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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
26 January 2017 (26.01.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/015064 A1

(51) International Patent Classification:
A61K 38/00 (2006.01) *C12N 15/09* (2006.01)

(21) International Application Number:
PCT/US2016/042341

(22) International Filing Date:
14 July 2016 (14.07.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/194,726 20 July 2015 (20.07.2015) US

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,

[Continued on next page]

WO 2017/015064 A1

(54) Title: DETECTION PHENOTYPING AND QUANTITATION OF CELLS WITH MULTIMERIC BINDING REAGENT

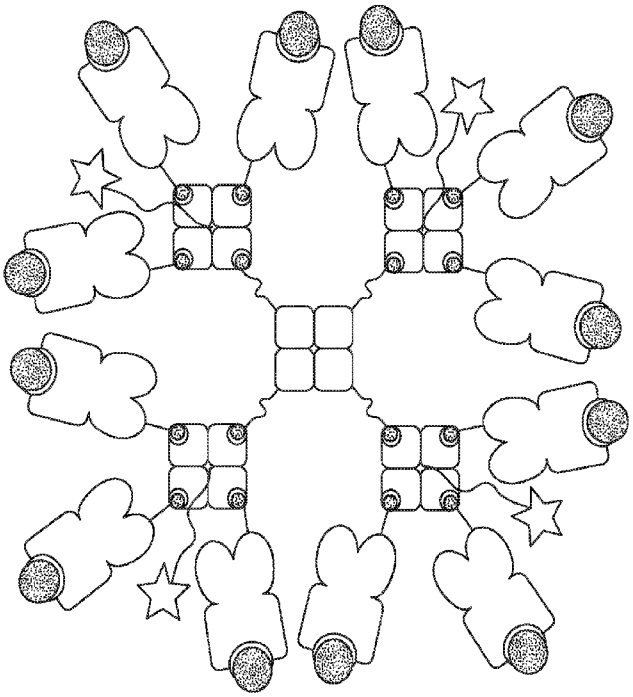

FIG. 1A

(57) Abstract: Methods and compositions are provided for labeling cells according to the specificity of a receptor of interes by contacting with a multimeric binding reagent. The multimeric binding reagent is based on a "tetrameric scaffold protein", which tetrameric scaffold protein has little or no affinity for biotin, and which comprises a C-terminal cysteine residue on one or more, usually all four of the polypeptides in the tetramer. The scaffold protein is modified by biotinylation at the terminal cysteines, to provide the central portion of the multimeric binding reagent. The biotinylated tetramer is combined with a high affinity biotin-binding protein tetramer. The complex thus generated has unfilled biotin-binding sites that can accommodate up to twelve biotin-tagged specific binding reagents, and is useful in various affinity selection and detection formats.

TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

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

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

DETECTION PHENOTYPING AND QUANTITATION OF CELLS WITH MULTIMERIC BINDING REAGENT

GOVERNMENT RIGHTS

[0001] This invention was made with Government support under contract AI106941 awarded by the National Institutes of Health. The Government has certain rights in the invention.

CROSS-REFERENCE

[0002] Pursuant to 35 U.S.C. § 119 (e), this application claims priority to the filing date of the United States Provisional Patent Application Serial No. 62/194,726 filed July 20, 2015, the disclosure of which application is incorporated herein by reference.

BACKGROUND

[0003] T lymphocytes compose a major part of the body's immune defenses against bacterial, viral and protozoal infection, and have been implicated in the rejection of cancerous cells. Numerous autoimmune syndromes have also been linked to antigen-specific T cell attack on various parts of the body. It is therefore of great interest to be able to track antigen-specific T cells during the course of these diseases. It would also be of great therapeutic benefit if T cells specific for a particular antigen could be enriched and then reintroduced in a disease situation, or selectively depleted in the case of an autoimmune disorder.

[0004] T cell receptors (TCR) detect antigens in the form of peptides bound to major histocompatibility complex (pMHC) molecules at the surface of antigen presenting cells. TCR-pMHC interactions determine the selection, development, differentiation, fate, and function of a T cell. However, TCRs bind monomeric pMHCs with very low binding affinities (K_d, ~1-200 μM, 1,000-200,000-fold weaker than a typical antibody–antigen interaction) and with fast dissociation rates (k_{off}, ~0.05 s^{-1}) in solution.

[0005] To increase the binding avidity and circumvent the problem of fast dissociation, pMHC tetramers were designed to detect antigen-specific T cells by conjugating four biotinylated pMHC monomers to a single fluorescent-labeled streptavidin. This fulfilled a critical need in both basic and clinical immunology to be able to identify and characterize often very rare specific T cells in a population. Subsequent improvements in sensitivity, manufacture and combinatorial labeling have made this methodology even more useful.

[0006] However there is a sharp drop-off in tetramer binding in the lower affinity range (~150 μM) so there have been a number of higher valency alternatives including pentamers, octamers, dextramers, and quantum dot (QD)-based multimers. Some of these clearly have improved sensitivity, and this is important to detect T cells with especially low affinities. For example, naïve T cells and thymocytes, which express low level and/or low affinity TCRs, show

little to no tetramer staining. Furthermore, αβ T cell and γδ T cells that do not bind antigen-specific tetramers can still produce significant antigen-specific cytokine responses. MHC class II tetramers are also problematic in cytometry by time-of-flight mass spectrometry (CyTOF), which is an advanced version of flow cytometry that can simultaneously measure more than 40 parameters on single-cells.

[0007] The present invention provides high affinity T cell receptor-specific reagents, having a simple structure that is straightforward and inexpensive to make, as well as being compatible with current tetramer technology and commercially available streptavidin conjugates The reagents can be used in various analysis formats, including flow cytometry and mass cytometry.

Publications

[0008] Altman, J.D. et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**, 94-96 (1996). Dolton, G. et al. Comparison of peptide-major histocompatibility complex tetramers and dextramers for the identification of antigen-specific T cells. *Clinical and experimental immunology* **177**, 47-63 (2014). Mallet-Designe, V.I. et al. Detection of low-avidity CD4+ T cells using recombinant artificial APC: following the antiovalbumin immune response. *Journal of immunology* **170**, 123-131 (2003). Guillaume, P. et al. Soluble major histocompatibility complex-peptide octamers with impaired CD8 binding selectively induce Fas-dependent apoptosis. *The Journal of biological chemistry* **278**, 4500-4509 (2003). Batard, P. et al. Dextramers: new generation of fluorescent MHC class I/peptide multimers for visualization of antigen-specific CD8+ T cells. *Journal of immunological methods* **310**, 136-148 (2006). Davis, M.M., Altman, J.D. & Newell, E.W. Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis. *Nat Rev Immunol* **11**, 551-558 (2011). Newell, E.W., Sigal, N., Bendall, S.C., Nolan, G.P. & Davis, M.M. Cytometry by time-of-flight shows combinatorial cytokine expression and virus-specific cell niches within a continuum of CD8+ T cell phenotypes. *Immunity* **36**, 142-152 (2012). Bendall, S.C. et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* **332**, 687-696 (2011). Tungatt, K. et al. Antibody stabilization of peptide-MHC multimers reveals functional T cells bearing extremely low-affinity TCRs. *Journal of immunology* **194**, 463-474 (2015). Fairhead, M. et al. SpyAvidin hubs enable precise and ultrastable orthogonal nanoassembly. *Journal of the American Chemical Society* **136**, 12355-12363 (2014).

SUMMARY OF THE INVENTION

[0009] Methods and compositions are provided for labeling cells according to the specificity of a receptor of interest. The labeling is performed by contacting a cell population comprising the cells of interest with a multimeric binding reagent comprising a plurality of specific binding

reagents. The multimeric binding reagent is based on a "tetrameric scaffold protein", which tetrameric scaffold protein has little or no affinity for biotin, and which comprises a C-terminal cysteine residue on one or more, usually all four of the polypeptides in the tetramer. The scaffold protein is modified by biotinylation at the terminal cysteines, to provide the central portion of the multimeric binding reagent. The biotinylated tetramer is combined with a high affinity biotin-binding protein tetramer, usually avidin, streptavidin, neutravidin, etc. As shown in Figure 1, the complex thus generated has unfilled biotin-binding sites that can accommodate up to twelve biotin-tagged specific binding reagents. This complex may be referred to as a "dodecameric scaffold", in reference to the twelve specific binding reagents that can potentially be bound to the scaffold.

[0010] In some embodiments of the invention, the dodecameric scaffold is combined with one or a plurality of different biotin-tagged specific binding reagents. Binding reagents having a low affinity for the cognate ligand particularly benefit from the dodecameric structure. In some embodiments, the biotin-tagged specific binding reagent is am MHC-peptide complex. In some such embodiments the MHC protein component of the complex is a Class I MHC protein. In other such embodiments the MHC protein component is a Class II MHC protein. The MHC protein can be empty of peptides, or can be complexed with a peptide of interest. Other biotin tagged specific binding reagents can be used with the scaffold, e.g. antibodies and fragments thereof, peptides or proteins, including epitopes; nucleic acids; carbohydrates; lectins; and the like. The specific binding reagents bound to a single dodecamer scaffold can be the same or different. The dodecameric scaffold complexed with specific binding reagents may be referred to as a "multimeric binding reagent", or as a "dodecameric binding reagent".

[0011] In certain specific embodiments, the tetrameric scaffold is comprised of biotin binding proteins, *e.g.* avidin, streptavidin, *etc.* that have been modified to substantially ablate binding to biotin while retaining the tetrameric structure, and have been further modified to provide an unpaired terminal cysteine residue. In some embodiments, such a tetrameric scaffold is comprised of a modified streptavidin. The modified streptavidin may comprise, relative to a reference full length sequence, the amino acid modifications N23A, S27D, and C-terminal cysteine. The modified streptavidin may further comprise the amino acid modification S45A.

[0012] In some embodiments, a composition comprising a dodecameric scaffold of the invention is provided. The composition may be provided, for example, in a kit format with instructions for use, reagents and buffers for binding to a biotin-tagged specific binding reagent of interest, and the like. In some embodiments, the kit further comprises at least one antigenic peptide that can be loaded onto the empty multimeric binding agent. In some embodiments, the kit comprises 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 different antigenic peptides. In some embodiments, the antigenic peptides are antigenic peptides of tumor antigens.

[0013] In some embodiments, a composition is provided comprising a dodecameric scaffold of the invention bound to biotin-tagged specific binding reagents of interest, which specific binding reagents include, without limitation, MHC proteins, which are optionally complexed with an antigenic peptide of interest. All or a portion of the open binding sites of the dodecamer may be bound to the biotin-tagged reagent. The composition may be provided as a pharmaceutical composition, as a reagent composition, etc., and may be included as a kit; in a unit dose format; and the like.

[0014] The multimeric binding reagents of the invention are useful in the study of cells, including without limitation T cells, in the detection of low affinity binding interactions. Various formats can be used for analytic studies, including quantitative analysis, receptor sequence analysis, and the like. An advantage of the reagents of the invention is the ability to readily use any biotin-labeled specific binding reagent. Another advantage of the reagents of the invention is the consistency of results obtained with high throughput analytic methods such as flow cytometry, including FACS, CyTOF, etc.

[0015] Some embodiments of this invention provide methods for staining, detecting, and isolating cells, the methods comprising a step of contacting a population of cells with a multimeric binding reagent as described herein, wherein the multimer comprises a detectable label, and performing an assay to detect a cell binding the multimer. In other embodiments, method are provided for the isolation of cells that bind a multimer as described herein, the methods comprising the steps of contacting a population of cells with a multimeric binding reagent as provided herein, for example, with a dodecamer comprising a plurality of MHC-peptides and a detectable label, optionally, detecting a cell binding the multimer, and isolating the cell binding the multimer. In some embodiments, the detectable label is a fluorophore or heavy metal ion. In some embodiments, detecting is by fluorescent microscopy, flow cytometry, CyTOF, *etc.* In some embodiments, detecting is by isolating the cell binding the multimer. In some embodiments, detecting comprises quantifying a number of cells binding the multimer. In some embodiments, detecting comprises quantifying a number of cells binding the multimer as a ratio to a number of cells of the population of cells that do not bind the multimer.

[0016] Exemplary is the analysis of low affinity T cell receptors (TCRs), such as those that typically predominate in tumor specific responses and autoimmune diseases, which escape detection by traditional tetramer staining. The high binding avidity of the multimeric binding reagents can also be utilized to identify low frequency and/or low affinity $\gamma\delta$ T cells. The high avidity of binding further allows sequencing of TCRs, for highly sensitive phenotyping. The high binding avidity is useful in the screening of TCR ligands in high throughput technology, e.g. as developed with CyTOF, in yeast based screening methods, etc. Other usages include the evaluation of vaccine efficacy, the study of allogeneic and superantigen interactions with TCRs, and the analysis of the T cell repertoire.

[0017] The multimeric binding reagents of the invention are useful in the manipulation of T-cell responses in vivo and in vitro and to develop targeted immunotherapy. In such embodiments, an effective dose of a multimeric binding reagent, which comprises an MHC-peptide of interest, is brought into contact with a population of T cells. The dose and route of administration is chosen to activate a T cell receptor of interest. Targets may include T cells that specifically react to a tumor; that react to a pathogen, e.g. virus, protozoan, bacteria, etc. Targets may also include suppressive T cells, e.g. Treg cells, that act on undesirable immune reactions, including without limitation autoimmune diseases. In such embodiments, the methods comprising a step of contacting a population of cells expressing a T-cell receptor with an multimeric binding agent as described herein under conditions suitable for the multimer to bind to the T-cell receptor and for a time sufficient for the T-cell receptor/MHC molecule interaction to activate a T-cell expressing the T-cell receptor and binding the multimeric binding agent. In such methods the population of cells expressing a T-cell receptor is contacted with an multimeric binding agent provided herein under conditions suitable for the multimer to bind to the T-cell receptor and for a time sufficient for the T-cell receptor/MHC molecule interaction to activate a T-cell expressing the T-cell receptor and binding the multimer. In other embodiments the population of cells expressing a T-cell receptor is contacted with an multimeric binding agent provided herein under conditions suitable for the multimer to bind to the T-cell receptor and to render the T-cell non-responsive to an antigen of interest.

[0018] Some aspects of this invention provide methods for the production of dodecamer scaffolds that comprise providing a modified tetrameric protein comprising terminal cysteines, biotinylated the cysteine residues; combining the scaffold thus produced with biotin-binding tetramers; and binding to biotin-labeled specific binding reagent under conditions suitable for formation of the complex. In some such aspects the biotin-labeled specific binding reagent is a peptide-loaded MHC protein. In some embodiments the MHC protein comprises a detectable label.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] Figure 1. Generation of a pMHC dodecamer. (A) Molecular structure of a pMHC dodecamer. A tetrameric scaffold protein was site-specifically conjugated to four biotins that bind to four fluorescent/metal-labeled streptavidin molecules. These four streptavidins further bind to twelve biotinylated pMHCs and form a pMHC dodecamer. (B) SDS-PAGE of the unbiotinylated and the biotinylated tetrameric scaffold protein under non-denaturing conditions. (C) SDS-PAGE of the unbiotinylated and the biotinylated monomeric scaffold protein under denaturing conditions to break the tetramer into monomers.

[0020] Figure 2. Antigen-specific stains of transgenic 5C.C7 naïve T cells using different pMHC multimers. Dodecamers, tetramers, dextramers and QD-multimers were made using IE^k

monomer containing the specific MCC peptide recognized by the 5C.C7 TCR (solid dots and lines) or an irrelevant CLIP peptide (dashed dots and lines). Splenocytes were stained with an antibody cocktail (see methods) and specific (MCC) or control (CLIP) dodecamers, tetramers, dextramers and QD multimers. Histograms are gated on naïve T cells and show representative stains at 100 nM (A, C, E, and G), and the curves (B, D, F, and H) summarize the mean fluorescence intensities at different concentrations. (A-B) T cells stained by PE labeled dodecamers (red) and A555-labeled dodecamers (purple). (C-D) Staining comparison between PE-labeled dodecamers (red) and tetramers (black). (D). (E-F) Staining comparison between FITC-labeled dodecamers (red) and dextramers (blue). (G-H) Staining comparison between PE-labeled dodecamers (red) and QD605-labeled multimers (golden).

[0021] Figure 3. Biophysical properties of dodecamers and tetramers binding to TCRs. (A) Dissociation kinetics of pMHC multimers binding to 5C.C7 naïve T cells at 4°C. The half-lives ($t_{1/2}$) of a dodecamer and a tetramer were determined using real-time flow cytometry in the presence of saturating amounts of anti-IE^k blocking antibody. (B) Temperature dependence of MHC multimer staining. (C) Disruption of cytoskeleton using latrunculin A reduces pMHC multimer binding.

[0022] Figure 4. Antigen-specific staining of human influenza-specific CD4+ T cells (A) or CMV-specific CD8+ T cells (B) by dodecamers and tetramers. Dodecamers and tetramers were made using HLA-DR4 loaded with either a hemagglutinin peptide HA306 or an irrelevant VIM peptide (A) or with HLA-A2 loaded with either a CMV peptide or an irrelevant HIV peptide (B) (6). The influenza-specific CD4+ T cells were enriched before FACS analyses using a protocol developed by Jenkins et al. (A). The pseudocolor plots show representative staining of influenza-specific CD4+ T cells (A) or CMV-specific CD8+ T cells (B) in human PBMCs by dodecamers and tetramers at different concentrations. The percentages of T cells staining positive are labeled in each panel.

[0023] Figure 5. Staining of rare and low-affinity T cells by dodecamers. (A) 5C.C7 αβ transgenic CD4+CD8+ double-positive thymocytes were stained by specific (MCC) and control (CLIP) dodecamers (red) and tetramers (green) at different concentrations. (B) 5C.C7 β-chain CD4+CD8+ double-positive thymocytes were stained by specific (MCC) dodecamers and tetramers at different concentrations. The percentages of pMHC multimer positive T cells are indicated in each panel.

[0024] Figure 6. The application of dodecamers in single-cell mass cytometry. (A and B) Naive 5C.C7 T cells were stained with 20 nM (A) or 100 nM (B) metal-labeled dodecamers and tetramers and analyzed by CyTOF. Dodecamers and tetramers were made using IEk MHC loaded with the specific MCC peptide recognized by the 5C.C7 TCR or an irrelevant CLIP peptide. (C and D). Cytokine analysis by CyTOF. Tetramer (+) T cells and tetramer (−) dodecamer (+) T cells were stimulated by either 1 μM MCC peptide and CH27 cells (C) or PMA

+ Ionomycin (D). Stimulated cells were intracellularly stained with a metal-labeled antibody mixture and analyzed by CyTOF. Data were shown as mean fluorescence intensity (MFI) after subtracting background staining.

[0025] Figure 7. Dose-dependent staining of 5C.C7 naive T cells by different pMHC multimers. (A-C) Splenocytes were stained with an antibody mixture (Materials and Methods) and specific (MCC) dodecamers, tetramers, and dextramers. Histograms are gated on naive T cells. (A) Naive T cells stained with PE-labeled dodecamers and A555-labeled dodecamers at 30 nM (red), 100 nM (blue), and 300 nM (orange). (B) Staining comparison between PE-labeled dodecamers and tetramers at 0.3 nM (red), 1 nM (blue), 3 nM (orange), 10 nM (light green), 30 nM (dark green), 100 nM (brown), and 300 nM (purple). (C) Staining comparison between PE-labeled dodecamers and dextramers at 3 nM (red), 10 nM (blue), 30 nM (orange), 100 nM (light green), and 300 nM (dark green). (D) Nonspecific binding of QD multimers. Splenocytes were stained with an antibody mixture (Materials and Methods) and specific (MCC) or control (CLIP) QD multimers. Histograms are gated on naive T cells and show staining by MCC or CLIP QD multimers at 1 nM (red), 3 nM (blue), 10 nM (orange), 30 nM (light green), and 100 nM (dark green).

[0026] Figure 8. Antigen-specific staining of Flu-specific (A) and CMV-specific (B) CD8+ T cells by dodecamers and tetramers at different concentrations at room temperature (22 °C). Total human PBMCs were stained with an antibody mixture (Materials and Methods) and specific (HLA-A2:Flu or HLA-A2:CMV) dodecamers and tetramers or control (PE–streptavidin). Plots are gated on CD8+ T cells.

[0027] Figure 9. Background staining analysis. Dodecamers and tetramers were made using HLA-A2 loaded with either a CMV peptide or an irrelevant HIV peptide. The pseudocolor plots show representative background staining of CD4+ T cells in total human PBMCs by dodecamers and tetramers at different concentrations.

[0028] Figure 10. Staining of TCR+ CD3+ thymocytes of 5C.C7 αβ transgenic mice by dodecamers and tetramers. Total 5C.C7 thymocytes were collected from transgenic mouse thymuses and stained with an antibody mixture (Materials and Methods) and specific (MCC) or control (CLIP) dodecamers and tetramers. TCR+ CD3+ thymocytes were gated from other cells. The pseudocolor plots (A) and histograms (B) show the staining of TCR+ CD3+ thymocytes at 10, 30, 90, and 270 nM.

[0029] Figure 11. Representative gating of CD4+ CD8+ double-positive thymocytes of 5C.C7 αβ transgenic mice. (A) Thymocytes were stained by live/dead aqua stain to identify live cells. (B-E) Plots are gated on CD4+ CD8+ double-positive thymocytes. (F) Representative staining of CD4+ CD8+ double-positive thymocytes by 30 nM MCC-IEk dodecamer. (G) Staining of CD4+ CD8+ double-positive thymocytes of 5C.C7 αβ transgenic mice by dodecamers and tetramers. Total 5C.C7 thymocytes were collected from transgenic mouse thymuses and

stained with an antibody mixture (Materials and Methods) and specific (MCC) or control (CLIP) dodecamers and tetramers. Plots are gated on CD4+ CD8+ thymocytes. The histograms show the staining of CD4+ CD8+ thymocytes at 10, 30, and 90 nM.

[0030] Figure 12. (A) Staining of naive 5C.C7 β-chain T cells by dodecamers and tetramers. 5C.C7 β-chain splenocytes were stained with an antibody mixture (Materials and Methods) and specific (MCC) dodecamers and tetramers. Plots are gated on naive 5C.C7 β-chain T cells. The pseudocolor plots show the staining of naïve 5CC.7 β-chain T cells at 10, 30, and 90 nM. (B) Staining of TCR+ CD3+ 5C.C7 β-chain thymocytes by dodecamers and tetramers. Total 5C.C7 β-chain thymocytes were stained with an antibody mixture (Materials and Methods) and specific (MCC) dodecamers and tetramers. Plots are gated on TCR+ CD3+ 5C.C7 β-chain thymocytes. The pseudocolor plots show the staining of TCR+ CD3+ 5C.C7 β-chain thymocytes at 10, 30, and 90 nM.

[0031] Figure 13. Antigen-specific staining of γδ TCR-expressing cells (Top row) and γδ-negative cells (Bottom row) by dodecamers and tetramers. The pseudocolor plots show representative staining at 1 nM.

[0032] Figure 14. Antigen-specific staining of γδ TCR-expressing cells by dodecamers and dextramers. γδ TCR-expressing cells (Top two rows) and γδ-negative cells (Bottom two rows) were stained with dodecamers and dextramers at 0.1, 0.3, and 1 nM.

[0033] Figure 15. Representative analysis of antibody and pMHC dodecamer-stained cells using CyTOF. (A) DNA content stained by an iridium-191/193 interchelator is used to identify individual cells. (B) The cells are also gated for uniformity in the length of signal received by the ion detector and by exclusion of a live-dead viability stain. (C–G) Plots are gated on naive 5C.C7 T cells. (H) Representative staining of naive 5C.C7 T cells by a 100-nM metal-labeled specific MCC-IEk dodecamer.

