activated large and blasting B cell, secreting copious amount of antibody. Both surface IgG and B220/CD45R are down regulated. CD138 and TACI become highly expressed on the surface of the plasma cell (Tellier et al., Eur J Immunol 47(8): 1276-1279 (2017)). As the plasma cell terminally differentiates, the ability to divide is lost. The plasma cell has a short lifespan of a few days to weeks unless sequestered in highly specialized long-term survival niches. The diversity of the plasma cell compartment is low; the *in-vivo* selection process having focused in on a relatively small number of high affinity clones to mount an effective and rapid immune response to antigen.

[0004] While hybridoma generation is capable of sampling the immune repertoire, the process is highly inefficient, with successful immortalization rates in the range of 0.01%-0.001%. This inefficiency of fusion can be counteracted by increasing the number of cells for fusion using larger immunization cohorts or by working with larger animals such as rats. In view of the foregoing, there is a need for methods for enhanced hybridoma generation.

SUMMARY

[0005] The present disclosure provides enhanced hybridoma generation (EHG) methods which provide benefits over those known in the art by virtue of the removal of IgM-positive (IgM+) B cells, the large scale, the high efficiency, and the specificity of the repertoire of the hybridomas produced. Advantageously, the presently disclosed EHG methods provide an enrichment for IgG-positive (IgG+) memory B cells with efficient bead-based removal of IgM+ B cells. IgM class-switches in culture to IgG+ B cells and dilute out the IgG+ Ag+ memory B cell population with irrelevant or low affinity B cell clones. IgM+ B cells divide faster than IgG+ B cell clones. By removing the vast majority of the IgM+ B cells before culture and then culturing the IgG+ B cell enriched population (to allow them to make hundreds of clonal copies before fusion), a large portion of the clones of interest are immortalized. The result is a large hybridoma pool that facilitates the identification of rare Ag+ clones. The presently disclosed EHG methods provide a large-scale bulk culture. Liters of bulk cultures with hundreds of thousands of highly selected IgG+ memory B cells enables large scale deep repertoire mining of larger numbers of animals. Additionally, in the prior art methods, only a very small number of antigen-binding clones are identified whereas the EHG of the present disclosure routinely identifies hundreds to thousands of unique binders. The EHG methods of the present disclosure also comprise B cell immortalization before identification of antigen binding clones. Hybridoma supernatant is screened. This order represents a reversed order of the steps of prior art methods (see, e.g., Steenbakkers et al., J Immunol Methods 152 (1): 69-77 (1992)) where B cells are screened for antigen-binding before they are immortalized. The order of immortalization followed by identification of antigen binding clones of the present disclosure enables high efficiency of identifying antigen-binding clones. In the presently disclosed methods, an unlimited supply of cell supernatant (SN) is available for screening and characterization after fusion. The assay sensitivity at time of SN screening is not limiting. Hybrid cultures can be grown to high concentrations of antibody. In the

presently disclosed EHG methods, the ability to identify antigen-binding clones is not limited by B cell antibody secretion rate and rare binders can be identified by deep mining the hybrid pool. The deep mining by large scale liquid handling plating and Ag+ FACS sorting can be carried out.

[0006] Accordingly, the present disclosure provides enhanced hybridoma generation methods useful in antibody discovery. In exemplary examples, the method of generating hybridomas comprises (a) preparing an enriched population of IgG-positive (IgG+) memory B cells from cells obtained from secondary lymphoid organs of one or more immunized non-human animals, wherein the enriched population is substantially devoid of IgM+ B-cells, (b) bulk-culturing at large-scale the enriched population to obtain an expanded population, and (c) fusing cells of the expanded population with myeloma cells to obtain hybridomas. In exemplary examples, (i) less than or about 10% of the enriched population are IgM-positive (IgM+) B cells and/or (ii) the ratio of the IgG+ memory B cell count to IgM+ B cell count of the enriched population is greater than about 0.5, optionally, greater than about 1 or greater than about 2. Without being bound to a particular theory, the hybridomas produced by the presently disclosed methods allow for deep mining of the immune repertoire of an immunized non-human animal without limitations imposed by B cell antibody secretion rate and/or low abundance of rare B cells. Advantageously, the hybridomas produced by the presently disclosed methods more fully represent the immune repertoire of immunized non-human animals. In exemplary examples, the percentage of the immortalized IgG+ memory B cell repertoire captured by the presently disclosed methods is maximized and, in various instances, is at least about 15% (e.g., at least about 20% or at least about 25%) of the repertoire produced by the immunized animals. The presently disclosed methods further allow for an unlimited supply of hybridoma supernatant that may be screened for antigen-specific antibodies. In exemplary examples, the presently disclosed methods routinely yield hundreds, if not thousands, of antigen-specific hybridomas producing antigen-specific antibodies. The presently disclosed methods furthermore lead to higher fusion efficiencies, yielding a highly efficient capture of B cell clones in the hybridoma pool.

