



(51) International Patent Classification:

C12N 5/078 (2010.01) C12Q 1/6869 (2018.01)

C12M 3/00 (2006.01)

(21) International Application Number:

PCT/US2023/025308

(22) International Filing Date:

14 June 2023 (14.06.2023)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

63/352,497 15 June 2022 (15.06.2022) 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, CV, CZ, DE, DJ, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG,
KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY,
MA, MD, MG, MK, MN, MU, 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, ST, SV, SY, TH,
TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS,
ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every

kind of regional protection available): ARIPO (BW, CV,
GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST,
SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ,
RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ,
DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT,
LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE,
SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN,
GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

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

(54) Title: A 3-DIMENSIONAL HUMAN IMMUNE ORGANOID SYSTEM FOR HIGH THROUGHPUT SCREENING

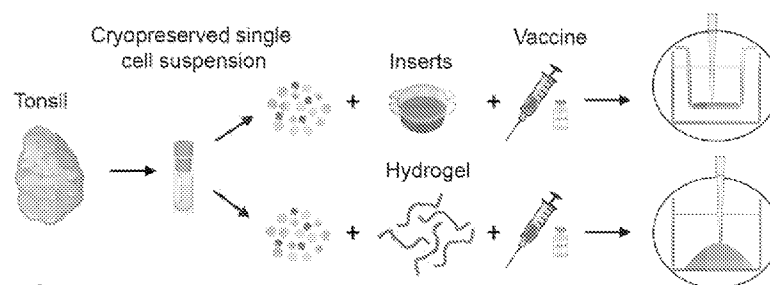


FIG. 1A

(57) Abstract: There is a tremendous need for advanced human model systems to screen new immunomodulatory candidates in an economical and robust manner, which is addressed by the present disclosure. Compositions and methods are provided for in vitro immune organoid cultures that can be adapted to large screening assays for flexible downstream immunological readout.



A 3-DIMENSIONAL HUMAN IMMUNE ORGANOID SYSTEM FOR HIGH THROUGHPUT SCREENING

BACKGROUND

[0001] Vaccination is an important strategy to induce protective immunity against diverse infectious diseases. The clinical benefits of vaccination include reduced severity of illness, limited transmissibility, and often reduction in mortality, though not everyone mounts a protective response. Immunosuppressed individuals are a particularly vulnerable group and comprise up to 2.7% of adults in the United States, the vast majority of which are receiving immunosuppressive medications for malignancy, autoimmune disease or organ transplantation. These patients demonstrate highly variable humoral responses to many vaccines including seasonal influenza, SARS-CoV2, and pneumococcus. The magnitude by which different medications impair adaptive immune responses is also highly variable and dependent on the specific mechanisms of action of the drug. Temporary discontinuation or timing of the vaccination around dosing immunotoxic medications is often considered, though these practices have been associated with disease flares and are no longer recommended by some clinical guidelines. Moreover, medication regimens rarely consist of single agents in isolation, so patients and clinicians are often reliant on observational clinical studies to guide decision making, which is time consuming and often impractical.

[0002] The neutralizing antibodies and effector cell subsets recruited by vaccines evolve through complex, coordinated interactions largely within the germinal centers of secondary lymphoid organs. Antigen-presenting cells display peptide-MHC complexes to CD4+ T cells that then go on to activate naïve and memory B cells. Through both follicular and extrafollicular reactions, activated B cells can undergo isotype class switching, mutate their BCRs through somatic hypermutation and differentiate into proliferative, high affinity antibody-secreting cells called plasmablasts (PBs).

[0003] Given this complexity, it is not surprising that different drug compounds with both known and unknown immunomodulatory effects can impact immune responses. Accordingly, regulatory agencies mandate that all new human pharmaceuticals are evaluated for immunotoxicity during the drug development process and prior to clinical testing. The methods typically employed, however, rely on animal models that have increasingly recognized limitations in recapitulating human immunobiology. Thus, there is a need for improved, human *in vitro* assays to rapidly screen compounds during drug development, specifically using relevant antigenic stimuli in an integrated immunological environment.

[0004] Organoids hold much potential in replicating diverse aspects of human biology. For example, an organoid model of the human adaptive immune system using discarded secondary lymphoid tissue from patients undergoing tonsillectomy has been developed. These dissociated cell cultures reaggregated upon stimulation with various vaccine antigens and showed many of

the hallmarks of germinal center reactions including the secretion of high-affinity antibodies. Most critically, immune organoids depleted of selected functional populations do not generate responses, reaffirming that the organoid system captures the complexity of the integrated human immune system that is missing from conventional cell culture.

[0005] Preclinical vaccine candidates, adjuvants and immune modulating compounds are typically screened within simplified *in vitro* culture systems and non-human animal models prior to focused development. This is costly and requires large biological input, which is challenging to scale for high-throughput utility and reproducibility. The outputs of such screening assays are also usually fixed for few pre-selected readouts, limiting their flexibility in detecting impacts on diverse aspects of immunobiology. There is a tremendous need for advanced human model systems to screen new immunomodulatory candidates in an economical and robust manner, which is addressed by the present disclosure.

SUMMARY

[0006] Compositions and methods are provided for *in vitro* immune organoid cultures that can be adapted to large screening assays for flexible downstream immunological readouts. The cultures comprise one or a plurality of hydrogel droplets comprising embedded immune cells including B cells, suspended in culture medium. In some embodiments a hydrogel droplet fills the well. In some embodiments a droplet is suspended in media in a well. The embedded cells exhibit hypermutation maturation, affinity maturation, plasmablast differentiation, class switching recombination, and antigen-specific antibody production. Results can be read out by harvesting the immune organoids for flexible readouts, including, without limitation, high dimensional imaging, flow cytometry, DNA/RNA sequencing, antibody binding assays, antigen binding assays, etc., including, for example, antibody sequence, presence of different Fc regions in antibodies, quantitative antibody production, antibody binding characteristics, and the like.

[0007] Immune cells for embedding in hydrogel may comprise, without limitation, a suspension of T cells, B cells and antigen-presenting cells (APC). The APCs can comprise B-cells or dendritic cells. The dendritic cells can comprise follicular dendritic cells. The B-cells can comprise CD38⁺ B-cells. The B-cells can comprise CD27⁺ B-cells. The T-cells can comprise $\alpha\beta$ CD8⁺ T-cells, $\alpha\beta$ CD4⁺ T-cells, $\gamma\delta$ T cells, etc. In some embodiments the immune cells are obtained by creating a cell suspension from a tissue comprising germinal centers, e.g. lymph node tissue. In some embodiments the lymph node tissue is tonsil tissue. In some embodiments the tissue is spleen tissue, adenoid tissue, thymus tissue, etc.

[0008] In some embodiments, the hydrogel comprising embedded immune cells further comprise an antigen of interest. The antigen can be a peptide, protein or fragment thereof, polysaccharides, lipids, nucleic acids, or other biomolecules. The protein can be a viral protein, a growth factor, a cancer related protein, or an auto-immune disease related protein. The carbohydrate can be a

bacterial coat protein or a fragment thereof. In some embodiments the antigen of interest is a vaccine composition, which may or may not comprise an adjuvant.

[0009] The immune organoid cultures are shown herein to respond to vaccines, e.g. live attenuated influenza vaccine (LAIV), with expansion of plasmablasts that release high affinity, flu-specific IgG antibodies, and show other features of class switching and somatic hypermutation. The immune organoid cultures are shown to be useful in screening a panel of immunosuppressive medications for their impacts on development of a vaccine response, demonstrating the utility of the cultures to evaluate compounds during drug development and assessment.

[0010] In some embodiments, culture plates comprising a plurality of wells, e.g. 12, 24, 48, 96, 384, 1536, etc. are provided, where the wells comprise culture media and one or a plurality of hydrogel droplets comprising embedded immune cells. The size of droplet may be varied according to the size of the well. This matrix finds use in high throughput screening of an agent of interest for an effect on immune function, including without limitation antibody production, such as vaccines, immunosuppressive agents, biologics altering immune responsiveness, adjuvants, candidate drugs suspected of affecting immune responsiveness, and the like.

[0011] In an embodiment, methods are provided for screening immune responsiveness with an agent of interest, the method comprising obtaining a sample comprising dissociated immune cells, e.g. lymph node cells; embedding the immune cells in a hydrogel droplet to form an immune organoid, optionally in the presence of an antigen of interest; maintaining the hydrogel immune organoid in culture for a period of time sufficient to observe the immune response, collecting droplets from the culture, and reading out an indicia of immune responsiveness, e.g. antibody sequences, cellular proliferation, cellular death, antibody expression, expression of markers on immune cells, etc. The source of immune cells is optionally an individual patient.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] The features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0013] FIGS. 1A-1E. Establishment of 3D immune organoids. (A) Overview of methodologies used to prepare immune organoids. (B) Continuous, live imaging at indicated time points of 3D immune organoids in brightfield mode showing distinct changes in overall structure during early polymerization followed by later reaggregation. Samples were imaged at 2.5x magnification, scale bar = 500 μ m. (C) Immune organoids were stimulated with LAIV added directly within the hydrogel matrix (fixed) versus to the surrounding media (diffusion), and HA-specific IgG antibodies measured at day 7. n = 3 replicates for a single biological donor. (D) Immune organoids were prepared at high (5×10^6) and low (1×10^6) cell input for hydrogels produced at both 2 mM

and 5 mM final maleimide concentrations to adjust the stiffness. Flu-specific IgG antibodies were measured at day 7. (E) Time course of HA-specific IgG antibodies from unstimulated and LAIV-stimulated immune organoids. n = 4 biological replicates.

[0014] FIGS. 2A-2E. Characterization of plasmablast phenotypes in 3D immune organoids. (A) Gating strategy for identifying B-cell populations in immune organoids. Live, CD19⁺ CD3⁻ cells were separated by CD27 and CD38 into naïve, memory (MBC), pre-germinal center (pre-GCB), germinal center (GCB) and plasmablast (PB) populations. (B) scRNA-seq UMAP plot of day 0 vs day 7 organoid PBs. (C) Differential expression plots of genes used to classify B cell clusters (x-axis) and their corresponding histograms showing distributions across each cluster (y-axis). (D) scRNA-seq UMAP plot showing naïve and PB clusters in day 0 and day 7 LAIV-stimulated organoids. (E) Relative gene expression kinetic plots of selected genes along pseudotime from RNA trajectory.

[0015] FIGS. 3A-3E. Evolution of B cells in 3D immune organoids. (A) scRNA-seq UMAP plot identifying clonally expanded (>2) BCR sequences. (B) Enumeration of clonal expansion across B cell clusters from day 0 and day 7 LAIV-stimulated organoids. (C) scRNA-seq UMAP plot showing evolution from day 0 to day 7 organoids and their corresponding cluster distributions. (D) Differential isotype use across naïve and PB clusters for day 0 and day 7 LAIV-stimulated organoids. (E) SHM frequency for IGHM and IGHG1 isotypes across naïve and PB clusters.

[0016] FIGS. 4A-4I. Comparison of immune organoids grown on permeable membranes and 3D hydrogels. (A) Organoids from biological replicates (n = 4) were prepared using inserts and hydrogels, and Flu-specific IgG antibodies detected at day 7 in unstimulated and LAIV-stimulated conditions. *p < 0.05. (B) scRNA-seq UMAP plot of PBs from organoids grown on inserts vs hydrogels, and their corresponding distributions across each cluster. (C) scRNA-seq UMAP plot showing clonal expansion (>2) across organoid methodologies. (D-G) SHM quantification across the most frequently detected isotypes for organoids grown on inserts or hydrogels compared to day 0. (H) Isotype distributions for organoids grown on inserts or hydrogels compared to day 0 tonsil cells. (I) Hierarchical clustering of BCR repertoire overlap for stimulated and unstimulated organoids compared to day 0.

[0017] FIGS. 5A-5H. Miniaturized 3D immune organoids for drug screening. (A) Assay design for miniaturized 3D immune organoid screening and subsequent validation of identified "hits" using larger cultures for cellular in-depth phenotyping. (B) Brightfield microscopy and DAPI stained images for miniaturized 3D immune organoids grown in 96 well plates. Size bar = 500 μ m. (C) Validation study for HA-specific IgG antibodies measured at day 10 by organoids generated from 12 biological samples (x-axis). 95% confidence levels are shown across biological samples and grouped by replicates (n = 2). (D, E) Immune organoids were stimulated with LAIV in the presence of indicated drugs and HA-specific IgG antibodies detected in the supernatant at day 10. Unstimulated and vehicle-treated, LAIV-stimulated organoids show the control conditions for drug comparisons. Infliximab at 20, 10, 5 μ g/mL; etanercept at 5, 2.5,

1.25 µg/mL; vedolizumab at 50, 5, 0.5 µg/mL. Quantitative data show means +/- standard deviation across 4 biological replicates. *p < 0.05, **p < 0.005, ****p < 0.0001. (F, G, H) Immune organoids were stimulated with LAIV in the presence of immunosuppressive medications, and B and T cell frequencies at day 5 were measured by flow cytometry. Quantitative data are from 4 replicates from a single donor and show mean +/- standard deviation. *p < 0.05, **p < 0.005, ****p < 0.0001.

[0018] FIGS. 6A-6D. (A) Viability of cells recovered from immune organoids. Quantitative data are mean +/- standard deviation for n = 5 separate organoids. (B-D) Tonsil immune cells were labeled with CFSE and spiked into unlabeled suspensions at indicated frequencies. CFSE positive cells were then detected on day 3 for recovery.

[0019] FIGS. 7A-7B. (A) Cell frequencies determined by flow cytometry for naïve, memory (Bmem), pre-germinal center B cells (pre-GCB), germinal center B cells (GCB) and plasmablasts (PB) in unstimulated and LAIV-stimulated immune organoids at day 5. (B) Pearson correlation plot for HA-specific IgG antibodies detected by ELISA and PB frequencies in LAIV-stimulated 3D immune organoids.