[0034] Figure 16. (A and B) Isolation of tetramer (+) T cells and tetramer (−) dodecamer (+) T cells by fluorescence-activated cell sorting. Splenocytes isolated from a 5C.C7 single β-chain transgenic mouse were stained with APC-Cy7–labeled anti-CD19, anti-CD11b, anti-Gr-1, and anti-F4/80 antibodies and followed by anti-APC magnetic beads for cell selection. (A) Negatively selected T cells were stained with live/dead aqua stain, pacific blue-labeled anti-TCRβ antibody, FITC labeled anti-CD4 antibody, and PE-labeled tetramer (90 nM). Aqua (−) APC-Cy7 (−) pacific blue (+) FITC (+) PE (+) cells were sorted as tetramer (+) cells. (B) Tetramer (−) T cells were further stained with PE-labeled dodecamer (90 nM). Aqua (−) APC-Cy7 (−) pacific blue (+) FITC (+) PE (+) cells were sorted as tetramer (−) dodecamer (+) cells. (C-F) Cytokine analysis by CyTOF. Tetramer (+) T cells and tetramer (−) dodecamer (+) T cells were stimulated by either 1 μM MCC peptide and CH27 cells (C and D) or PMA + Ionomycin (E and F). Stimulated cells were stained with a metal-labeled antibody mixture (Materials and

Methods) and analyzed by CyTOF. The expression of IFN-γ, PRF, GZMB, IL-10, IL-2, IL-4, TNF-α, IL-6, and IL-17 in the same T cells are shown in each panel.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

[0035] High avidity multimeric binding compositions are provided, which find use, for example, in detection, quantitation, characterization, separation and activation of cells according to specificity of a receptor. In some embodiments the receptor is a T cell antigen receptor, which may be an αβ receptor, or a γδ receptor. T cells can be CD8+, CD4+, thymocytes, etc. Variants of the binding complex for different binding partners are easily produced.

Definitions

[0036] *Tetrameric scaffold protein*. Multimeric binding reagents of the invention are based on a "tetrameric scaffold protein". This tetrameric scaffold protein is comprised of 4 polypeptide chains, which form a stable tetramer and which comprise a C-terminal cysteine residue on one or more, usually all four of the polypeptides in the tetramer. The tetrameric protein naturally or by sequence modification has little or no affinity for biotin. The scaffold protein is modified by biotinylation at the terminal cysteines. The biotinylated tetramer provides the central portion of the multimeric binding reagent, and may be referred to herein as the central scaffold.

[0037] In certain specific embodiments, the tetrameric scaffold protein is comprised of proteins which are derived from biotin-binding proteins, *e.g.* avidin, streptavidin, *etc.* but which have been modified to substantially ablate binding to biotin while retaining the tetrameric structure. The tetrameric scaffold protein is further modified to provide an unpaired terminal cysteine residue. In some embodiments, such a tetrameric scaffold is comprised of a modified streptavidin. The modified streptavidin may comprise, relative to a reference full length sequence, the amino acid modifications N23A, S27D, and C-terminal cysteine. The modified streptavidin may further comprise the amino acid modification S45A.

[0038] In certain embodiments the tetrameric scaffold protein comprises or consists of the amino acid sequence of SEQ ID NO:1:

AEAGITGTWYAQLGDTFIVTAGADGALTGTYEAAVGNAESRYVLTGRYDSAPATDGSG
TALGWTVAWKNNYRNAHSATTWSGQYVGGAEARINTQWLLTSGTTEANAWKSTLVG
HDTFTKVKPSAASC

[0039] The central scaffold is combined with a high affinity biotin-binding protein tetramer, usually avidin, streptavidin, neutravidin, etc. As shown in Figure 1, the complex thus generated has unfilled biotin-binding sites that can accommodate up to twelve biotin-tagged specific binding reagents. This complex may be referred to as a "dodecameric scaffold", in reference to the twelve specific binding reagents that are potentially be bound to the scaffold.

[0040] In some embodiments of the invention, the dodecameric scaffold is combined with one or a plurality of different biotin-tagged specific binding reagents. Binding reagents having a low affinity for the cognate ligand particularly benefit from the dodecameric structure. In some embodiments, the biotin-tagged specific binding reagent is am MHC-peptide complex. In some such embodiments the MHC protein component of the complex is a Class I MHC protein. In other such embodiments the MHC protein component is a Class II MHC protein. The MHC protein can be empty of peptides, or can be complexed with a peptide of interest. Other biotin tagged specific binding reagents can be used with the scaffold, e.g. antibodies and fragments thereof, peptides or proteins, including epitopes; nucleic acids; carbohydrates; lectins; and the like. The specific binding reagents bound to a single dodecamer scaffold can be the same or different. The dodecameric scaffold complexed with specific binding reagents may be referred to as a "multimeric binding reagent", or as a "dodecameric binding reagent".

[0041] *Specific binding member*, as used herein refers to a member of a specific binding pair, i.e. two molecules where one of the molecules through chemical or physical means specifically binds to the other molecule. The complementary members of a specific binding pair are sometimes referred to as a ligand and receptor.

[0042] In addition to antigen and antibodies and antibody fragments (e.g., Fab, F(ab)'2, single chain antibodies, diabodies, etc.), receptors, proteins binding a ligand, aptamers, and adnectins, peptide-MHC antigen and T cell receptor pairs; complementary nucleotide sequences (including nucleic acid sequences used as probes and capture agents in DNA hybridization assays); peptide ligands and receptor; autologous monoclonal antibodies, and the like. The specific binding pairs may include analogs, derivatives and fragments of the original specific binding member. For example, an antibody directed to a protein antigen may also recognize peptide fragments, chemically synthesized peptidomimetics, labeled protein, derivatized protein, etc. so long as an epitope is present.

[0043] Typically, in the context of cells, cell culture, or processing of living cells (e.g. staining, FACS sorting), a binding molecule is able to form a binding interaction with a binding partner that is strong enough to be stable under physiological conditions or under the conditions typically encountered during cell processing. In some embodiments of the invention, a binding molecule binds its binding partner with a low affinity, e.g. less than about 10^{-9} Kd, less than about 10^{-8} Kd, less than about 10^{-7} Kd, less than about 10^{-6} Kd affinity, which binding interactions can significantly benefit from the high avidity of the binding complexes of the invention. The term "ligand" is art-recognized and refers to a binding partner of a binding molecule. Ligands can be, for example, proteins, peptides, nucleic acids, small molecules, and carbohydrates. Avidins, for example, streptavidin, are non-limiting examples of binding molecules that can bind a ligand, in this case, for example, biotin.

[0044] Immunological specific binding pairs include antigens and antigen specific antibodies or T cell antigen receptors. Recombinant DNA methods or peptide synthesis may be used to produce chimeric, truncated, or single chain analogs of either member of the binding pair, where chimeric proteins may provide mixture(s) or fragment(s) thereof, or a mixture of an antibody and other specific binding members. Antibodies and T cell receptors may be monoclonal or polyclonal, and may be produced by transgenic animals, immunized animals, immortalized human or animal B-cells, cells transfected with DNA vectors encoding the antibody or T cell receptor, etc. The details of the preparation of antibodies and their suitability for use as specific binding members are well-known to those skilled in the art.

[0045] The term "conjugated," as used herein, refers to an entity, molecule, or moiety that is stably associated with another molecule or moiety via a covalent or non-covalent bond. In some embodiments, the conjugation is via a covalent bond, for example, in the case of a peptide tag conjugated to an MHC protein via fusion of the peptide to a heavy chain of the MHC protein, or covalent binding of a fluorochrome to an MHC protein. In other embodiments, the conjugation is via a non-covalent interactions, for example, via hydrogen bonding, van der Waals interactions, hydrophobic interactions, magnetic interactions, or electrostatic interactions.

[0046] The term “label” may refer to any detectable moiety on an MHC protein, multimeric scaffold, peptide, etc. Labels include peptide tags, e.g. myc tag, his tag, etc., to enzyme substrates, radioisotopes, fluorochromes, heavy metal labels, and the like. Included, for example, are a fluorophore, a phycobilin, phycoerythrin or allophycocyanine, a nanocrystal, a quantum dot (Qdot), a magnetic particle, or a nanoparticle. The terms "quantum dot" and "Qdot," as used herein, refer to fluorescent inorganic semiconductor nanocrystals in which the excitons are confined in all three spatial dimensions and which are useful as detectable agents in some embodiments of the invention.

[0047] Metal labels (e.g., Sm^{152}, Tb^{159}, Er^{170}, Nd^{146}, Nd^{142}, and the like) can be detected (e.g., the amount of label can be measured) using any convenient method, including, for example, nano-SIMS, by mass cytometry (see, for example: U.S. patent number 7,479,630; Wang et al. (2012) Cytometry A. 2012 Jul;81(7):567-75; Bandura et. al., Anal Chem. 2009 Aug 15;81(16):6813-22; and Ornatsky et. al., J Immunol Methods. 2010 Sep 30;361(1-2):1-20. An advantage of the multimeric binding reagent of the invention is its utility in mass cytometry. Mass cytometry is a real-time quantitative analytical technique whereby cells or particles are individually introduced into a mass spectrometer (e.g., Inductively Coupled Plasma Mass Spectrometer (ICP-MS)), and a resultant ion cloud (or multiple resultant ion clouds) produced by a single cell is analyzed (e.g., multiple times) by mass spectrometry (e.g., time of- flight mass spectrometry). Mass cytometry can use elements (e.g., a metal) or stable isotopes,

attached as label moieties to a detection reagent (e.g., an antibody and/or a nucleic acid detection agent).

[0048] The term "linker," as used herein, refers to a chemical structure between two molecules or moieties or between a molecule and a moiety, thus linking the two. In some embodiments, the linker is covalently bound to both linked elements. In some embodiments, the linker is covalently bound to one, but not the other linked element. In some embodiments, the linker is non-covalently bound to one or both elements. Linkers may be peptides or non-peptides, e.g. using maleimide alkylation chemistry.

[0049] *T cell receptor*, refers to the antigen/MHC binding heterodimeric protein product of a vertebrate, e.g. mammalian, TCR gene complex, including the human TCR α, β, γ and δ chains. For example, the complete sequence of the human β TCR locus has been sequenced, as published by Rowen et al. (1996) Science 272(5269):1755-1762; the human α TCR locus has been sequenced and resequenced, for example see Mackelprang et al. (2006) Hum Genet. 119(3):255-66; see a general analysis of the T-cell receptor variable gene segment families in Arden Immunogenetics. 1995;42(6):455-500; each of which is herein specifically incorporated by reference for the sequence information provided and referenced in the publication.

[0050] In some embodiments the specific binding member is one or, more usually, a pair of biotinylated, soluble MHC proteins, e.g. a Class II α/β pair, or a Class I/β2 microglobulin pair. Biotin-labeled MHC proteins can be prepared as conventional in the art, e.g. by enzymatic biotinylation of monomeric MHC proteins comprising a C-terminal biotinylation sequence peptide (BSP). MHC-peptide staining reagents have been described, including Streptamers, desthiobiotin (DTB) multimers, pentamers (Proimmune Inc., FL, USA). Non-phycobilin-based multimeric binding agents have also been developed, for example Quantum dots loaded with MHC class I-peptide complexes, which allow simultaneous use of multiple MHC class I-peptide specificities in polychrome flow cytometry, Cy5-labeled dimeric, tetrameric and octameric MHC class I-peptide complexes, dextramers (Immudex, Copenhagen, Denmark) and dimeric MHC-peptide-immunoglobulin (Ig) fusion proteins.

[0051] *MHC Proteins.* Major histocompatibility complex proteins (also called human leukocyte antigens, HLA, or the H2 locus in the mouse) are protein molecules expressed on the surface of cells that confer a unique antigenic identity to these cells. MHC/HLA antigens are target molecules that are recognized by T-cells and natural killer (NK) cells as being derived from the same source of hematopoietic reconstituting stem cells as the immune effector cells ("self") or as being derived from another source of hematopoietic reconstituting cells ("non-self"). Two main classes of HLA antigens are recognized: HLA class I and HLA class II.

[0052] The MHC proteins used in methods of the invention may be from any mammalian or avian species, e.g. primate sp., particularly humans; rodents, including mice, rats and hamsters; rabbits; equines, bovines, canines, felines; etc. Of particular interest are the human HLA proteins, and the murine H-2 proteins. Included in the HLA proteins are the class II subunits HLA-DPα, HLA-DPβ, HLA-DQα, HLA-DQβ, HLA-DRα and HLA-DRβ, and the class I proteins HLA-A, HLA-B, HLA-C, and β_2-microglobulin. Included in the murine H-2 subunits are the class I H-2K, H-2D, H-2L, and the class II I-Aα, I-Aβ, I-Eα and I-Eβ, and β_2-microglobulin.

[0053] The MHC binding domains are typically a soluble form of the normally membrane-bound protein. The soluble form is derived from the native form by deletion of the transmembrane domain. Conveniently, the protein is truncated, removing both the cytoplasmic and transmembrane domains. In some embodiments, the binding domains of a major histocompatibility complex protein are soluble domains of Class II alpha and beta chain. In some such embodiments the binding domains have been subjected to mutagenesis and selected for amino acid changes that enhance the solubility of the single chain polypeptide, without altering the peptide binding contacts.

[0054] An “allele” is one of the different nucleic acid sequences of a gene at a particular locus on a chromosome. One or more genetic differences can constitute an allele. An important aspect of the HLA gene system is its polymorphism. Each gene, MHC class I (A, B and C) and MHC class II (DP, DQ and DR) exists in different alleles. Current nomenclature for HLA alleles are designated by numbers, as described by Marsh et al.: Nomenclature for factors of the HLA system, 2010. *Tissue Antigens* **75**:291-455, herein specifically incorporated by reference. For HLA protein and nucleic acid sequences, see Robinson et al. (2011), The IMGT/HLA database. Nucleic Acids Research 39 Suppl 1:D1171-6, herein specifically incorporated by reference.

[0055] The numbering of amino acid residues on the various MHC proteins and variants disclosed may be made to be consistent with the full length polypeptide. Boundaries can be set to either be the end of the MHC peptide binding domain, and the end of the Beta2/Alpha2/Alpha3 domains as judged by structure and/or sequence for the ‘full length’ MHCs.

[0056] In some embodiments, of this invention, a multimeric binding complex of the invention comprises a genetically engineered MHC molecule. In some embodiments, an MHC molecule as provided herein comprises an extracellular domain of a naturally occurring MHC molecule, or a genetically engineered derivative thereof, but is devoid of all or part of the transmembrane domain or domains. In some embodiments, MHC class II molecules are provided that comprise a leucine zipper in place of the transmembrane domain in order to achieve dimerization of α and β chains. Genetically engineered MHC proteins, for example, MHC molecules lacking transmembrane domains, MHC molecules comprising leucine zippers, single chain MHC molecules or MHC molecules fused to an antigenic peptide, are also included in the scope of

the term "MHC molecule". In some embodiments, the term "MHC molecule" refers to a complete molecule, for example, an MHC heavy chain type α (genetically engineered or not) that is associated with a molecule of β2 microglobulin in the case of an MHC class I molecule, or an MHC heavy chain type α (genetically engineered or not) that is associated with an MHC heavy chain type β (genetically engineered or not), for example, via leucine zipper interaction. In some embodiments, the term "MHC molecule" refers to a single component of an MHC molecule, for example, to an MHC heavy chain (e.g. type α or type β, genetically engineered or not), or to a β2 microglobulin.

[0057] The term "monomeric MHC molecule," "MHC monomer," and "MHC molecule monomer," as used herein, refer to a single MHC molecule, for example, to a single MHC heavy chain, a single MHC heavy chain associated with a β2 microglobulin, or a heterodimer of an MHC type α heavy chain and an MHC type β heavy chain. The term "MHC multimer," as used herein, refers to a plurality of MHC molecules associated with each other.

[0058] *MHC context*. The function of MHC molecules is to bind peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T cells. Thus T cell receptor recognition can be influenced by the MHC protein that is presenting the antigen. The term MHC context refers to the recognition by a TCR of a given peptide, when it is presented by a specific MHC protein.

[0059] *Class II HLA/MHC*. Class II binding domains generally comprise the α1 and α2 domains for the α chain, and the β1 and β2 domains for the β chain. Not more than about 10, usually not more than about 5, preferably none of the amino acids of the transmembrane domain will be included. The deletion will be such that it does not interfere with the ability of the α2 or β2 domain to bind peptide ligands.

[0060] In some embodiments, the binding domains of a major histocompatibility complex protein are soluble domains of Class II alpha and beta chain. In some such embodiments the binding domains have been subjected to mutagenesis and selected for amino acid changes that enhance the solubility of the single chain polypeptide, without altering the peptide binding contacts.

[0061] In certain specific embodiments, the binding domains are an HLA-DR allele. The HLA-DRA protein can be selected, without limitation, from the binding domains of DRA*01:01:01:01; DRA*01:01:01:02; DRA*01:01:01:03; DRA*01:01:02; DRA*01:02:01; DRA*01:02:02; and DRA*01:02:03. The HLA-DRA binding domains can be combined with any one of the HLA-DRB binding domains, e.g. DRB4, DRB15, etc. In other embodiments the Class II binding domains are an H2 protein, e.g. I-Aα, I-Aβ, I-Eα and I-Eβ.

[0062] *Class I HLA/MHC*. For class I proteins, the binding domains may include the α1, α2 and α3 domain of a Class I allele, including without limitation HLA-A, HLA-B, HLA-C, H-2K, H-2D, H-2L, which are combined with β_2-microglobulin. Not more than about 10, usually not more than about 5, preferably none of the amino acids of the transmembrane domain will be included. The deletion will be such that it does not interfere with the ability of the domains to bind peptide ligands.

[0063] In certain specific embodiments, the binding domains are HLA-A2 binding domains, e.g. comprising at least the alpha 1 and alpha 2 domains of an A2 protein. A large number of alleles have been identified in HLA-A2, including without limitation HLA-A*02:01:01:01 to HLA-A*02:478, which sequences are available at, for example, Robinson et al. (2011), The IMGT/HLA database. Nucleic Acids Research 39 Suppl 1:D1171-6. Among the HLA-A2 allelic variants, HLA-A*02:01 is the most prevalent. In certain specific embodiments, the binding domains are HLA-B57 binding domains, e.g. comprising at least the alpha1 and alpha 2 domains of a B57 protein.

[0064] The α and β subunits may be separately produced and allowed to associate in vitro to form a stable heteroduplex complex, or both of the subunits may be expressed in a single cell. An alternative strategy is to engineer a single molecule having both the α and β subunits. A "single-chain heterodimer" can be created by fusing together the two subunits using a short peptide linker, e.g. a 15 to 25 amino acid peptide or linker. See Bedzyk et al. (1990) J. Biol. Chem. 265:18615 for similar structures with antibody heterodimers. The soluble heterodimer may also be produced by isolation of a native heterodimer and cleavage with a protease, e.g. papain, to produce a soluble product.

[0065] The MHC heterodimer will bind an antigenic peptide in the groove formed by the two membrane distal domains, either α2 and α1 for class I, or α1 and β1 for class II. The bound peptide can be substantially homogenous, that is, there will be less than about 10% of the bound peptides having an amino acid sequence different from the desired sequence, usually less than about 5%, and more usually less than about 1%.

[0066] Conditions that permit folding and association of the subunits and peptide are known in the art, see for example Ramachandiran et al. (2007) J Immunol Methods 319(1-2): 13–20; Leisner et al. (2008) PLoS One. 3(2):e1678; He et al. (2005) World J Gastroenterol. 11(27):4180-7; each herein specifically incorporated by reference. As one example of permissive conditions, roughly equimolar amounts of solubilized α and β subunits are mixed in a solution of urea. Refolding is initiated by dilution or dialysis into a buffered solution without urea. Peptides are loaded into empty class II heterodimers at about pH 5 to 5.5 for about 1 to 3 days, followed by neutralyzation, concentration and buffer exchange. However, it will be readily understood by one of skill in the art that the specific folding conditions are not critical for the practice of the invention.

[0067] *Peptide ligands of the TCR* are peptide antigens against which an immune response involving T lymphocyte antigen specific response can be generated. Such antigens include antigens associated with autoimmune disease, infection, foodstuffs such as gluten, etc., allergy or tissue transplant rejection. Antigens also include various microbial antigens, e.g. as found in infection, in vaccination, *etc.*, including but not limited to antigens derived from virus, bacteria, fungi, protozoans, parasites and tumor cells. Tumor antigens include tumor specific antigens, *e.g.* immunoglobulin idiotypes and T cell antigen receptors; oncogenes, such as p21/ras, p53, p210/bcr-abl fusion product; etc.; developmental antigens, *e.g.* MART-1/Melan A; MAGE-1, MAGE-3; GAGE family; telomerase; etc.; viral antigens, e.g. human papilloma virus, Epstein Barr virus, etc.; tissue specific self-antigens, e.g. tyrosinase; gp100; prostatic acid phosphatase, prostate specific antigen, prostate specific membrane antigen; thyroglobulin, α-fetoprotein; *etc.*; and self-antigens, *e.g.* her-2/neu; carcinoembryonic antigen, muc-1, and the like.

[0068] The peptide ligand is usually from about 8 to about 20 amino acids in length, usually from about 8 to about 18 amino acids, from about 8 to about 16 amino acids, from about 8 to about 14 amino acids, from about 8 to about 12 amino acids, from about 10 to about 14 amino acids, from about 10 to about 12 amino acids. For example, peptides may be from about 6 to 12 amino acids in length for complexes with class I MHC proteins, usually from about 8 to 10 amino acids. The peptide will be from about 6 to 20 amino acids in length for complexes with class II MHC proteins, usually from about 10 to 18 amino acids. The peptides may have a sequence derived from a wide variety of proteins.

[0069] The peptides may be prepared in a variety of ways. Conveniently, they can be synthesized by conventional techniques employing automatic synthesizers, or may be synthesized manually. Alternatively, DNA sequences can be prepared which encode the particular peptide and may be cloned and expressed to provide the desired peptide. In this instance a methionine may be the first amino acid. In addition, peptides may be produced by recombinant methods as a fusion to proteins that are one of a specific binding pair, allowing purification of the fusion protein by means of affinity reagents, followed by proteolytic cleavage, usually at an engineered site to yield the desired peptide. The peptides may also be isolated from natural sources and purified by known techniques, including, for example, chromatography on ion exchange materials, separation by size, immunoaffinity chromatography and electrophoresis.

[0070] "Suitable conditions" shall have a meaning dependent on the context in which this term is used. That is, when used in connection with binding of a T cell receptor to an MHC-peptide complex, the term shall mean conditions that permit a TCR to bind to a cognate peptide ligand. When this term is used in connection with nucleic acid hybridization, the term shall mean

conditions that permit a nucleic acid of at least 15 nucleotides in length to hybridize to a nucleic acid having a sequence complementary thereto. When used in connection with contacting an agent with a cell, this term shall mean conditions that permit an agent capable of doing so to enter a cell and perform its intended function. In one embodiment, the term "suitable conditions" as used herein means physiological conditions.

[0071] The term “specificity” refers to the proportion of negative test results that are true negative test result. Negative test results include false positives and true negative test results.

[0072] The term “sensitivity” is meant to refer to the ability of an analytical method to detect small amounts of analyte. Thus, as used here, a more sensitive method for the detection of amplified DNA, for example, would be better able to detect small amounts of such DNA than would a less sensitive method. “Sensitivity” refers to the proportion of expected results that have a positive test result.

