particle to a denaturing wash. Thus, the method of the first aspect may comprise the steps of:

a. Providing a phagocytatable particle;

b. Tightly associating a candidate antigen polypeptide to the particle;

c. Subjecting the particle with the associated polypeptide to a denaturing wash resulting in an endotoxin level low enough to not interfere with any of the subsequent steps (d-h) of the method;

d. Providing a viable antigen-presenting cell;

e. Contacting the washed particle with the antigen-presenting cell under conditions allowing phagocytosis of the particle by the antigen-presenting cell;

f. Providing a T-cell sample to be assayed comprising viable T-cells;

g. Contacting the T-cell sample with the antigen-presenting cell contacted with the particle under conditions allowing specific activation of T-cells in response to an antigen presented by an antigen-presenting cell; and

h. Determining the degree of T-cell activation in the T-cell sample.

[0025] The washed particle, the antigen presenting cell and the T-cell sample may be contacted concurrently, in the same container.

Candidate antigen polypeptide

[0026] The candidate antigen polypeptide preferably comprises at least 50 amino-acids, even more preferably at least 75 amino-acids, most preferably at least 100 amino-acids. The candidate antigen polypeptide may be in form of a Protein Epitope Signature Tag (PrEST). Large protein libraries consisting of PrESTs already exist, for example the library created within the Human Atlas Project (Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, et al. *Proteomics. Tissue-based map of the human proteome. Science.* 2015;347(6220):1260419). PrESTs contain amino-acid sequences unique for the corresponding full length protein, and have been used in the large scale Human Atlas Project for generating antibodies to most human proteins. The use of several PrESTs for the same human protein increases the chance of finding relevant T-cell epitopes. The antigen polypeptides being larger than the fragments presented by APCs also ensures that a wide variety of epitopes of each antigen will be presented, since the degradation of the antigen by the APCs is not a uniform process. In other words, the use of polypeptide antigens being of certain size is enabled by utilizing the phagocytic route of the APCs and allows improved detection of antigens with use of fewer antigen polypeptides, as opposed to presenting the antigens in short peptides that are capable of binding to MCH-receptors directly without cellular processing.

[0027] The candidate antigen polypeptide may be derived from an antigen known or suspected to be associated with disease. For example, the candidate antigen polypeptide is derived from a polypeptide being:

a. known to be highly expressed in a tissue or cell affected in an autoimmune disease; Examples being Proinsulin, Myelin-associated proteins.

b. known to be associated with a neoplastic disease; Examples being Estrogen receptor, Epidermal growth factor receptor, Cyclin-dependent kinase 1.

c. known to be associated with an autoimmune disease; Examples: Myelin Oligodendrocyte Protein, Myelin Basic Protein, Transaldolase.

d. known to be associated with an infectious disease; Examples: Viral capsid antigen, bacterial enterotoxins.

5 e. known to be associated with allergy or similar hypersensitivity; Examples: Can f1, Equ c1, Fel d1.

10 f. a known tumour antigen; a neoantigen formed by common mutations in e.g. p53, ERBB2 (Erb-B2 Receptor Tyrosine kinase 2), PIK3CA (Phosphatidylinositol-4,5-bisphosphate 3-kinase)

15 g. a known modified protein, such citrullinated, variant, or phosphorylated protein.

[0028] The T-cell sample and the candidate antigen polypeptide are derived from the same species or from different species. For instance, for studying autoimmunity or neoplastic disease, the T-cell sample and the candidate antigen polypeptide are preferably derived from the same species. For studying infectious disease, the T-cell sample is from the host whereas the candidate antigen polypeptide is derived from pathogens of interest. For studying allergy or similar hypersensitivity, the T-cell sample is from the subject of interest whereas the candidate antigen polypeptide is derived from a different species known or suspected to elicit allergic or other hypersensitive reaction in the subject.

30 **[0029]** The T-cell sample may be from a human and the candidate antigen polypeptide sequence may be derived from a human.

Particle properties

35 **[0030]** The particle is phagocytatable by the antigen-presenting cell (APC; discussed below). The APCs can phagocytose particles of many different materials and shapes. In contrast, the size of the particles is limiting for phagocytosis. Too small of a size and the cells will not be triggered to phagocytose the particle. Too large of a size and the cell will not be able to phagocytose the particle as it will not fit in the cell.

40 **[0031]** The particles may have a largest dimension of less than 5.6 μm , preferably less than 4 μm , more preferably less than 3 μm , even more preferably in the interval 0.5-2 μm or most preferably about 1 μm . The particles may be substantially spherical, in which case the dimensions would refer to diameter.

45 **[0032]** Size similar to that of bacteria facilitates complete phagocytosis by APCs. This to ensure the antigen gets degraded by APCs and subsequently presented to T-cells via MHCII. The optimal size depends on the type of specific APC and can be determined by routine experimentation (see Example 5, Figure 5)

50 **[0033]** The particle may have paramagnetic properties, which facilitates the denaturing wash discussed below by permitting the particles to be collected and/or held in place by a magnet. However, it is also possible to per-

form the washes by other means, such as by holding the particles in a column, or sedimenting the particles by gravity or by centrifugation.

Association of the antigen polypeptide and the particle

[0034] The antigen polypeptide is associated to the particle in a manner that allows performing a denaturing wash as discussed below without dissociating the antigen from the particle.

[0035] One possible way of associating the polypeptide to the particle is shown in **Example 1**. However, the precise manner of association is not critical for the methods of the invention. Preferably, the candidate antigen polypeptide is covalently linked to the particle. Alternatively, the candidate antigen polypeptide may be linked to the particles via a metal chelate.

[0036] For example, particles linked with a metal chelating ligand, such as iminodiacetic acid can bind metal ions such as Cu^{2+} , Zn^{2+} , Ca^{2+} , Co^{2+} or Fe^{3+} . These metal chelates can in turn bind proteins and peptides containing for example histidine or cysteine with great strength. Thus, particles with metal chelates can non-covalently adsorb peptides/proteins, in a manner which allows stringent washing to reduce the amount of LPS and other contaminating components in the bound peptides/proteins.

Denaturing wash

[0037] The denaturing wash is performed to reduce the level of endotoxin in the candidate antigen polypeptide to a level that does not interfere with the later step of determining the degree of antigen-specific T-cell activation.

[0038] Since endotoxins associate tightly with polypeptides, performing a denaturing wash which at least transiently disrupts the secondary and tertiary structure is highly advantageous and in many cases essential to dissociate the endotoxin from the polypeptide. In the present application, even very low quantities of endotoxin cause interference.

[0039] The antigen-presenting cells will after uptake degrade the antigen polypeptide to small fragments, thus both transient and irreversible denaturation (disruption of the 3D-structure) can be tolerated. Association with the particles as disclosed by the present invention allow the handling of denatured proteins which may otherwise become insoluble or aggregate.

[0040] As shown in **Example 2 and Figure 1**, the particular manner of denaturing wash is not critical in the context of the present invention. For instance, the denaturing wash may involve subjecting the associated polypeptide to a high pH, to a low pH, to a high temperature, to a denaturing agent or a combination thereof. Preferably, the denaturing wash involves subjecting the associated polypeptide to a high pH or to a strong denaturing agent such as 8M urea or 6M guanidine-HCl. Most

preferably, the denaturing wash involves subjecting the associated polypeptide to a high pH of at least 13.0, more preferably at least 14.0, most preferably at least 14.3. The denaturing may involve subjecting the associated polypeptide to a wash with 1-5M, preferably 1-3M, more preferably 1.5-2.5M, most preferably 2M strong alkali, such as NaOH or KOH, preferably NaOH.

[0041] Advantageously, the denaturing wash is such that it degrades or inactivates endotoxins, in addition to flushing them away.

[0042] The wash may comprise a single wash or several repeated washes, such as 2, 3, 4 or 5 washes.

[0043] The denaturing wash must result in an endotoxin level in the candidate antigen polypeptide that is compatible with a method for assaying antigen-specific T-cell activation as disclosed herein, such that the endotoxin does not result in a too high interference for the antigen-specific T-cell activation to be adequately detected. The level of remaining endotoxin may conveniently be tested using a monocyte activation assay (IL-1 β /IL-6 FluoroSpot) as described herein in example 2. The tolerable level of endotoxin depends on the circumstances and in particular the antigen concentration. However, the endotoxin amount in the antigen preferably is such that in the T-cell activation assay, the final concentration of endotoxin is less than 100 pg/ml (i.e. 0-100 pg/ml), preferably less than 50 pg/ml, more preferably less than 25 pg/ml and most preferably less than 10 pg/ml. Methods for measuring endotoxin in a sample are known, for example the LAL assay.