[0020] [0001] FIGS. 8A-8B. Immune organoids were grown as floating droplets in the indicated hydrogel conditions at high and low cell densities, and HA-specific IgG (A) and IgM (B) antibodies were measured at day 7 following LAIV stimulation. Data are shown as serial dilution curves for one representative experiment.

[0021] FIGS. 9A-9C. Cell frequencies measured by flow cytometry for (A) Naïve, (B) memory B cell and (C) GCB populations recovered from miniaturized 3D immune organoids stimulated with LAIV in the presence of indicated drugs.

[0022] FIGS. 10A-10B. Immune organoid arrays facilitate plasmablast activation during LAIV immunization. A) Single cell RNA-seq and UMAP visualization for plasmablasts isolated from immune organoids at baseline day 0 or day 7 following immunization with LAIV. B) Plasmablasts from LAIV immunized organoids display higher interferon stimulated genes score (ISG) compared to plasmablasts from baseline day 0.

[0023] FIG. 11. Miniaturized human immune organoid arrays replicate known biological variability in humoral responses. Impact of age on immune organoid responses to LAIV. Immune organoid arrays from older donors show less HA-specific antibody responses compared to younger donors.

DETAILED DESCRIPTION OF THE EMBODIMENTS

[0024] Before the present methods and compositions are described, it is to be understood that this invention is not limited to particular method or composition described, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0025] Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limits of that range is also specifically disclosed. Each smaller range between any stated value or intervening value in a stated range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included or excluded in the range, and each range where either, neither or both limits are included in the smaller ranges is also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the invention.

[0026] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, some potential and preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. It is understood that the present disclosure supercedes any disclosure of an incorporated publication to the extent there is a contradiction.

[0027] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the peptide" includes reference to one or more peptides and equivalents thereof, e.g. polypeptides, known to those skilled in the art, and so forth.

[0028] The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention. Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

[0029] As used herein, compounds which are "commercially available" may be obtained from commercial sources including but not limited to Acros Organics (Pittsburgh PA), Aldrich Chemical (Milwaukee WI, including Sigma Chemical and Fluka), Apin Chemicals Ltd. (Milton Park UK), Avocado Research (Lancashire U.K.), BDH Inc. (Toronto, Canada), Bionet (Cornwall, U.K.), Chemservice Inc. (West Chester PA), Crescent Chemical Co. (Hauppauge NY), Eastman Organic Chemicals, Eastman Kodak Company (Rochester NY), Fisher Scientific Co. (Pittsburgh PA), Fisons Chemicals (Leicestershire UK), Frontier Scientific (Logan UT), ICN Biomedicals, Inc. (Costa Mesa CA), Key Organics (Cornwall U.K.), Lancaster Synthesis (Windham NH), Maybridge Chemical Co. Ltd. (Cornwall U.K.), Parish Chemical Co. (Orem UT), Pfaltz & Bauer, Inc. (Waterbury CN), Polyorganix (Houston TX), Pierce Chemical Co. (Rockford IL), Riedel de Haen

AG (Hannover, Germany), Spectrum Quality Product, Inc. (New Brunswick, NJ), TCI America (Portland OR), Trans World Chemicals, Inc. (Rockville MD), Wako Chemicals USA, Inc. (Richmond VA), Novabiochem and Argonaut Technology.

[0030] The term "subject," as used herein, generally refers to any animal or living organism. Animals can be mammals, such as humans, non-human primates, rodents such as mice and rats, dogs, cats, pigs, sheep, rabbits, and others. Animals can be fish, reptiles, or others. Animals can be neonatal, infant, adolescent, or adult animals. A human may be an infant, a toddler, a child, a young adult, an adult or a geriatric. A human can be more than about 1, 2, 5, 10, 20, 30, 40, 50, 60, 65, 70, 75, or about 80 years of age. In some embodiments a subject, e.g. a source of immune cells, is a human.

[0031] A "sample" in the context of the present teachings refers to any biological sample that is isolated from a subject, generally a sample comprising immune cells. A typical sample is obtained from a lymphoid organ, e.g. lymph node, spleen, thymus, etc., where tonsils are a convenient source of lymphoid tissue. A sample may be obtained from a tissue comprising germinal centers.

[0032] *Immune cells.* The immune organoids of the disclosure comprise sufficient immune cells to provide a readout of interest. Generally, the immune cells include B cells, T cells and antigen presenting cells. The APCs can comprise B-cells, macrophages, and/or dendritic cells. The dendritic cells can comprise follicular dendritic cells, pDC. The B-cells can comprise, for example, CD3⁻ B-cells, CD45⁺ B-cells, CD19⁺ B-cells, CD38⁺ B-cells, CD38⁻ B-cells, or CD27⁺ B-cells. The B-cells can comprise transitional B-cells, naïve B-cells, plasma B-cells, memory B-cells, pre-GC B cells, GC B cells, plasmablasts. The T-cells can comprise, for example, helper T-cells, follicular helper T-cells, follicular regulatory T-cells, cytotoxic T-cells, memory T-cells, regulator T-cells, natural killer T-cells, mucosal associated invariant T-cells, gamma delta T-cells. The T-cells can comprise CD8⁺ T-cells, CD4⁺ T-cells. Cells obtained from human tonsils are of interest.

[0033] The term "inflammatory" response is the development of a humoral (antibody mediated) and/or a cellular response, which cellular response may be mediated by antigen-specific T cells or their secretion products), and innate immune cells. An "immunogen" is capable of inducing an immunological response against itself in the systems of the disclosure.

[0034] *Antigens.* The immune organoids are responsive to antigen, and undergo an immune response in response to antigen, e.g. hypermutation maturation, affinity maturation, plasmablast differentiation, class switching recombination, and antigen-specific antibody production. The antigen can be any substance that is bound by an antibody. Antigen can be originated from the environment or formed inside the body. The antigen can be a peptide, protein or fragment thereof,

polysaccharides, lipids, nucleic acids, or other biomolecules. The protein can be a viral protein, a bacterial protein, a growth factor, a cancer related protein, a cancer related peptide, an auto-immune disease related protein, an auto-immune disease related peptide, or fragment thereof.

[0035] The antigen can be a protein or fragment thereof. The protein can be a viral protein, a growth factor, a cancer related protein, a bacterial antigen, a fungal antigen, or an auto-immune disease related protein. The viral protein can be derived from a virus. The viral protein can be derived from, for example, a helical virus, a polyhedral virus, a spherical virus, or a complex virus. The antigen can be a virus. The virus can be, for example, a coronavirus or a flu virus. In some embodiments the antigen is a vaccine or vaccine candidate.

[0036] The term "vaccine", as used herein, is defined in accordance with the pertinent art and relates to a composition that induces or enhances the protective immunity of an individual to a particular disease caused by a pathogen. Without wishing to be bound by theory, it is believed that a protective immunity arises from the generation of neutralizing antibodies, or from the activation of cytotoxic cells of the immune system, or both. In order to induce or enhance a protective immunity, a vaccine comprises as an immunogenic antigen a part of the pathogen causing said disease or a nucleic acid molecule encoding this immunogenic antigen. Upon contact with the immunogenic antigen, the immune system of the individual is triggered to recognize the immunogenic antigen as foreign and to destroy it. The immune system subsequently remembers the contact with this immunogenic antigen, so that at a later contact with the disease-causing pathogen an easy and efficient recognition and destruction of the pathogen is ensured.

[0037] Vaccines known and used in the art include, for example, inactivated pathogen vaccines; live-attenuated pathogen vaccines; messenger RNA (mRNA) vaccines; subunit, recombinant, polysaccharide, and conjugate vaccines; toxoid vaccines; and viral vector vaccines. Inactivated vaccines use a killed version of the pathogen that causes a disease, e.g. Hepatitis A, influenza, rabies, etc. Live vaccines use an attenuated form of the pathogen that causes a disease, e.g. measles, mumps, rubella (MMR combined vaccine), rotavirus, smallpox, chickenpox, yellow fever. mRNA vaccines encode pathogen proteins that trigger an immune response, e.g. SRS-CoV2. Subunit, recombinant, polysaccharide, and conjugate vaccines use specific pathogen molecules, e.g. Hib (Haemophilus influenzae type b), Hepatitis B, HPV (Human papillomavirus), Bordetella pertussis, pneumococcal disease, meningococcal disease, Varicella Zoster virus. Toxoid vaccines use a toxin made by the pathogen, e.g. diphtheria, and tetanus. Viral vector vaccines use a modified version of a different virus as a vector to deliver sequences encoding pathogen protein. Several different viruses have been used as vectors, including influenza, vesicular stomatitis virus (VSV), measles virus, and adenovirus. Viral vectors are in use currently for SARS-CoV2.

[0038] The term “adjuvant” generally refers to a composition that increases the humoral or cellular immune response of an individual. Adjuvants of interest stimulate the immune system, and increase responsiveness or durability of response to a co-administered antigen. Immune responses of the organoid cultures as described herein can be enhanced by the use of an adjuvant or derivative thereof. Adjuvants can comprise, for example, aluminum salts, Freund’s adjuvant, Poly-IC, Poly-ICLC, MDP, MPL, CpG ODN, Virosome, MF59, AS01, Flagellin, R837/R848, AS04, AS02, AS03, mineral adjuvants such as aluminum hydroxide, phosphate adjuvants, calcium phosphate adjuvants, imiquimod, ISA51, or any combination thereof.

[0039] The terms “biomarker,” “biomarkers,” “marker” or “markers” for the purposes of the invention refer to, without limitation, proteins together with their related metabolites, mutations, variants, polymorphisms, modifications, fragments, subunits, degradation products, elements, and other analytes or sample-derived measures. Markers include expression levels of a gene of interest. Markers can also include combinations of any one or more of the foregoing measurements, including temporal trends and differences. Broadly used, a marker can also refer to an immune cell subset. Markers include, for example, proteins expressed on the surface of immune cells, cytokines expressed by immune cells, antibodies synthesized by immune cells, and the like.

[0040] To “analyze” includes determining a set of values associated with a sample by measurement of a marker or readout and comparing the measurement against measurement in a sample or set of samples in comparison to controls, across dilutions, etc. The markers of the present teachings can be analyzed by any of various conventional methods known in the art. To “analyze” can include performing a statistical analysis, e.g. normalization of data, determination of statistical significance, determination of statistical correlations, clustering algorithms, and the like.

[0041] *Cell Culture Conditions.* The hydrogel droplets comprising immune cells are suspended in culture medium. The culture medium may comprise of AIM V, IMDM, MEM, DMEM, RPMI 1640, Alpha Medium or McCoy’s Medium, or an equivalent known culture medium component. The medium may comprise a serum component which can comprise human serum, fetal bovine serum, or horse serum. The medium may comprise B-cell activating factor (BAFF). The medium may comprise an antibiotic to prevent microbial growth. The medium may comprise basal media supplements which can comprise nonessential amino acids, sodium pyruvate, insulin/selenium/transferrin cocktail, growth factors, hormones, or cytokines. The media can be supplemented with fetal calf serum. The media can be replaced every one day, two days, three days, or four days.

[0042] *Hydrogels.* The term "hydrogel" as used herein refers to a network of polymer chains that are water-insoluble, sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels can contain over 99% water and may comprise natural or synthetic polymers, or a combination thereof. In other instances, hydrogels may contain other percentages of water, as described herein. Hydrogels also possess a degree of flexibility due to their significant water content.

[0043] The term polymer as used herein refers to a single compound species or a mixture of compound species that may be cross-linked to form a polymer. Such precursor compounds include, but are not limited to, such as a dextran-based gel, a commercial gel such as VITROLIFE™; poly(ethylene glycol), polyaliphatic polyurethanes, polyether polyurethanes, polyester polyurethanes, polyethylene copolymers, polyamides, polyvinyl alcohols, polypropylene glycol, polytetramethylene oxide, polyvinyl pyrrolidone, polyacrylamide, poly(hydroxyethyl acrylate), and poly(hydroxyethyl methacrylate), collagen, hyaluronic acid, chitosan, dextran, agarose, alginate, protein polymers, methylcellulose, dextrans, and the like. When polymerized, the polymer will be inert with respect to any cell or cell line in contact with the polymer. A polymerizing agent may be used, e.g. maleimide.

[0044] In some embodiments of the invention, the hydrogel composition may comprise a polymer (or combination of polymers) selected from the group consisting of: a dextrose, a poly vinyl alcohol, a poly(ethylene glycol), a polyaliphatic polyurethane, a polyether polyurethane, a polyester polyurethane, a polyethylene copolymer, a polyamide, a polyvinyl alcohol, a polypropylene glycol, a polytetramethylene oxide, a polyvinyl pyrrolidone, a polyacrylamide, a poly(hydroxyethyl acrylate), a poly(hydroxyethyl methacrylate), poly(glycolic acid), poly(DL-lactic-co-glycolic acid), polyhydroxyalkanoate, poly(4-hydroxybutyrate), sulfonated polymers, polygluconic acid, poly(acrylic acid), polyphosphazenes, polysaccharides, proteins, collagen, elastin, alginate, fibrin, fibronectin, laminin, hyaluronic acid, or another biologically-occurring polymer, or copolymers formed from monomers of one or more of these.

[0045] The elasticity of the hydrogel may be influenced by a variety of factors, including, but not limited to, the branching of the polymer, the concentration of polymer, and the degree of cross-linking. For example, where a polymer comprises PEG or cyclodextrin cross-linkers, the length of the PEG monomer and the branching, e.g. 2 arm, 4 arm, 8-arm, the level of cross-linking and the like may be varied to achieve the desired elasticity. Hydrogels may be chemically cross-linked, e.g. by functionalizing the polymer with thiol-reactive groups and cross-linking with maleimide; or various other methods as known in the art.

