



Chapter 15

Tfh-Mediated and Tfr-Suppressed Antigen-Driven IgG and IgE Assays

Rachel L. Clement, Benjamin L. Hanson, and Peter T. Sage

Abstract

T Follicular helper (Tfh) cells stimulate, whereas T follicular regulatory (Tfr) cells inhibit, effector B cell responses. Although new tools have been developed to assess the functional roles of Tfh and Tfr cells in vivo, methods to assess mechanisms have been limited. One such limitation has been the ability of in vitro functional assays to recapitulate robust germinal center-like responses. Although previous in vitro Tfh-mediated and Tfr-suppressed assays to assess antibody regulation have been developed, these classically have relied on polyclonal stimulation. To understand Tfh and Tfr cell functionality, more robust assays that utilize specific antigen are needed. Here we describe an in vitro approach for sensitively and quantitatively assessing the capacity of Tfh and Tfr cells to regulate B cell responses in an antigen-driven system. These assays allow the study of Tfh and Tfr cells in specific disease contexts, such as IgG production after vaccination or IgE responses during allergic airway disease.

Key words T follicular regulatory cells, TFR, T follicular helper cell, T_{FH}, Treg, B cell, Antibody, Suppression

1 Introduction

T follicular helper (Tfh) cells have essential roles in stimulating B cell responses from within germinal centers (GCs) resulting in the production of high-affinity antibodies [1]. T follicular regulatory (Tfr) cells suppress Tfh-mediated antibody responses [2–4]. Newly developed in vivo models have shown a strong regulatory role of Tfr cells, but other studies have shown more complex roles in controlling autoimmunity and foreign antigen-specific antibody responses [4–7]. Although in vivo perturbation strategies have been essential in demonstrating the functionality of Tfr (and Tfh) cells, assessing mechanisms of function have been more elusive. One hurdle has been the ability to assess functionality of Tfr and Tfh cells on a per-cell basis with synchronized timing. We have

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previously developed in vitro assays to assess Tfh stimulation, and Tfr inhibition, of B cell responses using polyclonal stimulation. These assays have been used to determine the roles of PD-1 and CTLA-4 on Tfr functionality, as well as epigenetic and metabolic changes in B cells after suppression [8–11].

One limitation in the previously developed in vitro functional assays has been the lack of antigen to stimulate Tfh cells in the system. The use of antigen may stimulate a more physiologically relevant Tfh and Tfr interaction with B cells leading to an enhanced germinal center like response. In addition, the use of antigen may promote expansion and/or differentiation of effector Tfh cells that may be less competitive during polyclonal stimulation. In support of this, we have recently shown that house dust mite (HDM) driven in vitro Tfh assays promote Tfh13 cells, resulting in robust IgE responses [5]. Here we provide a protocol to assess in vitro functionality of Tfh and Tfr cells in a system that is stimulated with specific antigen. We provide examples of this assay in settings of NP-OVA vaccination and HDM-driven allergic airway disease. Although these disease settings are used as examples, many different antigens may be used to uncover the functionality of Tfh, Tfr, or B cells in unique disease settings such as autoimmunity, infection, and transplant rejection.

2 Materials

2.1 HDM Solution

1. House Dust Mite extract (HDM) diluted to 1 mg/ml in sterile PBS (*see Note 1*).
2. Sterile PBS.

2.2 NP-OVA Emulsion

1. 4-Hydroxy-3-nitrophenylacetyl-conjugated ovalbumin (NP-OVA) with a NP:Ova ratio of 15-30:1, diluted to 10 mg/ml in PBS.
2. Complete Freund's Adjuvant *Mycobacterium tuberculosis* H37RA.
3. 3-way stopcock.
4. Sterile PBS.

2.3 Mice

1. 10–20 *Foxp3*^{GFP} reporter mice (*see Note 2*). WT mice can also be used (*see Note 3*).
2. P200 pipet (for HDM assay only).
3. Isoflurane vaporizer (for HDM assay only).
4. Isoflurane (for HDM assay only).
5. Chamber with tubing (for HDM assay only).
6. Charcoal canister (for HDM assay only).

2.4 Cell Purification

1. Anti-CD4 magnetic positive selection beads and LS columns. Magnetic separation kits can be found from many suppliers; here we will describe the protocol from Miltenyi. Kits from other manufacturers may use different protocols.
2. Anti-CD19 magnetic positive selection beads and LS columns.
3. Flow Cytometry (FACs) buffer: PBS, 1% FBS, 1 mM EDTA.
4. Conjugated antibodies: anti-CD4 PerCPCy5.5 (RM4-5), anti-CD19 APC/Cy7 (6D5), anti-ICOS PE (15 F9), anti-CXCR5 Biotin (2G8), streptavidin BV421. For non-FoxP3 reporter mice, anti-GITR FITC (DTA-1) may be used.
5. Aria (BD Biosciences) or Astrios (Beckman Coulter) cell sorter.
6. Accuri cytometer (BD Biosciences), Countess II FL (Thermo-fisher) or other suitable cell counter.