Antigen-presenting cell (APC) and the T-cell sample

[0044] In the context of the present invention, the APC is a professional antigen presenting cell, such as a monocyte/macrophage or a dendritic cell. The APC may be a primary cell or an immortalized cell.

[0045] The APC must be compatible with the T-cells of the T-cell sample, such that they are capable of presenting antigens to the T-cells in an antigen-specific context (MHC restricted) that the T-cells can react to. The APC and the T-cell sample are preferably obtained from the same species and donor-matched with respect to MHC receptors. However, use of genetically engineered APCs from a different species is also envisioned.

[0046] If the antigen-presenting cell and the T-cell sample are derived from the same individual, any potential for a mismatch between the APC and the T-cells is avoided.

[0047] The antigen-presenting cell and the T-cell sample may be derived from the same blood sample, which is advantageous from a practical point of view. The antigen-presenting cell and the T-cell sample may be derived from a PBMC-sample from the same individual. Obtaining PBMC from peripheral blood samples is a routine protocol, which provides a handy source for both APCs and T-cells at the same time and from the same individual.

[0048] The PBMC sample may be freshly used or subjected to freezing. The possibility of using frozen cells is of great practical advantage from a logistical point of view.

[0049] The T-cell sample may be derived from a tumour, preferably a lymphatic vessel in a tumour.

[0050] The T-cell sample may also be derived from ascites.

[0051] The T-cell sample may comprise whole PBMCs including both CD4+ and CD8+ T-cells, purified T-cell populations, or PBMCs depleted of (a) particular T-cell population(s).

Determining the degree of T-cell activation

[0052] The method may further comprise the step of comparing the degree of T-cell activation to a relevant reference, whereby a higher degree of T-cell activation in the sample compared to the reference leads to the conclusion that the candidate antigen results in antigen-specific T-cell activation in the sample. The reference is critical for defining "activation", so degree of activation is preferably defined in comparison to reference samples. The reference samples may for example be samples from normal healthy individuals when analysing patient samples, samples from normal tissue when analysing tumour or tumour draining lymph node samples or samples from before a treatment when following treatment effects. The reference samples are used to set the threshold for diagnostic/prognostic conclusion, and determine antigen specific positive i.e. activation, or negative i.e. downregulation.

[0053] Determining the degree of T-cell activation in the T-cell sample may involve determining the *fraction* of activated T-cells in the sample, i.e. the number of activated T-cells in relation to the total T-cells in the sample. The fraction of activated cells in relation to the fraction of activated cells in the reference sample will provide a measure of the magnitude of T-cell activation to specific antigens. Preferably, in each analysis the level of spontaneous activation ("background activation level") in individual samples is determined in samples incubated without antigen, i.e. negative control. The background activation may be compensated for in the analysis; fractions of activation are calculated on net values where the fraction of activation to negative control is subtracted.

[0054] As shown in **Example 3**, determining the degree of T-cell activation in the T-cell sample may be performed using an ELISpot/FluoroSpot-technique or a proliferation assay (i.e. thymidine incorporation). However, other assays and techniques suitable for the purpose are also known and may also be used. Determining the degree of T-cell activation in the T-cell sample may comprise determining the fraction of T-cells contacted with the antigen-presenting cell responding by secretion of Interferon gamma (IFN- γ), Interleukin 17 (IL-17), Interleukin 22 (IL-22) or a combination thereof, e.g. by means of an ELISpot or FluoroSpot assay. Other relevant analytes (combination(s) of cytokines) can be used for ana-

lysing specific diseases or conditions. For example, when analysing allergic patients, Th2 cytokines rather than Th1/Th17 cytokines may be analysed. T-reg cytokines may be of interest in cancer, allergy and when following vaccine treatments. Thus the FluoroSpot technique enables analysis of relevant activation profiles to various candidate antigen peptides in various conditions. This adds information on e.g. heterogeneity between patients, disease severity and progression.

[0055] As seen in **Example 3**, the determination of IL-17 and/or IL-22 secretion is surprisingly better than IFN- γ for differentiating MS patients from healthy controls. Thus, determining the degree of T-cell activation in the T-cell sample preferably comprises determining the fraction of T-cells contacted with the antigen-presenting cell responding by secretion of IL-17 and/or IL-22, and the method is for diagnosing multiple sclerosis, or following the course of multiple sclerosis. In other words, a T-cell sample derived from a subject having MS or suspected to have MS is preferably analysed for IL-17 and/or IL-22 secretion in response to candidate antigens.

Multiplex analysis

[0056] As shown in **Example 4**, several candidate antigens may be assayed against the same T-cell sample. Preferably, at least 10 candidate antigens are assayed against the same T-cell sample. With the inventive method additional samples do not necessarily increase the laboratory work by much. Herein lies a strength of the inventive method, that it is easy to screen for a large number of antigens without much effort. An advantage of this is for example that being able to perform a large screening of possible autoantigens increases the chance of finding true autoantigens.

Diagnosing autoreactivity

[0057] The method of the first aspect may be a method for diagnosing autoreactivity in a subject comprising the steps of:

a. Providing a phagocytatable particle having a candidate auto-antigen peptide tightly associated thereto, wherein the candidate auto-antigen has been subjected to a denaturing wash subsequent to association to the particle resulting in an endotoxin level low enough to not interfere with the subsequent steps (b-e) of the method;

b. Providing a peripheral blood mononuclear cell (PBMC) sample from the subject comprising viable T-cells and viable antigen-presenting cells;

c. Contacting the PBMC sample with the particles under conditions allowing phagocytosis of the particles by antigen-presenting cells and allowing specific activation of T-cells in response to an antigen pre-

sented by an antigen-presenting cell;

d. Quantitating the fraction of activated T-cells in the contacted PBMC sample; and

e. Comparing the quantitated fraction to comparably quantitated fraction from a healthy subject, whereby higher fraction of activated T-cells in the subject is indicative of autoreactivity against the auto-antigen in the subject.

[0058] As shown in **Example 4**, the present invention provides a powerful tool to quickly screen for a large variety of autoantigens at the same time, and is not limited to MS. A screenable candidate library of proteins can readily be designed depending on disease and patients.

[0059] The method can be used for other inflammatory diseases in addition to autoimmune conditions, as long as antigen specific T-cells is a feature of the disease and the antigen library is adapted to the disease in question.

Method for reducing endotoxin in a polypeptide sample

[0060] In a second aspect, the present invention provides a method for reducing endotoxin in a polypeptide sample, comprising the steps of:

a. Tightly associating the polypeptide to a particle, preferably a phagocytatable particle; and

b. Subjecting the particle with the associated polypeptide to a denaturing wash to reduce endotoxins in the sample, wherein the denaturing wash results in an endotoxin level low enough to not interfere with a method for assaying antigen-specific T-cell activation in response to the polypeptide sample.

[0061] The polypeptide may be a candidate antigen polypeptide as described above in the context of the first aspect. It is particularly preferred that the polypeptide comprises at least 50 amino-acids.

[0062] The particle may be as described above in the context of the first aspect. It is particularly preferred that the particle has paramagnetic properties. It is particularly preferred that the particle has a largest dimension in the interval 0.5-2 μm .

[0063] The association between polypeptide and the particle may be as described above in the context of the first aspect. It is particularly preferred that the association is covalent or by metal chelation.

[0064] The denaturing wash may be as described above in the context of the first aspect and may involve subjecting the associated polypeptide to a high pH, to a low pH, to a high temperature, to a denaturing agent (such as urea, guanidine hydrochloride or the like) or a combination thereof. A high pH wash is particularly preferred, most preferably pH>14.

[0065] The method preferably reduces the level of endotoxin to such levels, that the remaining endotoxin amount in the polypeptide sample is such that in a T-cell activation assay performed on the sample, the final concentration of endotoxin is less than 100 pg/ml (i.e. 0-100 pg/ml), preferably less than 50 pg/ml, more preferably less than 25 pg/ml and most preferably less than 10 pg/ml.