[0046] The elasticity of the hydrogel may be selected to have physiological parameters. Elasticity may be measured by any convenient method, as is known in the art. For example see, inter alia, Kaletunc et al. (1991) Food Hydrocolloids 5:237-247; Krall and Weitz (1998) Physical Review Letters 80:778-781; Melekaslan et al. (2003) Polymer Bulletin 50:287-294; each herein incorporated by reference. For example, a shear rheometer may be used to measure the elasticity

of the substrate. The elasticity range for a hydrogel may be 0.001-250 kPa, and may be from about 0.01 to 10 kPa.

[0047] As used herein, the term "theranosis" refers to the use of results obtained from a diagnostic method to direct the selection of, maintenance of, or changes to a therapeutic regimen, including but not limited to the choice of one or more therapeutic agents, changes in dose level, changes in dose schedule, changes in mode of administration, and changes in formulation. Diagnostic methods used to inform a theranosis can include any that provides information on the state of a disease, condition, or symptom.

[0048] The terms "therapeutic agent", "therapeutic capable agent" or "treatment agent" are used interchangeably and refer to a molecule or compound that confers some beneficial effect upon administration to a subject, including vaccines and vaccine adjuvants. The beneficial effect includes induction of a therapeutic immune response, enablement of diagnostic determinations; amelioration of a disease, symptom, disorder, or pathological condition; reducing or preventing the onset of a disease, symptom, disorder or condition; and generally counteracting a disease, symptom, disorder or pathological condition.

[0049] As used herein, "treatment" or "treating," or "palliating" or "ameliorating" are used interchangeably. These terms refer to an approach for obtaining beneficial or desired results including but not limited to a therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant any therapeutically relevant improvement in or effect on one or more diseases, conditions, or symptoms under treatment. For prophylactic benefit, the compositions may be administered to a subject at risk of developing a particular disease, condition, or symptom, or to a subject reporting one or more of the physiological symptoms of a disease, even though the disease, condition, or symptom may not have yet been manifested.

[0050] The term "effective amount" or "therapeutically effective amount" refers to the amount of an agent that is sufficient to effect beneficial or desired results. The therapeutically effective amount will vary depending upon the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. The term also applies to a dose that will provide an image for detection by any one of the imaging methods described herein. The specific dose will vary depending on the particular agent chosen, the dosing regimen to be followed, whether it is administered in combination with other compounds, timing of administration, the tissue to be imaged, and the physical delivery system in which it is carried.

[0051] "Suitable conditions" shall have a meaning dependent on the context in which this term is used. That is, when used in connection with an antibody, the term shall mean conditions that

permit an antibody to bind to its corresponding antigen. When used in connection with contacting an agent to 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.

Generation of Immune Organoid cultures

[0052] Immune cells, particularly an interactive population of immune cells comprising B cells, T cells and antigen presenting cells, are dissociated and embedded in a hydrogel matrix, then maintained in culture in contact with an antigen of interest and/or an agent of interest for testing. The immune cells are capable of productive interaction with each other, and may be obtained from an individual human. In some embodiments the immune cells are obtained from a tissue, particularly a tissue comprising germinal centers, e.g. lymph node, tonsils, adenoids, spleen, etc. The tissue may be a biopsy, or surgically removed tissue, e.g. from a tonsillectomy.

[0053] A tissue comprising immune cells of interest is dissociated into individual cells, e.g. mechanical dissociation by sectioning the tissue and passing the suspension through a filter. The cells are optionally fractionated, e.g. on Ficoll density gradient separation, and the collected cells were washed and resuspended in medium, e.g. at a concentration from about 10^7 to 10^9 cells/ml. The cells may be used fresh, or frozen.

[0054] The dissociated cells are resuspended at a high density, e.g. from about 10^8 to about 10^9 cells/ml in media supplemented with BAFF. The suspension of cells is then combined with hydrogel, to a final cell concentration of from about 10^5 , 2×10^5 , 3×10^5 , 4×10^5 , 5×10^5 , 6×10^5 , 7×10^5 , 8×10^5 , 9×10^5 , 10^6 , 2×10^6 , 3×10^6 , 4×10^6 , 5×10^6 , 6×10^6 , 7×10^6 , 8×10^6 , 9×10^6 embedded in hydrogel. The concentration of hydrogel is selected to maintain viability of the cells in culture for periods of from about 12 hours to about 4 weeks, e.g. from about 1 to about 21, from about 1 to about 14, from about 1 to about 10 days.

[0055] For larger cultures the hydrogel is directly plated into wells. For miniaturized immune organoids, individual hydrogel droplets of from 10 to about 25 μ l, from about 10 to about 20 μ l, from about 10 to about 15 μ l in volume. Individual droplets are distributed into wells containing media.

[0056] The hydrogel may comprise an antigen of interest, e.g. conjugated to the hydrogel matrix, or embedded with the cells in the hydrogel. The dose of antigen may be any amount sufficient to elicit an immune response; and may be provided for a dose response over a series of concentrations. The antigen may be a vaccine, e.g. live virus, killed virus, subunit, mRNA, viral vector, etc. as known in the art.

[0057] The cultures may be contacted with an agent of interest, e.g. an agent suspected of acting on immune cells as a modulator, i.e. immune suppressant, immune enhancer, and the like.

[0058] The immune organoids are maintained in culture for a period of time sufficient to observe a response of interest, e.g. hypermutation maturation, affinity maturation, plasmablast

differentiation, class switching recombination, antigen-specific antibody production, etc. Typically a detectable response is after about 7 days exposure to antigen, although some responses are seen after 4 days, as shown in the Examples.

[0059] Results can be read out by harvesting the immune organoids for flexible readouts, including, without limitation, high dimensional imaging, flow cytometry, DNA/RNA sequencing, antibody binding assays, antigen binding assays, etc., including, for example, antibody sequence, presence of different Fc regions in antibodies, quantitative antibody production, antibody binding characteristics, and the like.

[0060] For example, the binding specificity of antibodies produced by the organoid can be detected by ELISA, or other suitable antibody binding assays, e.g. to determine the specificity and expression of antibodies that bind to the antigen of interest. For affinity determination, standard methods such as biacore can be used. ELISA assays can also be used to determine class switching, e.g. by screening for the presence of IgG isotypes.

[0061] Cells can be retrieved from organoids by digestion of the hydrogel, e.g. dextranase for dextran-based hydrogels, and analyzed by flow cytometry for the expression of markers, e.g. markers associate with lymphocyte activation, lymphocyte maturation, and the like. Such markers include, without limitation, CD4, CD8, CD3, CD19, CD38, CD27, HLA-DR, etc.

[0062] Various sequencing methodologies are known and used in the art for single cells, including single cell RNA sequencing. Examples of next generation and high-throughput sequencing include, for example, massively parallel signature sequencing, polony sequencing, 454 pyrosequencing, Illumina (Solexa) sequencing with HiSeq, MiSeq, and other platforms, SOLiD sequencing, ion semiconductor sequencing (Ion Torrent), DNA nanoball sequencing, heliscope single molecule sequencing, single molecule real time (SMRT) sequencing, MassARRAY®, and Digital Analysis of Selected Regions (DANSR™). See, e.g., Stein RA (1 September 2008). "Next-Generation Sequencing Update". Genetic Engineering & Biotechnology News 28 (15); Quail, Michael; Smith, Miriam E; Coupland, Paul; Otto, Thomas D; Harris, Simon R; Connor, Thomas R; Bertoni, Anna; Swerdlow, Harold P; Gu, Yong (1 January 2012). "A tale of three next generation sequencing platforms: comparison of Ion torrent, pacific biosciences and illumina MiSeq sequencers". BMC Genomics 13 (1): 341 ; Liu, Lin; Li, Yinhu; Li, Siliang; Hu, Ni; He, Yimin; Pong, Ray; Lin, Danni; Lu, Lihua; Law, Maggie (1 January 2012). "Comparison of Next-Generation Sequencing Systems". Journal of Biomedicine and Biotechnology 2012: 1-11; Qualitative and quantitative genotyping using single base primer extension coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MassARRAY®). Methods Mol Biol. 2009;578:307-43; Chu T, Bunce K, Hogge WA, Peters DG. A novel approach toward the challenge of accurately quantifying fetal DNA in maternal plasma. Prenat Diagn 2010;30: 1226-9; and Suzuki N, Kamataki A, Yamaki J, Homma Y. Characterization of circulating DNA in healthy human plasma. Clinica chimica acta; international journal of clinical chemistry

2008;387:55-8). Similarly, software programs for primary and secondary analysis of sequence data are well-known in the art.

[0063] A kit may be provided, and include one or more containers housing one or more of the components provided in this disclosure and instructions for use. Specifically, such kits may include one or more compositions described herein, along with instructions describing the intended application and the proper use and/or disposition of these compositions. Kits may contain the components in appropriate concentrations or quantities for running various experiments.

[0064] In some embodiments, culture plates comprising a plurality of wells, e.g. 12, 24, 48, 96, 384, 1536, etc. are provided, where the wells comprise culture media and one or a plurality of hydrogel droplets comprising embedded immune cells. This matrix finds use in high throughput screening of an agent of interest for an effect on immune function, including without limitation antibody production, such as vaccines, immunosuppressive agents, biologics altering immune responsiveness, adjuvants, candidate drugs suspected of affecting immune responsiveness, and the like.

Screening

[0065] In an embodiment, methods are provided for screening immune responsiveness with an agent of interest, the method comprising obtaining a sample comprising dissociated immune cells, e.g. lymph node cells; embedding the immune cells in a hydrogel droplet to form an immune organoid, optionally in the presence of an antigen of interest; maintaining the hydrogel immune organoid in culture for a period of time sufficient to observe the immune response, e.g. in the presence of a candidate agent, collecting droplets from the culture, and reading out an indicia of immune responsiveness, e.g. antibody sequences, antibody expression, expression of markers on immune cells, etc. The source of immune cells is optionally an individual patient.

[0066] Candidate agents of interest are biologically active agents that encompass numerous chemical classes, primarily organic molecules, which may include organometallic molecules, inorganic molecules, genetic sequences, etc. An important aspect of the invention is to evaluate candidate drugs, candidate vaccines, and protein-based therapeutics, for an effect on an immune response. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, frequently at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules, including peptides, polynucleotides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

[0067] Included are pharmacologically active drugs, genetically active molecules, etc. Compounds of interest include chemotherapeutic agents, anti-inflammatory agents, hormones or hormone antagonists, ion channel modifiers, and neuroactive agents. Exemplary of pharmaceutical agents suitable for this invention are those described in, "The Pharmacological Basis of Therapeutics," Goodman and Gilman, McGraw-Hill, New York, N.Y., (1996), Ninth edition, under the sections: Drugs Acting at Synaptic and Neuroeffector Junctional Sites; Drugs Acting on the Central Nervous System; Autacoids: Drug Therapy of Inflammation; Water, Salts and Ions; Drugs Affecting Renal Function and Electrolyte Metabolism; Cardiovascular Drugs; Drugs Affecting Gastrointestinal Function; Drugs Affecting Uterine Motility; Chemotherapy of Parasitic Infections; Chemotherapy of Microbial Diseases; Chemotherapy of Neoplastic Diseases; Drugs Used for Immunosuppression; Drugs Acting on Blood-Forming organs; Hormones and Hormone Antagonists; Vitamins, Dermatology; and Toxicology, all incorporated herein by reference. Also included are toxins, and biological and chemical warfare agents, for example see Somani, S. M. (Ed.), "Chemical Warfare Agents," Academic Press, New York, 1992).

[0068] Compounds, including candidate agents, are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds, including biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

[0069] Test compounds include all of the classes of molecules described above, and may further comprise samples of unknown content. Of interest are complex mixtures of naturally occurring compounds derived from natural sources such as plants. While many samples will comprise compounds in solution, solid samples that can be dissolved in a suitable solvent may also be assayed. Samples of interest include environmental samples, e.g. ground water, sea water, mining waste, etc.; biological samples, e.g. lysates prepared from crops, tissue samples, etc.; manufacturing samples, e.g. time course during preparation of pharmaceuticals; as well as libraries of compounds prepared for analysis; and the like. Samples of interest include compounds being assessed for potential therapeutic value, i.e. drug candidates.

[0070] The term samples also includes the fluids described above to which additional components have been added, for example components that affect the ionic strength, pH, total protein concentration, etc. In addition, the samples may be treated to achieve at least partial fractionation or concentration. Biological samples may be stored if care is taken to reduce

degradation of the compound, e.g. under nitrogen, frozen, or a combination thereof. The volume of sample used is sufficient to allow for measurable detection, usually from about 0.1 μ l to 1 ml of a biological sample is sufficient.

[0071] Agents are screened for biological activity by adding the agent to at least one and usually a plurality of wells, where an antigen may be present, usually in conjunction with wells lacking the agent. The change in readout in response to the agent is measured, desirably normalized, and the resulting readout may then be evaluated by comparison to reference wells, which may be a control in the assay or may be a previously obtained reference.

[0072] The agents are conveniently added in solution, or readily soluble form, to the medium of cells in culture. The agents may be added in a flow-through system, as a stream, intermittent or continuous, or alternatively, adding a bolus of the compound, singly or incrementally, to an otherwise static solution. In a flow-through system, two fluids are used, where one is a physiologically neutral solution, and the other is the same solution with the test compound added. The first fluid is passed over the cells, followed by the second. In a single solution method, a bolus of the test compound is added to the volume of medium surrounding the cells. The overall concentrations of the components of the culture medium should not change significantly with the addition of the bolus, or between the two solutions in a flow through method.

[0073] Preferred agent formulations do not include additional components, such as preservatives, that may have a significant effect on the overall formulation. Thus preferred formulations consist essentially of a biologically active compound and a physiologically acceptable carrier, e.g. water, ethanol, DMSO, etc. However, if a compound is liquid without a solvent, the formulation may consist essentially of the compound itself.

[0074] A plurality of assays may be run in parallel with different agent concentrations to obtain a differential response to the various concentrations. As known in the art, determining the effective concentration of an agent typically uses a range of concentrations resulting from 1:10, 1:3, etc. or other log scale, dilutions. The concentrations may be further refined with a second series of dilutions, if necessary. Typically, one of these concentrations serves as a negative control, i.e. at zero concentration or below the level of detection of the agent or at or below the concentration of agent that does not give a detectable change in the phenotype.