[0007] Accordingly, the present disclosure additionally provides methods of generating hybridomas. In exemplary examples, the method comprises (a) preparing an enriched population of IgG+ memory B cells from cells obtained from secondary lymphoid organs of one or more immunized non-human animals, wherein less than about 10%, optionally, less than about 5%, of the cells of the enriched population are IgM+ B cells; (b) bulk-culturing the enriched population to obtain an expanded population; and (c) fusing cells of the expanded population with myeloma cells to obtain hybridomas. In exemplary examples, less than 2.5% or less than 1% of the cells of the enriched population are IgM+ B cells. In various examples, the method comprises removing greater than about 90% (e.g., greater than 95%, greater than 98%, greater than 99%) IgM+ cells and/or positively selecting for IgG+ cells to obtain the enriched population. In exemplary examples, (i) less than or about 10% of the enriched population are IgM-positive (IgM+) B cells and/or (ii) the ratio of the IgG+

memory B cell count to IgM+ B cell count of the enriched population is greater than about 0.5, optionally, greater than about 1 or greater than about 2. In exemplary examples, the IgM+ B cell count of the enriched population is smaller than the IgM+ B cell count and the ratio of the IgG+ memory B cell count to IgM+ B cell count of the enriched population is greater than 1 or greater than 2. In various examples, the method comprises bulk-culturing the enriched population at a density of about 350 B220-positive B cells per mL to about 700 B220-positive B cells per mL. In exemplary examples, bulk-culturing the enriched population is initiated with a seeding density of about 350 B220-positive B cells per mL to about 700 B220-positive B cells, optionally, about 600 B220-positive cells per mL to about 650 B220-positive cells per mL. In exemplary instances, the method comprises fusing cells of the expanded population with myeloma cells at a ratio of B cells to myeloma cells within the range of 1:1 to 1:4, e.g., 1:1.0, 1:1.5, 1:2.0, 1:2.5, 1:3.0, 1:3.5, or 1:4.0. In exemplary examples, all cells of the expanded population (and thus all B-cells of the expanded population) are combined with myeloma cells. For instance, B cells of the enriched population or expanded population are not selected for fusing with myeloma cells based on production of antibodies which bind to an antigen. Also, for example, B cells of the enriched population or expanded population are not assayed for the production of antigen-specific antibodies prior to fusing with myeloma cells. In various instances, the method comprises screening hybridomas for production of antibodies and, optionally, screening sera obtained from the immunized animals for production of antigen-specific antibodies. In exemplary instances, the only screening for production of antigen-specific antibodies which occurs after harvesting lymphoid organs from immunized animals is the screening of hybridomas.

[0008] The present disclosure also provides methods of screening for hybridomas expressing antigen-expressing antibodies, comprising generating hybridomas in accordance with the present disclosure, culturing hybridomas in individual wells, and screening the supernatant of each well for antigen-specific antibodies. In various examples, about 1 to about 10 or about 1 to about 5 different clones of hybridomas are cultured in a single well.

[0009] Further provided are methods of producing antigen-specific antibodies, comprising generating hybridomas in accordance with the present disclosure, culturing hybridomas in individual wells, screening the supernatant of each well for antigen-specific antibodies to identify the hybridomas expressing antigen-specific antibodies; and expanding the culture of the identified hybridomas to produce antigen-specific antibodies.

[0009a] In one aspect, the present disclosure relates to a method of generating hybridomas, comprising: a) preparing an enriched population of IgG-positive (IgG+) memory B cells from cells obtained from secondary lymphoid organs of one or more immunized non-human animals, wherein: i) less than or about 10% of the enriched population are IgM-positive (IgM+) B cells, and/or ii) the ratio of the IgG+ memory B cell count to IgM+ B cell count of the enriched population is greater than 0.5,

optionally, greater than 1 or greater than 2; b) bulk-culturing the enriched population to obtain an expanded population; and c) fusing cells of the expanded population with myeloma cells to obtain hybridomas.