10 Polypeptide collection

[0066] In a third aspect, the present invention provides a collection of multiple different polypeptides tightly associated to phagocytatable particles, wherein the polypeptides have been subjected to denaturing wash subsequent to association to the particles, having an endotoxin level low enough to not stimulate monocytes activation to a degree that interferes with a method for assaying antigen-specific T-cell activation in response to a polypeptide of the collection. A FluoroSpot assay measuring IL-1 β and IL-6 responses in monocytes provides a sensitive functional assay of endotoxins. By stimulating PBMCs containing monocytes with washed beads the efficiency of LPS removal is assessed.

[0067] The tolerable level of endotoxin in the collection depends on the polypeptide concentration, but is preferably such that in a T-cell activation assay, the final concentration of endotoxin is less than 100 pg/ml (i.e. 0-100 pg/ml), preferably less than 50 pg/ml, more preferably less than 25 pg/ml and most preferably less than 10 pg/ml.

[0068] At least one of the polypeptides in the collection may be derived from a bacterial expression system, such as *E. coli*.

[0069] The collection may comprise at least 100 different polypeptides in the present assay format. Using the FluoroSpot assay, one 96-well FluoroSpot plate allows for 48 tests to be done in duplicates simultaneously. If more polypeptides are present, either a pooling of two or more polypeptides can be done to fit it into one plate, or more plates can be used. In **example 4**, 125 different polypeptides were used.

[0070] The polypeptides may be selected candidate antigen polypeptides as described above in the context of the first aspect or may represent large libraries of polypeptides from a tissue or whole organism protein library.

[0071] The particles may be as described above in the context of the first aspect.

[0072] The association between polypeptides and the particles may be as described above in the context of the first aspect. It is particularly preferred that the association is covalent. It is particularly preferred that the particles are paramagnetic and having a largest dimension in the interval 0.5-2 μm .

[0073] The denaturing wash may be as described above in the context of the first aspect and may involve subjecting the associated polypeptide to a high pH, to a

low pH, to a high temperature, to a denaturing agent or a combination thereof.

[0074] Sets of particles with a particular polypeptide may be designed for particular applications/diseases and provided in separate containers.

[0075] The collection may be manufactured by way of the method of the second aspect.

[0076] The term "comprising" is to be interpreted as including, but not being limited to. All references are hereby incorporated by reference. The arrangement of the present disclosure into sections with headings and sub-headings is merely to improve legibility and is not to be interpreted limiting in any way, in particular, the division does not in any way preclude or limit combining features under different headings and subheadings with each other.

EXAMPLES

Example 1: Polypeptide coupling to beads

[0077] Covalently coupled polypeptides to beads containing free carboxylic acid groups. In this example Dynabeads® MyOne™ Carboxylic Acid (ThermoFischer Scientific) was used (1 μm diameter spheres). The bicinchoninic acid, BCA, protein assay was used to measure protein concentration before and after coupling to ensure successful coupling. Several polypeptides were tested and an average of 48.7 μg (mean: 48.7, SD: 20.5, N=10) was coupled per 1 mg beads. According to manufacturer's instruction, 50 μg polypeptide can be coupled per 1 mg beads, indicating that the efficiency of the coupling done in this example was high.

Example 2: Endotoxin removal by denaturing washes

[0078] To test endotoxin removal with different types of denaturing washes, four different denaturing washing conditions were tested: (a) High pH (2M NaOH pH 14.3), (b) Heat (95°C) and denaturing agents ((c) 8M Urea and (d) 6M guanidine hydrochloride). The level of remaining endotoxin was tested using a monocyte activation assay (IL-1β/IL-6 FluoroSpot) as monocyte activity is strongly increased by endotoxin and thus monocyte activation can be used for monitoring remaining LPS.

[0079] All tested types of denaturizing washes managed to significantly lower the amount of monocyte activation compared to the unwashed beads (see figure 1) (P<0.0001 for all washes). The denaturing washes were so effective the washed beads stimulated the cells to a degree comparable to that of the negative control. Wash with 2M NaOH resulted in the lowest amount of endotoxin, followed closely by 8M urea.

Example 3: T-cell activation assays

FluoroSpot

[0080] FluoroSpot assay was performed with samples from 16 Multiple Sclerosis Patients and 9 healthy controls. PBMCs from patients and controls were incubated together with a suspected autoantigen in the FluoroSpot plate. As seen in **Figure 3**, several of the MS patients showed significantly higher number of activated cells (panel a). The MS patients had a mean number of 37.4 (95%CI 13.0-52.9) IL-22 secreting activated cells while the healthy controls had a mean number of 3.3 (95%CI 0.97-5.6) activated cells. P value of difference = 0.0062. For IL-17 secreting activated cells the number was 14.7 (CI95% 5.3-24.1) in patients compared to 0.28 (CI95% -1.4-2.0), P value of difference <0.0007 (panel b). For IFN_γ secreting cells however the difference was not statistically significant with a mean number of 80.0 (CI95% 22.3-138) for patients versus 33 (CI95% 7.1-59.0) P=0.495 for controls (panel c). Significance calculated using Mann-Whitney-U test.

[0081] These results show that the activation of T-cells with antigen coupled to beads can be measured with the FluoroSpot assay, and comparisons of patients vs. healthy controls can be made. Surprisingly the classic marker for T-cell activation, IFN_γ, showed the weakest difference in the patient vs control comparison. Both IL-17 and IL-22 however showed a strong significant difference between patients and controls, indicating that these might be more suitable cytokines to analyse when looking for autoantigens in MS.

Proliferation assay

[0082] Proliferation assay with Thymidine incorporation. Splenocytes from ovalbumin (OVA) immunized mice were incubated with OVA or BSA coupled beads to measure antigen specific proliferation. The proliferation of the cells incubated with beads was expressed as stimulation index (SI). (As seen in **figure 2** the cells incubated with OVA-beads had an increase in proliferation with an SI of 2.69 (95%CI 1.6 - 3.78, P<0.005). The cells incubated with BSA failed to give an increase in proliferation with an SI of 0.9 (95%CI 0.7-1.1, P=0.37).

[0083] These results show that the activation of immune cells (splenocytes in this example) with antigen coupled to beads can be measured by a proliferation assay. It also shows that the proliferation observed is antigen specific, as the OVA coupled beads stimulated proliferation, but the BSA coupled beads completely failed to induce proliferation. The experiment shows that antigen coupled to beads can be used for *in vitro* stimulation of antigen-specific T-cell proliferation. Thymidine incorporation is just one method to measure proliferation, other methods for measuring proliferation will thus also be applicable, e.g. CFSA dilution and BrdU incorporation assays.

Example 4: Autoantigen screen in MS against a library of 125 proteins

[0084] Identification of autoantigens in Multiple Sclerosis; Measuring T-cell activation to a large number of PrESTs coupled to beads, using IFN γ /IL-22/IL-17A FluoroSpot as assay for T-cell activation. A screening like this identifies possible autoantigens by detecting those antigens that stimulate a higher T-cell response in PBMCs from MS patients compared to healthy controls, and helps decide which antigen to analyse further.

[0085] As seen in **Figure 4**, in this screening of 16 patients and 9 healthy controls, statistically significant difference between mean patient and mean control number activated cells could be seen when analysed using a Two-Way ANOVA. For IL-22 secreting activated T-cells difference was seen for 5 antigens, antigen #6 (P<0.0001), antigen #18 (P<0.0001), antigen #23 (P<0.0001), antigen #29 (P<0.0001) and antigen #33 (P<0.05) (**panel a**). For IL-17 secreting T-cells a difference between patients and controls could be seen for the same 5 antigens, antigen #6 (P<0.05), antigen #18 (P<0.0001), antigen #23 (P<0.01), antigen #29 (P<0.0001) and antigen #33 (P<0.05) (**panel b**). For IFN γ a difference could be seen only for antigen #18 (p<0.0001) (**panel c**).

[0086] These results show that a method of the present invention can be used as an autoantigen screening, a tool to identify possible new autoantigens in MS.

Example 5: Identification of suitable bead size for phagocytatable beads

[0087] Proliferation assay with Thymidine incorporation was used to test the effect of bead size on antigen-specific T-cell activation. Splenocytes from ovalbumin (OVA) immunized mice were stimulated with OVA coupled beads of different sizes to measure antigen specific proliferation. As seen in **Figure 5**, cells incubated with OVA-beads with a diameter of 0.2 μ m showed increase in proliferation with a mean SI of 4.1 (95%CI 2.4-5.8, P=0.007). The cells incubated with OVA-beads with a diameter of 1 μ m showed increase in proliferation with a mean SI of 8.4 (95%CI 6.1-10.6, P<0.005). The cells incubated with OVA-beads with a diameter of 5.6 μ m failed to stimulate proliferation, mean SI 1.1 (95%CI 0.4-2.7, P=0.876).