[0075] Various methods can be utilized for quantifying the readout, as previously discussed. Depending upon the readout chosen, parameters may be measured using such immunoassay techniques as radioimmunoassay (RIA) or enzyme linked immunosorbance assay (ELISA), homogeneous enzyme immunoassays, and related non-enzymatic techniques; sequencing, flow cytometry, etc. These techniques utilize specific antibodies as reporter molecules, which are particularly useful due to their high degree of specificity for attaching to a single molecular target. Cell readouts for proteins and other cell determinants can be obtained using fluorescent or otherwise tagged reporter molecules. Cell based ELISA or related non-enzymatic or fluorescence-based methods enable measurement of cell surface parameters and secreted

parameters. Capture ELISA and related non-enzymatic methods usually employ two specific antibodies or reporter molecules and are useful for measuring parameters in solution. Flow cytometry methods are useful for measuring cell surface and intracellular parameters, as well as shape change and granularity and for analyses of beads used as antibody- or probe-linked reagents. Readouts from such assays may be the mean fluorescence associated with individual fluorescent antibody-detected cell surface molecules or cytokines, or the average fluorescence intensity, the median fluorescence intensity, the variance in fluorescence intensity, or some relationship among these.

[0076] Flow cytometry may be used to quantitate parameters such as the presence of cell surface proteins or conformational or posttranslational modification thereof; intracellular or secreted protein, where permeabilization allows antibody (or probe) access, and the like. Brefeldin A is commonly utilized to prevent secretion of intracellular substances. Flow cytometry methods are known in the art, and described in the following: Flow Cytometry and Cell Storing (Springer Lab Manual), Radbruch, Ed., Springer Verlag, 2000; Ormerod, Flow Cytometry, Springer Verlag, 1999; Flow Cytometry Protocols (Methods in Molecular Biology, No 91), Jaroszeski and Heller, Eds., Humana Press, 1998; Current Protocols in Cytometry, Robinson et al., eds, John Wiley & Sons, New York, N.Y., 2000. The readouts of selected parameters are capable of being read simultaneously, or in sequence during a single analysis, as for example through the use of fluorescent antibodies to cell surface molecules. Both single cell multiparameter and multicell multiparameter multiplex assays, where input cell types are identified and parameters are read by quantitative imaging and fluorescence and confocal microscopy are used in the art, see Confocal Microscopy Methods and Protocols (Methods in Molecular Biology Vol. 122.) Paddock, Ed., Humana Press, 1998. These methods are described in U.S. Pat. No. 5,989,833 issued Nov. 23, 1999.

Computer systems

[0077] The present disclosure provides computer systems for implementing methods provided herein. A computer system includes a central processing unit (CPU, also "processor" and "computer processor" herein), which can be a single core or multi core processor, or a plurality of processors for parallel processing. The computer system also includes memory or memory location (e.g., random-access memory, read-only memory, flash memory), electronic storage unit (e.g., hard disk), communication interface (e.g., network adapter) for communicating with one or more other systems, and peripheral devices, such as cache, other memory, data storage and/or electronic display adapters. The computer system can be operatively coupled to a computer network ("network") with the aid of the communication interface. The network can be the Internet, an internet and/or extranet, or an intranet and/or extranet that is in communication with the Internet. The network in some cases is a telecommunication and/or data network. The network can include one or more computer servers, which can enable distributed computing, such as

cloud computing. The network, in some cases with the aid of the computer system, can implement a peer-to-peer network, which may enable devices coupled to the computer system 1001 to behave as a client or a server.

[0078] The CPU can execute a sequence of machine-readable instructions, which can be embodied in a program or software. The instructions may be stored in a memory location, such as the memory. The instructions can be directed to the CPU, which can subsequently program or otherwise configure the CPU to implement methods of the present disclosure. Examples of operations performed by the CPU can include fetch, decode, execute, and writeback. The CPU can be part of a circuit, such as an integrated circuit. One or more other components of the system can be included in the circuit. In some cases, the circuit is an application specific integrated circuit (ASIC).

[0079] The storage unit can store files, such as drivers, libraries and saved programs. The storage unit can store user data, e.g., user preferences and user programs. The computer system in some cases can include one or more additional data storage units that are external to the computer system, such as located on a remote server that is in communication with the computer system through an intranet or the Internet.

[0080] The computer system can communicate with one or more remote computer systems through the network. For instance, the computer system can communicate with a remote computer system of a user (e.g., remote cloud server). Examples of remote computer systems include personal computers (e.g., portable PC), slate or tablet PC's (e.g., Apple® iPad, Samsung® Galaxy Tab), telephones, Smart phones (e.g., Apple® iPhone, Android-enabled device, Blackberry®), or personal digital assistants. The user can access the computer system via the network.

[0081] Methods as described herein can be implemented by way of machine (e.g., computer processor) executable code stored on an electronic storage location of the computer system, such as, for example, on the memory or electronic storage unit. The machine executable or machine readable code can be provided in the form of software. During use, the code can be executed by the processor. In some cases, the code can be retrieved from the storage unit and stored on the memory for ready access by the processor. In some situations, the electronic storage unit can be precluded, and machine-executable instructions are stored on memory. The code can be pre-compiled and configured for use with a machine having a processor adapted to execute the code, or can be compiled during runtime. The code can be supplied in a programming language that can be selected to enable the code to execute in a pre-compiled or as-compiled fashion.

[0082] Aspects of the systems and methods provided herein, such as the computer system, can be embodied in programming. Various aspects of the technology may be thought of as “products” or “articles of manufacture” typically in the form of machine (or processor) executable code and/or associated data that is carried on or embodied in a type of machine readable medium. Machine-

executable code can be stored on an electronic storage unit, such as memory (e.g., read-only memory, random-access memory, flash memory) or a hard disk. "Storage" type media can include any or all of the tangible memory of the computers, processors or the like, or associated modules thereof, such as various semiconductor memories, tape drives, disk drives and the like, which may provide non-transitory storage at any time for the software programming. All or portions of the software may at times be communicated through the Internet or various other telecommunication networks. Such communications, for example, may enable loading of the software from one computer or processor into another, for example, from a management server or host computer into the computer platform of an application server. Thus, another type of media that may bear the software elements includes optical, electrical and electromagnetic waves, such as used across physical interfaces between local devices, through wired and optical landline networks and over various air-links. The physical elements that carry such waves, such as wired or wireless links, optical links or the like, also may be considered as media bearing the software. As used herein, unless restricted to non-transitory, tangible "storage" media, terms such as computer or machine "readable medium" refer to any medium that participates in providing instructions to a processor for execution.

[0083] Hence, a machine readable medium, such as computer-executable code, may take many forms, including but not limited to, a tangible storage medium, a carrier wave medium or physical transmission medium. Non-volatile storage media include, for example, optical or magnetic disks, such as any of the storage devices in any computer(s) or the like, such as may be used to implement the databases, for example, shown in the drawings. Volatile storage media include dynamic memory, such as main memory of such a computer platform. Tangible transmission media include coaxial cables; copper wire and fiber optics, including the wires that comprise a bus within a computer system. Carrier-wave transmission media may take the form of electric or electromagnetic signals, or acoustic or light waves such as those generated during radio frequency (RF) and infrared (IR) data communications. Common forms of computer-readable media therefore include for example: a floppy disk, a flexible disk, hard disk, magnetic tape, any other magnetic medium, a CD-ROM, DVD or DVD-ROM, any other optical medium, punch cards paper tape, any other physical storage medium with patterns of holes, a RAM, a ROM, a PROM and EPROM, a FLASH-EPROM, any other memory chip or cartridge, a carrier wave transporting data or instructions, cables or links transporting such a carrier wave, or any other medium from which a computer may read programming code and/or data. Many of these forms of computer readable media may be involved in carrying one or more sequences of one or more instructions to a processor for execution.

[0084] The computer system can include or be in communication with an electronic display that comprises a user interface (UI) for providing, for example, an electronic output of identified gene fusions. Examples of UI's include, without limitation, a graphical user interface (GUI) and web-based user interface. Methods and systems of the present disclosure can be implemented by

way of one or more algorithms. An algorithm can be implemented by way of software upon execution by the central processing unit.

[0085] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g. amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

EXPERIMENTAL

Evaluating drug immunotoxicity on vaccine responses using human immune organoid arrays

[0086] To develop an adaptive immune model that could be used for a drug screening platform, we re-engineered an earlier model to be grown in miniaturized, hydrogel-based cultures within 96-well plates. These immune organoids responded to the live attenuated influenza vaccine (LAIV) with expansion of plasmablasts that released high affinity, flu-specific IgG antibodies and showed other features of class switching and somatic hypermutation. We used these immune organoids in arrays to screen a panel of approved immunosuppressive medications used in autoimmune disease and solid organ transplant for their impacts on flu-vaccination. We found that while several directly impacted PBs and antibody responses, others had minimal effects, demonstrating that immune organoids can be used to evaluate compounds during the drug development process.

RESULTS

[0087] *Establishing human immune organoids in a hydrogel matrix.* B cells in culture show strong dependencies on cell density, antigen immobilization, and adhesion to CD40L co-stimulation, so we first aimed to equip our immune organoid model with flexibility and scale by adding 3-dimensional organization. We established a pipeline to generate mixed single cell suspensions of tonsil tissue obtained from patients undergoing clinically indicated tonsillectomy and implemented modifications in the preparation of organoids (Table 1 for donor characteristics, FIG. 1A). First, we created discrete structure by incorporating tonsil single-cell suspensions directly in a polymerized dextran-based hydrogel matrix. Second, we stimulated the cells using antigen embedded within the hydrogel rather than in the surrounding media by diffusion. Third, we tested a range of cell densities and hydrogel parameters to empirically identify conditions most permissive to antigen-specific responses while maintaining background quiescence.

[0088] During organoid preparation, single cells initially spread evenly, but settled in niches early within the matrix that evolved over time in culture. Continuous brightfield imaging of live organoids revealed two distinct phases in organization. Within 15 minutes the hydrogel polymerized, and cells were dispersed (FIG. 1B). By 24 hours, discrete cellular aggregates began to form that continued to expand with increased density. We verified that these cultures had no negative impact on the overall viability of the organoids (FIG. 6A). We focused further on the capacity of these preparations to model adaptive immune responses. Antigen-specific B cells can range in frequencies from 0.01-1% for flu-specific antibody secreting cells in human tonsil. To further determine the technical capacity of immune organoids to recover such rare cell populations, we spiked in limiting frequencies of CFSE labeled tonsil cells and quantified recovered B and T cell frequencies following organoid culture. We found strong linear relationships for CFSE-labeled cells recovered down to 0.01% of total cells for both B and T lymphocytes in both permeable membrane and hydrogel-based organoids (FIG. 1B-1D). The CD45⁺ populations, in contrast, were far less frequent and undetectable at spike-in frequencies below 1% across all preparations.

[0089] Next, we simulated adaptive immune responses by immunizing the organoids with the LAIV, which induces mucosal immune responses in humans. By 7 days of culture, there was increased hemagglutinin (HA)-specific IgG antibodies detected in the supernatants of organoid cultures, but not in unstimulated conditions (FIG. 1C). 3D organoids responded more strongly when immunized by embedding the antigen directly within the hydrogel matrix compared with addition into the surrounding media (FIG. 1D). The hydrogel matrix itself also showed a clear impact on antibody responses. At high gel stiffness induced by increasing the maleimide crosslinking concentration, flu-specific IgG antibodies were detected in organoids with high- but not low cell density (FIG. 1E). The dependency on cell density was lost when maleimide was reduced to decrease the stiffness, suggesting a threshold for optimal B cell responses.

[0090] In summary, hydrogel structures are conducive to maintain immune organoid growth and can be used to study even rare antigen-specific B and T cell frequencies found within the physiological range.

[0091] *B cell evolution in 3D human immune organoids.* The identification of antigen-specific, IgG isotype antibodies in LAIV-stimulated 3D organoids suggested evolution of antibody secreting cells. Indeed, stimulation with LAIV resulted in the expansion of pre-GCBs, GCBs and PBs, with a concomitant reduction in both naïve and memory compartments (FIG. 2A, FIG. 7A). Day 7 PB frequencies correlated strongly with antigen-specific antibody responses, suggesting that they constitute the dominant antibody secreting cells in the 3D organoid culture system (FIG. 7B). To further explore these temporal changes, we sorted CD27-CD38- naïve B cells and CD27+CD38+ PBs from baseline day 0 tonsil suspensions and compared them to PBs sorted from day 7 LAIV-stimulated 3D organoids using single cell RNA sequencing (scRNA-seq, FIG. 2A, 2B). We began by characterizing the cellular heterogeneity in these populations. Manual classification of 13,249 total cells using differentially expressed genes revealed 6 clusters (FIG.

2C). Importantly, all CD27-CD38- naïve sorted cells clustered distinctly (FIG. 2B). Consistent with previous reports, these cells showed highest *CD20*, *HLA-DRA* and *LTB*, confirming their naïve phenotype. We focused on the plasmablast populations, which were further composed of 5 distinct subclusters. Two populations of proliferating cells, the Cycling-1 and Cycling-2 subclusters, expressed the highest cell cycle and cytoskeletal genes including *TUBA1B* and *CDC20*. Committed PBs expressed high *ATF5*, a transcription factor that enforces the plasmablast phenotype by promoting the final stages of activated B cell survival. Lastly, there were two populations that differed most strongly by isotype use- the IgM-High PB subcluster, and another collection of non-cycling Mature PBs marked by variable BCR gene and isotype use. We fit a trajectory across the clusters beginning with the Naïve B cells and ending with Mature PBs and mapped differential expression of several genes involved in germinal center responses (FIG. 2E). Consistent with B cell evolution, progression through pseudotime was accompanied by a reduction in naïve markers *IGHD*, *CD20*, and *HLA-DRA*, whereas germinal center B and PB markers *IGHG1* and *AICDA* progressively increased.