2.5 Assay Plating

1. 96-well round bottom culture plates (*see Note 4*).
2. Specific antigen (HDM or NP-OVA) (*see Note 5*).
3. R10 media: RPMI, 2 mM L-Glutamine, 10% heat-inactivated fetal calf serum, 100 U/ml Pen-Strep, 1 mM HEPES, 50 μ M beta-mercaptoethanol.

2.6 Antibody ELISA

1. Maxisorp ELISA Plates.
2. Rat anti-mouse IgE (R35-92) or goat anti-mouse Ig coating antibody (1010-01) (*see Note 6*).
3. Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG or IgE (*see Note 7*).
4. Mouse IgE (C38-2), and Mouse IgG (B1-8) standards.
5. Phosphatase substrate, plus diethanolamine (DEA) buffer.
6. Plate reader or alternative.

2.7 Flow Cytometry

1. Conjugated antibodies (in addition to above): anti-IA BV421 (M5.114.15.2), anti-GL7 FITC, anti-IgG1 PE (A85-1), and IgE Pacific Blue (R35-72). Anti-IgE (R35-72) for blocking surface bound IgE.
2. FoxP3 Fix/Perm and Perm/Wash buffer kit.

3 Methods**3.1 Preparation of HDM**

1. Dilute the HDM in PBS to a final concentration of 10 μ g/60 μ l of PBS.

3.2 Preparation of NP-OVA/CFA Vaccine

1. Add 250 μ l of 10 mg/ml NP-OVA to 2.25 ml PBS. Take up into a 5 ml syringe. Add 3-way stop-cock and expunge air.
2. Thoroughly mix H37RA CFA and take up 2.5 ml into a 5 ml syringe. Add to other end of 3-way stop-cock.

3. Emulsify by sequentially pushing each syringe to mix contents into neighboring syringe. Mix thoroughly, but cool to 4 °C if syringes become warm (excessive heat from friction will break down the antigen). Continue mixing until emulsion becomes somewhat difficult to move (~20 min of mixing). Check emulsion by putting a small amount in PBS and make sure it does not partially dissolve (it should have the consistency of toothpaste).

3.3 Immunization of Mice with HDM

1. Place mouse in an approved isoflurane chamber and administer isoflurane according to institutional and laboratory-specific anesthesia policies. When mouse is sufficiently anesthetized and breathing slows, take mouse out of the chamber and drip 60 µl of HDM solution on mouse nostrils alternating ~5 µl per nostril in rapid succession. Challenge every 2 days for 7 days (*see Note 8*).

3.4 Tfh, Tfr, and B Cell Sorting

1. Harvest draining lymph node(s) from the challenged mice on day 7 (*see Note 9*). For HDM-challenged mice mediastinal lymph node should be harvested. For NP-OVA vaccination, inguinal lymph nodes should be harvested (depending on placement of subcutaneous vaccine).
2. To generate a single cell suspension, mash the LNs through a pre-wet 70 µm cell strainer. Wash the top of the strainer with ice-cold FACs buffer thoroughly (*see Note 10*).
3. Perform CD4-positive enrichment according to manufacturer's protocol and collect flowthrough from the column. Keep flowthrough on ice. Purity of CD4+ T cells should be ~95%.
4. Perform CD19-positive bead enrichment on the flowthrough using manufacturer's protocol. Purity should be ~98%. If purity is above 99%, CD19+ cells may be used in assay without the need for cell sorting.
5. For flow cytometry sorting, incubate CD4-enriched cells with anti-CD4, anti-CD19, anti-ICOS (each diluted 1:200), and anti-CXCR5 biotin (diluted 1:100) in FACS buffer for 30 min on ice.
6. Wash twice by adding excess FACs buffer and centrifuging cells at $350 \times g$ for 6 min at 4 °C and removing the supernatant.
7. Incubate cells with streptavidin BV421 at 1:400 in FACS buffer for 15 min on ice. Wash 2× with excess FACs buffer as in **step 6**.
8. Incubate CD19-enriched cells with anti-CD19 and anti-CD4 at 1:200 in FACs buffer for 30 min on ice. Wash 2× with FACs buffer.