[0088] These results show that antigen coupled to beads of different sizes can stimulate cell proliferation. The beads with a diameter of about 1 μ m seems to be most efficient in regards to cell stimulation but beads down to a size of 0.2 μ m still works. It is reasonable to predict that beads of sizes larger than 1 μ m also work, although as the diameter comes close to 5.6 μ m the beads completely fail to stimulate the cells. It is reasonable to assume that 1 μ m is an optimal size, since it is similar to the size of bacteria. Our immune system has evolved to phagocytose and react to microorgan-

isms/particles of this size. A normal antigen presenting cell has a size in the range 10-15 μ m.

MATERIALS AND METHODS

Example 1

[0089] Covalent coupling of proteins to paramagnetic beads containing free carboxylic acid groups.

[0090] In this example Dynabeads[®] MyOne[™] Carboxylic Acid with 1 μ m diameter (ThermoFischer Scientific) were used and the coupling procedure was carried out according to the manufacturers protocol (Two-Step procedure using NHS (N-Hydroxysuccinimide) and EDC (ethyl carbodiimide)).

[0091] Beads were washed twice with MES-Buffer (25mM MES (2-(N-morpholino)ethanesulfonic acid), pH 6). The carboxylic acid groups were then activated by adding 50 mg/ml NHS (N-Hydroxysuccinimide) and 50mg/ml EDC (N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide) in MES-buffer to the beads and incubated for 30 minutes in room temperature. The beads were collected with a magnet and the supernatant was removed and the beads washed twice with MES-buffer. The protein was diluted in MES-buffer to a concentration of 1 mg/ml, total 100 ug and added to the beads and incubated for 1h in room temperature. The beads were collected with a magnet and the supernatant was removed and saved for protein-concentration measurement. The non-reacted activated carboxylic acid groups were quenched with 50 mM Tris pH 7,4 for 15 minutes. The beads were then washed with PBS pH 7.4 and then stored in -80°C.

[0092] To measure the amount of protein coupled to the beads, a BCA protein assay kit (Pierce BCA Protein Assay Kit, ThermoFisher Scientific) was used to measure the protein concentration of the protein before coupling as well as in the supernatant after coupling. The BCA-assay was used according to the manufacturer's protocol.

Example 2

[0093] Endotoxin removal by denaturing washes.

[0094] Beads were coupled with a recombinant protein produced in *E. coli*. The protein-coupled beads were washed at several different denaturing conditions to ensure removal of endotoxin. The remaining endotoxin was measured using a monocyte reactivity assay (IL1B/IL6 FluoroSpot-assay, MABTECH, Sweden).

[0095] For endotoxin removal, the beads were washed with one of 3 different wash-buffers, 2M NaOH pH 14.3, 8M Urea or 6 M Guanidine (Guanidine-HCl), all in sterile water at RT, or they were incubated in PBS at 95°C. The beads were suspended in the buffer and shaken for 4 min, collected with a magnet and the supernatant removed. This was repeated 3 times. The heat treated beads were put in PBS pH 7.4 and put in a heating block at 95°C for 5 minutes, then collected with a magnet and

the supernatant removed. This was repeated 3 times. The beads were then washed 3 times with sterile PBS to remove any remaining wash-buffer.

[0096] To measure endotoxin levels, a monocyte reactivity assay was used (IL-1 β /IL-6 FluoroSpot-assay, MABTECH, Sweden). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation. Cells were suspended in cRPMI (RPMI 1640 medium containing 10% fetal calf serum, 1% L-glutamine and 1% penicillin-streptomycin). 3,000,000 beads were added to each well in the FluoroSpot plate in quadruplicates for each type of bead and each type of wash. Unwashed beads, LPS and medium were used as controls. The cells were added to the FluoroSpot plate at a concentration of 10,000 and 5,000 cells in 200 μ l cRPMI cells per well (2 wells of each cell concentration and each type of bead). The plate was incubated for 18h in a humidified environment at 37°C and 5% CO₂. The plate was then developed according to the manufacturer's protocol and read in a FluoroSpot-reader.

Example 3

FluoroSpot

[0097] The FluoroSpot assay for analyzing patient vs control T-cell activation versus a suspected autoantigen in MS was run with an IFN γ /IL17/IL22 FluoroSpot (MABTECH). The protocol was exactly as described in Example 4, although here just analyzing the response to one antigen instead of the whole library of 125 proteins.

Proliferation assay

[0098] Proliferation assay with thymidine incorporation with splenocytes from ovalbumin sensitized mice. As stimuli, ovalbumin (SigmaAldrich) and BSA (SigmaAldrich) coupled to beads (Dynabeads[®] MyOne[™] Carboxylic Acid) were used.

[0099] Mice were immunized to ovalbumin via monthly injections of 100 μ g ovalbumin (Sigma) adsorbed to aluminium hydroxide. Three months after the first injection the mice were killed and spleens harvested. Splenocytes were prepared by standard procedures, as described in Thunberg et al. 2009, Allergy 64:919.

[0100] The cells were incubated in cRPMI either with ovalbumin coupled beads or BSA coupled beads (10 beads per cell) for 5 days. All cells were incubated for 6 days in a humidified atmosphere with 6 % CO₂ at 37°C. One μ Ci/well [³H] thymidine was added to cell cultures for the final 18 hours of incubation. Mean counts per minute (cpm) obtained from stimulated triplicates were divided by mean cpm values from un-stimulated cells and expressed as stimulation indices (SI). SI-values \geq 2.0 are generally considered positive.

Example 4

[0101] An autoantigen screening against a library of 125 proteins in Multiple Sclerosis using protein coupled beads (Dynabeads[®] MyOne[™] Carboxylic Acid (ThermoFischer Scientific)) as stimuli and FluoroSpot (IFN γ /IL17/IL22 FluoroSpot, MABTECH) as assay for measuring for T-cell activation.

[0102] Peripheral blood mononuclear cells (PBMCs) from a total of 18 multiple sclerosis patients and 9 healthy controls were used. The 125 different proteins consisted of a wide variety of human-specific PrESTs from the Human Atlas Project. The PrESTs were coupled to beads according to manufacturer's protocol (as described in example 1).

[0103] PBMCs were isolated from venous blood samples (taken in BD Vacutainer EDTA-tubes) by Ficoll-Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation according to standard protocol. The cells were frozen in freezing medium (45% FCS, 45% RPMI, 10% DMSO) and stored in -150°C.

[0104] The 125 different PrESTs were pooled into 45 different pools to fit the 96-well format of the FluoroSpot and subsequently washed in 2M NaOH to remove endotoxin (as described in Example 2). 3,000,000 beads were added to each well of the FluoroSpot plate, each different pool was run in duplicates. Anti-CD3 was used as positive control, medium only and medium with naked beads were used as negative controls.

[0105] The PBMCs were thawed in a water bath at 37°C and washed in cRPMI (RPMI 1640, 10% FCS, 1% L-glut and 1% Penicillin-Streptavidin). 250,000 cells in 200 μ l cRPMI were added to each well and the plate was then incubated for 44h in a humidified environment at 37°C and 5% CO₂. After incubation, the plate was developed according to the manufacturer's protocol (Mabtech) and read in a FluoroSpot reader.

Example 5

[0106] Splenocytes from Ovalbumin sensitized mice and Ovalbumin coupled beads were used to evaluate the effectiveness of differently sized beads. Paramagnetic beads with a diameter of 5.6 μ m, 1 μ m and 0.2 μ m with carboxylic acid on their surface were coupled with ovalbumin or bovine serum albumin according to the protocol in Example 1.

[0107] To test the effectiveness of the beads to stimulate antigen specific T-cell activation a proliferation assay (with ³H thymidine incorporation) was used. Bead concentration in relation to cell concentration was 1:1 for the 5,6 μ m beads, 10:1 for the 1 μ m beads and 500:1 for the 0.2 μ m beads. Total protein concentration during the incubation with the cells was calculated to 125ng/ml, 160ng/ml and 160ng/ml for the 5.6 μ m, 1 μ m and 0.2 μ m respectively. The proliferation assay was run the same way as in example 3.