[0092] *Immune organoids undergo SHM and isotype switching.* Antigen specific B cell responses *in vivo* are characterized by clonal expansion, isotype switching and somatic hypermutation (SHM). To determine whether these immune organoids stimulated with LAIV could recapitulate these hallmarks of adaptive immunity during culture, we integrated the single cell gene expression data with single cell BCR sequencing (scVDJ-seq). As expected, the PB populations all showed strikingly greater clonal expansion compared to naïve cells (FIG. 3A). 86% of naïve clonal groups were composed of unexpanded, single clones (FIG. 3B). In contrast, Cycling-1, Cycling-2, IgM-High and both Committed and Mature plasmablast clusters showed higher clonality, many of which were substantially expanded.

[0093] We enumerated the frequencies of PBs from day 0 and day 7 organoids across the different clusters (FIG. 3C). Interestingly, mature and committed cells accounted for 73% of day 0 PBs, whereas these groups made up only 56% of day 7 organoid PBs. BCR sequences from these clusters at day 0 were dominated by IgG1, IgG2, IgA1 and IgA2 isotypes with high frequencies of SHM, consistent with the tonsil as mucosal secondary lymphoid tissue (FIG 3D, 3E). In contrast, PBs from day-7 LAIV-stimulated organoids differed, showing expansion of the cycling and IgM-High populations. These three clusters accounted for 39.1% of day 7 organoid PBs compared to just 14.6% from day 0, suggesting that they may result from B cells stimulated directly within the organoid environment. Interestingly, these populations also showed unique isotypes compared to day 0 PBs. For example, the Cycling 1 population accounted for only 4.3% of day 0 PBs and were mostly of IgG2, IgA1 and IgA2 isotypes. However, the day 7 organoid Cycling-1 cluster contained over three times as many cells at 13.5% and were mostly IgG1 with abundant SHM. This pattern of increased expansion of highly mutated, IgG1 BCR sequences was also evident across the other day-7 organoid clusters, including the Cycling, Committed and Mature PB populations.

[0094] There were also substantial changes evident within the IgM compartment. Day 7 organoids demonstrated increased numbers of IgM PBs across all clusters compared to day 0. Importantly, this was accompanied by increased frequency of SHM, suggesting maturation of these subsets. This was particularly evident within the cycling populations, where there was a large increase in mutated IgM+ BCRs. Moreover, the IgM-High cluster, which increased in frequency from 5.6% in day 0 PBs to 16.8% in day 7 organoids, also showed a large increase in SHM.

[0095] In summary, integrated scRNA-seq and scVDJ-seq analysis showed that PBs clearly evolved during culture, with expansion of highly mutated IgM and IgG1 BCR sequences evident after 7 days of LAIV stimulation. These results further demonstrate that immune organoids recapitulate critical *in vivo* B cell responses of clonal expansion, isotype switching and SHM during antigen activation.

[0096] *Comparison of immune organoids prepared with 3D hydrogels versus Transwell cultures.* Our initial goal was to improve the scale and flexibility of the immune organoid system as a screening platform, while still maintaining the essence of the initial model. To that end, we extensively benchmarked immune organoids grown in hydrogels compared to Transwells to ensure that the mode of preparation did not impact the underlying biology. Flu-specific IgG antibody responses were similar when standardizing for number of cells and volume of media, with slightly lower background in the hydrogel organoid cultures potentially reflecting improved cellular quiescence (FIG. 4A). These similarities in antibody responses were also evident in the PB subsets. Comparison of day 7 PBs from organoids grown in hydrogel versus Transwell inserts using scRNAseq also showed similar cluster distributions and degrees of clonal expansion (FIG. 4B, 4C).

[0097] There were no changes in the degree of SHM between the two organoid models across the most frequent BCR isotypes detected (FIG. 4D-4G). More detailed evaluation of differential isotype-use across all PB clusters again confirmed expansion in IgM and IgG1 with reduced IgG2 and IgA from both day-7, LAIV-stimulated organoids compared to day 0 cells; but importantly, isotype-use did not differ based on the method of organoid preparation (FIG. 4D-4H).

[0098] We further compared the BCR repertoires of immune organoids using bulk BCR heavy chain sequencing. Using hierarchical clustering to compare repertoire similarities, organoid samples most clearly separated based on antigen stimulation and not on the method of preparation (FIG. 4I). BCR repertoires were most highly similar between day-7 LAIV-stimulated PBs from permeable inserts and hydrogel samples. Naïve cells from day 0, day-7 membrane and day-7 hydrogel samples also clustered together. Finally, day 0 PBs formed a separate group, further supporting the evolution of B cell populations over time during immune organoid culture.

[0099] *Screening medications for immunotoxicity in miniaturized immune organoids.* Given the ability of hydrogel-based organoids to recapitulate human *in vivo* adaptive immune responses to the LAIV vaccine, we next looked to scale the system for compound-screening. We empirically

tested other commercially available hydrogel matrices with diverse gel stiffnesses and functionalized adhesion molecules for LAIV-induced IgG and IgM antibodies (FIG. 8A, 8B). We found that plating embedded single-cell tonsil suspensions at high density within hydrogels as floating droplets was most conducive to the 96 well plate screening format, enabling temporal surveillance of the media for antibody measurements without disturbing the cells in culture. (FIG. 5A). To fully characterize the operating characteristics of the 96-well plate format, we profiled miniaturized immune organoids from twelve separate tissue donors in replicates by stimulation with LAIV and measured HA-specific IgG antibodies in the culture media at day 10 (FIG. 5C). Importantly, there was extremely low background in HA-specific IgG antibodies in unstimulated organoids. Eleven of the twelve donors responded with greater than 5-fold induction in HA-specific antibodies following LAIV stimulation, and 8 donor samples responded with greater than 10-fold change over baseline. Interestingly, we found that while there was substantial biological heterogeneity, there was limited technical variability. Replicate organoids from the same donor responded to LAIV with similar magnitude, however separate donors displayed a wide range of HA-specific IgG antibodies. To accommodate this, further screening assays employed biological replicates from at least four separate donors.

[00100] *Immunosuppressive drugs inhibit plasmablast expansion during LAIV vaccination.* To determine whether miniaturized immune organoids could be used to evaluate compounds for immunotoxicity, we cultured samples in the presence of drugs that are implicated in impaired vaccine responses. We chose well characterized immunosuppressive medications with diverse mechanisms of action on different cellular populations. Methotrexate (MTX) and mycophenolate (MPA) are antiproliferative agents that antagonize folate metabolism and *de novo* purine biosynthesis, respectively. Hydroxychloroquine (HCQ) is an immunomodulator that interferes with antigen presentation by altering lysosomal pH. Cyclosporin A (CsA) is a calcineurin inhibitor that blocks the downstream consequences of T cell receptor activation. While these medications are widely utilized clinically and highly effective in the management of autoimmune diseases and solid organ transplant, they have also been found to variably impact immunization responses to the influenza, pneumococcal and SARS-CoV2 vaccines.

[00101] The miniaturized 3D immune organoid setup allowed us to screen these compounds all at the same time across various concentrations in multiple donors. Organoids grown in the presence of MTX, MPA and CsA each had significantly reduced HA-specific antibody responses to LAIV stimulation in a concentration-dependent manner (FIG. 5D). MPA displayed the strongest inhibition, with 2.1-fold reduction in HA-specific IgG evident at low concentrations, consistent with its well documented clinical effects on vaccine responses. Interestingly, HCQ, which is not previously known to impact influenza-vaccine efficacy, showed no significant effects at even 10uM. CsA exhibits its immunosuppressive effect dominantly via the T cell compartment and inhibited the LAIV response in immune organoids at higher concentrations.

[00102] Anti-TNF biologics have emerged as highly efficacious medications in the treatment of various autoimmune diseases including rheumatoid arthritis, ankylosing spondylitis and inflammatory bowel disease. While their clinical use has increased dramatically, the potential for immunotoxicity and adverse impacts on vaccines has remained controversial. We tested infliximab, a humanized anti-TNF IgG1 monoclonal antibody, and etanercept, a TNF receptor-IgG1 Fc fusion protein for their effects on the LAIV response and found no impact. 3D organoids grown in the presence of clinically relevant concentrations of infliximab (20, 10, 5 $\mu\text{g/mL}$) and etanercept (5, 2.5, 1.25 $\mu\text{g/mL}$) still produced HA-specific antibodies, similar to organoids stimulated with LAIV in the presence of vehicle or IgG1 isotype controls (FIG. 5E). Vedolizumab, a monoclonal antibody directed against $\alpha 4\beta 7$ integrin that inhibits gut-homing lymphocytes used in inflammatory bowel disease, also had no impact (50, 5, 0.5 $\mu\text{g/mL}$).

[00103] The absence of HA-specific antibodies from organoids grown in the presence of immunosuppressive conditions could result from broad cellular toxicity. Alternatively, it may relate to selective inhibition of key cellular mediators of the adaptive immune response. To answer this question, we validated the previous screening assays for immunosuppressive medications using the lowest effective concentrations in replicate organoids and profiled the lymphocyte populations using flow cytometry at day 5 post stimulation. Consistent with the ELISA results, MTX and MPA strongly inhibited LAIV-induced PB expansion, whereas HCQ had no effect (Figure 5F). CsA had an intermediate effect, again consistent with only mild reduction in the HA-specific IgG response seen previously. Importantly, the impact on PBs were not uniform across all B cell populations. There were still reductions in the naïve and memory populations, and similar increases in the pre-GCB and GCB cells seen in all LAIV stimulated organoids grown in the presence of immunosuppressive medications, suggesting that the antibody response was not the result of non-selective cell death (FIG. 9A). There were similar effects within the T cell populations during LAIV stimulation. MTX, MPA and CsA-treated organoids all had significantly reduced effector CD8 T cell expansion induced by LAIV, with slightly lower impact on the CD4 T cell pool suggesting these cells may activate more strongly from the surrounding cytokine environment than antigen-dependent responses. (FIG. 5G, 5H). However, CD4 and CD8 T cells from immune organoids stimulated with PMA/ionomycin in the presence of immunosuppressive compounds still produced IFN γ and IL-2, again suggesting that drug effects on antibody responses relate to selective inhibition, not widespread cytotoxicity.

[00104] In summary, our immune organoid screening platform can be used to detect the effects of clinically relevant immunosuppressive medications on human vaccine responses, and mechanistically characterize selectively impacted immune populations.

[00105] Here we describe a new approach to evaluate drug compounds for immunosuppressive effects on vaccine responses. We show that tonsil cell suspensions could be miniaturized into

discrete, organoids arrayed within 96-well plates, providing a rapid, reproducible human platform to screen many candidate molecules within a single assay.

[00106] The evaluation of compounds for toxic effects on the immune system is a central component to drug development. The conventional approach involves a general weight of evidence review to determine the need for specific immunotoxicology assessment, which is based on the mechanism of action, drug disposition, and intended patient population. When indicated, further testing is conducted on broad arms of the immune system, and several assays have been developed for antigen-specific humoral immunity. The T cell dependent antibody response (TDAR) is one method endorsed by regulatory authorities and involves measuring antibody responses after immunizing animals with sheep erythrocytes (SRBC) or keyhole limpet hemocyanin (KLH) antigens. While these methods are available, they are time and labor intensive, require multiple replicate animals for testing and may not fully reflect relevant human immunity. Other human B cell and lymph node models have therefore been adapted, though these have employed patient derived PBMC samples or complex bioreactor methodologies for culture growth that may not reflect germinal center dynamics. Moreover, previous readouts for such systems have relied on proliferation or cell frequencies. In contrast, our method using human tonsil immune organoids incorporates secondary lymphoid populations with secreted antigen-specific antibodies as a key read out. HA-specific IgG antibodies in our assay correlated strongly with antigen-expanded PB populations, and single cell sequencing revealed evidence of isotype changes and somatic hypermutation within these cells during time in culture, suggesting that our immune organoid model is better equipped to recapitulate relevant *in vivo* adaptive immune biology.

[00107] The functionality of our screening platform was evidenced by the ability to both conduct assays on supernatants to detect antibodies, and subsequent cellular phenotyping on selected organoids for further in-depth characterization. In this way, we defined the populations directly impacted by common immunosuppressive medications with diverse mechanisms of action in autoimmune disease and solid organ transplantation. Accordingly, measuring the impact of these medications on B cell populations and antibody reactions to the influenza vaccine informs much more than general cellular toxicity, but rather elaborates on how compounds can interact with emerging populations during a dynamic antigen response.

[00108] The ability to scale immune organoids for screening directly benefited from adapting growth conditions to 3D culture from permeable membrane inserts. Patient derived tumor spheroids and other organoid models have consistently shown enhanced cellular communication and nutrient exchange compared to conventional monolayer tissue culture. Incorporating tonsil suspensions into a discrete hydrogel structure also had the added technical benefits for designing customized, larger scale screening assays that would be otherwise challenging using commercially available permeable inserts. In this way, the 3D model provides added flexibility in

designing a screening assay to evaluate multiple conditions across various immunological endpoints.

[00109] In summary, we established a fully human screening tool to evaluate the impacts of drug compounds on vaccine responses in a rapid, reproducible manner. The platform can be utilized in the characterization of vaccines and drug panels to better inform patient care, and to efficiently screen for other types of drugs or biologics that might impair or enhance vaccine responses.

MATERIALS AND METHODS

[00110] *Study Design.* The goal of this study was to develop a miniaturized human immune organoid platform to screen the impacts of drugs on vaccine responses. We began by re-engineering our tonsil immune organoid model by incorporating the cells within hydrogels as 3D structures. This required extensive validating and optimization for plating, growth and stimulatory conditions. We used flow cytometry to measure cell frequencies and developed a custom ELISA assay for influenza and hemagglutinin-specific antibodies. We surveyed the effects of technical and biological variability on these readouts. Formal preliminary power analysis on 12 biological samples for these assays using an alpha of 0.05 with an empirically measured effect size of 2.7 required 4 samples each to obtain 80% power. The establishment of a 96-well plate screen also permitted the use of multiple concentrations per compound for testing.