[0009b] In another aspect, the present disclosure relates to a method of generating hybridomas producing antigen-specific antibodies, comprising: a) immunizing one or more non-human animal(s) with an immunogen; b) harvesting secondary lymphoid organs from the immunized non-human animal(s); c) preparing a single-cell suspension (SCS) from the secondary lymphoid organs harvested from each immunized non-human animal; d) preparing a pooled SCS by combining all SCSs prepared in (c); e) removing greater than 95% IgM+ cells from the pooled SCS and/or positively selecting for surface IgG+ cells from the pooled SCS to obtain an enriched population of IgG-positive (IgG+) memory B cells, wherein: i) less than or about 10% of the enriched population are IgM-positive (IgM+) B cells and/or ii) the ratio of the IgG+ memory B cell count to IgM+ B cell count of the enriched population is greater than 0.5, optionally, greater than 1 or greater than 2, f) bulk-culturing the enriched population to obtain an expanded population; g) fusing cells of the expanded population with myeloma cells to obtain hybridomas; and h) identifying the hybridomas producing antigen-specific antibodies by culturing single hybridomas in individual wells and screening the supernatant of each well for antigen-specific antibodies.

[0009c] In another aspect, the present disclosure relates to a method of screening for hybridomas expressing antigen-specific antibodies, comprising: a) generating hybridomas in accordance with any one of the methods of any one of the preceding claims, b) culturing single hybridomas in individual wells; and c) screening the supernatant of each well for antigen-specific antibodies.

[0009d] In another aspect, the present disclosure relates to a method of screening for hybridomas expressing antigen-specific antibodies, comprising: a) an enriched population of IgG+ memory B cells from cells obtained from secondary lymphoid organs of one or more immunized non-human animals, wherein less than about 5% of the cells of the enriched population are IgM+ B cells; b) bulk-culturing the enriched population to obtain an expanded population; c) fusing cells of the expanded population with myeloma cells to obtain hybridomas; d) culturing single hybridomas in individual wells; and e.

screening the supernatant of each well for antigen-specific antibodies.

[0009e] In another aspect, the present disclosure relates to a method of producing antigen-specific antibodies, comprising: a) preparing an enriched population of IgG+ memory B cells from cells obtained from secondary lymphoid organs of one or more immunized non-human animals; b) bulk-culturing the enriched population to obtain an expanded population; c) fusing cells of the expanded population with myeloma cells to obtain hybridomas; d) culturing single hybridomas in individual wells; e) screening the supernatant of each well for antigen-specific antibodies to identify the hybridomas expressing antigen-specific antibodies; and f) expanding the culture of the hybridomas identified in (e) to produce antigen-specific antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] Figure 1A illustrates an exemplary EHG method of the present disclosure and the following text details the EHG method.

WHAT IS CLAIMED IS:

1. A method of generating hybridomas, comprising
    a. preparing an enriched population of IgG-positive (IgG+) memory B cells from cells obtained from secondary lymphoid organs of one or more immunized non-human animals, wherein:
        i. less than or about 10% of the enriched population are IgM-positive (IgM+) B cells, and/or
        ii. the ratio of the IgG+ memory B cell count to IgM+ B cell count of the enriched population is greater than 0.5, optionally, greater than 1 or greater than 2;
    b. bulk-culturing the enriched population to obtain an expanded population; and
    c. fusing cells of the expanded population with myeloma cells to obtain hybridomas.
2. The method of claim 1, further comprising (a) immunizing one or more non-human animal(s) with an immunogen, (b) harvesting secondary lymphoid organs from the immunized non-human animal(s), optionally, about 3 to about 5 days post-immunization, (c) preparing a single-cell suspension (SCS) from the secondary lymphoid organs harvested from each immunized non-human animal, and/or (d) preparing a pooled SCS by combining the SCS from the secondary lymphoid organs of more than one immunized non-human animals.
3. The method of claim 1 or 2, wherein:

    (A) the enriched population of IgG+ memory B cells are prepared from a single-cell suspension of cells obtained from the secondary lymphoid organs;

    (B) the enriched population is prepared by removing IgM+ cells and/or selecting for IgG+ cells;

    (C) the enriched population is prepared by a negative selection of IgM+ cells and/or a positive selection for IgG+ cells;