Claims

1. A method for assaying antigen-specific T-cell activation, comprising the steps of:
 - a. Providing a phagocytatable particle, having a candidate antigen polypeptide tightly associated thereto, wherein the particle with the associated polypeptide has been subjected to a denaturing wash resulting in an endotoxin level low enough to not interfere with the subsequent steps;
 - b. Providing a viable antigen-presenting cell;
 - c. Contacting the washed particle with the antigen-presenting cell under conditions allowing phagocytosis of the particle by the antigen-presenting cell;
 - d. Providing a T-cell sample to be assayed comprising viable T-cells;
 - e. Contacting the T-cell sample with the antigen-presenting cell contacted with the particle under conditions allowing specific activation of T-cells in response to an antigen presented by an antigen-presenting cell; and
 - f. Determining the degree of T-cell activation in the T-cell sample.
2. The method according to claim 1, wherein the method further comprises the steps (a') tightly associating a candidate polypeptide to a phagocytatable particle and/or (a'') subjecting a candidate antigen associated with a particle to a denaturing wash.
3. The method according to any of the preceding claims, wherein the particle has a largest dimension in the interval 0.5-2 μm .
4. The method according to any of the preceding claims, wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a high pH, such as at least pH 14.
5. The method according to any of the preceding claims, wherein the particle has paramagnetic properties.
6. The method according to any of the preceding items, wherein the candidate antigen polypeptide is covalently linked to the particle.
7. The method according to any of the preceding claims, wherein the antigen-presenting cell and the T-cell sample are derived from the same individual.
8. The method according to any of the preceding claims, wherein the washed particle, the antigen presenting cell and the T-cell sample are contacted concurrently.
9. The method according to any of the preceding claims, wherein the method is for diagnosing multiple sclerosis, or following the course of multiple sclerosis, and comprises determining the degree of T-cell activation in the T-cell sample comprises determining the fraction of T-cells responding by secretion of IL-17 and/or IL-22.
10. The method according to any of the preceding claims, wherein the candidate antigen polypeptide comprises at least 50 amino-acids.
11. A method for according to any of the preceding claims, for diagnosing autoreactivity in a subject comprising the steps of:
 - a. Providing a phagocytatable particle having a candidate auto-antigen peptide tightly associated thereto, wherein the candidate auto-antigen has been subjected to a denaturing wash subsequent to association to the particle resulting in an endotoxin level low enough to not interfere with the subsequent steps;
 - b. Providing a peripheral blood mononuclear cell (PBMC) sample from the subject comprising viable T-cells and viable antigen-presenting cells;
 - c. Contacting the PBMC sample with the particles under conditions allowing phagocytosis of the particles by antigen-presenting cells and allowing specific activation of T-cells in response to an antigen presented by an antigen-presenting cell;
 - d. Quantitating the fraction of activated T-cells in the contacted PBMC sample; and
 - e. Comparing the quantitated fraction to comparably quantitated fraction from a healthy subject, whereby higher fraction of activated T-cells in the subject is indicative of autoreactivity against the auto-antigen in the subject.
12. A method for reducing endotoxin in a polypeptide sample, comprising the steps of:
 - a. Tightly associating the polypeptide of the sample to a particle; and
 - b. Subjecting the particle with the associated polypeptide to a denaturing wash to reduce endotoxins in the polypeptide sample; wherein the denaturing wash results in an endotoxin level low enough to not interfere with a method for assaying antigen-specific T-cell activation in response to the polypeptide sample.
13. The method according to claim 12 wherein the denaturing wash involves subjecting the particle with the associated polypeptide to a high pH.
14. A collection of multiple different polypeptides tightly

associated to phagocytatable particles, wherein the polypeptides have been subjected to denaturing wash subsequent to association to the particles resulting in an endotoxin level low enough to not interfere with a method for assaying antigen-specific T-cell activation in response to a polypeptide of the collection. 5

15. The collection according to claim 14 wherein the particles have paramagnetic properties and have a largest dimension in the interval 0.5-2 μm . 10