[0073] The term “reproducibility” as used herein refers to the general ability of an analytical procedure to give the same result when carried out repeatedly on aliquots of the same sample.

[0074] Sequencing platforms that can be used in the present disclosure include but are not limited to: pyrosequencing, sequencing-by-synthesis, single-molecule sequencing, second-generation sequencing, nanopore sequencing, sequencing by ligation, or sequencing by hybridization. Preferred sequencing platforms are those commercially available from Illumina (RNA-Seq) and Helicos (Digital Gene Expression or “DGE”). “Next generation” sequencing methods include, but are not limited to those commercialized by: 1) 454/Roche Lifesciences including but not limited to the methods and apparatus described in Margulies et al., Nature (2005) 437:376-380 (2005); and US Patent Nos. 7,244,559; 7,335,762; 7,211,390; 7,244,567; 7,264,929; 7,323,305; 2) Helicos BioSciences Corporation (Cambridge, MA) as described in U.S. application Ser. No. 11/167046, and US Patent Nos. 7501245; 7491498; 7,276,720; and in U.S. Patent Application Publication Nos. US20090061439; US20080087826; US20060286566; US20060024711; US20060024678; US20080213770; and US20080103058; 3) Applied Biosystems (e.g. SOLiD sequencing); 4) Dover Systems (e.g., Polonator G.007 sequencing); 5) Illumina as described US Patent Nos. 5,750,341; 6,306,597; and 5,969,119; and 6) Pacific Biosciences as described in US Patent Nos. 7,462,452; 7,476,504; 7,405,281; 7,170,050; 7,462,468; 7,476,503; 7,315,019; 7,302,146; 7,313,308; and US Application Publication Nos. US20090029385; US20090068655; US20090024331; and US20080206764. All references are herein incorporated by reference. Such methods and apparatuses are provided here by way of example and are not intended to be limiting.

METHODS

[0075] A multimeric binding reagent of the invention is based on a "tetrameric scaffold protein", as defined above, which tetrameric scaffold protein has little or no affinity for biotin, and which comprises a C-terminal cysteine residue on one or more, usually all four of the polypeptides in the tetramer. The scaffold protein is modified by biotinylation at the terminal cysteines, to provide the central portion of the multimeric binding reagent. In certain specific embodiments, the tetrameric scaffold is comprised of biotin binding proteins, *e.g.* avidin, streptavidin, *etc.* that have been modified to substantially ablate binding to biotin while retaining the tetrameric structure, and have been further modified to provide an unpaired terminal cysteine residue. In some embodiments, such a tetrameric scaffold is comprised of a modified streptavidin. The modified streptavidin may comprise, relative to a reference full length sequence, the amino acid modifications N23A, S27D, and C-terminal cysteine. The modified streptavidin may further comprise the amino acid modification S45A.

[0076] Biotinylation, also called biotin labeling, is most commonly performed through chemical means, although enzymatic methods could be used. Chemical methods provide greater flexibility in the type of biotinylation needed than enzymatic approaches and can be performed both in vitro and in vivo. Enzymatic methods require the co-expression of bacterial biotin ligase and an exogenously expressed protein of interest that is modified to carry a biotin acceptor peptide, which provides a more uniform biotinylation than chemical methods and can be cell compartment-specific.

[0077] Biotinylation reagents typically used in the methods of the invention comprise a reactive moiety that crosslinks the biotinylation reagent to a cysteine sulfhydryl group. The distance between this reactive moiety and the biotin molecule can be adjusted to increase the availability of biotin for avidin binding, increase the solubility of the reagent or to make the biotinylation reversible. The structure from the site of cysteine binding to the end of the biotin molecule is the spacer arm. The ability of avidin/streptavidin to bind to biotin molecules on biotinylated proteins is dependent upon the availability of biotin without steric hindrance from multiple biotins on the same protein. Longer spacer arms can enhance the detection sensitivity of the target protein, because more biotin molecules are available for reporter-conjugated avidin binding. Various groups can be used as a spacer arms, as known in the art.

[0078] As used herein, the term biotin may also refer to modified versions of biotin, such as cleavable biotin, iminobiotin and desthiobiotin. Iminobiotin is a cyclic guanidino analog of biotin that is elutable from avidin because of its weaker binding affinity for avidin that is pH-dependent. Iminobiotin-tagged proteins bind to avidin conjugates at pH 9, but the avidin-iminobiotin complexes dissociate at pH 4 to allow the captured protein to be purified without denaturation. Desthiobiotin is a single-ring, sulfur-free analog of biotin that binds to streptavidin with nearly equal specificity but less affinity than biotin

[0079] The biotinylated tetramer is combined with a high affinity biotin-binding protein tetramer, usually avidin, streptavidin, neutravidin, etc. The complex thus generated has unfilled biotin-binding sites that can accommodate up to twelve biotin-tagged specific binding reagents. This complex may be referred to as a "dodecameric scaffold", in reference to the twelve specific binding reagents that can potentially be bound to the scaffold.

[0080] In some embodiments of the invention, the dodecameric scaffold is combined with one or a plurality of different biotin-tagged specific binding reagents. Binding reagents having a low affinity for the cognate ligand particularly benefit from the dodecameric structure. In some embodiments, the biotin-tagged specific binding reagent is am MHC-peptide complex or a T cell receptor heterodimer, e.g. a soluble $\alpha\beta$ heterodimer, or a soluble $\gamma\delta$ heterodimer. In some such embodiments the MHC protein component of the complex is a Class I MHC protein. In other such embodiments the MHC protein component is a Class II MHC protein. The MHC protein can be empty of peptides, or can be complexed with a peptide of interest. Other biotin tagged specific binding reagents can be used with the scaffold, e.g. antibodies and fragments thereof, peptides or proteins, including epitopes; nucleic acids; carbohydrates; lectins; and the like. The specific binding reagents bound to a single dodecamer scaffold can be the same or different. The dodecameric scaffold complexed with specific binding reagents may be referred to as a "multimeric binding reagent", or as a "dodecameric binding reagent".

[0081] In some embodiments, a method is provided for the generation of a multimeric binding complex, in which a dodecameric scaffold is contacted with a biotin tagged specific binding reagent. In some embodiments, recombinant MHC class II molecules are produced in soluble form, e.g. by insect expression systems, such as Drosophila S2 cells or baculovirus and sf9 cells. In some embodiments, the MHC proteins are fused to a leucine zipper to add in dimerization. In some embodiments, empty MHC molecules are first isolated and then loaded with an antigenic peptide of interest. Such peptide-loaded MHC molecules can then be isolated and used in the production of the multimeric binding complex. In some embodiments, for example, in embodiments where the binding interaction between the peptide of interest and an MHC molecule is of low strength, peptides can be fused to the N-terminus of the β chain via a flexible linker. Such fusions of MHC chains and antigenic peptides, resulting in the production of recombinant, peptide-loaded MHC molecules, are well known to those of skill in the art.

[0082] In some embodiments, the MHC molecule is sufficiently stable without peptide cargo (e.g. HLA DRB1*0101 or DRB1*0401) to allow the production of empty MHC molecules and assembly into the multimeric binding complex. In some such embodiments, the MHC monomer is loaded after isolation or purification with the peptide of interest. In some embodiments, the MHC monomer is first incorporated into a multimeric binding complex as provided herein and subsequently loaded with a peptide of interest. The efficiency of peptide loading strongly depends on its binding strength to the respective MHC molecule. If the binding is below a

critical threshold, peptide loading is inefficient and the resulting complexes are of limited stability, both physically and conformationally.

[0083] Some aspects of this invention provide methods and reagents for the generation of peptide-loaded MHC molecule, for example, MHC class II molecules, that include the use of a tag conjugated to the antigenic peptide. Alternatively tags can be provided on the MHC protein component, or other portions of the complex, as desired.

[0084] In some embodiments MHC molecules, or multimeric binding complexes, comprise a tag that allows isolation or purification, usually by a method that can be carried out under non-denaturing conditions, for example, by certain chromatography methods (e.g., affinity chromatography or ion exchange chromatography). In some embodiments, the tag can be removed, for example, by cleaving a linker that connects the tag to the antigenic peptide, and methods for tag removal from tagged peptide-loaded MHC molecules. Affinity tags are well known to those of skill in the art and examples of peptide tags include, but are not limited to, biotin carboxylase carrier protein (BCCP) tags, myc-tags, calmodulin-tags, FLAG-tags, hemagglutinin (HA)-tags, polyhistidine tags, also referred to as histidine tags or His-tags, maltose binding protein (MBP)-tags, nus-tags, glutathione-S-transferase (GST)-tags, green fluorescent protein (GFP)-tags, thioredoxin-tags, S-tags, Softags (e.g., Softag 1, Softag 3), strep-tags, biotin ligase tags, FlAsH tags, V5 tags, and SBP-tags.

[0085] The multimeric binding complex, once assembled, can be suspended in a suitable medium for use in binding procedures.

[0086] Variations that are designed in the assembly of the multimeric binding complexes include, for example, coexpression of the specific binding members with chaperone proteins, biotinylation of the expressed specific binding members during synthesis, modifications of peptide linkers used to dock covalent peptides in the coexpressed MHC groove, or the use of cleavable CLIP peptides, which take advantage of the natural role of invariant chain residues (CLIP) that are a functional intermediate in loading peptides into class II molecules.

[0087] Methods of the invention include the staining, detection, isolation and/or activation of cells using a multimeric binding reagent of the invention. In some embodiments, the method comprises contacting a population of cells with a multimeric binding reagent, for example, conjugated to MHC or T cell receptors. In some embodiments, the multimeric binding reagent comprises a detectable label, for example, a fluorophore, heavy metal tag, etc.

[0088] The methods include quantifying the number of T cells expressing a specific TCR in a cell population, for example, in a cell population obtained from a subject. In some embodiments, the quantity of cells, for example, of T-cells expressing a specific TCR, is compared to a reference quantity. In some embodiments, the comparison of the quantity of T-cells expressing a specific TCR in a subject to a reference quantity is used to determine an

immune reaction in the subject. In some embodiments, the reference quantity is a quantity measured or expected in a healthy subject or in healthy subjects, or a quantity measured in the subject prior to a clinical intervention, for example, prior to a vaccination, and a quantity in the subject that is higher than the reference is indicative of an immune response in the subject, whereas a quantity in the subject that is lower than the reference is indicative of depletion of a specific T-cell population.

[0089] Accordingly, multimeric binding agents as provided herein are useful, for example, for monitoring immune responses in subjects, either in response to a clinical intervention, for example, a vaccination, or as a result of a disease or condition, for example, a hyperproliferative disease in the subject. In some embodiments, the clinical intervention is a vaccination against a tumor antigen. In some embodiments, the vaccination is a vaccination administered after surgical removal of a tumor expressing the tumor antigen. In some embodiments, the clinical intervention is an intervention aimed to suppress a function of the immune system, for example, by depleting a specific population of T-cells. In some embodiments, the subject is a subject having an autoimmune disease.

[0090] Multimeric binding agents provided herein can be employed alone or in combination with other binding and/or staining agents. For example, in some embodiments, multimeric binding agents provided herein are used to stain T-cells in combination with staining the cells for an additional antigen, for example, with a staining for intracellular cytokine staining, or such markers as CD3, CD4, CD8, CD25, CD117, CD91, FoxP3, and the like as known in the art.

[0091] The T cells may be from any source, usually having the same species of origin as the MHC heterodimer. The T cells may be from an in vitro culture, or a physiologic sample. For the most part, the physiologic samples employed will be blood or lymph, but samples may also involve other sources of cells, particularly where T cells may be invasive. Thus other sites of interest are tissues, or associated fluids, as in the brain, lymph node, neoplasms, spleen, liver, kidney, pancreas, tonsil, thymus, joints, synovia, and the like. The sample may be used as obtained or may be subject to modification, as in the case of dilution, concentration, or the like. Prior treatments may involve removal of cells by various techniques, including centrifugation, using Ficoll-Hypaque, panning, affinity separation, using antibodies specific for one or more markers present as surface membrane proteins on the surface of cells, or any other technique that provides enrichment of the set or subset of cells of interest.

[0092] The binding reagent is added to a suspension comprising T cells of interest, and incubated for a period of time sufficient to bind the available cell surface receptor. The incubation will usually be at least about 5 minutes and usually less than about 30 minutes. It is desirable to have a sufficient concentration of labeling reagent in the reaction mixture, so that labeling reaction is not limited by lack of labeling reagent. The appropriate concentration is determined by titration. The medium in which the cells are labeled will be any suitable medium

as known in the art. If live cells are desired a medium will be chosen that maintains the viability of the cells. A preferred medium is phosphate buffered saline containing from 0.1 to 0.5% BSA. Various media are commercially available and may be used according to the nature of the cells, including Dulbecco's Modified Eagle Medium (dMEM), Hank's Basic Salt Solution (HBSS), Dulbecco's phosphate buffered saline (dPBS), RPMI, Iscove's medium, PBS with 5 mM EDTA, etc., frequently supplemented with fetal calf serum, BSA, HSA, etc.

[0093] Staining can be performed through a wide range of temperatures. In some embodiments, staining is performed at a temperature between 0-37° C. In some embodiments, staining is performed at 37° C. In some embodiments, staining is performed at 0-4° C. It will be appreciated by those of skill that binding at low temperatures (e.g., 0-4° C.) tends to be slow, necessitating extended periods of time for staining as compared to staining at higher temperatures. In some embodiments, staining is performed at ambient temperature, e.g. at 20-30° C., preferably at 22-25° C. In some embodiments, staining is performed in the presence of EDTA (e.g., 5 mM) and/or sodium azide (e.g., 0.02%) to inhibit cell activation. In some embodiments, staining is performed for about 10 minutes, about 15 minutes, about 20 minutes, about 25 minutes, about 30 minutes, or about 20-45 minutes.

[0094] Multimer concentration is an important factor in achieving maximum staining efficiency, and, while exemplary multimeric binding agent concentrations are provided herein, it will be appreciated by those of skill that it is preferable to test a range of concentrations, for example, in the range of about 5-50 nM (about 2.5-25 μg/ml), or, in the case of low affinity binding, in higher concentration ranges, for example, in the range of about 5-100 nM (about 2.5-50 μg/ml).

[0095] In some embodiments in which a cell is contacted with multimeric binding agent, binding is facilitated by desialylation of the cell. Desialylation is a process by which sialyl groups on the cell surface are removed or modified. Methods and reagents for desialylating a cell are described in detail elsewhere herein and additional methods are well known to those of skill in the art. For example, in some embodiments, a cell is contacted with a desialylating agent, e.g. neuraminidase, in order to achieve desialylation. Desialylating agents are, in some embodiments, enzymes, while, in other embodiments, chemicals are used to effect desialylation. Enzymes known to desialylate cell surfaces are, for example, neuraminidases. Methods and conditions suitable for desialylation of cells by contacting them with a neuraminidase are well known to those of skill in the art.

[0096] In some embodiments, staining is increased by inhibiting TCR down modulation with the protein kinase inhibitor dasatinhib. In some embodiments, scarce antigen-specific cells can be enriched by combination of fluorescence-based methods as described herein with a non-fluorescent-based isolation method, for example, with MACS using magnetic beads coated with an antibody against an epitope of the carrier molecule.

[0097] In some embodiments, the method includes a step of detecting the multimeric binding reagent bound to a cell, for example, to a surface receptor (e.g., a T-cell receptor or an MHC protein complex) of a cell. The method performed to detect the binding reagent depends, of course, on the nature of the detectable label comprised in the multimer. For example, in some embodiments, where the detectable label is a fluorophore, suitable methods for detection are fluorescence microscopy, cytometry, or FACS. The reagents of the invention find particular use in heavy metal labeling and detection by mass cytometry.

[0098] Detection of T cells is of interest in connection with a variety of conditions associated with T cell activation. Such conditions include autoimmune diseases, e.g. multiple sclerosis, myasthenia gravis, rheumatoid arthritis, type 1 diabetes, graft vs. host disease, Grave's disease, etc.; various forms of cancer, e.g. carcinomas, melanomas, sarcomas, lymphomas and leukemias. Various infectious diseases such as those caused by viruses, e.g. HIV-1, hepatitis, herpesviruses, enteric viruses, respiratory viruses, rhabdovirus, rubeola, poxvirus, paramyxovirus, morbillivirus, etc. are of interest. Infectious agents of interest also include bacteria, such as Pneumococcus, Staphylococcus, Bacillus. Streptococcus, Meningococcus, Gonococcus, Eschericia, Klebsiella, Proteus, Pseudomonas, Salmonella, Shigella, Hemophilus, Yersinia, Listeria, Corynebacterium, Vibrio, Clostridia, Chlamydia, Mycobacterium, Helicobacter and Treponema; protozoan pathogens, and the like. T cell associated allergic responses may also be monitored, e.g. delayed type hypersensitivity or contact hypersensitivity involving T cells.

[0099] Of interest are conditions having an association with a specific peptide or MHC haplotype, where the subject reagents may be used to track the T cell response with respect to the haplotype and antigen. A large number of associations have been made in disease states that suggest that specific MHC haplotypes, or specific protein antigens are responsible for disease states. However, direct detection of reactive T cells in patient samples can be difficult. Detection and quantitation with the subject reagents allows such direct detection, including detection with MHC Class II complexes, detection of $\gamma\delta$ T cell, etc. For example, the activity of cytolytic T cells against HIV infected $CD4^+$ T cells may be determined with the subject methods. The association of diabetes with the DQB1*0302 allele may be investigated by the detection and quantitation of T cells that recognize this MHC protein in combination with various peptides of interest. The presence of T cells specific for peptides of myelin basic protein in conjunction with MHC proteins of multiple sclerosis patients may be determined. The antigenic specificity may be determined for the large number of activated T cells that are found in the synovial fluid of rheumatoid arthritis patients. It will be appreciated that the subject methods are applicable to a number of diseases and immune-associated conditions.

[00100] Human class II multimeric binding complexes containing autoantigenic specificities can be used to study T cell responses in a variety of diseases, including type 1 diabetes (T1D),

celiac disease, pemphigus vulgaris, rheumatoid arthritis, multiple sclerosis, and uveitis. Unlike analyses in infectious pathogen and allergen studies, in which most of the responding T cells have a high-avidity binding to the appropriate MHC complex, MHC binding to peripheral T cells in the context of autoimmunity displays a much wider spectrum of relative strength of interaction. In addition, the frequency of autoreactive CD4 T cells specific for a particular autoantigen-MHC complex is quite low in peripheral blood, often less than five per million lymphocytes, requiring large sample volumes and careful handling for reliable detection.

[00101] Particularly relevant to the analysis of low-avidity T cell responses in autoimmunity is the definition of a precise peptide-binding register for docking within the class II molecule. The presence of more than one binding register within long peptides generated during Ag processing can result in presentation of pMHC ligands to distinct TCR, with potentially different roles in selection and autoreactivity. The reagents of the invention may include differential peptide registers for the identification of such distinctions.

[00102] Because of the high specificity and sensitivity of TCR recognition for pMHC, binding studies can also be designed to identify unconventional antigenic epitopes. For example, many protein therapeutics that are administered to patients are potentially immunogenic. Analysis of the T cell response in these cases, using reagents containing pMHC specificities from the therapeutic molecule can readily identify the specific epitopes that trigger immunogenicity.

[00103] A number of methods for detection and quantitation of labeled cells are known in the art. Flow cytometry and mass cytometry are a convenient means of enumerating cells that are a small percent of the total population. Fluorescent microscopy may also be used. Various immunoassays, e.g. ELISA, RIA, etc. may be used to quantitate the number of cells present after binding to an insoluble support. Flow cytometry or magnetic selection may also be used for the separation of a labeled subset of T cells from a complex mixture of cells. The cells may be collected in any appropriate medium which maintains the viability of the cells, usually having a cushion of serum at the bottom of the collection tube. Various media are commercially available as described above. The cells may then be used as appropriate.

[00104] In some embodiments, methods include isolating specific cells or cell populations. In general, useful reagents for isolation methods comprise a detectable label and the methods include a step of staining the target cell population as described in more detail elsewhere herein. In some embodiments including the isolation of cells, a method of cell separation is employed that allows for the enrichment or the isolation of homogenous populations of cells based on the cells binding the employed multimer, for example, the employed multimeric binding agent. Such methods are well known in the art and include, for example, FACS and MACS.

[00105] Some embodiments of this invention provide isolated cells or cell populations, for example, isolated native, or non-activated T-cell populations obtained by using a multimeric binding agent or a method as provided herein. In some embodiments, an isolated cell is provided that has been contacted with a multimeric binding agent provided herein and isolated from a cell population based on the cell binding the multimeric binding agent, for example, by a method for detection and/or isolation described in more detail elsewhere herein. In some embodiments, the cell is a T-cell. In some embodiments, the T-cell is a native T-cell, or a T-cell that has not undergone TCR-mediated cell activation. In some embodiments, the cell is a T-cell recognizing a tumor antigen. In some embodiments, the cell is a T-cell expressing a TCR that binds a tumor antigen with high affinity. In some embodiments, the cell is a therapeutically valuable cell. In some embodiments, the cell is expanded in vitro after isolation, and used in a therapeutic method. In some embodiments, the therapeutic method includes a step of administering the cell to a subject in need thereof, for example, a subject having a tumor or having an elevated risk of developing a tumor expressing a tumor antigen. In some embodiments, a subject at risk of developing a tumor expressing a tumor antigen is a subject which was diagnosed to have such a tumor and has undergone surgical removal of the tumor.

[00106] The isolation of antigen specific T cells finds a wide variety of applications. The isolated T cells may find use in the treatment of cancer as in the case of tumor-infiltrating lymphocytes. Specific T cells may be isolated from a patient, expanded in culture by cytokines, antigen stimulation, etc., and replaced in the autologous host, so as to provide increased immunity against the target antigen. A patient sample may be depleted oft cells reactive with a specific antigen, to lessen an autoimmune response.

[00107] The DNA sequence of single T cell receptors having a given antigen specificity can be determined by isolating single cells by the subject separation method. Conveniently, flow cytometry may be used to isolate single T cells in conjunction with single cell PCR amplification. In order to amplify unknown TCR sequences, ligation anchor PCR may be used. One amplification primer is specific for a TCR constant region. The other primer is ligated to the terminus of cDNA synthesized from TCR encoding mRNA. The variable region is amplified by PCR between the constant region sequence and the ligated primer.

[00108] In some aspects, this invention provides methods for the manipulation of T-cells. In some embodiments, the method includes a step of contacting a population of cells expressing a T-cell receptor with an multimeric binding agent as described herein under conditions suitable for the reagent to bind to the T-cell receptor and for a time sufficient for the T-cell receptor/MHC interaction to effect TCR-mediated T-cell activation. In some embodiments, the contacting is performed in vitro. In some embodiments, the contacting is performed ex vivo. In some embodiments, the contacting is performed in vivo. In some embodiments, the cells are

contacted with an multimeric binding agent for a time long enough to activate high-affinity TCR expressing T-cells, but not to activate low affinity TCR expressing T-cells. In some embodiments, the cells are cells from a subject having an autoimmune disease. In some embodiments, the cells are contacted with an multimeric binding agent that is loaded with an antigenic peptide recognized by T-cells that mediate an autoimmune disease. In some embodiments, the method further comprises measuring the quantity of the T-cells targeted by the multimeric binding agent, for example, by methods for the identification or detection of T-cells provided herein or otherwise known in the art.