[00111] *Human tonsil samples and subject consent.* All human tissue studies were reviewed, and ethics approval was granted by Stanford University Institutional Review Board (protocol IRB 60741 and IRB 30837). We collected human tonsils and prepared single cell suspensions as previously described. In this study, samples were taken from patients undergoing clinically indicated tonsillectomy for obstructive sleep apnea, hypertrophy or recurrent tonsillitis at Stanford Hospital. Written consent was obtained for all subjects, in addition to legal guardians for pediatric subjects. Importantly, medications were reviewed to ensure there was no previous use of immunosuppressive medications. Tonsil tissue was placed in saline after removal and subsequently transferred to Ham's F-12 media containing Normocin and penicillin/streptomycin for 30-60 minutes at 4°C for decontamination until further processing.

[00112] *Preparation of single cell suspensions from human tonsil tissue.* Tissue was cleaned, sectioned into 5 mm x 5 mm x 5 mm pieces and passed through a 100 μ M filter using a syringe plunger to generate a cell suspension. The membrane was washed with complete media (RPMI with glutamax, 10% FBS, 1X-penicillin/streptomycin, 1X Normocin (InvivoGen, 1X insulin/transferrin/selenium supplement (Gibco), 1X non-essential amino acids, 1X sodium pyruvate). Following Ficoll density gradient separation, collected cells were washed in complete medium, enumerated and distributed as 80-150 x 10⁶ cells/mL in individual cryovials in FBS + 10% DMSO. Frozen cells were stored at -140°C until use.

[00113] *Preparation of immune organoids.* Tonsil cells were thawed and washed in complete media. For organoids grown on membrane plates, washed cells were resuspended to 6x10⁷

cells/mL in complete media containing 0.5 $\mu\text{g/mL}$ human recombinant B-cell activation factor (BAFF, BioLegend). 100 μL cells were then distributed on 0.4 μM pore size permeable membranes (24-well size PTFE or polycarbonate membranes in 12 well standard culture plates; Corning). The lower chamber was filled with 1 mL complete media supplemented with 0.5 $\mu\text{g/mL}$ BAFF.

[00114] For larger 3D immune organoids, washed cells were gently resuspended at high density (8.33×10^8 cells/mL) in BAFF-supplemented complete media. 5×10^6 cells were then combined with dextran-based hydrogel components (TrueGel3D, Millipore Sigma) to final volume 30 μL and maleimide concentration 3 mM. Gels were plated in standard 12-well tissue culture plates and 1 mL complete media supplemented with 0.5 $\mu\text{g/mL}$ BAFF was added after 10 minutes, with the media exchanged after 1 hour.

[00115] For miniaturized 3D immune organoids, washed cells were resuspended to 5×10^8 cells/mL and 2.5×10^6 cells were combined with hydrogel components (VitroGel Organoid 1, The Well Bioscience). Individual 15 μL droplet suspensions were then distributed in standard 96-well V-bottom plates containing 150 μL prewarmed complete media supplemented with 0.5 $\mu\text{g/mL}$ BAFF. Culture media was supplemented on days 4, 7 and 10.

[00116] For antigen stimulation, PBS vehicle or LAIV (1 μL per organoid containing 1.6×10^4 to 1.6×10^5 fluorescent focus units per strain; FluMist Quadrivalent, Medimmune) was used for larger cultures, for 96-well plates 0.5 μL per organoid was used. The vaccine was added directly to the well containing cells for organoids grown in permeable inserts and embedded along with the cells in the hydrogel matrix for 3D organoids.

[00117] *Drug screening assay.* Drug compounds were prepared in concentrated stock solutions and serially diluted in BAFF-containing complete media to minimize solvent toxicity. Plate layout was maintained across experiments and a perimeter of blank wells filled with PBS was kept to avoid edge effects. Separate plates were prepared for different biological replicates to avoid contamination. Methotrexate, mycophenolic acid, hydroxychloroquine, and cyclosporin A were purchased commercially (Sigma Aldrich).

[00118] *ELISA for detection of influenza and HA-specific antibodies.* ELISA plates (Costar) were coated overnight at 4°C with 0.1 μg per well of season matched Fluzone Quadrivalent inactivated influenza vaccine (reported total HA content, Sanofi) or 0.05 μg per well recombinant influenza A H1N1 HA (A/California/7/2009, Invitrogen) diluted in coating buffer containing 100 mM bicarbonate, pH 9.6. Plates were then blocked in PBS containing 1% BSA, and culture supernatants were added at serial 10-fold dilutions. Horseradish peroxidase-conjugated secondary antibody against human IgG or IgM Fc (Invitrogen) were used for detection with 1-step Ultra TMB substrate solution (Thermo Fisher Scientific). After quenching with sulfuric acid, plates were read at 450 nm on a Cytation 7 (Agilent). Data were then compiled, visualized and

area under the curve (AUC) for each condition's dilution series computed in R using the flux package (v0.3.0).

[00119] *Flow cytometry.* Cells were retrieved from organoids grown in permeable membranes by rinsing in PBS. For larger 3D organoids, gels were first washed with PBS and then digested with dextranase recovery solution (Sigma-Aldrich) for 10 minutes at 37°C. Organoids grown in 96-well plates were washed, resuspended in recovery solution (The Well Bioscience) and gently pipetted several times to recover cells.

[00120] Recovered cells were washed in FACS buffer (0.5% BSA, 2 mM EDTA in PBS) and stained at 4°C with primary fluorophore conjugated antibodies in the presence of Fc block (BioLegend) and Live/Dead Fixable Blue stain (Thermo Fisher Scientific). Data was collected on a NovoCyte Penton Cytometer (Agilent) and analyzed using FlowJo 10 software (BD Biosciences). The following antibodies and dilutions were used, all from BioLegend: CD4-FITC (OKT4, 1:100), CD3-BV605 (OKT3, 1:100), CD45-BV711 (HI30, 1:100), CD8a-BV785 (RPA-T8, 1:100), CD19-APC-Cy7 (HIB19, 1:100), CD38-APC (HIT2, 1:100), CD27-E-Cy7 (O323, 1:100), HLA-DR-PE (L243, 1:100).

[00121] *Single cell RNA sequencing sample preparation.* CD27-CD38- and CD27+CD38+ live B cells were bulk sorted from day 0 control and day 7 LAIV-stimulated organoids prepared in both permeable inserts and 3D hydrogels from a single donor using a FACSria II instrument. 4 replicate organoids per condition were pooled in order to ensure sufficient cells could be sorted. Sorted cells were then washed and resuspended at 1000 cells/ μ L in PBS containing 0.04% BSA immediately followed by GEM capture on a 10X Genomics instrument. Separate gene expression and BCR libraries were prepared using the Chromium Single Cell 5' Reagent kits according to the manufacturer's instructions.

[00122] *Single cell RNA sequencing analysis.* Expression matrices from Cell Ranger were uploaded and processed using Seurat (v4.1.0). For quality control, cells were selected with <5% mitochondrial DNA and total gene counts between 200-3,000 for Naïve cells, 200-6,000 for PBs according to their distributions. Individual sample sets were then log-normalized and variable features identified separately (FindVariableFeatures, nfeatures = 2,000). Naïve cells and PBs from day 0 and PBs from day 7 organoids were then integrated (FindIntegrationAnchors, IntegrateData) using the default settings, before proceeding with scaling and PCA (npcs = 30). Cell cycle scores were regressed, followed by UMAP dimensionality reduction and clustering for downstream visualization (dims 1:30, resolution 0.5). Clusters were manually classified using the top differentially expressed genes (FindAllMarkers).

[00123] RNA trajectory analysis was performed using Monocle3 (v1.0.0). The combined, processed Seurat object was read into Monocle (as.cell_data_set), partitioned with UMAP, and fit with a principal graph (learn_graph). Cells were then reordered along pseudotime using the naïve cluster as the starting node. Differentially expressed genes were visualized along the trajectory using plot_genes_in_pseudotime (min_expr = 0.5).

[00124] *BCR analysis.* For single cell integrated gene expression analysis, filtered contig annotations containing VDJ alignments from Cell Ranger were loaded and processed using scRepertoire (v1.5.2)(43). Individual clonal sequences were grouped by V gene and >85% normalized Levenshtein distance using the nucleotide sequence (combineBCR), which was then merged with the Seurat object containing gene expression data (combineExpression).

[00125] For bulk sequencing, genomic DNA was isolated from flow sorted CD27-CD38- and CD27+CD38+ cells using the QIAamp DNA Micro kit (Qiagen), and bulk BCR sequencing was performed with the Immunoseq assay (Adaptive Biotechnologies, Seattle, WA).

[00126] *Statistical analysis.* All data are presented as mean +/- standard deviation unless otherwise indicated, and a minimum of three replicates performed for each experiment. Biological replicates were prioritized for drug testing as indicated above. Between group differences were calculated using one- or two-way analysis of variance (ANOVA) where appropriate, with subsequent Tukey post hoc analysis testing. Statistical analysis was performed using GraphPad Prism for Mac 9.0 (GraphPad software) and R (v4.1.3, R Foundation for Statistical Computing).

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Table 1

Donor	Sex	Age	Vaccination
BBENT16	M	29	Yes
IMD183	M	17	Yes
PED028	M	16	Yes
VIP14	M	23	No
VIP38	M	33	No
VIP67	M	40	Yes
VIP68	M	25	No
VIP70	M	46	No
VIP73	M	41	unknown
VIP74	F	19	unknown
VIP75	M	35	Yes
VIP81	F	50	unknown

[00170] The preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of the present invention is embodied by the appended claims.

WHAT IS CLAIMED IS:

1. An in vitro culture comprising:
one or a plurality of hydrogel droplets comprising embedded immune cells comprising B cells, suspended in culture medium, wherein the embedded cells exhibit one or more hypermutation maturation, affinity maturation, plasmablast differentiation, class switching recombination, and antigen-specific antibody production in response to antigen.
2. The in vitro culture of claim 1, wherein the hydrogel droplet comprises embedded antigen.
3. The in vitro culture of claim 1 or claim 2, wherein the embedded immune cells comprise B cells, T cells and antigen presenting cells.
4. The in vitro culture of any of claims 1-3, wherein the immune cells are dissociated cells from a germinal center.
5. The in vitro culture of any of claims 1-4, wherein the immune cells are dissociated cells from a lymph node, tonsils, spleen, thymus, adenoids.
6. The in vitro culture of any of claims 1-5, wherein the antigen is a peptide, protein or fragment thereof, polysaccharides, lipids, nucleic acids, or other biomolecule.
7. The in vitro culture of any of claims 1-6, wherein the antigen is a vaccine or vaccine candidate.
8. The in vitro culture of any of claims 1-7, wherein the culture is maintained for a period of from 1 to 28 days.
9. The in vitro culture of any of claims 1-8, wherein the culture is maintained for a period of from 4 to 10 days.
10. A method for screening immune responsiveness with an agent of interest, the method comprising:
obtaining a sample comprising dissociated immune cells;
embedding the immune cells in a hydrogel droplet to form an immune organoid in the presence of an antigen of interest;
maintaining the immune organoid in culture for a period of time sufficient to observe the immune response;

collecting droplets from the culture; and
reading out an indicia of immune responsiveness.

11. The method of claim 10, wherein the embedded cells exhibit one or more hypermutation maturation, affinity maturation, plasmablast differentiation, class switching recombination, and antigen-specific antibody production in response to antigen.

12. The method of claim 10 or 11, wherein the indicia of immune responsiveness is determined by one or more of high dimensional imaging, flow cytometry, DNA/RNA sequencing, antibody binding assays, antigen binding assays, etc., including, for example, antibody sequence, presence of different Fc regions in antibodies, quantitative antibody production, antibody binding characteristics.

13. The method of any of claims 10-12, wherein the embedded immune cells comprise B cells, T cells and antigen presenting cells.

14. The method of any of claims 10-13, wherein the immune cells are dissociated cells from a germinal center.

15. The method of any of claims 10-14, wherein the immune cells are dissociated cells from a lymph node, tonsils, spleen, thymus, adenoids.

16. The method of any of claims 10-15, wherein the antigen is a peptide, protein or fragment thereof, polysaccharides, lipids, nucleic acids, or other biomolecule.