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Figure 1

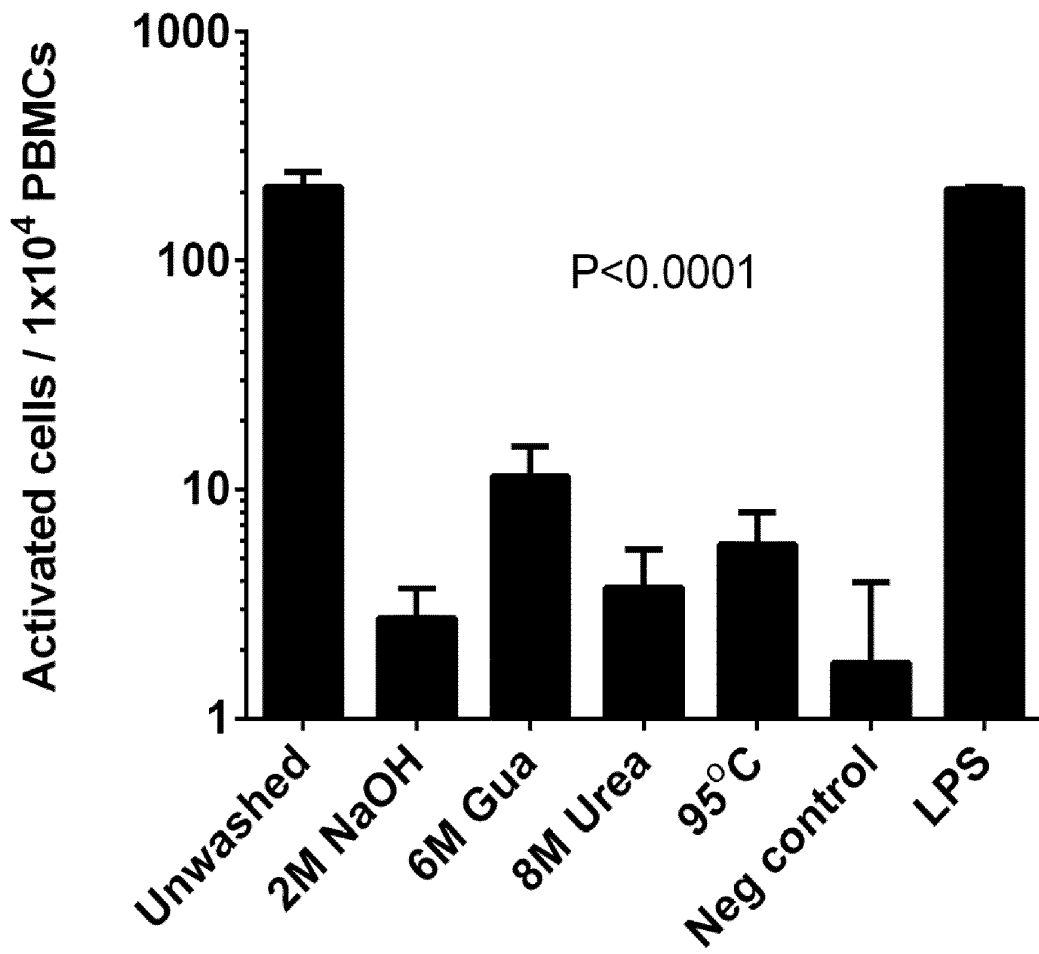


Figure 2

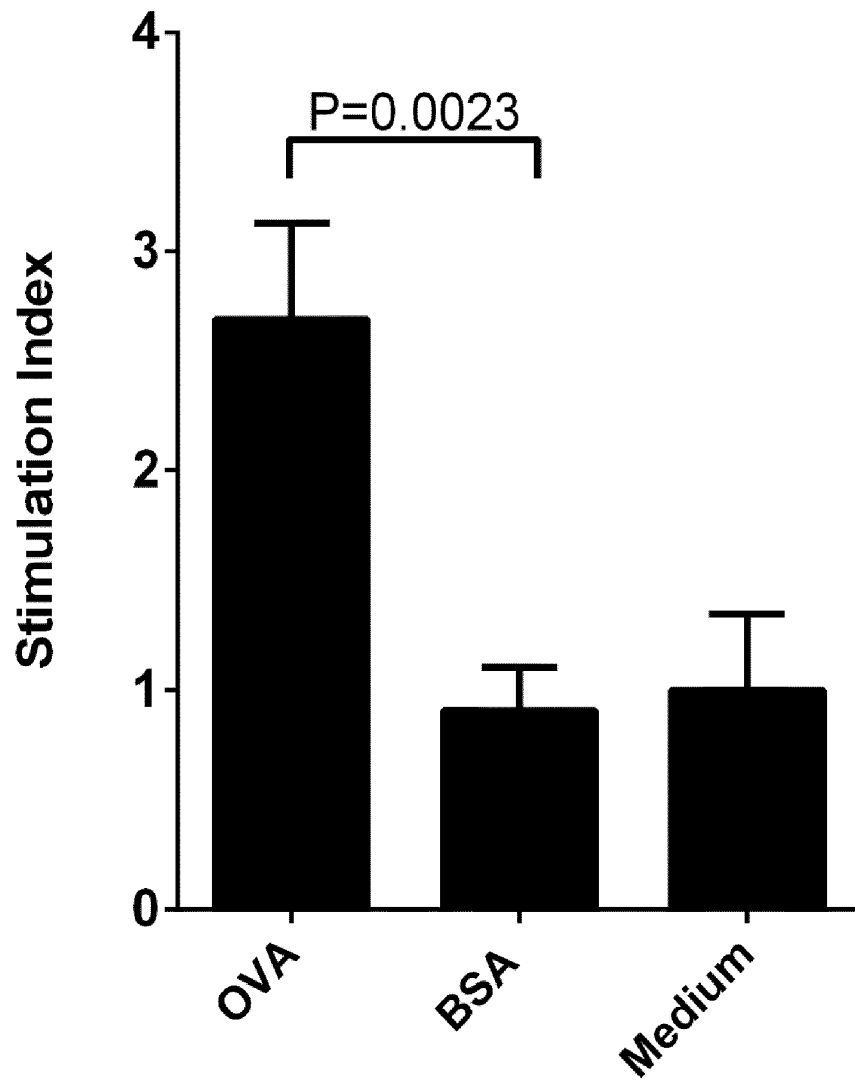


Figure 3a

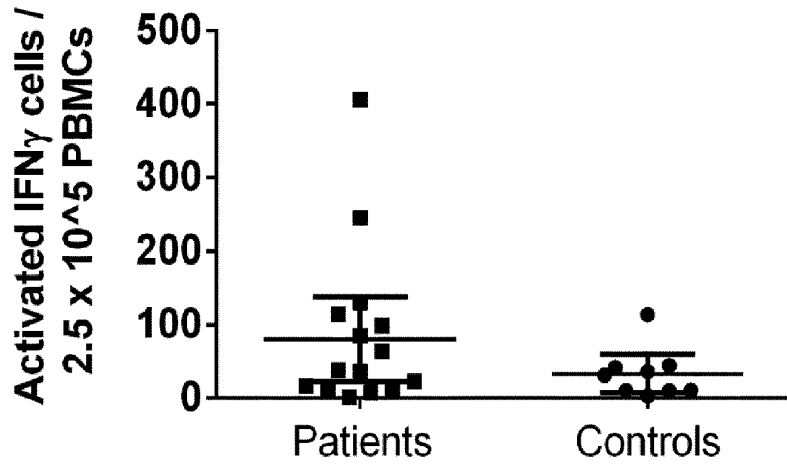


Figure 3b

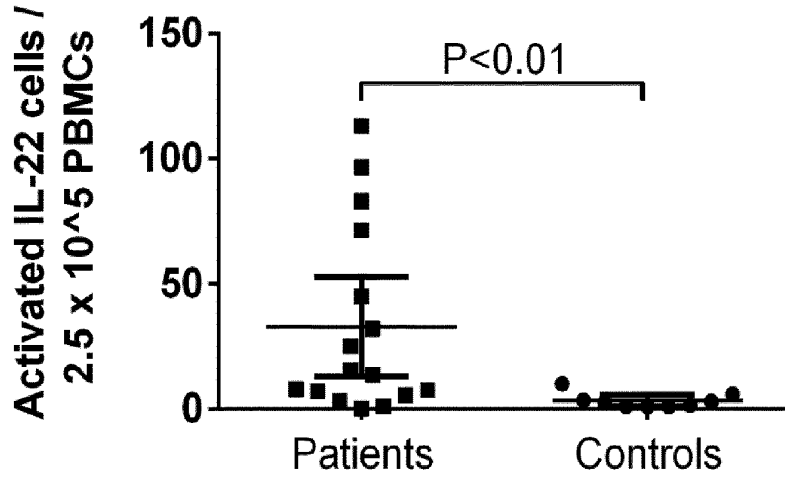
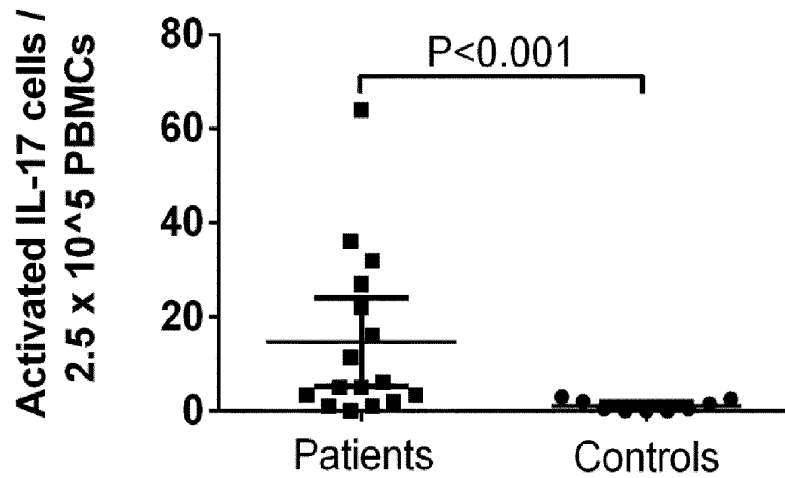
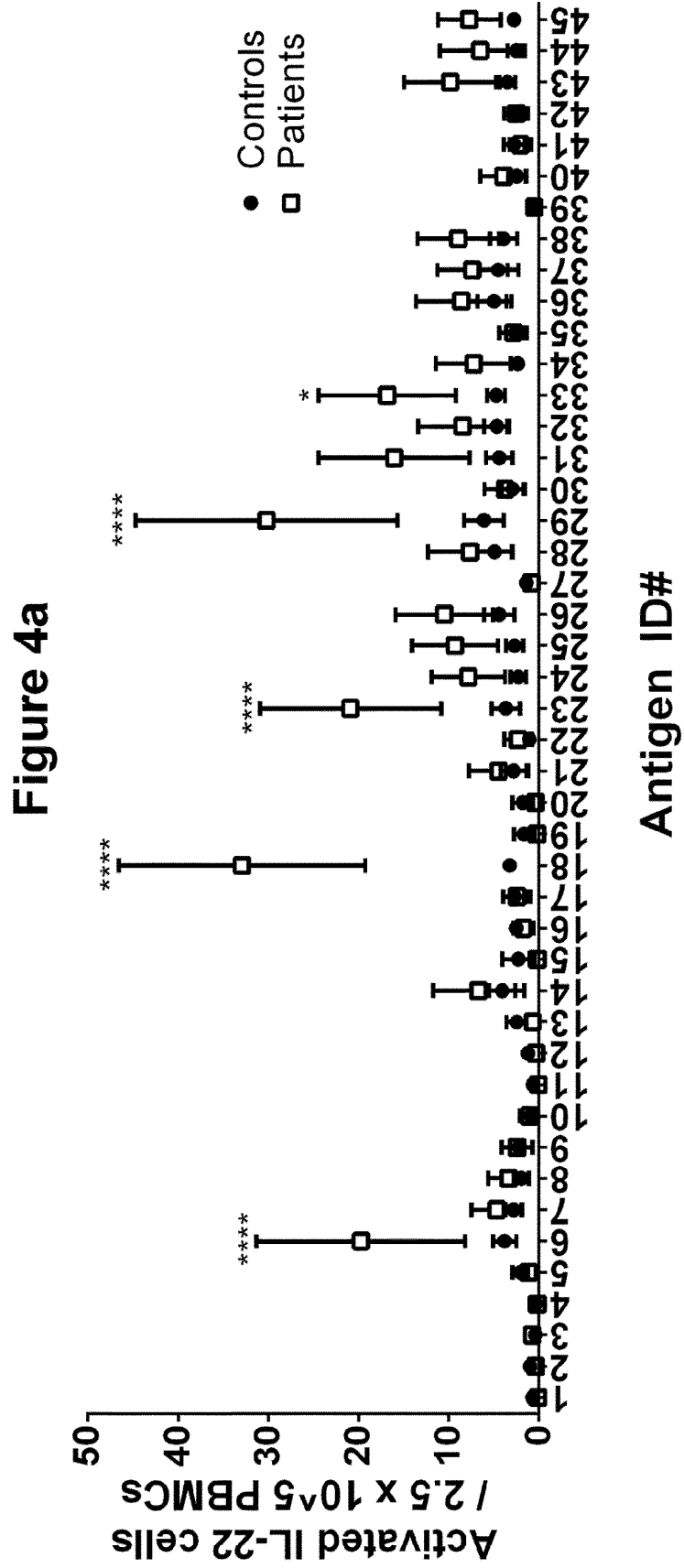
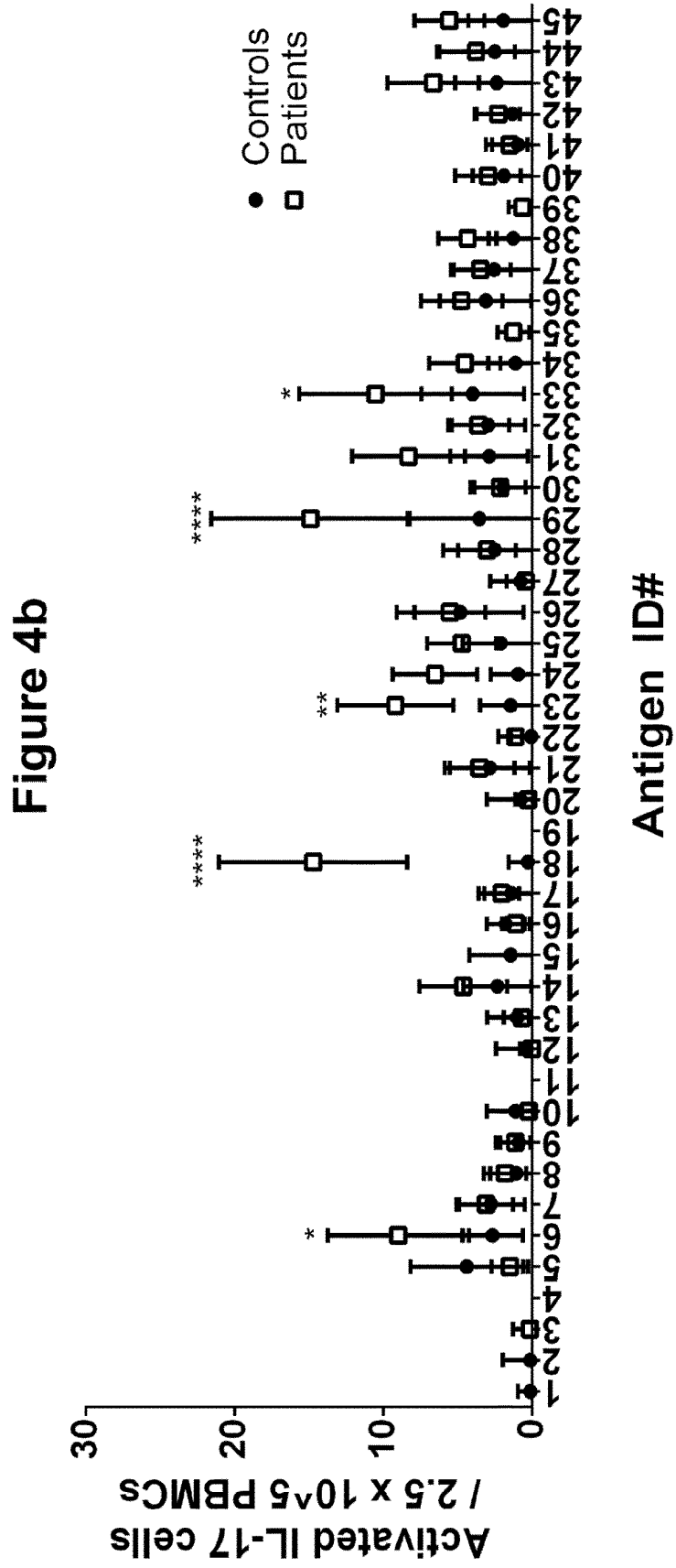