[00109] Inhibition of immune function may be achieved by inducing anergy of specific T cells, or by ablation of reactive T cells. The subject multimeric binding agents allow a therapy to be targeted to very specific subsets of T cells. The ability to inhibit immune system functions is known to be therapeutically useful in treating a variety of diseases such as atherosclerosis, allergies, autoimmune diseases, certain malignancies, arthritis, inflammatory bowel diseases, transplant rejection and reperfusion injury. Specific diseases of interest include systemic lupus erythematosus; rheumatoid arthritis; polyarteritis nodosa; polymyositis and dermatomyositis progressive systemic sclerosis (diffuse scleroderma); glomerulonephritis; myasthenia gravis; Sjogren's syndrome; Hashimoto's disease; Graves' disease; adrenalitis; hypoparathyroidism; pernicious anemia; diabetes; multiple sclerosis, and related demyelinating diseases; uveitis; pemphigus and pemphigoid cirrhosis; ulcerative colitis; myocarditis; regional enteritis; adult respiratory distress syndrome; local manifestations of drug reactions, such as dermatitis, etc.; inflammation-associated or allergic reaction patterns of the skin; atopic dermatitis and infantile eczema; contact dermatitis; psoriasis; lichen planus; allergic enteropathies; allergic rhinitis; bronchial asthma; transplant rejection, e.g. heart, kidney, lung, liver, pancreatic islet cell, etc.; hypersensitivity or destructive responses to infectious agents; poststreptococcal diseases, e.g. cardiac manifestations of rheumatic fever, and the like.

[00110] To ablate specific T cells, the subject multimeric binding agents may be conjugated to a toxin moiety. Various cytotoxic agents are known and have been used in conjunction with specific binding molecules. Illustrative of these compounds are ricin, abrin, diphtheria toxin, maytansinoids, cisplatin, and the like. Where there are two subunits, only the cytotoxic subunit may be used, e.g. the α-unit of ricin. The toxin is conjugated to the binding complex, generally by means of a cross-linker, or other conjugation that includes a disulfide bond. Toxin conjugates are disclosed in U.S. Pat. Nos. 5,208,020; 4,863,726; 4,916,213; and 5,165,923. The toxin conjugate is administered so as to specifically eliminate the target T cells without exerting significant toxicity against other cells.

[00111] Importantly, binding of MHC class I molecules to T-cell receptors can elicit T cell activation events, such as intracellular calcium mobilization, diverse tyrosine phosphorylation and endocytosis of MHC class I-peptide engaged TCR/CD8. For example, MHC class I-peptide

complex driven cell activation can induce death of effector cytotoxic T-cells (CTLs) via FasL-dependent apoptosis or severe mitochondrial damage. This can lead to changes in T-cell populations that are contacted with multimeric binding agents, for example, for cell staining, detection, or isolation, for example, by selective depletion of stained T-cells. In some circumstances, TCR-activation-mediated cell depletion can render isolation of a non-activated T-cell population impossible.

[00112] MHC class II multimer binding to CD4+ T-cells can also lead to T-cell activation and death, for example, of CD4+ effector cells. Accordingly, MHC class II multimers are useful in the staining of CD4+ T-cells and in the isolation of minimally manipulated or activated CD4+ T-cells.

[00113] Some aspects of this invention provide multimeric binding agents and methods for the use of such multimers to analyze the state of activation or differentiation of T-cells, for example, CD8+ and/or CD4+ T-cells. In some embodiments, homogenous populations of MHC dodecamers are provided for use in such methods. In some embodiments, the multimeric binding agents comprise linkers of defined length and flexibility. Binding studies with defined, homogenous populations of multimers can reveal differentiation- and activation-dependent differences, for example, differentiation- and activation-dependent changes in glycosylation and sialylation of T cell surface molecules involved in antigen recognition of the cells under study which can affect, for example, CD8 participation in MHC class I molecule binding and/or aggregation of TCR and CD8.

[00114] In some embodiments, MHC class I-peptide multimers, are provided that comprise a mutation in the α3 domain. In some embodiments, the mutation is a mutation that ablate CD8 binding, e.g. a D227K, T228A in human MHC and D227K, Q226A in mouse MHC molecules. In some embodiments, methods are provided that use such CD8 binding-deficient multimeric binding agents to stain, detect, and/or isolate CD8-independent T cells, which typically express high affinity TCRs.

[00115] For example, in some embodiments, CD8 binding-deficient multimeric binding agents are provided for the staining, detection, and/or isolation of CD8+ T cells expressing high-affinity TCRs specific for tumor antigens, for example, for MELAN-A/Mart-1, gp100, or tyrosinase. It is known to those of skill in the art that such tumor-antigen specific T-cell tend to express low affinity TCRs and that infrequent CD8+ T cells specific for tumor antigens expressing high affinity TCRs efficiently kill tumor cells. In some embodiments, the use of a MHC class I multimer as provided herein enables efficient identification and isolation of such rare cells with no or only minimal TCR activation, thus allowing for the isolation of native T-cell populations that cannot be isolated with conventional MHC class I multimers.

[00116] Further, in some embodiments, CD8 binding-deficient multimers are used to selectively induce FasL (CD95L) expression, resulting in apoptosis of antigen-specific CTLs.

[00117] The subject binding complexes may be administered to a host to induce anergy of the specific T cells. The binding complex will induce T cell anergy upon binding, because the co-stimulator molecules necessary for T cell activation are not present. The binding complexes are administered to individuals, preferably mammals, in a manner that will maximize the likelihood of the binding complexes reaching the targeted T cell and binding to it, and thereby inducing anergy. This in turn will inhibit the immune response mediated by that T cell.

[00118] The dose for individuals of different species and for different diseases is determined by measuring the effect of the binding complexes on the lessening of these parameters which are indicative of the disease being treated. The binding complexes will normally be administered parenterally, preferably intravenously. Doses of binding complexes in a mouse model will generally range from about 0.5-2 mg/host/week for from about 1 to 4 weeks. The dose of the binding complexes may have to be repeated periodically depending upon the particular disease.

[00119] When administered parenterally, the binding complexes will be formulated in an injectable dosage form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently non-toxic and non-therapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and Hanks' solution. Non-aqueous vehicles such as fixed oils and ethyl oleate may also be used. The vehicle may contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability, e.g. buffers and preservatives. The binding complexes is preferably formulated in purified form substantially free of aggregates and other proteins at concentrations of about 1-50 mg/ml. Suitable pharmaceutical vehicles and their formulations are described in Remington's Pharmaceutical Sciences, by E. W. Martin, which is incorporated herein by reference.

[00120] Analysis of T cell receptor expression and phenotyping, peptide specificity, and the like may be provided in a variety of media to facilitate their use. “Media” refers to a manufacture that contains the expression repertoire information of the present invention. The databases of the present invention can be recorded on computer readable media, *e.g.* any medium that can be read and accessed directly by a computer. Such media include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media. One of skill in the art can readily appreciate how any of the presently known computer readable mediums can be used to create a manufacture comprising a recording of the present database information. “Recorded” refers to a process for storing information on computer readable medium, using any such methods as

known in the art. Any convenient data storage structure may be chosen, based on the means used to access the stored information. A variety of data processor programs and formats can be used for storage, *e.g.* word processing text file, database format, etc.

[00121] As used herein, "a computer-based system" refers to the hardware means, software means, and data storage means used to analyze the information of the present invention. The minimum hardware of the computer-based systems of the present invention comprises a central processing unit (CPU), input means, output means, and data storage means. A skilled artisan can readily appreciate that any one of the currently available computer-based system are suitable for use in the present invention. The data storage means may comprise any manufacture comprising a recording of the present information as described above, or a memory access means that can access such a manufacture.

[00122] A variety of structural formats for the input and output means can be used to input and output the information in the computer-based systems of the present invention. Such presentation provides a skilled artisan with a ranking of similarities and identifies the degree of similarity contained in the test expression repertoire.

[00123] Search algorithms and sequence analysis may be implemented in hardware or software, or a combination of both. In one embodiment of the invention, a machine-readable storage medium is provided, the medium comprising a data storage material encoded with machine readable data which, when using a machine programmed with instructions for using said data, is capable of displaying any of the datasets and data comparisons of this invention. In some embodiments, the invention is implemented in computer programs executing on programmable computers, comprising a processor, a data storage system (including volatile and non-volatile memory and/or storage elements), at least one input device, and at least one output device. Program code is applied to input data to perform the functions described above and generate output information. The output information is applied to one or more output devices, in known fashion. The computer may be, for example, a personal computer, microcomputer, or workstation of conventional design.

[00124] Each program can be implemented in a high level procedural or object oriented programming language to communicate with a computer system. However, the programs can be implemented in assembly or machine language, if desired. In any case, the language may be a compiled or interpreted language. Each such computer program can be stored on a storage media or device (*e.g.*, ROM or magnetic diskette) readable by a general or special purpose programmable computer, for configuring and operating the computer when the storage media or device is read by the computer to perform the procedures described herein. The system may also be considered to be implemented as a computer-readable storage medium, configured with a computer program, where the storage medium so configured causes a

computer to operate in a specific and predefined manner to perform the functions described herein.

[00125] Further provided herein is a method of storing and/or transmitting, via computer, sequence, and other, data collected by the methods disclosed herein. Any computer or computer accessory including, but not limited to software and storage devices, can be utilized to practice the present invention. Sequence or other data can be input into a computer by a user either directly or indirectly. Additionally, any of the devices which can be used to sequence DNA or analyze DNA or analyze peptide binding data can be linked to a computer, such that the data is transferred to a computer and/or computer-compatible storage device. Data can be stored on a computer or suitable storage device (e.g., CD). Data can also be sent from a computer to another computer or data collection point via methods well known in the art (e.g., the internet, ground mail, air mail). Thus, data collected by the methods described herein can be collected at any point or geographical location and sent to any other geographical location.

Reagents and Kits

[00126] Also provided are reagents and kits thereof for practicing one or more of the above-described methods. The subject reagents and kits thereof may vary greatly. Reagents of interest include reagents specifically designed for use in the methods of the invention. Such a kit may comprise a library of polynucleotides encoding a tetrameric scaffold protein, sets of peptide ligans, MHC proteins of interest, and the like. In some embodiments an assembled scaffold protein, which may be biotinylated at the carboxy terminus, is provided. Reagents for labeling and multimerizing a TCR or an MHC protein can be included. In some embodiments the kit will further comprise a software package for analysis of a sequence database.

[00127] In addition to the above components, the subject kits will further include instructions for practicing the subject methods. These instructions may be present in the subject kits in a variety of forms, one or more of which may be present in the kit. One form in which these instructions may be present is as printed information on a suitable medium or substrate, *e.g.*, a piece or pieces of paper on which the information is printed, in the packaging of the kit, in a package insert, etc. Yet another means would be a computer readable medium, *e.g.*, diskette, CD, etc., on which the information has been recorded. Yet another means that may be present is a website address which may be used via the internet to access the information at a removed, site. Any convenient means may be present in the kits.

[00128] The function and advantage of these and other embodiments of the present invention will be more fully understood from the examples below. The following examples are intended to illustrate the benefits of the present invention, but do not exemplify the full scope of the invention.

[00129] While several embodiments of the present invention have been described and illustrated herein, those of ordinary skill in the art will readily envision a variety of other means and/or structures for performing the functions and/or obtaining the results and/or one or more of the advantages described herein, and each of such variations and/or modifications is deemed to be within the scope of the present invention. More generally, those skilled in the art will readily appreciate that all parameters, dimensions, materials, and configurations described herein are meant to be exemplary and that the actual parameters, dimensions, materials, and/or configurations will depend upon the specific application or applications for which the teachings of the present invention is/are used. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. It is, therefore, to be understood that the foregoing embodiments are presented by way of example only and that, within the scope of the appended claims and equivalents thereto, the invention may be practiced otherwise than as specifically described and claimed. The present invention is directed to each individual feature, system, article, material, kit, and/or method described herein. In addition, any combination of two or more such features, systems, articles, materials, kits, and/or methods, if such features, systems, articles, materials, kits, and/or methods are not mutually inconsistent, is included within the scope of the present invention.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Detection, phenotyping and quantification of antigen-specific T cells using a peptide-MHC dodecamer

[00130] The recognition of foreign peptide-MHCs by T cells is a central event in adaptive immunity that triggers antigen-specific immune responses against infections and cancer. To study antigen- specific T cells, we devised a peptide-MHC dodecamer that can sensitively detect and specifically stain these T cells, especially low-affinity and rare ones. This dodecamer technology is superior to most current peptide-MHC multimers, compatible with existing reagents, inexpensive to make, and easy to use. It has been successfully applied to studies of human and murine antigen-specific $\alpha\beta$ and $\gamma\delta$ T cells by flow cytometry and mass cytometry. Thus, this dodecamer constitutes an important tool for the investigation of antigen-specific T cells in basic and clinical research.

[00131] T-cell receptors (TCRs) detect antigens in the form of peptides bound to peptide-major histocompatibility complex (pMHC) molecules on the surface of antigen-presenting cells. TCR–pMHC interactions determine the selection, development, differentiation, fate, and function of a T cell. However, TCRs bind monomeric pMHCs with very low binding affinities (Kd, ~1–200 μM,

1,000- to 200,000-fold weaker than a typical antibody–antigen interaction) and with fast dissociation rates (koff, ~0.05 s−1) in solution. To increase the binding avidity and circumvent the problem of fast dissociation, we previously engineered a pMHC tetramer to detect antigen-specific T cells by conjugating four biotinylated pMHC monomers to a single fluorescent-labeled streptavidin. This fulfilled a critical need in both basic and clinical immunology to be able to identify and characterize often very rare specific T cells in a population. Subsequent improvements in sensitivity, manufacture, and combinatorial labeling have made this methodology even more useful. However, there is a sharp drop-off in tetramer binding in the lower affinity range (~150 μM) so there have been a number of higher valency alternatives including pentamers (ProImmune), lipid vesicles, octamers, dextramers, and quantum dot (QD)-based multimers. Some of these clearly have improved sensitivity, and this is important in detecting T cells with especially low affinities. For example, naive T cells and thymocytes, which express lowlevel and/or low-affinity TCRs, show little-to-no tetramer staining. Furthermore, αβ T cells and γδ T cells that do not bind antigen-specific tetramers can still produce significant antigenspecific cytokine responses. MHC class II tetramers are also problematic in cytometry by time-of-flight mass spectrometry (CyTOF), which is an advanced version of flow cytometry that can simultaneously measure more than 40 parameters on single cells. The extensive washing steps, harsh fixation conditions, complicated sample injection process, and sensitivity of the time-of-flight mass spectrometry make the low-avidity MHC class II tetramers unsuitable for CyTOF studies.

[00132] To augment the avidity of the tetramer, we have engineered a biotinylated scaffold protein linked to four streptavidin tetramers, each capable of binding three biotinylated pMHC monomers. We then used the resulting dodecamer (Greek for “12”) to detect low-affinity αβ and γδ T cells in blood and tissue samples from humans and mice. Compared with tetramers and at least some multimers, this construct has better sensitivity, stronger signal strength, higher binding avidity, and a much slower dissociation rate. For the various specificities that we have analyzed, it can identify two- to fivefold more specific T cells than an equivalent tetrameric reagent.

[00133] *Dodecamer Construction*. To overcome the limitations of current tetramer technology, we aimed to engineer a dodecamer with higher avidity to detect and quantify antigen-specific T cells, especially rare and low-affinity ones. To accomplish this, we added a cysteine residue at the C terminus of an inactive streptavidin subunit that has no biotin binding sites. After expression, refolding, and purification, a tetrameric scaffold protein was assembled bearing four terminal cysteines, which were subsequently biotinylated by 1-Biotinamido-4-[4’-(maleimidomethyl)cyclohexanecarboxamido] butane (BMCC-biotin) (molecular weight: 534). The scaffold protein with four biotin sites is the centerpiece of the dodecamer. As illustrated in Fig. 1A, a biotinylated scaffold protein associates with four commercial or homemade

fluorescent/metal-labeled streptavidin molecules, each of which further associates with three biotinylated pMHC monomers. Thus, a biotinylated scaffold protein allows the formation a pMHC dodecamer. The expression and biotinylation of the scaffold protein were confirmed by SDS/PAGE. This tetrameric scaffold protein has a molecular weight of ~53.4 and ~55.5 kDa before and after biotinylation (Fig. 1B). Consistently, a monomeric subunit has a molecular weight of ~13.3 and ~13.9 kDa before and after biotinylation (Fig. 1C). The increase in the molecular weight of the scaffold protein in both the tetrameric and the monomeric forms is consistent with the expected results (Fig. 1 B and C).

[00134] *Comparison of Dodecamers to Different Multimer Formats*. We first tested the functionality of our newly engineered dodecamers labeled with different fluorophores. The purified scaffold molecule was incubated with commercially available phycoerythrin (PE)-labeled streptavidin or Alexa555 (A555)-labeled streptavidin, and then incubated with biotinylated pMHC monomers containing specific moth cytochrome c (MCC) peptide or control human class II-associated invariant chain peptide (CLIP) to form pMHC dodecamers. Dodecamers labeled with either the large fluorescent protein PE ~250 kDa) or the small fluorescent dye A555 (~1 kDa) specifically stained antigen-specific naive T cells in preparations of transgenic 5C.C7 splenocytes. The PE-labeled dodecamer gave ~10 times better signal than the A555-labeled dodecamer as illustrated in Fig. 2A and Fig. 7A. This is expected because PE is a large fluorescent protein consisting of multiple fluorescent units whereas A555 is a small, singly fluorescent molecule. Both control dodecamers produced negligible nonspecific staining (Fig. 2 A and B). These data show that the dodecamer is compatible with different types of fluorophores for detecting antigen-specific T cells. We next compared dodecamers with tetramers. As shown in Fig. 2C, dodecamers gave a fivefold better signal than tetramers. Dodecamers yielded minimal background staining at 4 °C, comparable to tetramers over a broad range of concentrations (Fig. 2 C and D and Fig. 7B). We next compared dodecamers with dextramers (Fig. 2 E and F). Dextramers are hetereogeneous mixtures of polymers of different sizes conjugated to pMHC molecules. Unlike dextramers, dodecamers have a defined structure that allows precise calculation of their molar concentration to quantitatively study antigen-specific T cells. To directly compare dodecamer and dextramer staining, we had to rely on the molar concentration of the fluorescent streptavidin that was conjugated to these two multimers. Here we found a slight advantage in staining intensity (~20%) versus dextramers over a range of concentrations (Fig. 2 E and F and Fig. 7C). Finally, we compared dodecamers with QD-based multimers. We generated QD multimers by incubating QD–streptavidin conjugates with biotinylated pMHC monomers. We found that dodecamers stained significantly better than QD multimers. In addition, we observed that QD multimers produced high levels of nonspecific staining, whereas the level of nonspecific staining with dodecamers was negligible (Fig. 2 G and H and Fig. 7D). Overall, pMHC dodecamers gave excellent results.

[00135] *Binding Properties of Dodecamers*. To evaluate the binding stability of pMHC dodecamers, we measured the apparent dissociation rates of a tetramer and a dodecamer. For 5C.C7 naive T cells, a tetramer binds TCRs with a half-life of 5 min, whereas a dodecamer binds TCRs with a half-life of 90 min (Fig. 3A), which equates to a 16-fold slower dissociation rate for the dodecamer. This much slower dissociation rate is most likely responsible for much of the improved binding avidity of the dodecamer because most tetramer-dependent assays require much more than 5 min to execute. TCR–pMHC interactions are also highly dependent on temperature, so we also tested the effect of temperature on pMHC multimer staining. We found that temperature influences the binding of both the dodecamer and the tetramer and that the temperature dependence positively correlates with the binding valency of the multimer. The temperature dependence of the dodecamer showed an inverted V shape. The dodecamer staining rapidly increases from 4°C to 20°C, peaks at 20°C, and then quickly decreases from 20°C to 37°C (Fig. 3B). In comparison, tetramer binding is much less dependent on temperature. Tetramer staining slowly increases from 4°C to 25°C, peaks at 25°C, and then slightly decreases from 25°C to 37°C. The binding of both multimers decreases at temperatures exceeding 25°C, possibly due to TCR internalization, which occurs after T-cell activation dependent on the cytoskeleton. Because the cytoskeleton actively regulates TCR diffusion, binding, and internalization, we investigated the effect of latrunculin A, which disrupts actin filaments, on pMHC multimer staining. We found that latrunculin A significantly reduced the binding of both the dodecamer and the tetramer in a dose-dependent manner (Fig. 3C).

[00136] *Staining of Human αβ T Cells with Dodecamers*. One important use of pMHC multimers involves the detection of antigen-specific T cells from human blood samples. Tetramer staining has been the primary method for this over the past decade. To increase the efficiency of detection, T cells are usually purified from peripheral blood mononuclear cells (PBMCs), and the rare antigenspecific T cells are further enriched after tetramer staining. Dodecamers can also be used for T-cell enrichment, especially for rare and low-affinity T cells, such as CD4+ T cells specific for influenza hemagglutinin (HA). Compared with tetramers, dodecamers significantly augmented the enrichment efficiency. For example, at 10 nM, dodecamers already could identify 0.92% antigen specific T cells, but at 150 nM, tetramers could detect only 0.22% antigen-specific T cells. Dodecamers also had comparable nonspecific staining (as tetramers) for T-cell enrichment (Fig. 4A). For high-frequency antigen-specific T cells, dodecamers may even eliminate the need for enrichment in some cases, allowing direct staining and detection of antigen-specific T cells from human PBMCs at 4 °C (we have found that multimer staining generates higher backgrounds for human cells at room temperature) (Fig. 8). As shown in Fig. 4B, cytomegalovirus (CMV)-specific CD8+ T cells were easily detected by a cognate HLA-A2:CMV dodecamer from human PBMCs without enrichment. However, these CD8+ T cells were undetectable by a cognate HLA-A2:CMV tetramer at low concentrations (1–10 nM) at 4 °C

(Fig. 4B) [Note that HLA-A2:CMV tetramer detected antigen-specific CD8+ T cells at room temperature (Fig. 8B)]. Meanwhile, a control HLA-A2: HIV dodecamer produced negligible nonspecific staining similar to that of a control HLA-A2:HIV tetramer (Fig. 4B). Furthermore, the staining was specific to CD8+ T cells, and we detected negligible nonspecific bindings to CD4+ T cells in the PBMCs (Fig. 9). Overall, these results suggest that traditional tetramers may significantly underestimate the actual frequency of antigen specific T cells in the repertoire, which might be physiologically important in maintaining and mediating the effector function and homeostasis of the adaptive immune system.

[00137] *Detection of Rare and Low-Affinity T Cells by Dodecamers.* Because a dodecamer has a much higher pMHC valency compared with a tetramer, and is also much larger, we tested whether a dodecamer is able to stain rare and low-affinity αβ T cells that are usually difficult to detect with tetramers, such as thymocytes. The binding affinity of thymocytes has been generally recognized as a key determinant for T-cell selection. However, immature, "double positive" CD4+CD8+ thymocytes express 10- to 30-fold lower levels of TCR than mature T cells and consequently are refractory to tetramer staining. As expected, cytochrome c/I-Ek tetramers showed minimal staining of 5C.C7 TCR+CD3+ thymocytes even when used at high concentrations (~90 nM) (Fig. 10). In contrast, the dodecamer specifically stained 5C.C7 TCR+CD3+ thymocytes even at low concentrations (~10 nM) (Fig. 10). We further analyzed the CD4+CD8+ double-positive thymocyte population (Fig. 11). The dodecamer readily stained the rare and low-affinity CD4+CD8+ thymocytes, which were undetectable using the tetramer reagent (Fig. 5A and Fig. 11G). We further used this dodecamer to detect antigen-specific 5C.C7 β–chain transgenic splenic T cells and thymocytes, which all express the same TCRβ chain together with different TCRα chains, leading to differences in TCR affinity. Compared with the tetramer, the dodecamer detected significantly more specific cells in 5C.C7 β-chain naive T cells (Fig. 12A), TCR+CD3+ thymocytes (Fig. 12B), and CD4+CD8+ thymocytes (Fig. 5B). These data further indicate the utility of dodecamers in identifying populations of specific T cells that have been difficult to study because of low TCR densities or avidities.