17. The method of any of claims 10-16, wherein the antigen is a vaccine or vaccine candidate.

18. The method of any of claims 10-17, wherein the culture is maintained for a period of from 1 to 28 days.

19. The method of any of claims 10-18, wherein the culture is maintained for a period of from 4 to 10 days.

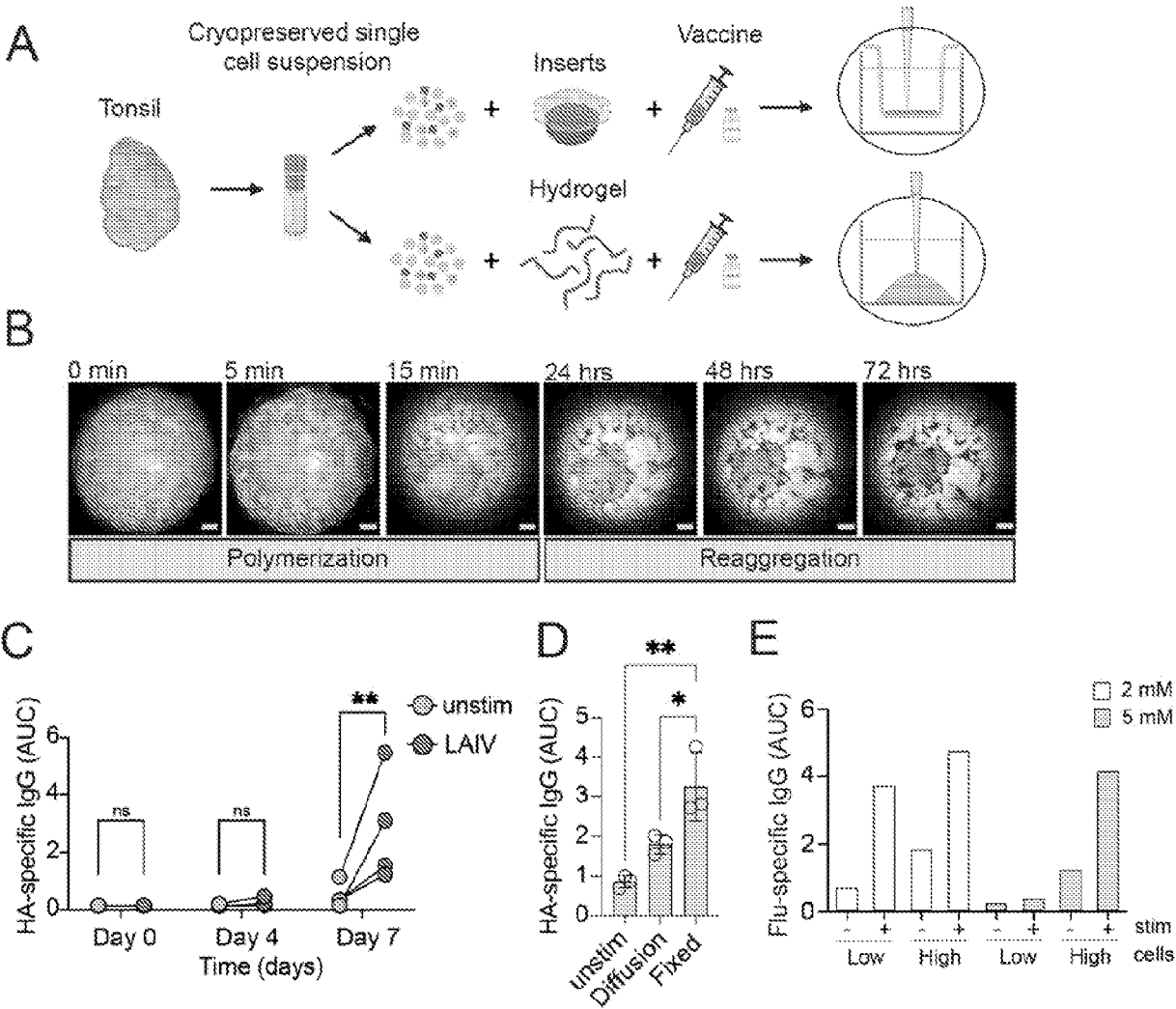


FIG. 1

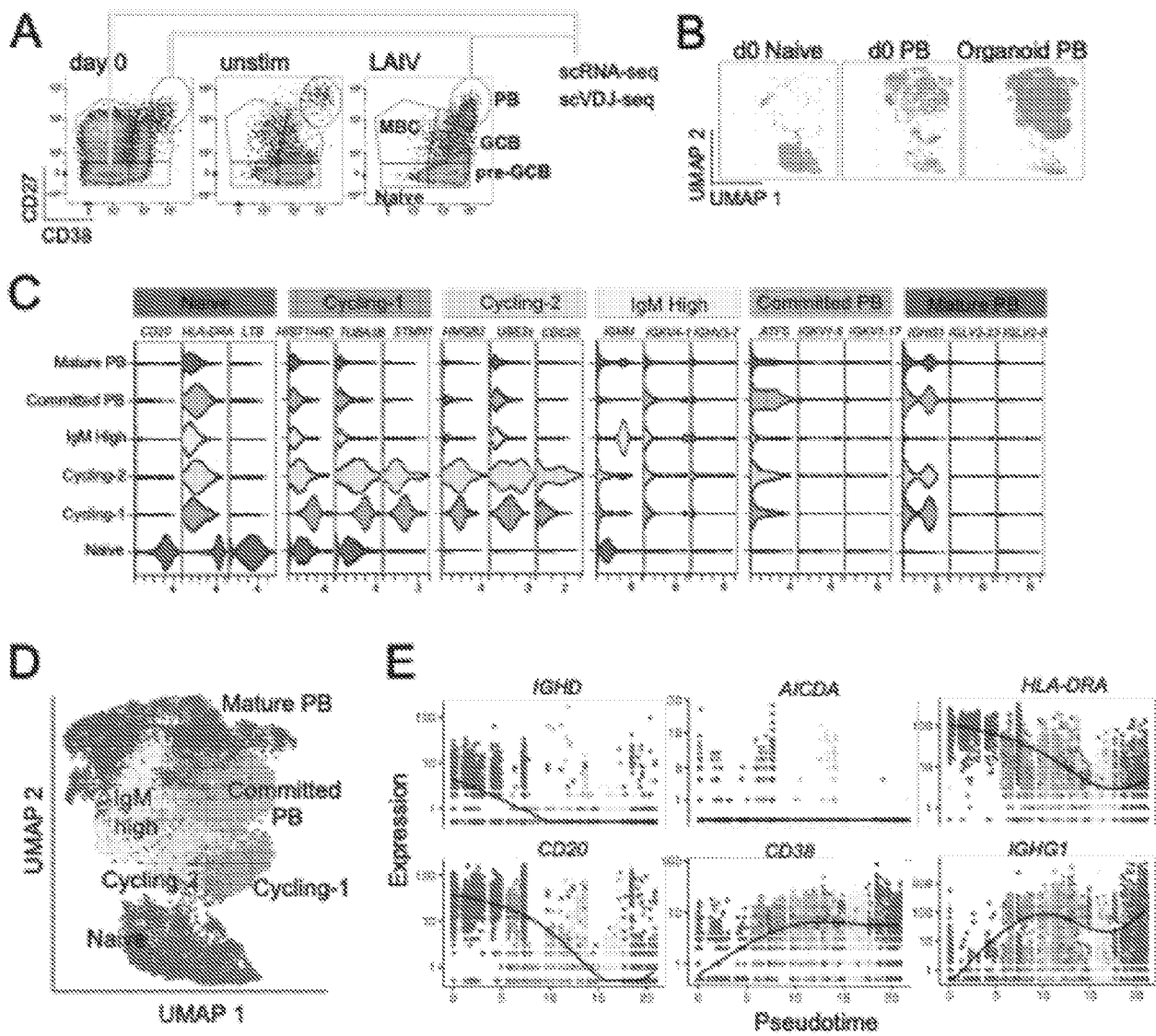


FIG. 2

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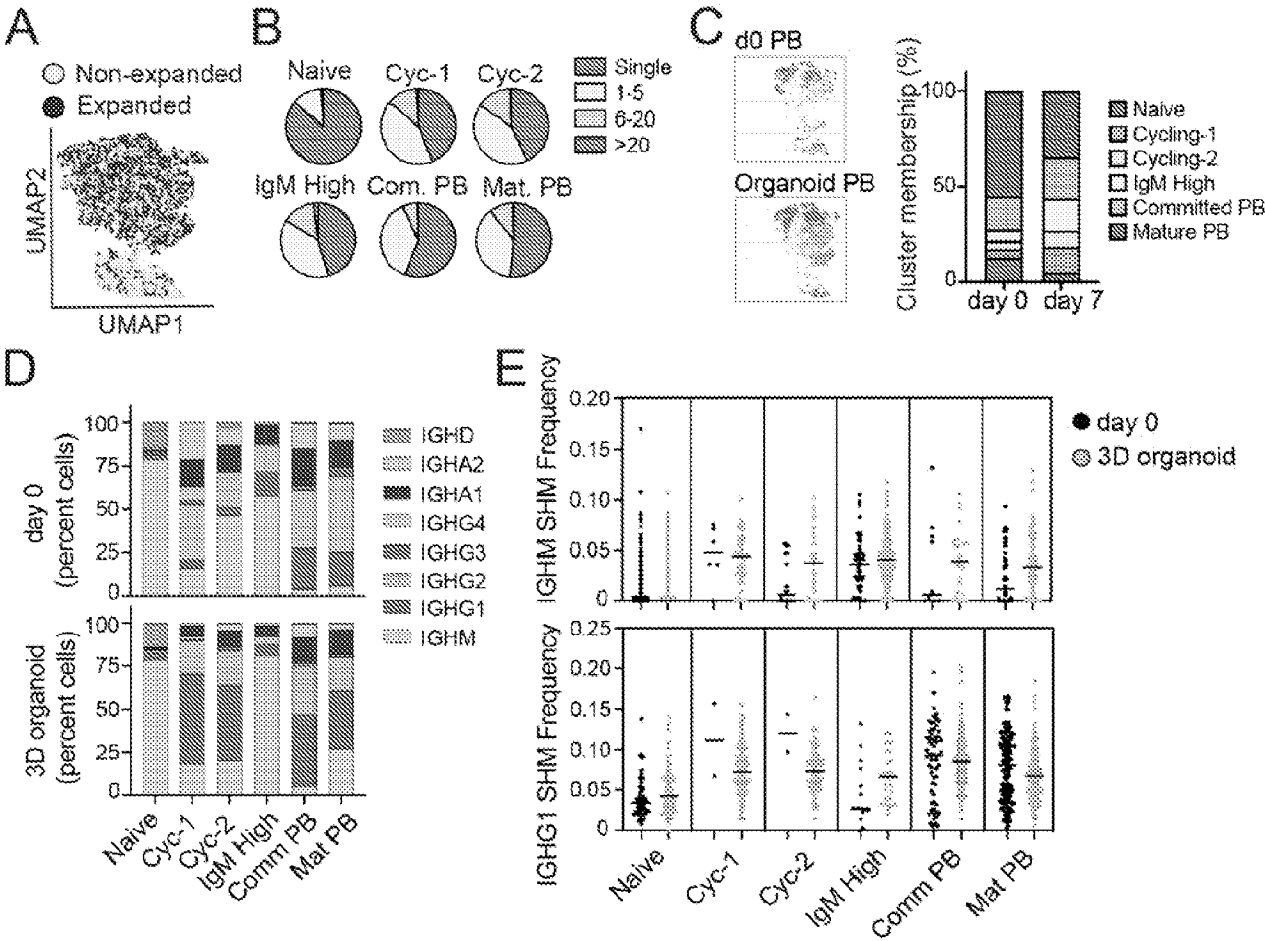


FIG 3

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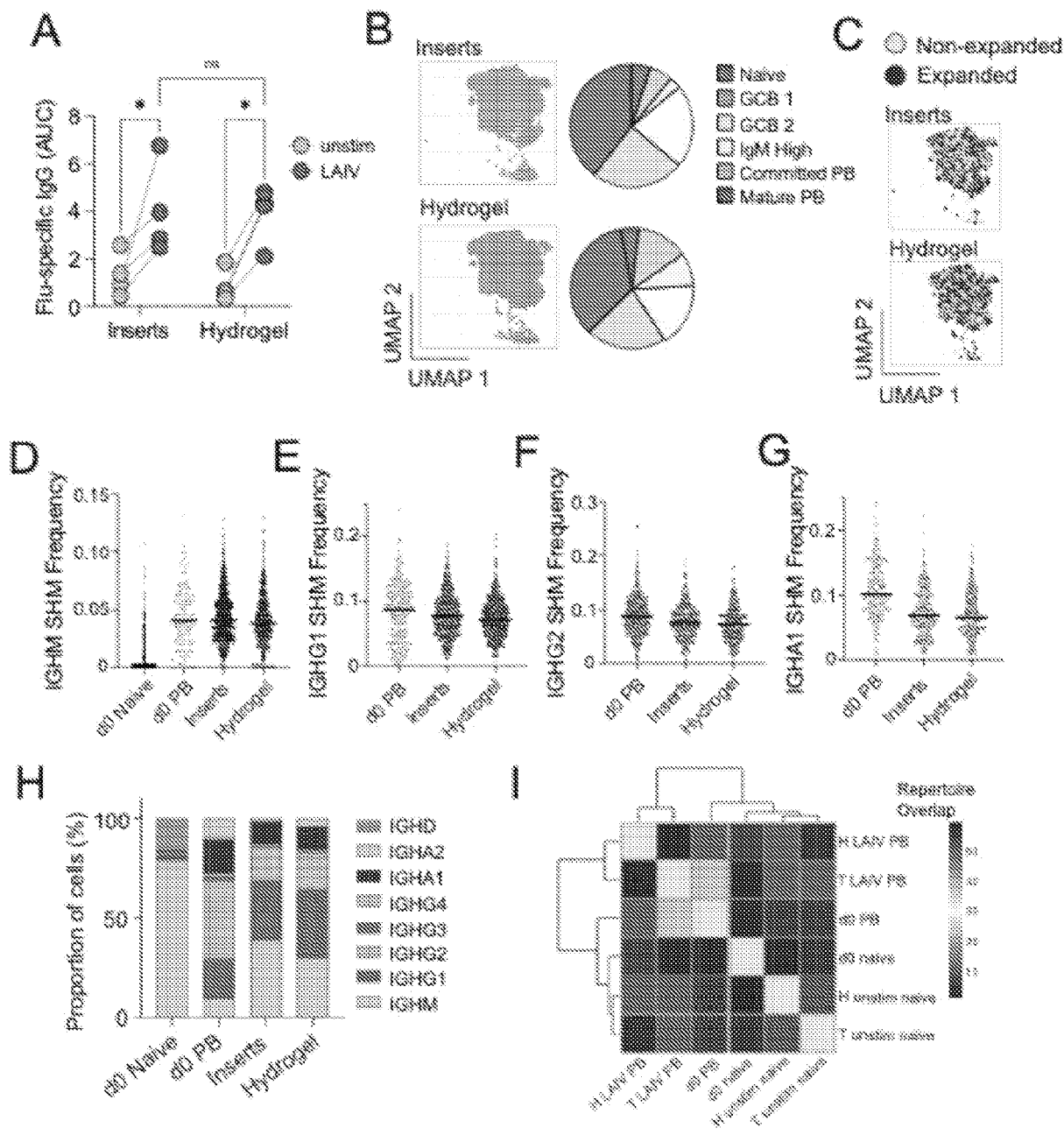