Figure 3c







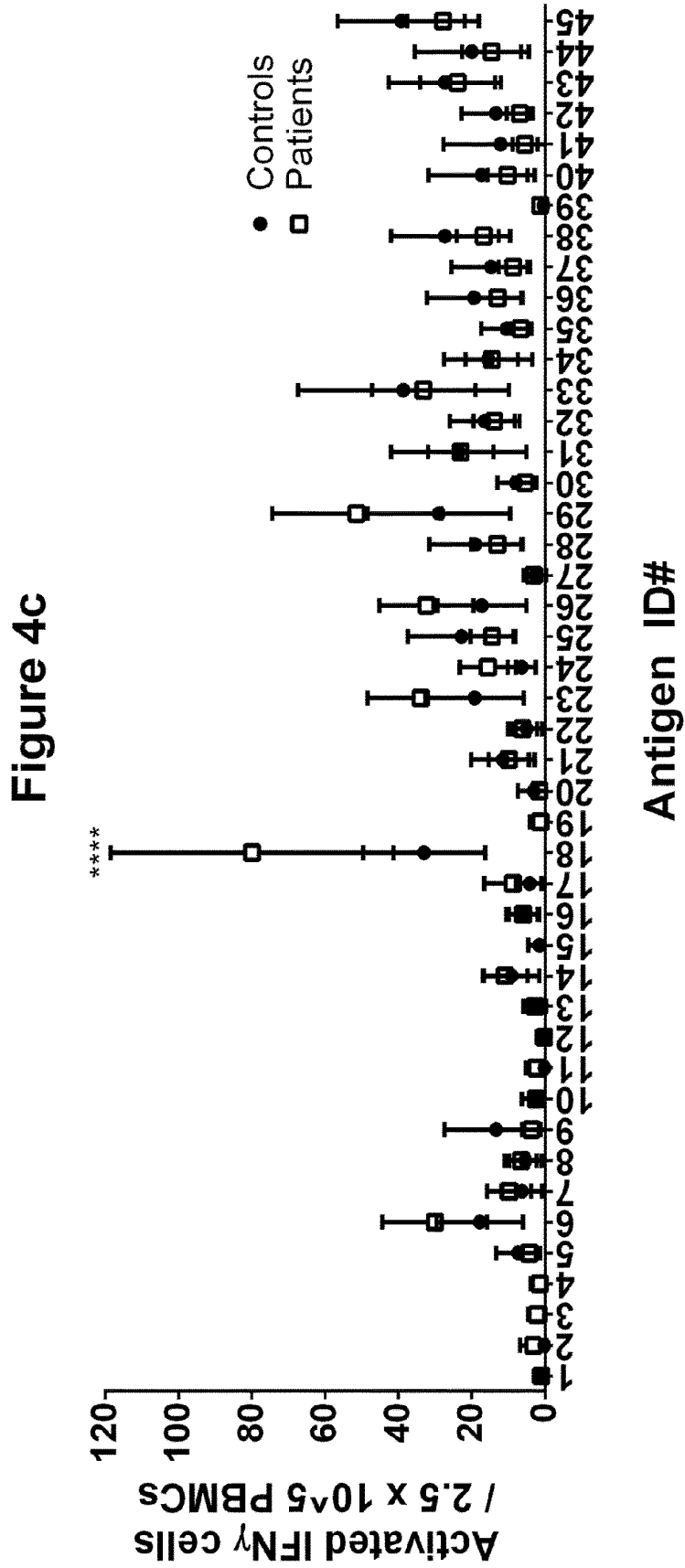
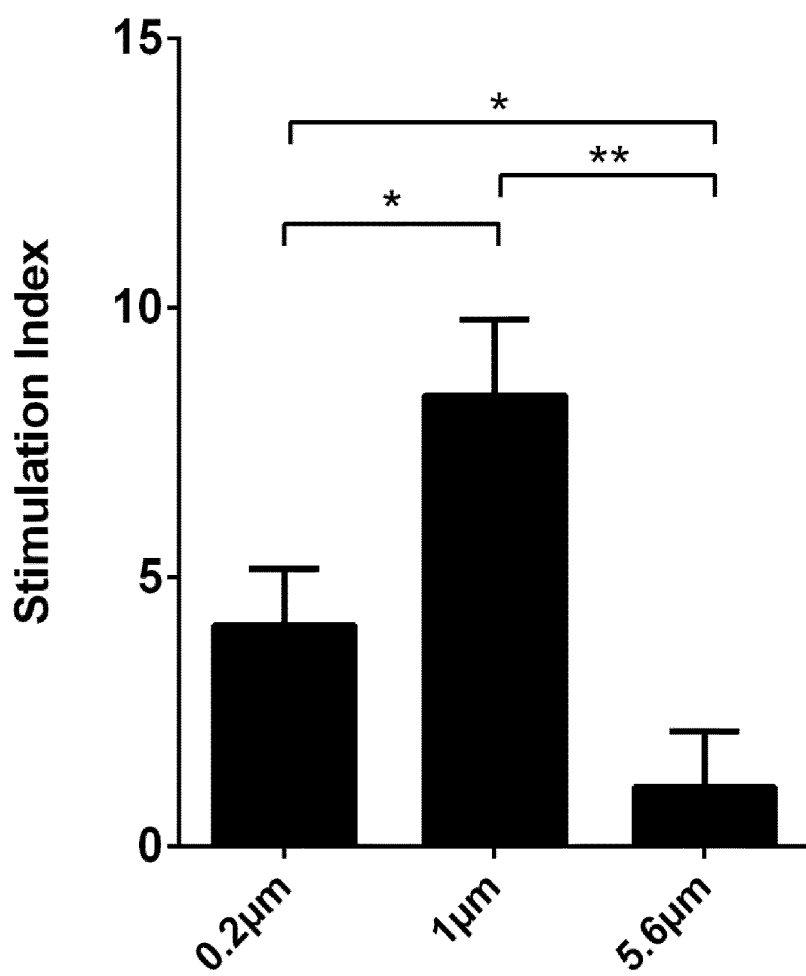