[00138] γδ T cells are a small subset of T cells that express γδ TCRs on their surface. We have also used dodecamers to successfully stain a T-cell line expressing δγ TCRs. We first compared staining with dodecamers to staining with tetramers. As expected, dodecamers stained γδ TCR-expressing T cells much better than tetramers (Fig. 13), which is consistent with our previous results for αβ T cells (Figs. 2–5). We further compared staining by dodecamers versus dextramers. Both dodecamers and dextramers showed comparable staining intensities (Fig. 14: compare top two rows), although dodecamers had the added benefit of less nonspecific binding (Fig. 14: compare bottom two rows).

[00139] *Application of Dodecamers to Single-Cell Mass Cytometry.* Single cell mass cytometry, also known as CyTOF, is a new type of flow cytometry in which antibodies coupled to heavy

metal isotopes are used to stain molecules of interest on cells. CyTOF can simultaneously measure more than 40 parameters on a single cell without the problem of overlapping excitation and emission spectra that is inherent to conventional fluorescence- based flow cytometry. Although we had some success in using MHC class I tetramers to analyze CD8+ T cells by CyTOF, the detection of antigen-specific CD4+ T cells using MHC class II tetramers has been variable, possibly due to the harsh experimental conditions needed for CyTOF measurements, such as extensive washing, cell fixation, and the complex sample introduction system of the machine. The high binding avidity of dodecamers may overcome these barriers, so we tested the application of dodecamers in CyTOF. 5C.C7 splenocytes were incubated with metal-labeled MHC class II tetramers or dodecamers and then analyzed by high-throughput single-cell CyTOF (Fig. 15). Dodecamers can readily stain antigen-specific 5C.C7 naive T cells with high specificity (Fig. 6A and B). Consistent with the results obtained by conventional flow cytometry, dodecamers stained significantly better than tetramers in CyTOF. The staining of the 20-nM dodecamer is better than that of the 100-nM tetramer in CyTOF (Fig. 6 A and B). Furthermore, to evaluate the function of low-affinity T cells detected by dodecamers but missed by tetramers, we sorted tetramer (+) T cells (Fig. 16A) and tetramer (−) dodecamer (+) T cells (Fig. 16B) from the splenocytes of a 5C.C7 β-chain mouse using flow cytometry. Sorted T cells were stimulated by either MCC peptide-pulsed CH27 cells (Fig. 6C) or phorbol 12-myristate 13-acetate (PMA)/Ionomycin (Fig. 6D) and then stained by ten metal-labeled antibodies to detect the effector cytokines. Tetramer (−) dodecamer (+) T cells showed a comparable cytokine expression profile to that of tetramer (+) T cells (Fig. 6 C and D and Fig. 16 C–F). The data highlighted the functional importance of low-affinity T cells missed by tetramers. Thus, dodecamers should be useful in CyTOF-based studies for detecting, phenotyping, quantifying, and analyzing antigen-specific T cells.

[00140] Peptide-MHC tetramers have had a major impact on the analysis of T cells in a wide variety of applications. Although various pMHC multimers have been developed, such as dimers, pentamers, octamers, dextramers, and polymers, tetramers remain the most common reagents for the profiling of antigen-specific T cells, mainly due to their simple structure, relatively high binding avidity, and low nonspecific staining. The development of T-cell enrichment methods in conjunction with advances in flow cytometry has further enabled the study of antigen-specific T cells using pMHC tetramers. However, tetramer technology is not perfect. The performance of tetramers has been less than adequate in the detection of antigen-specific CD4+ T cells, low-affinity αβ T cells, and rare γδ T cells, especially when analyzed by CyTOF. In these cases, the binding avidity of tetramers is probably insufficient. The maximum valency of a tetramer is four, but it is likely that only three would be able to bind TCRs on the cell surface at any one time because of the fourfold symmetry of the streptavidin molecule. To

increase this sensitivity, we have engineered a pMHC dodecamer, which has a maximum valency of 12 peptide-MHCs. This construct shows excellent sensitivity, high binding avidity, slow dissociation kinetics, and low background staining. It has a relatively simple structure and retains important features of a tetramer. Recent advances in MHC tetramer technology, such as photocleavable peptide exchange, tetramer-guided epitope mapping, T-cell enrichment, antibody stabilization staining, and combinatorial staining, can be easily used with dodecamer reagents. We can also make dodecamers for nonclassical MHC molecules and non-MHC molecules. The dodecamer is compatible with commercially available fluorescent/metal-labeled streptavidin, is easy to manufacture, and has advantages over standard tetramers and other multimers for many applications. MHC class I tetramers have been successfully applied to the study of CD8+ T cells. However, it has been difficult to apply MHC class II tetramers to the study of CD4+ T cells because it is more difficult to produce MHC class II molecules and the binding of the coreceptor CD4 to MHC class II is very weak. For cells from all sources, the greatly enhanced stability of dodecamers will make enrichment protocols more efficient (Fig. 4A) in capturing low-frequency and low affinity CD4+ T cells.

[00141] For cells from transgenic mice, our dodecamer technology can bypass cell enrichment steps and directly stain both monoclonal and polyclonal antigen specific CD4+ naive T cells (Figs. 2 and 3), CD3+TCR+ thymocytes (Fig. 10 and Fig. 12B), and even CD4+ CD8+ double-positive thymocytes (Fig. 5) from a large pool of different cells without any T-cell–enriching steps. Clearly, the high-binding valency of dodecamers compensates for the low affinity of TCRs and the weak binding of the CD4 coreceptor. Dodecamers also have significant advantages over most other multimers such as dextramers and QD multimers. Compared with dextramers, dodecamers have only a modest staining advantage (Fig. 2 E and F) but less nonspecific binding (Fig. 14). Compared with QD multimers, dodecamers are superior in both staining intensity and background (Fig. 2 G and H). We have successfully used dodecamers to stain antigen-specific $\gamma\delta$ T cells and human CD4+ and CD8+ $\alpha\beta$ T cells with significantly improved sensitivity over tetramers in flow cytometry (Fig. 4 and Figs. 8, 13, and 14). Dodecamers also showed significant advantages over tetramers in CyTOF applications (Fig. 6 A and B). Furthermore, using FACS we have purified low-affinity T cells by dodecamers that were missed by tetramers (Fig. 16A and B). The low-affinity tetramer (−) dodecamer (+) T cells showed comparably strong cytokine expression as the high-affinity tetramer (+) T cells in a CyTOF-based multiplex cytokine analysis (Fig. 6 C and D and Fig. 16C–F). These data highlight the functional importance of lower-affinity T cells that escape detection by tetramers. These experiments demonstrate the great potential of dodecamers in the analysis of antigen-specific T cells in both flow cytometry and mass cytometry. Because pMHC dodecamers can effectively identify functionally important, low-affinity T cells missed by tetramers (Fig. 6C and D), pMHC dodecamers are useful in the studies of other low-affinity T cells, such as those that

typically predominate in tumor-specific responses and autoimmune diseases, which escape detection by traditional tetramer staining. The dodecamers described here can also be used to manipulate T-cell responses in vivo and in vitro and to develop dodecamer-based targeted immunotherapy. Other variations along these lines involve putting two different pMHCs on one construct or mixing biotinylated costimulatory molecules together with specific pMHCs.

[00142] MHC dodecamers are useful in the study of T cells with low affinity TCRs, such as those that typically predominate in tumor specific responses and autoimmune diseases, which escape detection by traditional tetramer staining. The high binding avidity of dodecamers can also be utilized to identify low frequency and/or low affinity $\gamma\delta$ T cells. In conjunction with modern sequencing technologies, the high sensitivity of dodecamers allows new approaches to T cell phenotyping. The high binding avidity will greatly expedite the screening of TCR ligands in high throughput technology such as that developed in CyTOF. Dodecamers can be used to manipulate T-cell responses in vivo and in vitro and to develop dodecamer-based targeted immunotherapy. Other usages of dodecamers include the evaluation of vaccine efficacy, the study of allogeneic and superantigen interactions with TCRs, and the analysis of the T cell repertoire.

[00143] *Engineering of a scaffold protein*. A single cysteine was added at the C-terminus of an inactive streptavidin subunit that has no biotin-binding site, using a site-directed mutagenesis kit (Agilent) to create a scaffold protein monomer. Scaffold protein monomer was expressed in BL21 E. coli and refolded to form a tetrameric scaffold protein tagged with four cysteines, which was purified by fast protein liquid chromatography (FPLC). The four cysteines on a tetrameric scaffold protein were biotinylated using maleimide chemistry with EZ-Link BMCC-Biotin (Thermo Scientific).

[00144] Briefly, cysteine-tagged scaffold protein was treated with 10 mM TCEP for 10 minutes and followed by incubation with BMCC-Biotin at a 1:100 molar ratio overnight at room temperature. Excess BMCC-Biotin was removed using 7K MWCO Zeba Spin Desalting Columns (Thermo Scientific). Biotinylated scaffold protein was further purified by FPLC and then analyzed by SDS-PAGE.

[00145] *Generation of pMHC dodecamers and other multimers*. To generate pMHC dodecamers, biotinylated scaffold protein was mixed and incubated with fluorescently labeled streptavidin at a molar ratio of 1:4 for one hour at room temperature, followed by incubation with biotinylated pMHC at a molar ratio of 1:12 for an additional hour at room temperature. In some experiments, biotinylated scaffold protein was mixed with fluorescently labeled streptavidin at a molar ratio of 1:20 and the pentameric molecular complex was purified using FPLC before the addition of biotinylated pMHC. To generate QDmultimers, streptavidin-conjugated QD605 was

incubated with biotinylated pMHC at a 1:28 molar ratio for one hour at room temperature. To generate pMHC dextramers, fluorescently labeled streptavidin was incubated with biotinylated pMHC monomers at a molar ratio of 3.5:1. After one hour at room temperature, biotinylated dextramer was added at a molar ratio of 60:1 and the mixture was incubated for one hour or longer at room temperature prior to use.

[00146] *Cells*. TCRα/TCRβ transgenic 5C.C7 mice ($Rag2^{-/-}$ background) and TCRβ transgenic 5C.C7 (B10.BR background) mice were bred and maintained in the Research Animal Facility at Stanford University Department of Comparative Medicine Animal Facility (protocol 3540) in accordance with guidelines of the US National Institutes of Health. Mouse splenocytes and thymothytes were obtained and used for experiments. γδ TCR-expressing $58\alpha^{-}\beta^{-}$ cells and γδ negative $58\alpha^{-}\beta^{-}$ cells have been described. PBMCs from healthy blood donors were isolated by density centrifugation of leukocyte reduction shuttles from the Stanford Blood Center. For the purposes of pMHC multimer staining experiments, HLA-A2 positive donor samples were typed and identified by the Stanford Blood Center. These samples were also serotyped for cytomegalovirus (CMV) infection status. HLA-DR4 positive donors samples were typed by the Children's Hospital Oakland Research Institute. PBMCs were cryopreserved and stored in liquid nitrogen prior to use.

[00147] *Flow cytometry*. Mouse splenocytes and thymocytes were first incubated with 1% anti-CD16/CD32 Fc blocker, 10% rat serum, and 10% syrian hamster serum on ice for 30 minutes. Mouse cells were further stained with pMHC dodecamers, tetramers, dextramers, or QD605-based multimers, and co-stained with an antibody cocktail containing FITC-labeled anti-CD8, APC-Cy7-labeled anti-CD4, Alexa700-labeled anti-CD3, APC-labeled anti-TCRβ, pacific blue-labeled anti- CD19, anti-CD11b, anti-CD11C, anti-Gr1, and anti-F4/80, and aqua live/dead cell stain. Frozen human PBMCs were thawed and rested overnight5. To stain human CD8+ T cells, human PBMCs were incubated with pMHC dodecamers or tetramers, and co-stained with an antibody cocktail including FITC-labeled anti-CD8α, Alexa700-labeled anti-CD3, QD605-labeled anti-CD45RA, brilliant violet labeled anti-CD62L, PerCP/Cy5.5-labeled anti-CD4, anti-CD19, anti-CD33, and anti-CD14, and aqua live/dead cell stain. To profile rare influenza-specific CD4+ T cells, cells stained with pMHC dodecamers or tetramers were magnetically enriched and co-stained with a cocktail of antibodies containing PE/Texas Red labeled anti-CD4, PE/Cy5 non-CD4 exclusion markers and near-IR live/dead cell stain. Most cells were stained at 4°C though some were stained at other temperatures. To test the effect of temperature on pMHC multimer staining, mouse cells were incubated for one hour in a thermocycler at the indicated temperatures. In experiments with latrunculin A, mouse cells were pretreated with latrunculin A (0.1-10 μM) for one hour and then stained with pMHC tetramers or dodecamers and the mouse antibody cocktail for one additional hour at room temperature (22°C). Cells were washed and analyzed on a BD LSR II flow cytometer. To measure the

dissociation rates of tetramers and dodecamers, 5C.C7 splenocytes were incubated with 100 nM Alexa488-labeled tetramer or dodecamer for 1 hour at 22°C in the presence of PE-labeled anti-CD8, APC-Cy7- labeled anti-CD4, Alexa700-labeled anti-CD3, pacific blue-labeled anti-CD19, anti-CD11b, anti-CD11c, anti-Gr1, and anti-F4/80, and aqua live/dead cell stain. Cells were pelleted and resuspended in 200 μL FACS buffer in the presence of 100 μg/mL 14.4.1 anti-I-Ek MHC antibody on ice. The fluorescence intensities of 10,000 cells were measured at different time points after adding the anti-I-E^k MHC blocking antibody. Flow cytometry data were analyzed by FlowJo software. A first-order decay kinetic model was used to fit the mean fluorescence intensities at different time points and to obtain the off-rate, k_{off}, and the half-life, $t_{1/2}$.

[00148] *Mass cytometry*. Biotinylated I-E^k was refolded with either moth cytochrome c (MCC) peptide (amino acids 88–103, ANERADLIAYLKQATK) or a human class II-associated invariant chain peptide (CLIP) (amino acids 87-101, PVSKMRMATPLLMQA). For dodecamerization, biotinylated scaffold protein was incubated with metallabeled streptavidin (1:4 molar ratio) for one hour followed by incubation with pMHC monomers (1:12 molar ratio) for one more hour at room temperature. Tetramers were prepared as described. Each pMHC multimer was barcoded with two different metal labels. 5C.C7 mouse splenocytes were incubated with 1% anti-CD16/CD32 Fc blocker, 10% rat serum and 10% syrian hamster serum on ice for 30 minutes, stained by dodecamers or tetramers for one hour at room temperature, and then incubated with a metal-labeled antibody cocktail including anti-TCRβ (Nd143), anti-CD3 (Sm154), anti-CD4 (Nd146), anti-CD8 (Sm149), anti-CD11b (Eu153), anti-CD11c (Sm152), anti-CD19 (Cd112 and Cd114), anti- Gr1 (Nd145), and anti-F4/80 (Tb159). Splenocytes were washed with CyFACS buffer and stained with metal-labeled live/dead (La139) medium for 20 minutes on ice. Splenocytes were washed and fixed in 2% PFA CyPBS for one hour on ice. After treatment with permeabilization buffer (eBioscience), cells were washed, resuspended in milliQ water, and analyzed by CyTOF.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A tetrameric scaffold protein comprising:

four subunits of a tetramer forming protein, in which the protein has low affinity for biotin, and a c-terminal cysteine residue.

2. The tetrameric scaffold protein of Claim 1, wherein the protein is a biotin binding protein modified to (a) substantially ablate binding to biotin while retaining tetramer formation; and (c) provide a c-terminal cysteine.

3. The tetrameric scaffold protein of Claim 2, wherein the protein is derived from streptavidin.

4. The tetrameric scaffold protein of Claim 3, comprising the amino acid sequence set forth in SEQ ID NO:1.

5. The tetrameric scaffold protein of Claim 1, further comprising a biotin label at each terminal cysteine.

6. A dodecameric scaffold, comprising the tetrameric scaffold protein of Claim 5, and high affinity biotin-binding protein tetramers.

7. The dodecameric scaffold of Claim 6, wherein the high affinity biotin-binding protein tetramer is streptavidin.

8. A multimeric binding agent, comprising the dodecameric scaffold of Claim 6 or Claim 7; and one or more biotin-tagged specific binding reagents.

9. The multimeric binding agent of Claim 8, comprising 12 biotin-tagged specific binding reagents.

10. The multimeric binding agent of Claim 8 or Claim 9, wherein the biotin tagged specific binding reagent is selected from MHC proteins, T cell receptor, and antibody.

11. The multimeric binding agent of Claim 10, wherein the specific binding reagent is an MHC protein.

12. The multimeric binding agent of Claim 11, wherein the MHC protein is complexed with an antigenic peptide.

13. The multimeric binding agent of Claim 11, wherein the MHC protein is not complexed with an antigenic peptide.

14. The multimeric binding agent of Claim 11, wherein the MHC protein is a Class II heterodimer.

15. The multimeric binding agent of Claim 11, wherein the MHC protein is A class I protein.

16. The multimeric binding agent of Claim 11, comprising a detectable label.

17. The multimeric binding agent of Claim 16, wherein the label is a fluorochrome or a heavy metal.

18. A method of staining, detecting, or isolating cells, comprising contacting a cell population with a multimeric binding agent of any one of Claims 8-17, and determining the presence of the multimeric binding agent bound to cells.

19. The method of Claim 18, wherein the cell population comprises T cells.

20. The method of Claim 18, further comprising isolating cells bound to said multimeric binding agent.

21. A method of activating or anergizing T cells, the method comprising contacting a cell population comprising T cells with an effective dose of a multimeric binding agent of any one of Claims 8-17.

22. A kit comprising a tetrameric scaffold protein, dodecameric scaffold, or multimeric binding agent of any one of Claims 1-17.

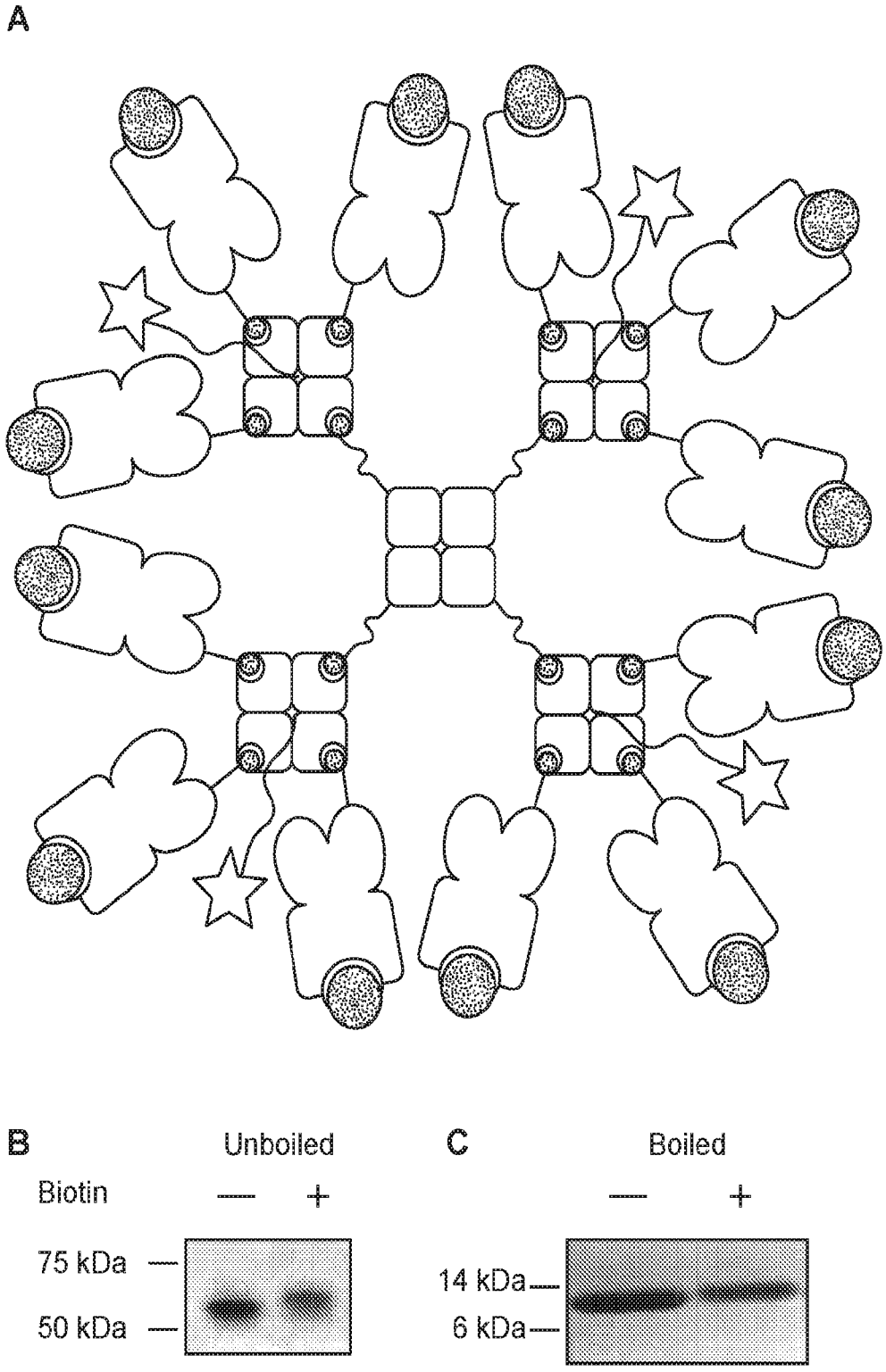

FIG. 1

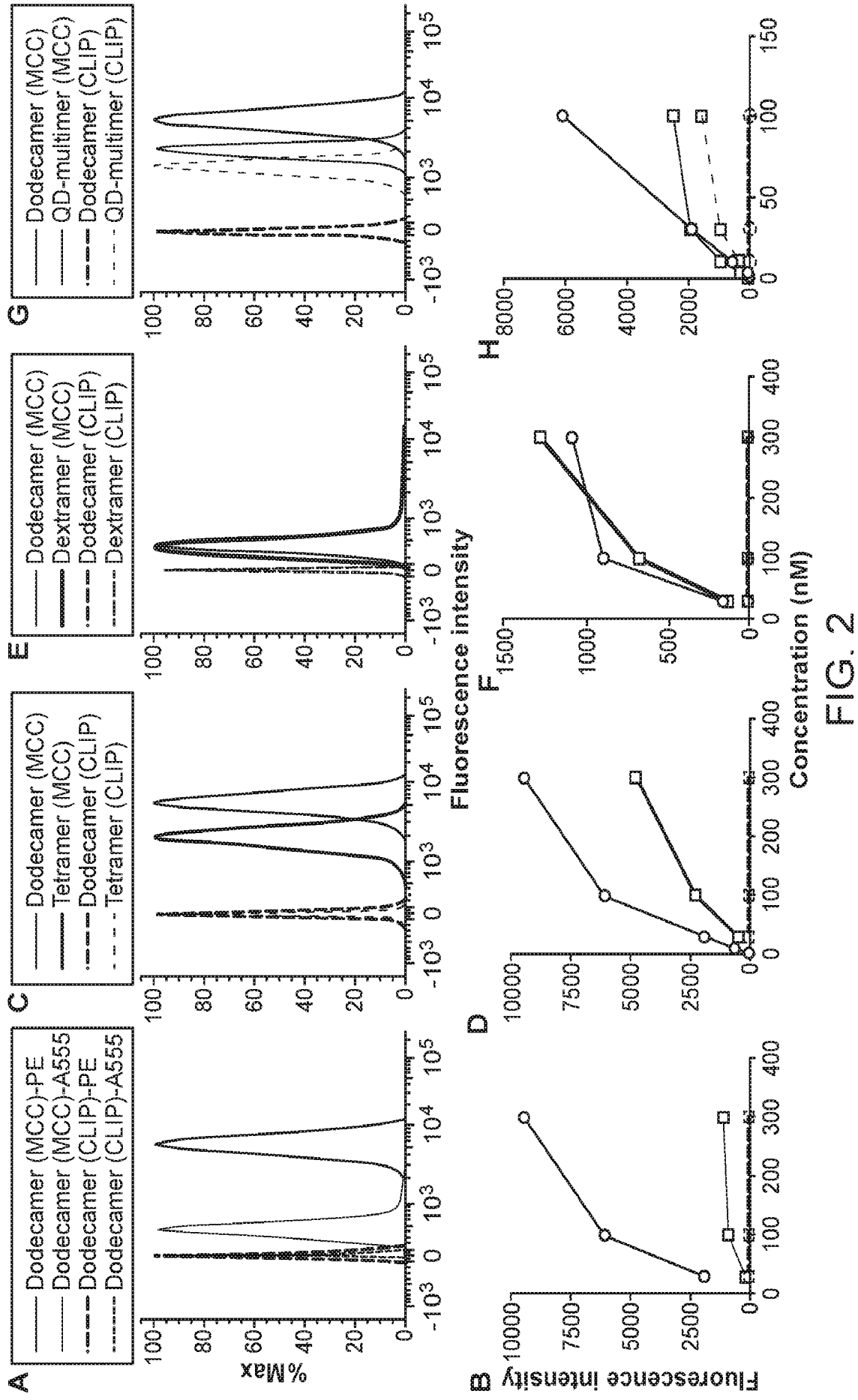
A
Dodecamer (MCC)-PE
Dodecamer (MCC)-A555
Dodecamer (CLIP)-PE
Dodecamer (CLIP)-A555
%Max
C
Dodecamer (MCC)
Tetramer (MCC)
Dodecamer (CLIP)
Tetramer (CLIP)
E
Dodecamer (MCC)
Dextramer (MCC)
Dodecamer (CLIP)
Dextramer (CLIP)
G
Dodecamer (MCC)
QD-multimer (MCC)
Dodecamer (CLIP)
QD-multimer (CLIP)
Fluorescence intensity
B
Fluorescence intensity
D
F
H
Concentration (nM)