FIG. 4

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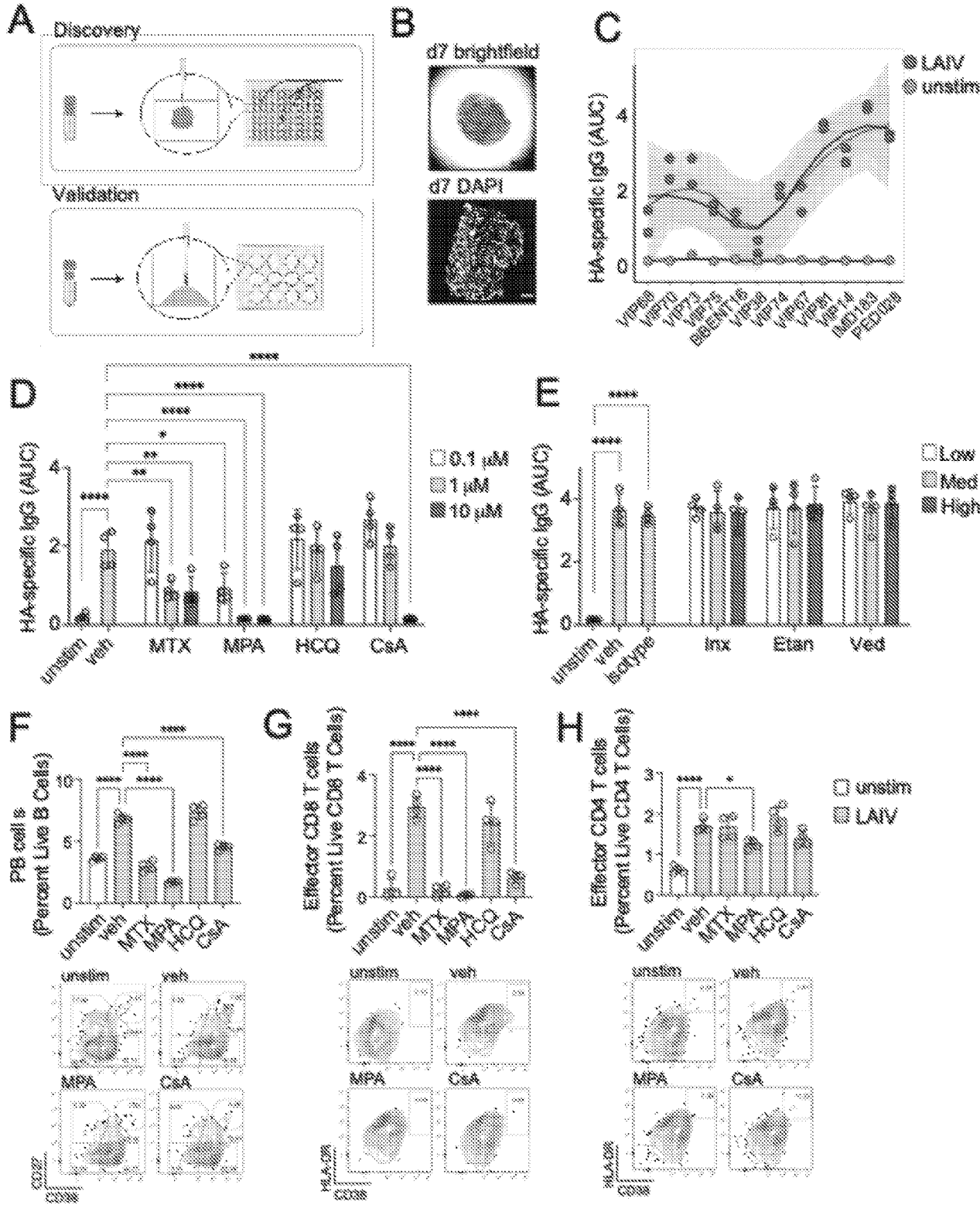


FIG. 5

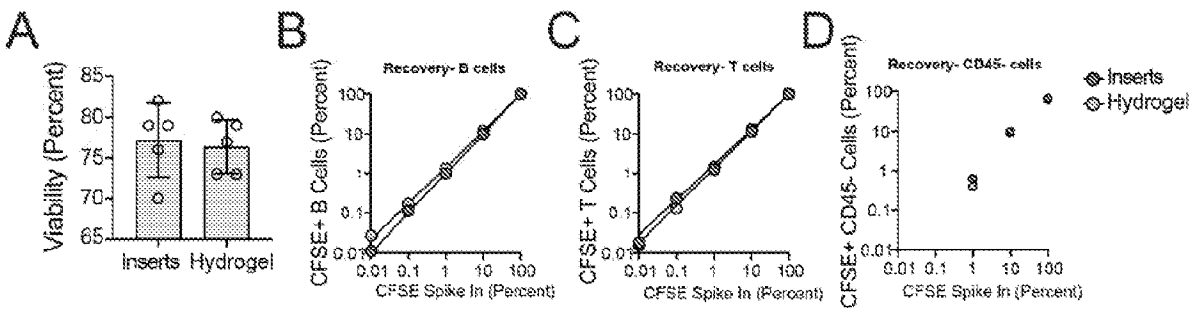


FIG. 6

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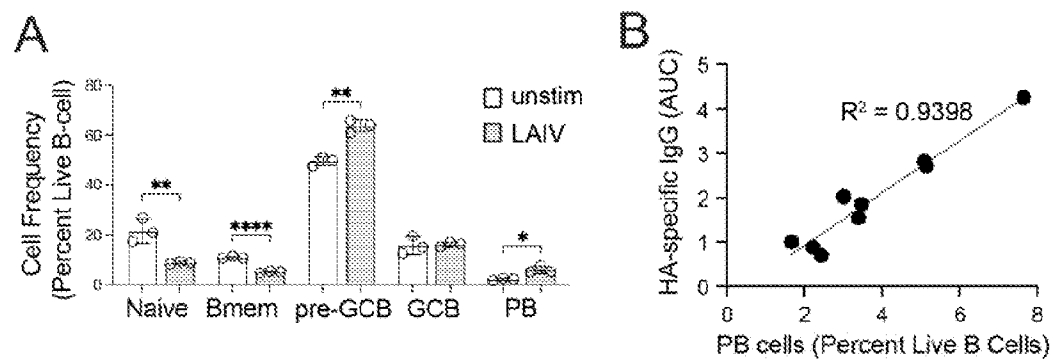


FIG. 7

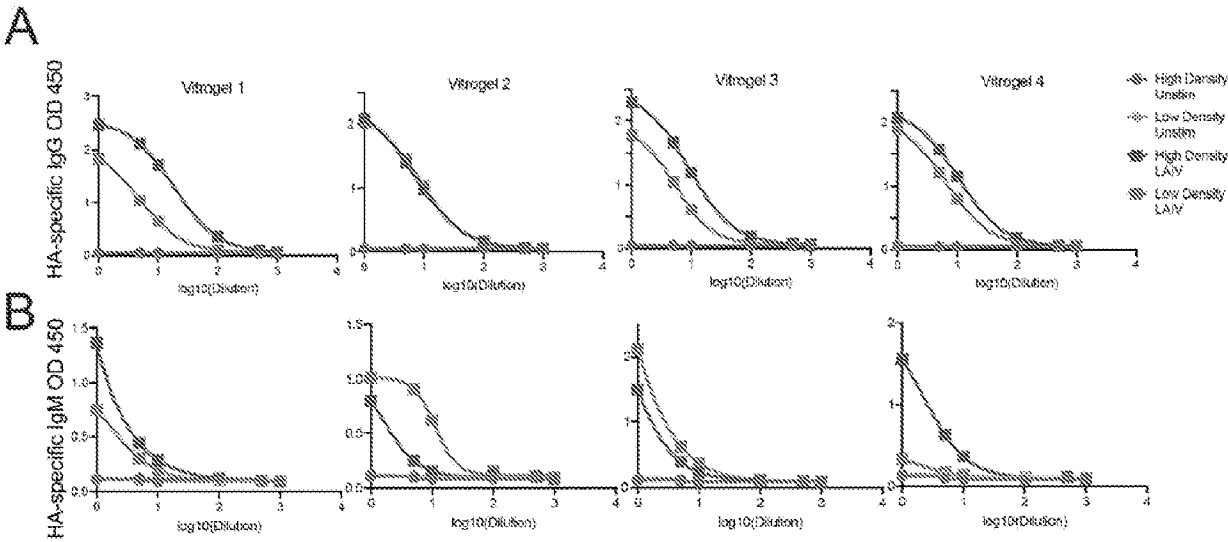


FIG. 8

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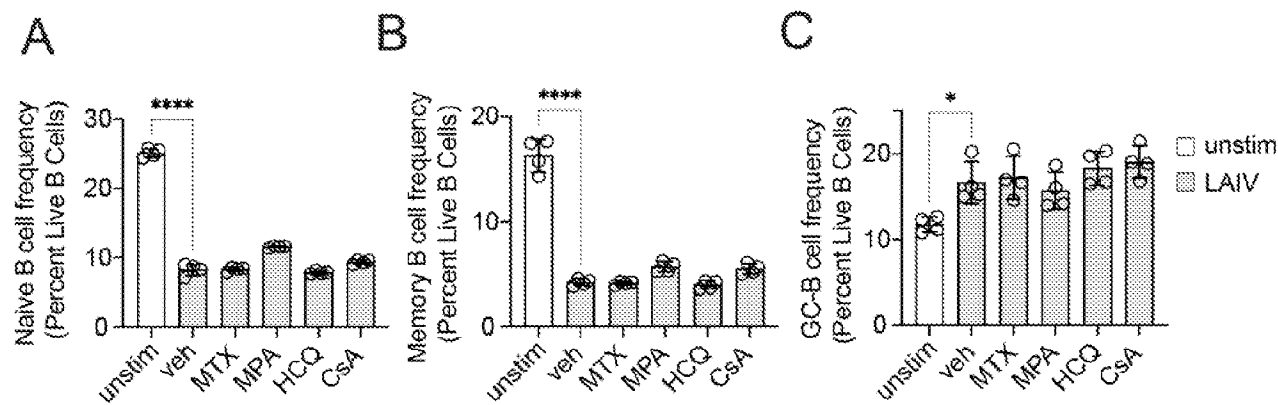


FIG. 9

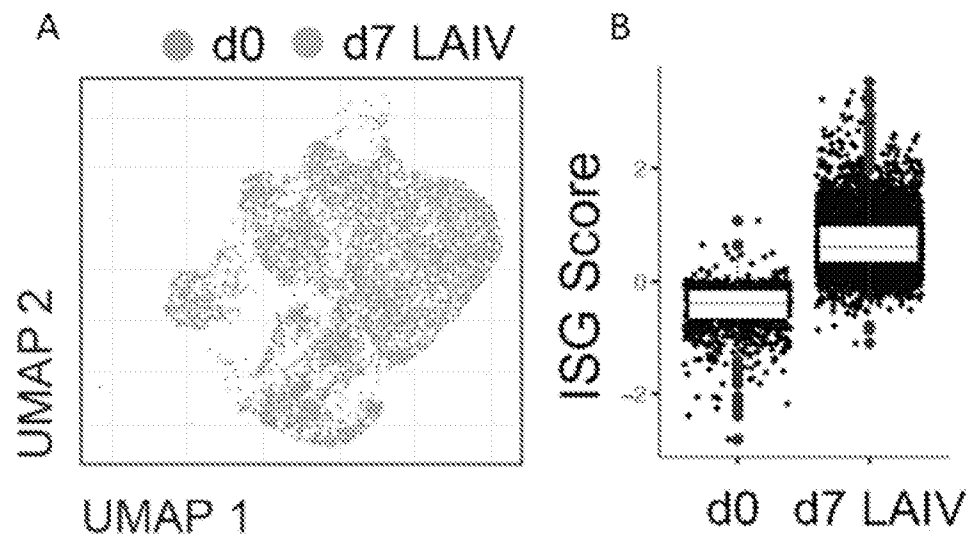


FIG. 10

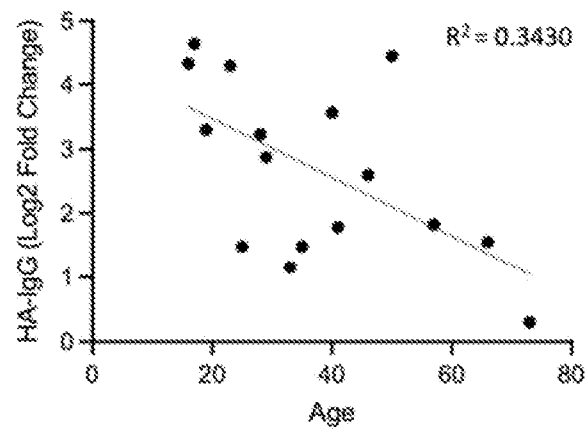


FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US23/25308

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. C12N 5/078; C12M 3/00; C12Q 1/6869 (2023.01)

ADD.

CPC - INV. C12N 5/0634; C12M 3/00; C12Q 1/6869

ADD. C12N 2513/00; C12Q 2563/159

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2018/0258422 A1 (GIGAGEN INC.) 13 September 2018; paragraphs [0007], [0049], [0055], [0057] [0075], [0076], [0078], [0094], [0109], [0120], [0121], [0133], [0149]	1, 3/1, 10-12
Y		2, 3/2
Y	US 2018/0085452 A1 (TOLEROGENICS S.A.R.L.) 29 March 2018; paragraphs [0037], [0038]	2, 3/2

☐ 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

"D" document cited by the applicant in the international application

"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

07 August 2023 (07.08.2023)

Date of mailing of the international search report

SEP 11 2023

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Authorized officer

Shane Thomas

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US23/25308

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.: 4-9, 13-19
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.