Figure 5





EUROPEAN SEARCH REPORT

Application Number
EP 15 20 0619

5

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	"Recombinant allergens for diagnosis and treatment" In: Hans Grönlund: "Diagnosis and treatment of IgE-mediated allergy: New approaches using recombinant allergens.", 6 May 2005 (2005-05-06), Intellecta Docucys, Nacka, Sweden, XP055258062, ISBN: 91-7140-373-6 * see abstract 3rd paragraph, page 14 "adjuvant preparation"; page 26 - page 29; figure 5 *	1-15	INV. G01N33/53 G01N33/564
X	S. THUNBERG ET AL: "Prolonged antigen-exposure with carbohydrate particle based vaccination prevents allergic immune responses in sensitized mice", ALLERGY, vol. 64, no. 6, 1 June 2009 (2009-06-01), pages 919-926, XP055258276, United Kingdom ISSN: 0105-4538, DOI: 10.1111/j.1398-9995.2008.01905.x * page 919 - page 921; figures *	1-15	TECHNICAL FIELDS SEARCHED (IPC) G01N
A	US 6 218 132 B1 (SPACK EDWARD G [US] ET AL) 17 April 2001 (2001-04-17) * claims; figures *	1-15	
A	WO 2005/010026 A2 (BECKMAN COULTER INC [US]; CHANG JENNIE CHYAN CHUU [US]; KASEY SUHA [US] 3 February 2005 (2005-02-03) * abstract; claims; figures *	1-15	
		-/--	
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 17 March 2016	Examiner Gonçalves Mauger, M
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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Application Number
EP 15 20 0619

5

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
X	HANS GRÖNLUND ET AL: "Carbohydrate-based particles: a new adjuvant for allergen-specific immunotherapy", IMMUNOLOGY, vol. 107, no. 4, 9 December 2002 (2002-12-09), pages 523-529, XP055258332, DOI: DOI: 10.1046/j.1365-2567.2002.01535. * page 523 - page 525; figures *	1-15	TECHNICAL FIELDS SEARCHED (IPC)
X	THERESA N ANDERSSON ET AL: "A novel adjuvant-allergen complex, CBP-rFel d 1, induces up-regulation of CD86 expression and enhances cytokine release by human dendritic cells in vitro", IMMUNOLOGY, vol. 113, no. 2, 23 August 2004 (2004-08-23), pages 253-259, XP055258364, DOI: 10.1046/j.1365-2567.2004.01943.x * page 253 - page 256; figures *	1-15	
X	T. NEIMERT-ANDERSSON ET AL: "Carbohydrate-based particles reduce allergic inflammation in a mouse model for cat allergy", ALLERGY, vol. 63, no. 5, 1 May 2008 (2008-05-01), pages 518-526, XP055258374, United Kingdom ISSN: 0105-4538, DOI: 10.1111/j.1398-9995.2008.01644.x * page 519 - page 521; figures *	1-15	
X	EP 1 356 826 A1 (BIOMAY PROD & HANDEL [AT]) 29 October 2003 (2003-10-29) * paragraph [0009] - paragraph [0011]; claims; examples *	1-15	
The present search report has been drawn up for all claims			
Place of search Munich		Date of completion of the search 17 March 2016	Examiner Gonçalves Mauer, M
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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EPO FORM 1503 03.02 (P04C01)

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ON EUROPEAN PATENT APPLICATION NO.

EP 15 20 0619

5

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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17-03-2016

10

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6218132 B1	17-04-2001	AU 718527 B2	13-04-2000
		AU 3138797 A	05-01-1998
		CA 2256693 A1	04-12-1997
		EP 0912895 A1	06-05-1999
		JP 2000512008 A	12-09-2000
		US 5750356 A	12-05-1998
		US 6218132 B1	17-04-2001
		WO 9745735 A1	04-12-1997

WO 2005010026 A2	03-02-2005	AT 488767 T	15-12-2010
		EP 1648919 A2	26-04-2006
		WO 2005010026 A2	03-02-2005

EP 1356826 A1	29-10-2003	AT 419874 T	15-01-2009
		AU 2003224104 A1	03-11-2003
		CA 2482973 A1	30-10-2003
		CN 1646173 A	27-07-2005
		EP 1356826 A1	29-10-2003
		EP 1496942 A1	19-01-2005
		ES 2318124 T3	01-05-2009
		JP 4814490 B2	16-11-2011
		JP 2005529880 A	06-10-2005
		RU 2329830 C2	27-07-2008
		US 2005095298 A1	05-05-2005
		WO 03089009 A1	30-10-2003

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

- **LINDSKOG M ; ROCKBERG J ; UHLEN M ; STERKY F.** Selection of protein epitopes for antibody production. *Biotechniques*, 2005, vol. 38 ((5)), 723-7 **[0016]**
- **UHLEN M ; FAGERBERG L ; HALLSTROM BM ; LINDSKOG C ; OKSVOLD P ; MARDINOGLU A et al.** Proteomics. Tissue-based map of the human proteome. *Science*, 2015, vol. 347 ((6220)), 1260419 **[0026]**
- **THUNBERG et al.** *Allergy*, 2009, vol. 64, 919 **[0099]**

专利名称(译)	T细胞反应平台		
公开(公告)号	EP3182125A1	公开(公告)日	2017-06-21
申请号	EP2015200619	申请日	2015-12-16
[标]发明人	TCER AB		
发明人	TCER AB		
IPC分类号	G01N33/53 G01N33/564		
CPC分类号	G01N33/53 G01N33/564		
外部链接	Espacenet		

摘要(译)

用于测定抗原特异性T细胞活化的方法，包括以下步骤：(a) 提供可吞噬颗粒，其具有与其紧密结合的候选抗原多肽，其中具有相关多肽的颗粒经历变性洗涤，产生内毒素水平足够低，不会干扰后续步骤；(b) 提供可行的抗原呈递细胞；(c) 在允许抗原呈递细胞吞噬颗粒的条件下使洗过的颗粒与抗原呈递细胞接触；(d) 提供待测定的T细胞样品，其包含活T细胞；(e) 使T细胞样品与与颗粒接触的抗原呈递细胞在允许T细胞响应抗原呈递细胞呈递的抗原的特异性活化的条件下接触；(f) 确定T细胞样品中T细胞活化的程度。减少多肽样品中内毒素的方法。与可吞噬颗粒紧密相关的多种不同多肽的集合，其中所述多肽在与颗粒结合后经历变性洗涤。