FIG. 2

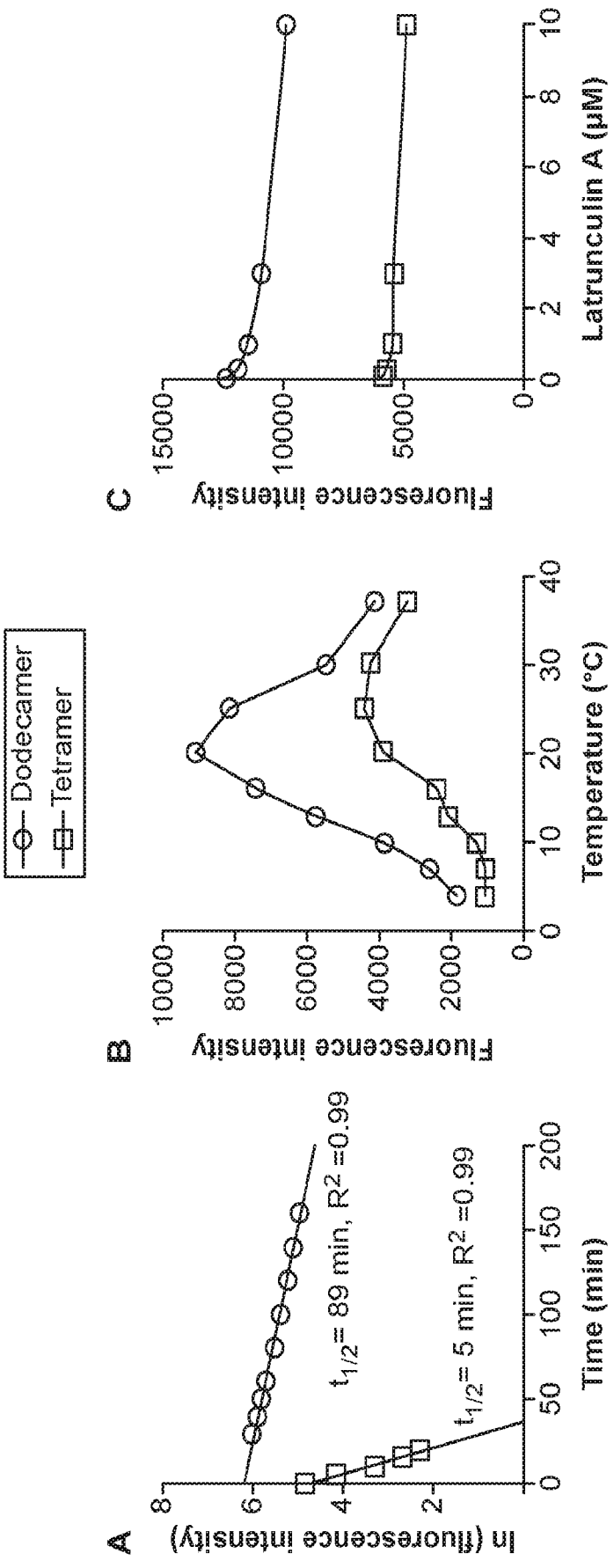
A
ln (fluorescence intensity)
8
6
4
2
0
50
100
150
200
Time (min)
$t_{1/2}$= 89 min, R^2 =0.99
$t_{1/2}$= 5 min, R^2 =0.99
B
Fluorescence intensity
10000
8000
6000
4000
2000
0
10
20
30
40
Temperature (°C)
Dodecamer
Tetramer
C
Fluorescence intensity
15000
10000
5000
0
2
4
6
8
10
Latrunculin A (μM)

FIG. 3

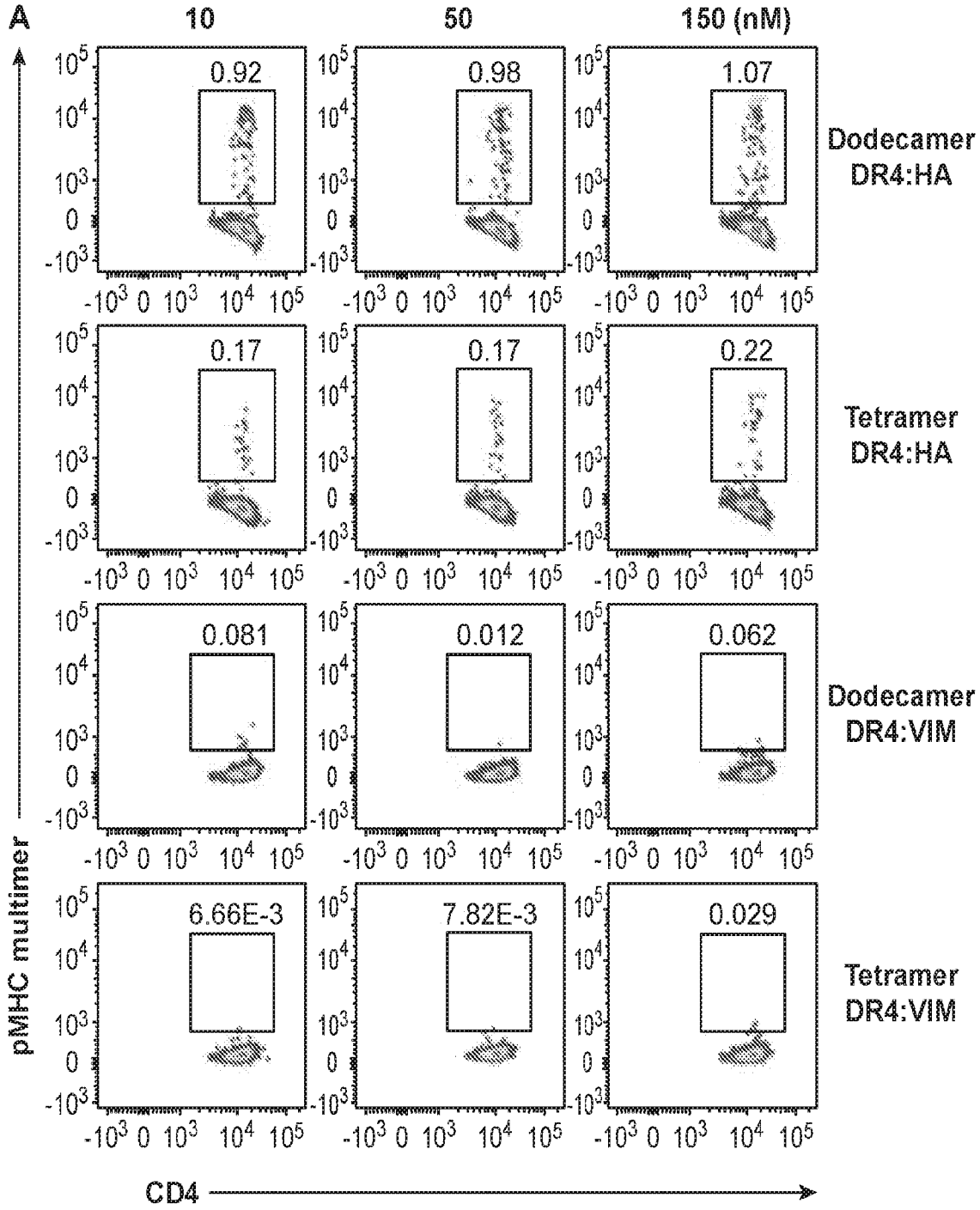
A
10
50
150 (nM)
0.92
0.98
1.07
Dodecamer DR4:HA
0.17
0.17
0.22
Tetramer DR4:HA
0.081
0.012
0.062
Dodecamer DR4:VIM
6.66E-3
7.82E-3
0.029
Tetramer DR4:VIM
pMHC multimer
CD4

FIG. 4

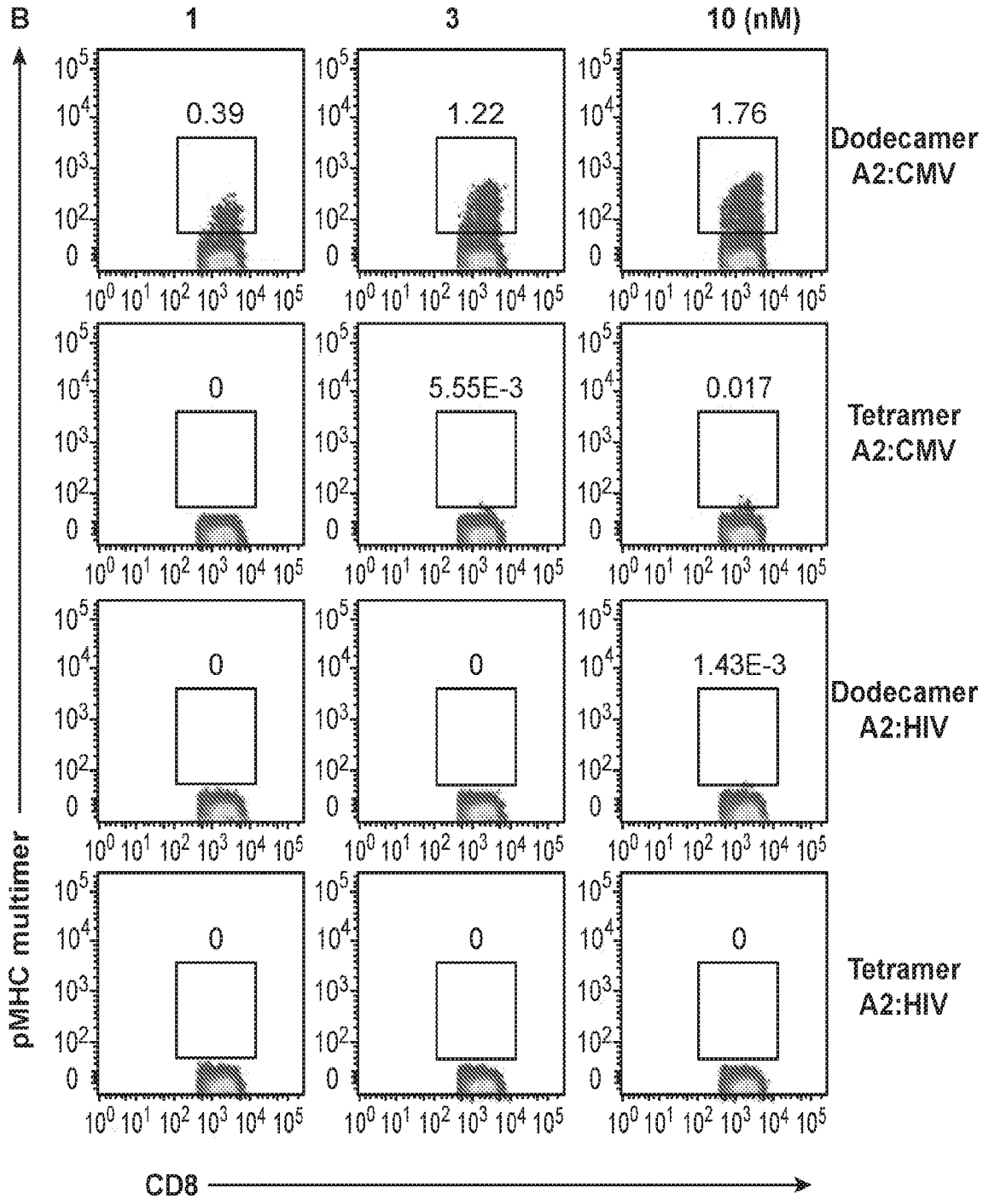
B
1
3
10 (nM)
0.39
1.22
1.76
Dodecamer A2:CMV
0
5.55E-3
0.017
Tetramer A2:CMV
0
0
1.43E-3
Dodecamer A2:HIV
0
0
0
Tetramer A2:HIV
pMHC multimer
CD8

FIG. 4 (Cont.)

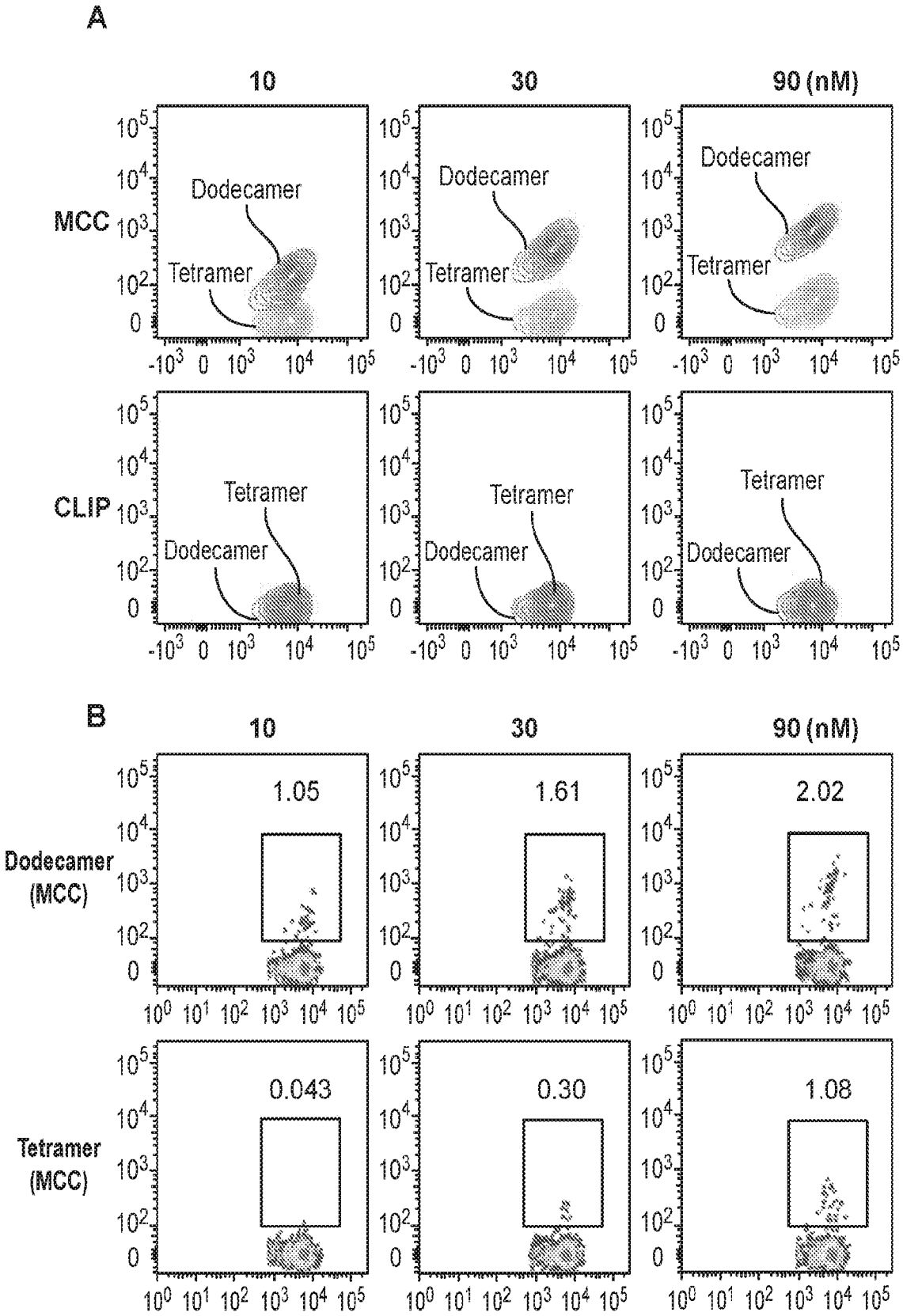
A
10
30
90 (nM)
MCC
CLIP
Dodecamer
Tetramer
B
Dodecamer (MCC)
Tetramer (MCC)
1.05
1.61
2.02
0.043
0.30
1.08

FIG. 5

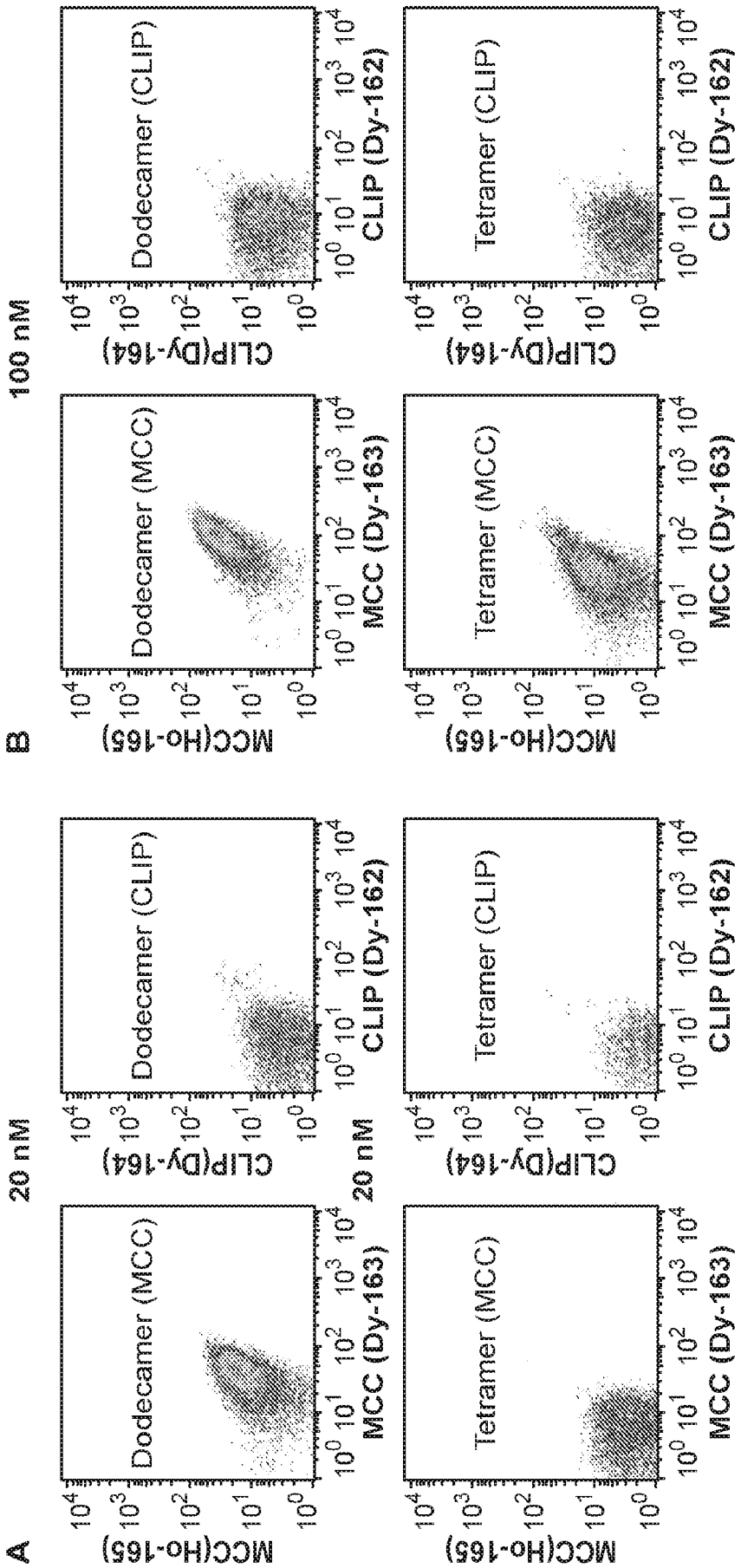
A
20 nM
B
100 nM
Dodecamer (MCC)
Dodecamer (CLIP)
Tetramer (MCC)
Tetramer (CLIP)
MCC(Ho-165)
CLIP(Dy-164)
MCC (Dy-163)
CLIP (Dy-162)

FIG. 6

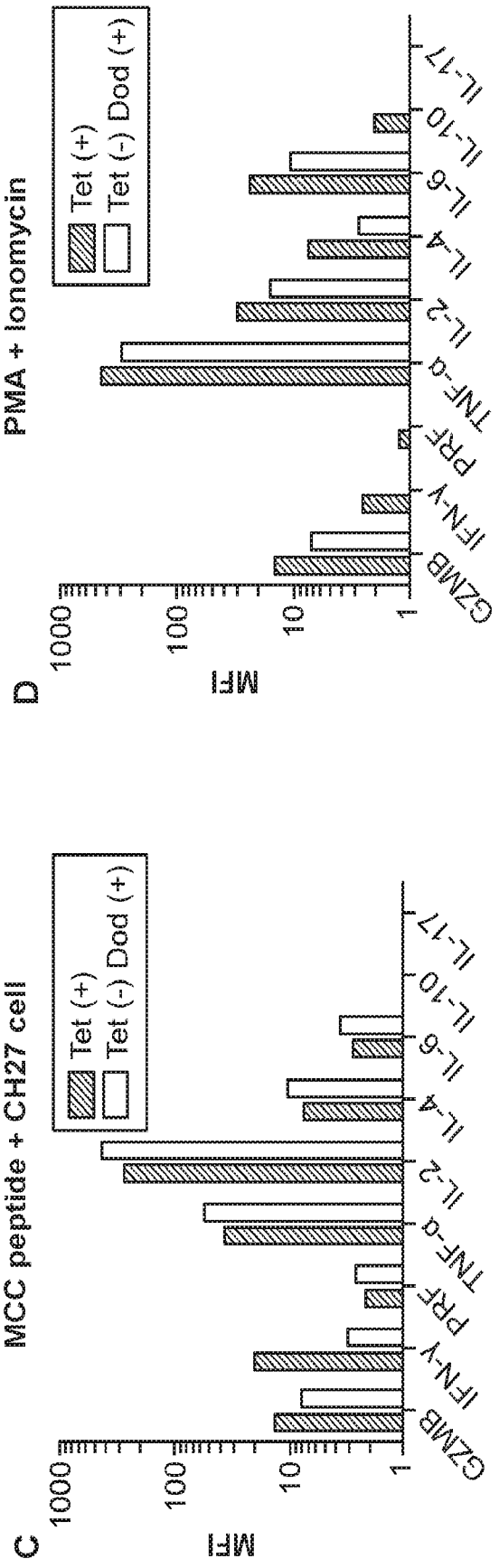
C
MCC peptide + CH27 cell
Tet (+)
Tet (-) Dod (+)
MFI
1000
100
10
1
GZMB
IFN-γ
PRF
TNF-α
IL-2
IL-4
IL-6
IL-10
IL-17
D
PMA + Ionomycin
Tet (+)
Tet (-) Dod (+)
MFI
1000
100
10
1
GZMB
IFN-γ
PRF
TNF-α
IL-2
IL-4
IL-6
IL-10
IL-17

FIG. 6 (Cont.)