    (D) the enriched population is prepared by removing non-B cells, red blood cells (RBCs), IgM+ cells, or a combination thereof, from a single cell suspension prepared from the secondary lymphoid organs, optionally, comprising removing non-B cells, RBCs, IgM+ cells, or a combination thereof, using antibodies specific to one or more cell surface markers expressed by the non-B cells, RBCs, or IgM+ cells; and/or

    (E) the enriched population is prepared by selecting for surface IgG+ cells, optionally, by using anti-IgG antibody-labeled beads, optionally, anti-human IgG antibody-labeled magnetic beads.
4. The method of any one of the preceding claims, wherein:

    (A) the secondary lymphoid organs are secondary lymphoid organs harvested from the

immunized non-human animal(s) about 3 to about 5 days post-immunization;

(B) the secondary lymphoid organs are secondary lymphoid organs harvested from at least 1, 2, 3, 4, or 5 immunized non-human animal(s);

(C) the secondary lymphoid organs are spleen and/or lymph nodes;

(D) the secondary lymphoid organs are the secondary lymphoid organs harvested from only select immunized non-human animals, optionally, wherein the select immunized non-human animals were selected based on post-immunization serum antibody titer level; and/or

(E) the non-human animals are mice or rats.

5. The method of claim 3(C) , wherein greater than 90% IgM+ B cells are removed upon the negative selection and/or the positive selection, optionally, wherein greater than 95% IgM+ B cells are removed upon the negative selection and/or the positive selection.
6. The method of claim 5 , wherein greater than 98% IgM+ B cells are removed upon the negative selection, optionally, wherein greater than 99% IgM+ B cells are removed upon the negative selection and the positive selection.
7. The method of claim 6 , wherein the cell surface markers are human IgM, CD90.2, Ly-6G GR.1, NK-1.1, CD3epsilon, CD4, CD8a, CD11b, and/or TER119.
8. The method of claim 6 or 7, wherein the antibodies are linked to biotin and the method comprises using streptavidin-labeled beads, optionally, streptavidin-labeled magnetic beads, to remove the non-B cells, RBCs, and/or IgM+ cells.
9. The method of claim 8 , wherein greater than 98% of the IgM+ cells present in the single cell suspension are removed.
10. The method of claim3(E), wherein greater than 99% of the IgM+ cells present in the single cell suspension are removed and/or wherein the ratio of the IgG+ memory B cell count to IgM+ B cell count increases by at least about 50-fold or at least about 100-fold.
11. The method of any one of the preceding claims, wherein:

(A) the method comprises bulk-culturing the enriched population of IgG+ cells with anti-human IgG antibody-labeled beads and feeder cells in a cell culture medium comprising rabbit T-cell supernatant

(B) bulk-culturing the enriched population is initiated with a seeding density of about 350 B220-positive B cells per mL to about 700 B220-positive B cells, optionally, about 600 B220-positive cells per mL to about 650 B220-positive cells per mL; and/or

(C) the enriched population is bulk-cultured in a volume of at least about 25 mL and less than or about 2 L.

12. The method of claim 11, wherein the feeder cells express CD40L and/or are gamma-irradiated.

13. The method of claim 12, wherein the feeder cells are gamma-irradiated, CD40L-positive EL4B5 feeder cells.
14. The method of any one of the preceding claims, wherein the myeloma cells are in a log phase growth stage and/or are P3 myeloma cells.
15. The method of claim 11(C), wherein the enriched population is bulk-cultured in a volume of about 50 mL to about 500 mL, optionally, about 100 mL to about 300 mL.
16. The method of any one of the preceding claims, comprising bulk-culturing the enriched population for at least about 4 days, at least about 5 days, or at least about 6 days.
17. The method of any one of the preceding claims, wherein:

    (A) the cells of the enriched population undergo at least about 6 or at least about 7 cell divisions to yield the expanded population;

    (B) all B cells of the expanded population are used for fusing with myeloma cells and/or all cells of the expanded population are combined with myeloma cells;

    (C) B cells are not selected for fusing with myeloma cells based on production of antibodies which bind to an antigen and/or B cells of the enriched population or the expanded population are not screened for production of antibodies which bind to an antigen; and/or

    (D) the cells of the expanded population are fused with myeloma cells by electrocell fusion (E) to obtain hybridomas, optionally, wherein cells of the expanded population are fused with myeloma cells in a volume greater than 10 mL per fusion event.
18. The method of any one of the preceding claims, wherein:

    (A) the method further comprises transferring the hybridomas and any unfused cells to selection medium, optionally, wherein the selection medium comprises hypoxanthine azaserine (HA);

    (B) the method further comprises storing hybridomas under freezing conditions;

    (C) the method further comprises screening the hybridomas for production of antibodies which bind to an antigen; and/or

    (D) the method further comprises culturing hybridomas in multiplate wells and screening the supernatant of each well for antigen-specific antibodies.
19. The method of claim18(C) and 18(D), wherein the screening comprises an immunoassay which detects binding of antibodies to the antigen.
20. The method of claim 19, wherein the immunoassay is a fluorescence activated cell sorting (FACS) analysis.
21. The method of any one of the preceding claims, wherein screening for cells producing antigen-specific antibodies occurs only after hybridomas are obtained, and not before hybridomas are

obtained and/or screening for antigen-specific antibodies occurs only before secondary lymphoid organs are harvested and after hybridomas are obtained.

22. The method of claim 21, wherein the screening for antigen-specific antibodies that occurs before secondary lymphoid organs are harvested comprises a titer analysis of serum obtained from live immunized animals.
23. A method of generating hybridomas producing antigen-specific antibodies, comprising
    a. immunizing one or more non-human animal(s) with an immunogen;
    b. harvesting secondary lymphoid organs from the immunized non-human animal(s);
    c. preparing a single-cell suspension (SCS) from the secondary lymphoid organs harvested from each immunized non-human animal;
    d. preparing a pooled SCS by combining all SCSs prepared in (c);
    e. removing greater than 95% IgM+ cells from the pooled SCS and/or positively selecting for surface IgG+ cells from the pooled SCS to obtain an enriched population of IgG-positive (IgG+) memory B cells, wherein:
        i. less than or about 10% of the enriched population are IgM-positive (IgM+) B cells and/or
        ii. the ratio of the IgG+ memory B cell count to IgM+ B cell count of the enriched population is greater than 0.5, optionally, greater than 1 or greater than 2,
    f. bulk-culturing the enriched population to obtain an expanded population;
    g. fusing cells of the expanded population with myeloma cells to obtain hybridomas; and
    h. identifying the hybridomas producing antigen-specific antibodies by culturing single hybridomas in individual wells and screening the supernatant of each well for antigen-specific antibodies.

24. A method of screening for hybridomas expressing antigen-specific antibodies, comprising
    a. generating hybridomas in accordance with any one of the methods of any one of the preceding claims,
    b. culturing single hybridomas in individual wells; and
    c. screening the supernatant of each well for antigen-specific antibodies.
25. A method of screening for hybridomas expressing antigen-specific antibodies, comprising
    a. preparing an enriched population of IgG+ memory B cells from cells obtained from secondary lymphoid organs of one or more immunized non-human animals, wherein less than about 5% of the cells of the enriched population are IgM+ B cells;
    b. bulk-culturing the enriched population to obtain an expanded population;

c. fusing cells of the expanded population with myeloma cells to obtain hybridomas;
d. culturing single hybridomas in individual wells; and
e. screening the supernatant of each well for antigen-specific antibodies.

26. A method of producing antigen-specific antibodies, comprising
    a. preparing an enriched population of IgG+ memory B cells from cells obtained from secondary lymphoid organs of one or more immunized non-human animals;
    b. bulk-culturing the enriched population to obtain an expanded population;
    c. fusing cells of the expanded population with myeloma cells to obtain hybridomas;
    d. culturing single hybridomas in individual wells;
    e. screening the supernatant of each well for antigen-specific antibodies to identify the hybridomas expressing antigen-specific antibodies; and
    f. expanding the culture of the hybridomas identified in (e) to produce antigen-specific antibodies.
27. The method of any one of the preceding claims, wherein the hybridomas obtained represent at least 15% of the IgG+ memory B cell repertoire produced by the immunized animals.
28. The method of claim 24, wherein the hybridomas obtained represent at least 10% of the IgG+ memory B cell repertoire produced by the immunized animals, optionally, at least 15 % of of the IgG+ memory B cell repertoire produced by the immunized animals.
29. The method of claim 28, wherein the hybridomas obtained represent at least 20% of the IgG+ memory B cell repertoire produced by the immunized animals.
30. The method of claim 29, wherein the hybridomas obtained represent at least 25% of the IgG+ memory B cell repertoire produced by the immunized animals.