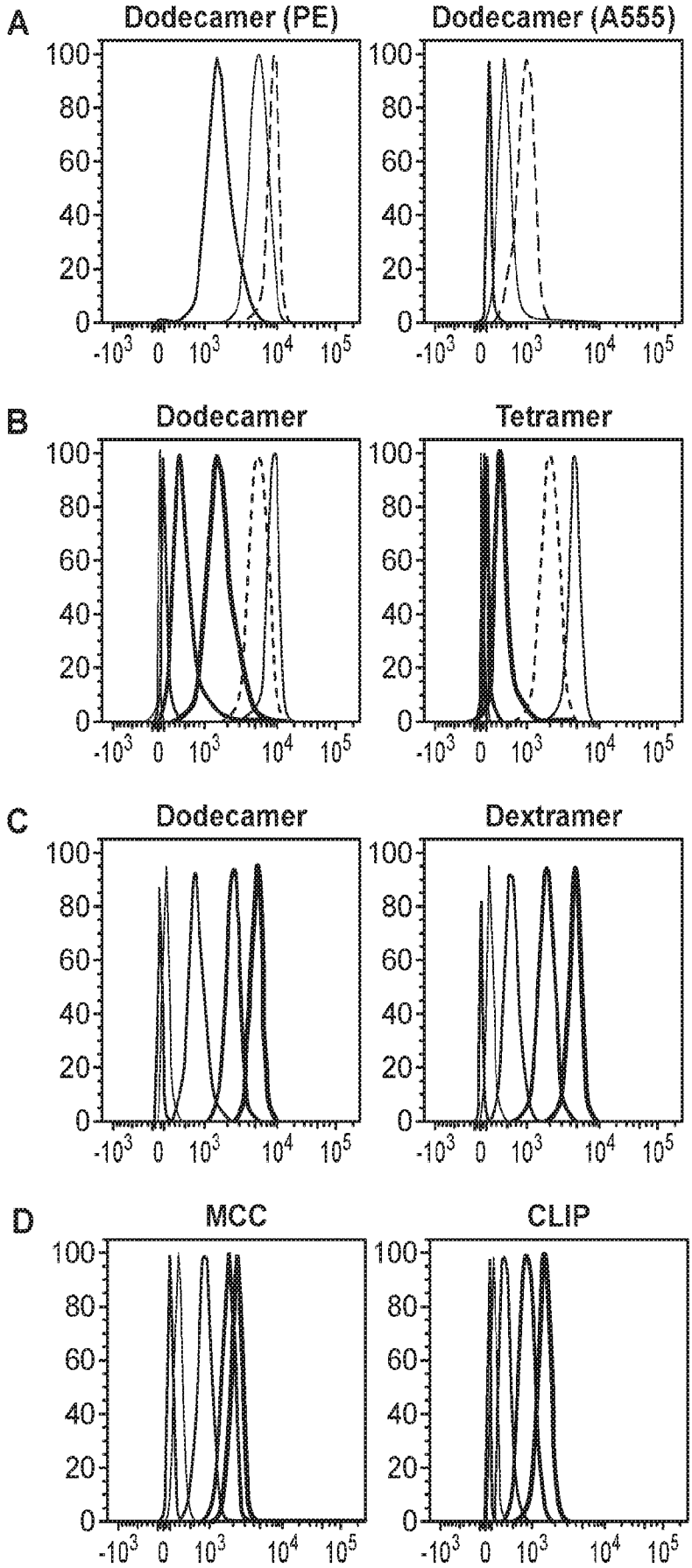
A
Dodecamer (PE)
Dodecamer (A555)
B
Dodecamer
Tetramer
C
Dodecamer
Dextramer
D
MCC
CLIP
100
80
60
40
20
0
-10^3
0
10^3
10^4
10^5

FIG. 7

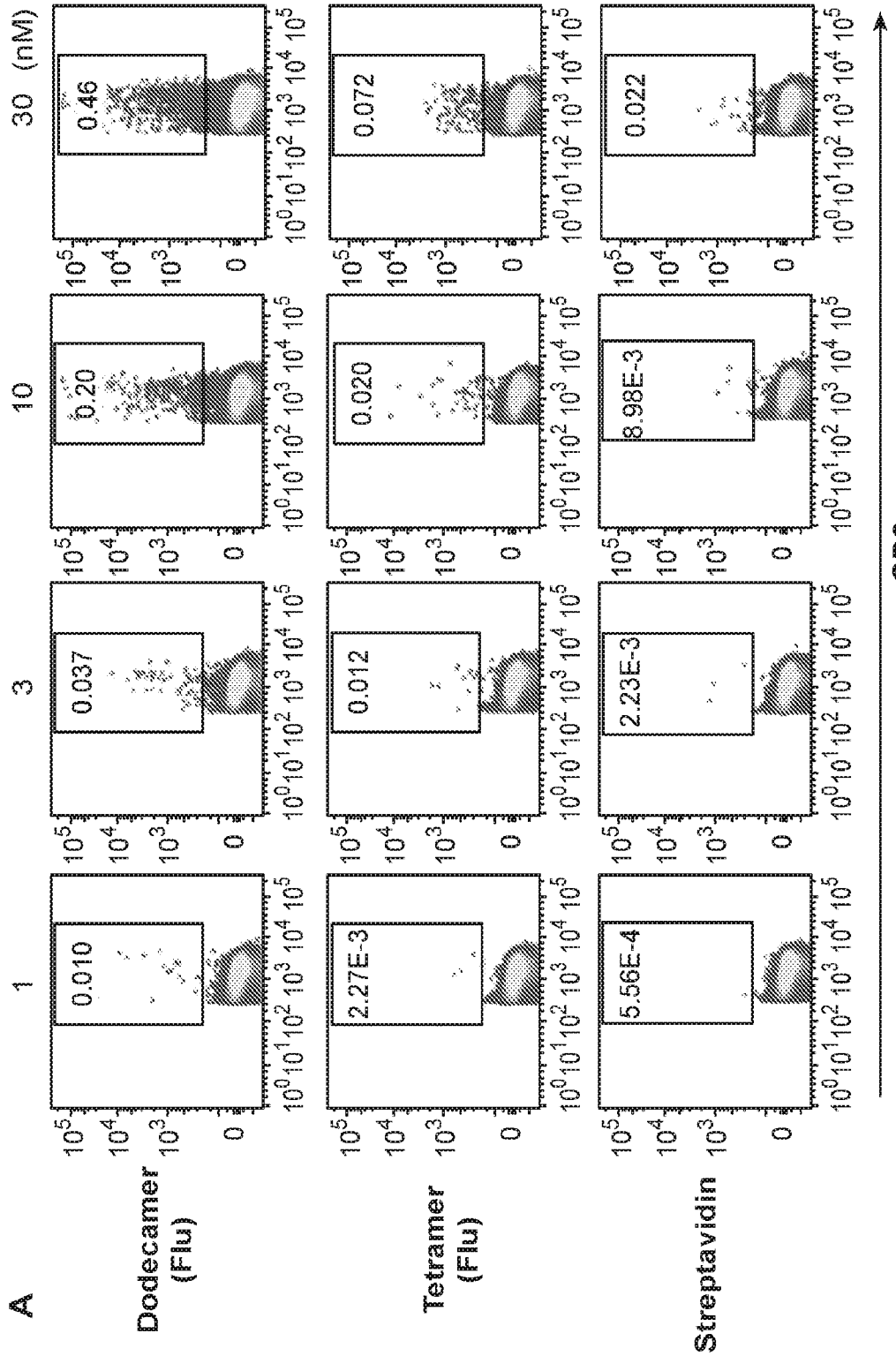
A
1
3
10
30 (nM)
Dodecamer (Flu)
Tetramer (Flu)
Streptavidin
0.010
0.037
0.20
0.46
2.27E-3
0.012
0.020
0.072
5.56E-4
2.23E-3
8.98E-3
0.022
CD8

FIG. 8

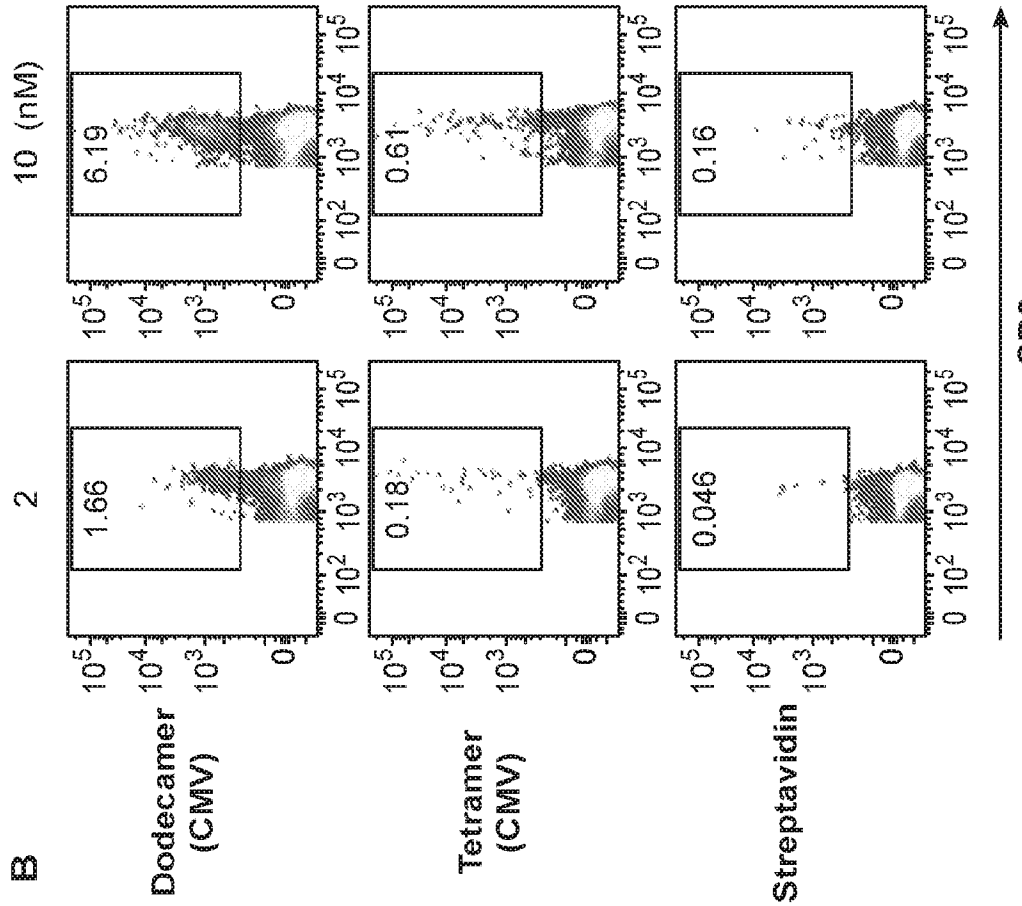
B
2
10 (nM)
Dodecamer (CMV)
Tetramer (CMV)
Streptavidin
1.66
6.19
0.18
0.61
0.046
0.16
CD8

FIG. 8 (Cont.)

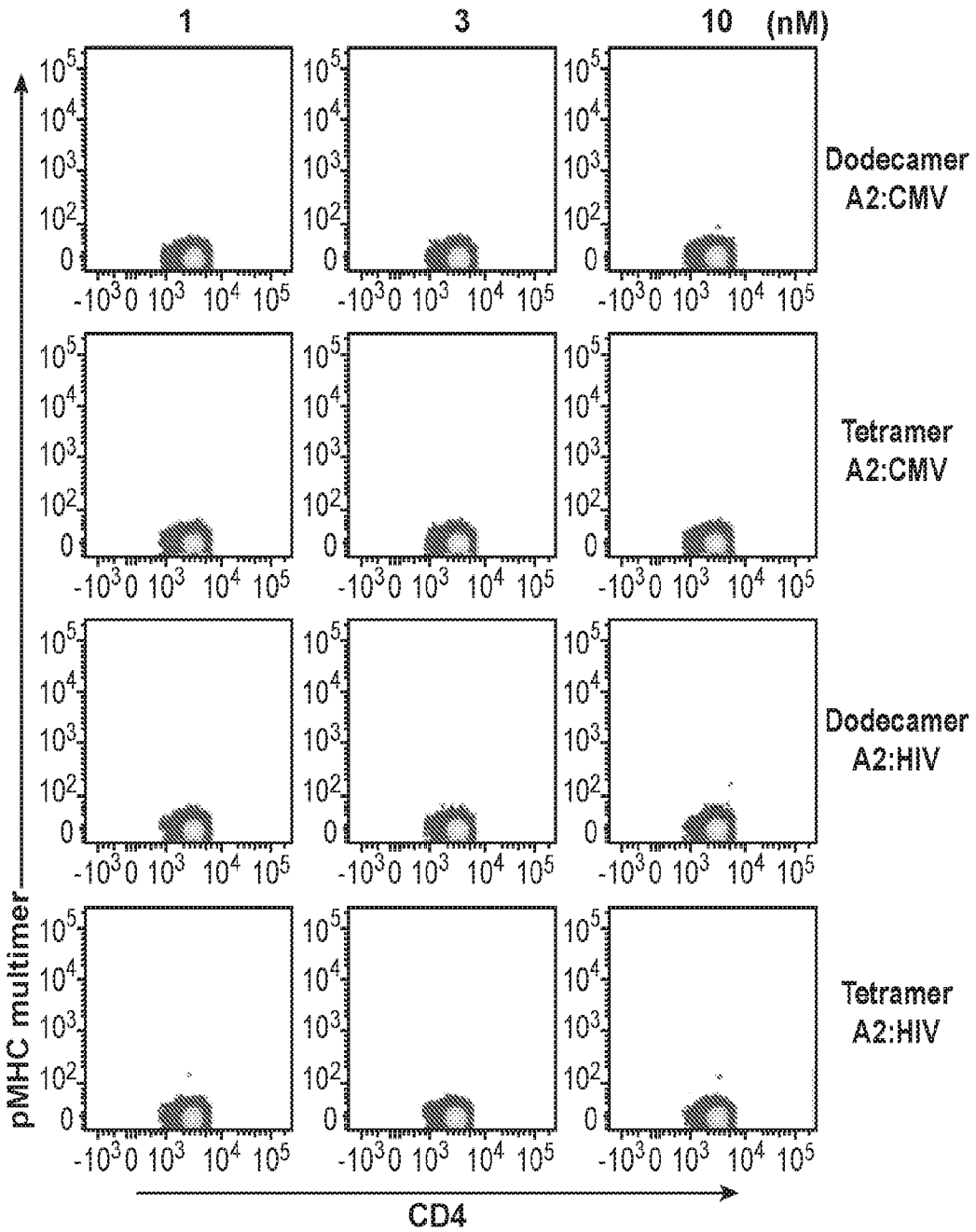
1
3
10
(nM)
Dodecamer A2:CMV
Tetramer A2:CMV
Dodecamer A2:HIV
Tetramer A2:HIV
pMHC multimer
CD4

FIG. 9

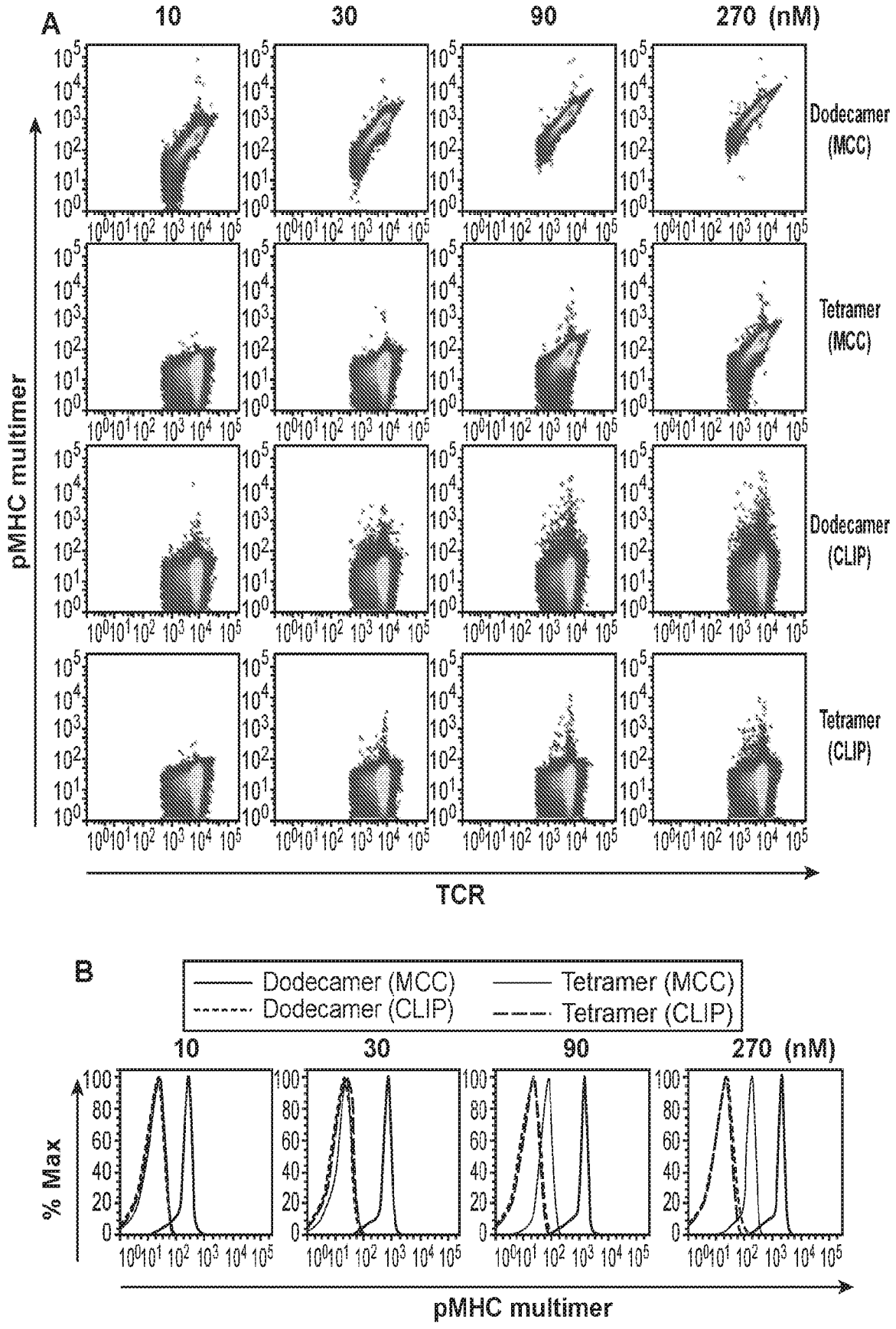
A
10
30
90
270 (nM)
Dodecamer (MCC)
Tetramer (MCC)
Dodecamer (CLIP)
Tetramer (CLIP)
pMHC multimer
TCR
B
Dodecamer (MCC)
Dodecamer (CLIP)
Tetramer (MCC)
Tetramer (CLIP)
10
30
90
270 (nM)
% Max
pMHC multimer

FIG. 10

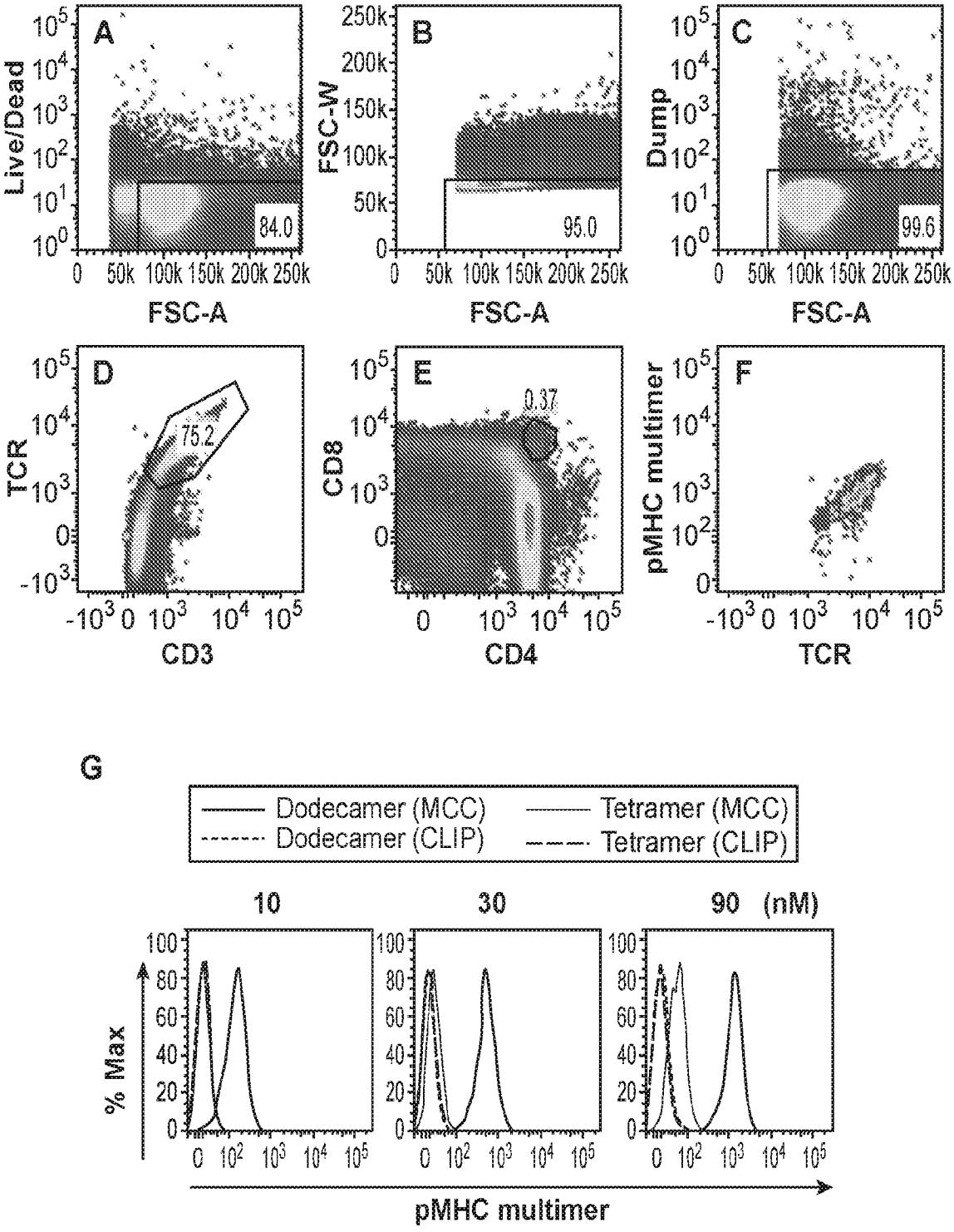
A
Live/Dead
FSC-A
84.0
B
FSC-W
FSC-A
95.0
C
Dump
FSC-A
99.6
D
TCR
CD3
75.2
E
CD8
CD4
0.37
F
pMHC multimer
TCR
G
Dodecamer (MCC)
Dodecamer (CLIP)
Tetramer (MCC)
Tetramer (CLIP)
10
30
90 (nM)
% Max
pMHC multimer

FIG. 11

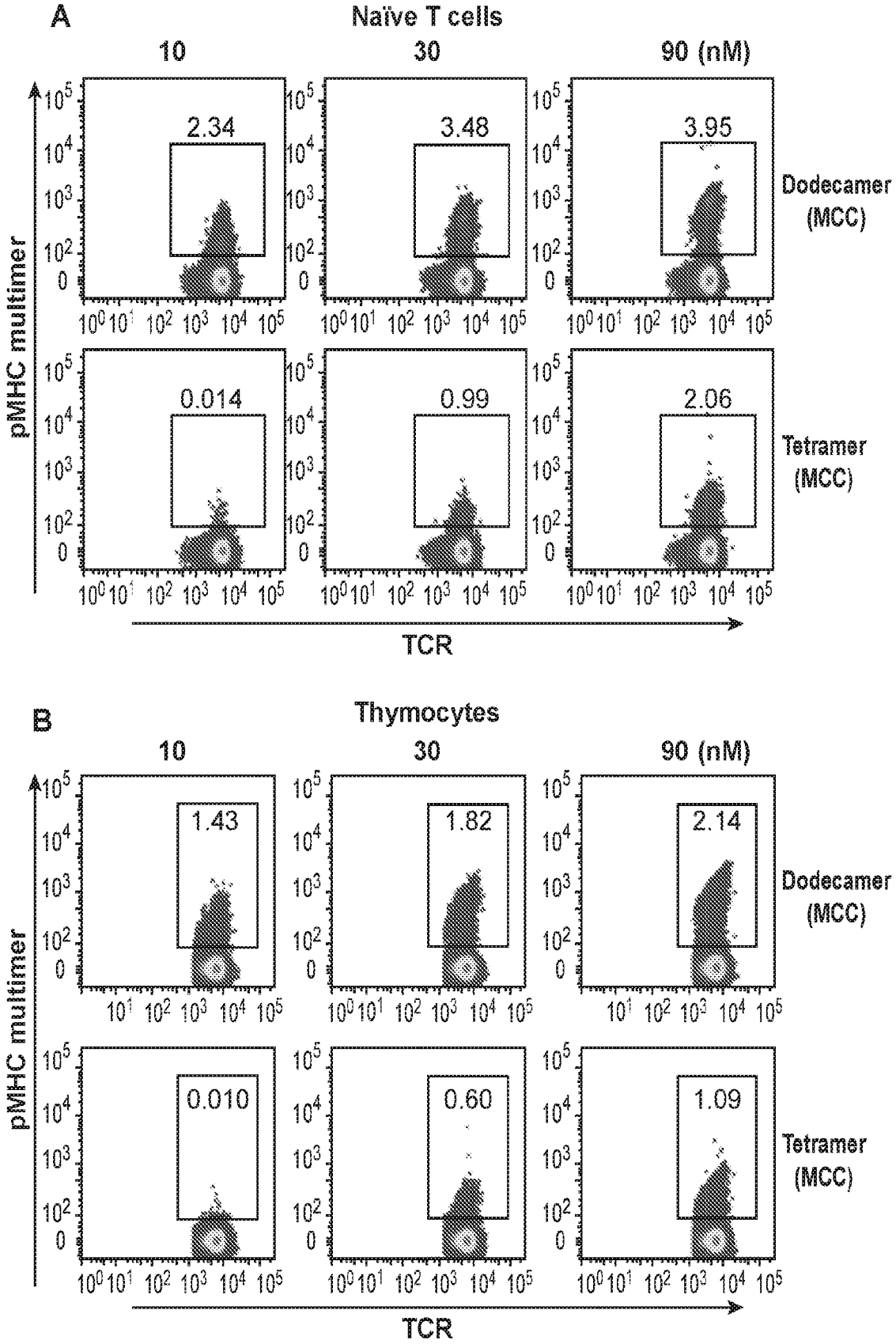
A
Naïve T cells
10
30
90 (nM)
2.34
3.48
3.95
0.014
0.99
2.06
Dodecamer (MCC)
Tetramer (MCC)
pMHC multimer
TCR
B
Thymocytes
10
30
90 (nM)
1.43
1.82
2.14
0.010
0.60
1.09
Dodecamer (MCC)
Tetramer (MCC)
pMHC multimer
TCR

FIG. 12

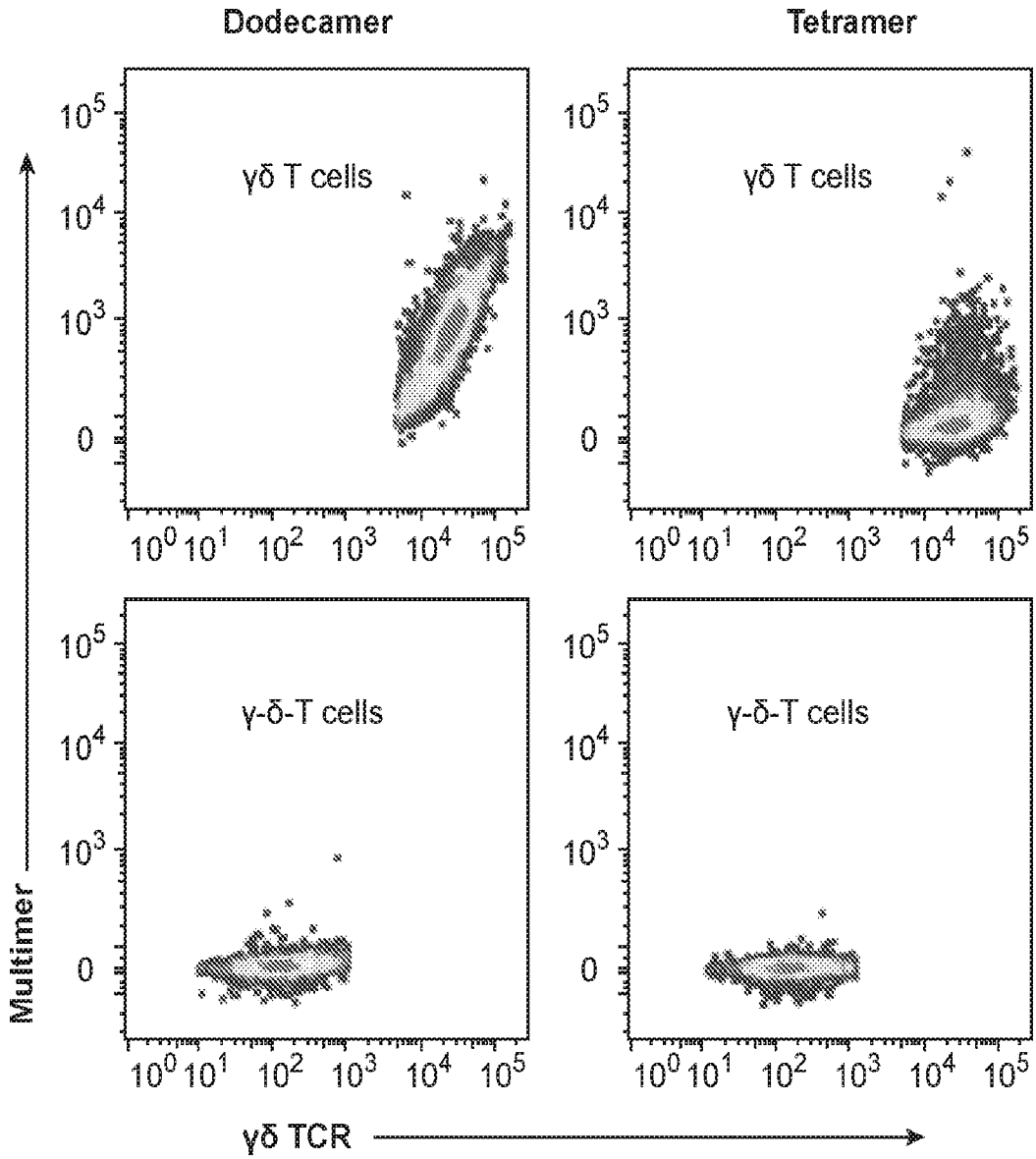
Dodecamer
Tetramer
γδ T cells
γδ T cells
γ-δ-T cells
γ-δ-T cells
Multimer
γδ TCR
10^5
10^4
10^3
0
10^0 10^1 10^2 10^3 10^4 10^5

FIG. 13

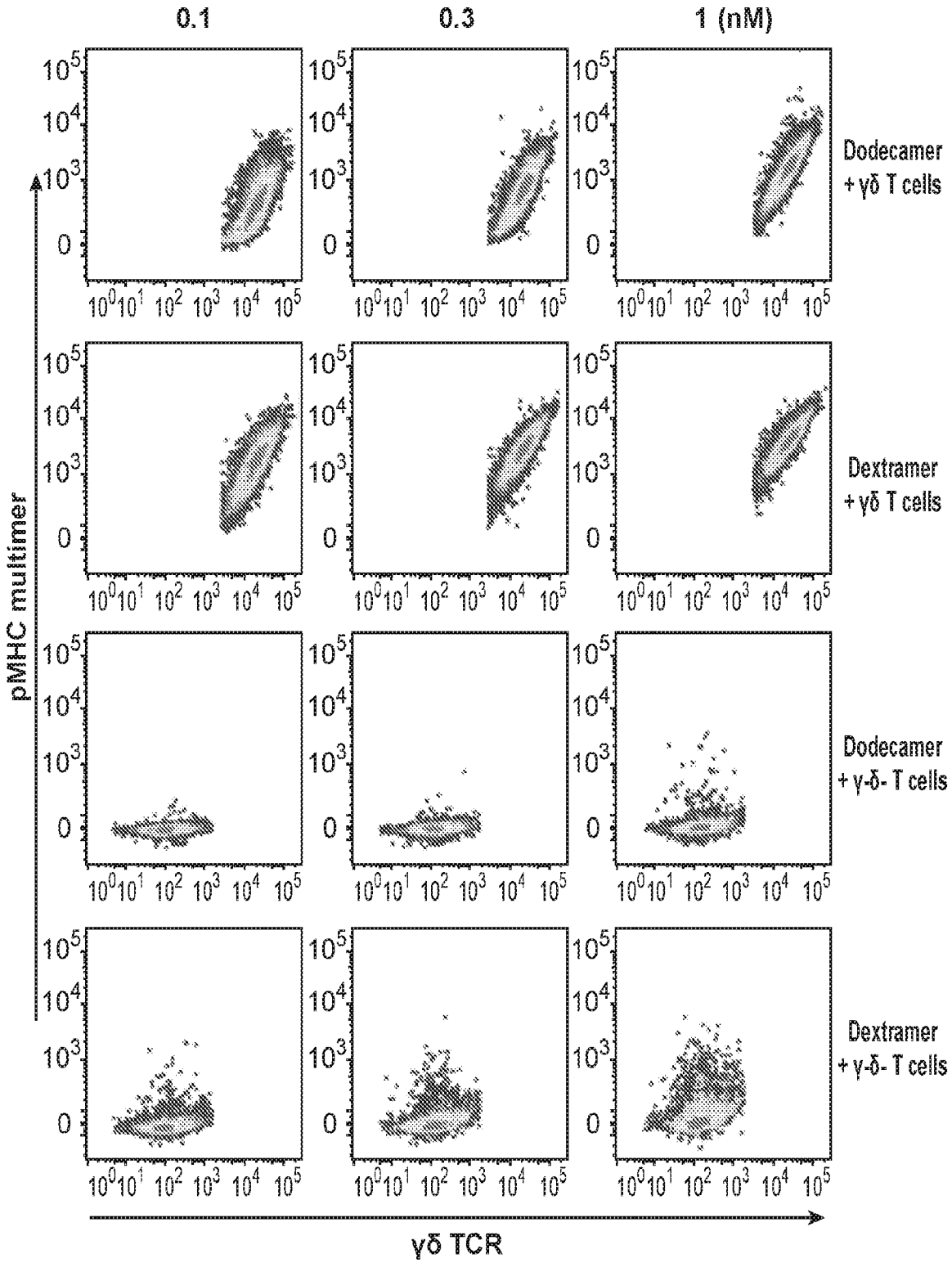
0.1
0.3
1 (nM)
Dodecamer + γδ T cells
Dextramer + γδ T cells
Dodecamer + γ-δ- T cells
Dextramer + γ-δ- T cells
pMHC multimer
γδ TCR

FIG. 14

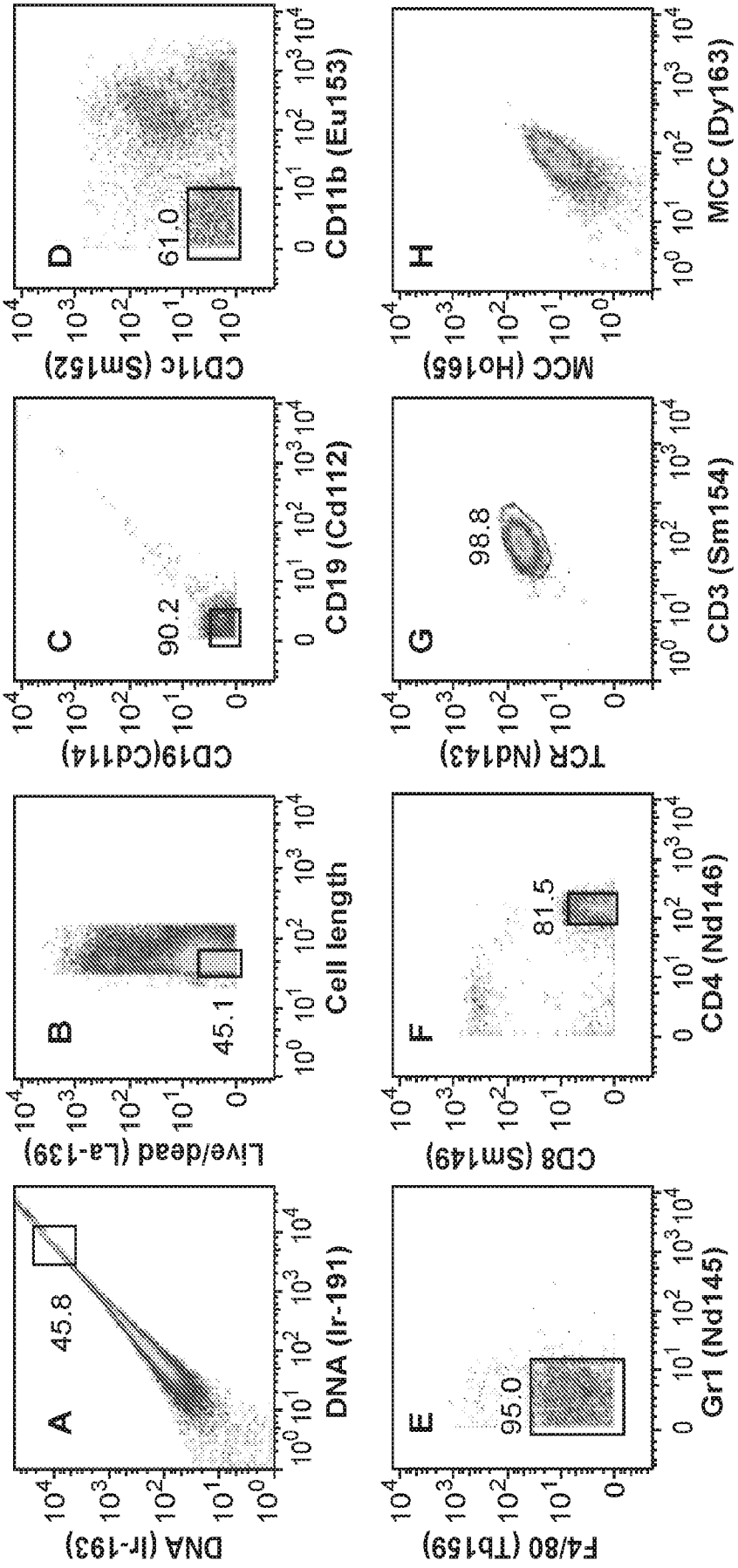
A
45.8
DNA (Ir-191)
DNA (Ir-193)
B
45.1
Cell length
Live/dead (La-139)
C
90.2
CD19 (Cd112)
CD19(Cd114)
D
61.0
CD11b (Eu153)
CD11c (Sm152)
E
95.0
Gr1 (Nd145)
F4/80 (Tb159)
F
81.5
CD4 (Nd146)
CD8 (Sm149)
G
98.8
CD3 (Sm154)
TCR (Nd143)
H
MCC (Dy163)
MCC (Ho165)

FIG. 15

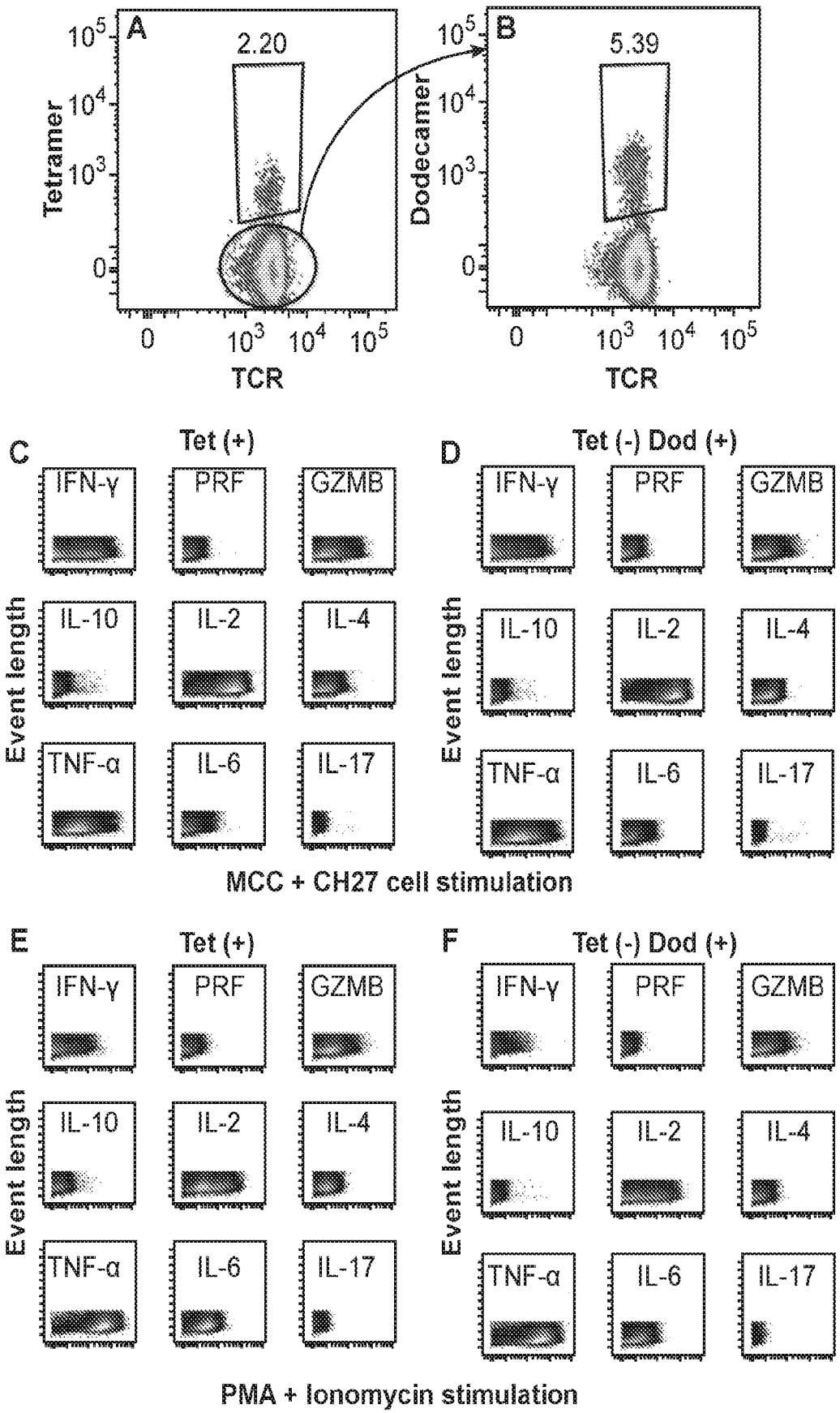
A
2.20
B
5.39
Tetramer
Dodecamer
TCR
TCR
C
Tet (+)
D
Tet (-) Dod (+)
IFN-γ
PRF
GZMB
IL-10
IL-2
IL-4
TNF-α
IL-6
IL-17
Event length
MCC + CH27 cell stimulation
E
Tet (+)
F
Tet (-) Dod (+)
PMA + Ionomycin stimulation

FIG. 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/042341

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 38/00; C12N 15/09 (2016.01)
CPC - C07K 4/00; C07K 14/00; C07K 2319/70; C12N 15/09 (2016.08)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC - A61K 38/00; C12N 15/09
CPC - C07K 4/00; C07K 14/00; C07K 2319/70; C12N 15/09

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/69.1; 530/300; 530/350 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Patents, Google Scholar, PubMed

Search terms used: tetramer scaffold scaffolding terminal cysteine biotin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	HUANG et al. "Detection, phenotyping, and quantification of antigen-specific T cells using a peptide-MHC dodecamer," Proc Natl Acad Sci USA, 15 March 2016 (15.03.2016), Vol. 113, Pgs. E1890-7 entire document	1-9
A	US 2008/0206136 A1 (GREENE et al) 28 August 2008 (28.08.2008) entire document	1-9
A	HYTÖNEN et al. "Dual-affinity avidin molecules," Proteins, 15 November 2005 (15.11.2005), Vol. 61, Pgs. 597-607. entire document	1-9
A	LEMPENS et al. "Noncovalent synthesis of protein dendrimers," Chem. Eur. J. 09 September 2009 (09.09.2009), Vol. 15, Pgs. 8760-7. entire document	1-9
A	US 2013/0337002 A1 (LANGE et al) 19 December 2013 (19.12.2013) entire document	1-9
A	US 2010/0329930 A1 (SALEMME et al) 30 December 2010 (30.12.2010) entire document	1-9

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
26 September 2016	14 OCT 2016
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/042341

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

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

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NO:1 was searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/042341

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

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

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

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

3. ☒ Claims Nos.: 10-22
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.