9. Sort Tfh (as CD4+ICOS+CXCR5+FoxP3–CD19–) and Tfr (as CD4+ICOS+CXCR5+FoxP3+CD19–) cells on a cell sorter using maximal purity settings and low pressure/flow rates as per Fig. 1.
10. Sort B Cells on cell sorter as CD19+CD4– (*see Note 11*).
11. Purity for both sorts should be ~99% (*see Note 12*).

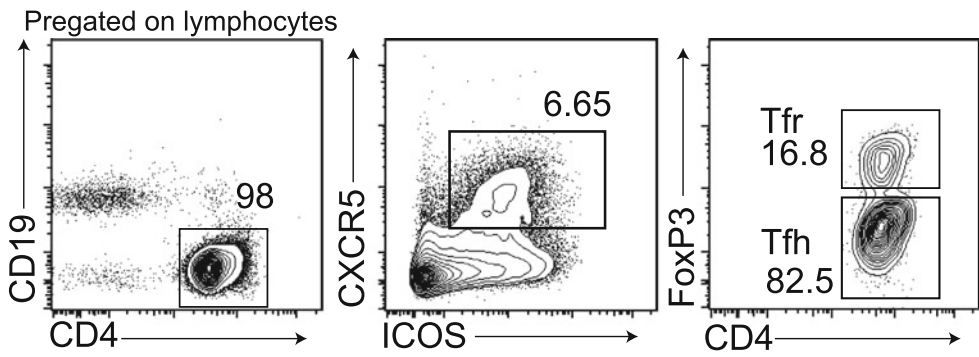
3.5 Cell Plating

1. Count sorted cells on an Accuri flow cytometer or Countess cell counter to get an accurate measurement on actual cell yield (which is typically ~20% lower than the theoretical yield from the cell sorter).
2. In a U-bottom 96-well culture dish, add 1×10^5 sorted B cells, 3×10^4 sorted TFH cells, and/or 3×10^4 TFR cells in 50 μ l R10 media. Experimental groups should include B cells alone (to assess the requirement of Tfh cells for B cell responses), B+TFH, and B+TFH+TFR cells (*see Note 13*).
3. Add 20 μ g/ml of HDM or NP-OVA to each well (depending on immunization strategy). Increase the volume of each well to 200 μ l with R10 media (Fig. 2).
4. Place the plate in a 37 °C incubator with 5% CO₂ for 6 days (*see Note 14*).

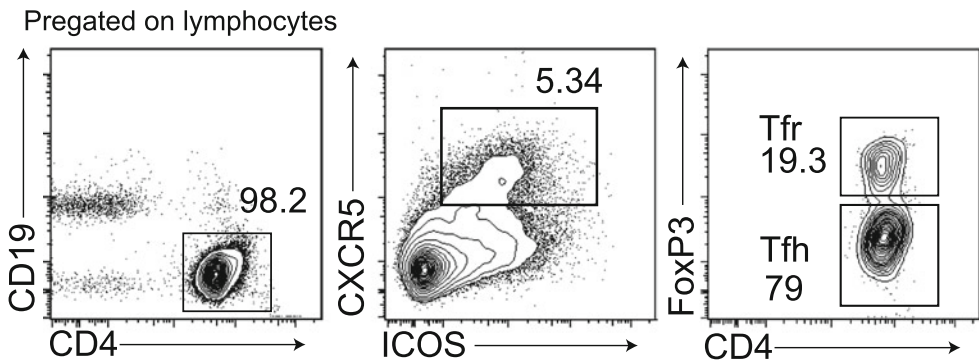
3.6 Flow Cytometry to Assess B Cell Function

1. After 6 days of culture, collect ~150 μ l of the supernatant for ELISA analysis (*see Note 15*).
2. Resuspend the cells in the remaining supernatant, transfer to a V bottom plate, and spin at $350 \times g$ for 5 min.
3. For HDM assays, remove supernatant and resuspend in FACS buffer containing anti-CD19 APC/Cy7, anti-CD4 PerCP-Cy5.5, anti-GL7 FITC, anti-FAS PE-Cy7, and IgE antibody (for blocking) diluted 1:200. For NP-OVA assays, resuspend in anti-CD19 APC/Cy7, anti-CD4 PerCP-Cy5.5, anti-GL7 FITC, anti-FAS PE-Cy7, and anti-IA-PB. Incubate on ice for 30 min.
4. Wash $2 \times$ with FACS buffer.
5. Add Fix/Perm from the FoxP3 intracellular staining kit; incubate 30 min on ice.
6. Wash $2 \times$ with Perm/Wash (dilution according to manufacturer's instructions) from FoxP3 intracellular kit.
7. Add anti-IgG1 PE and anti-IgE BV421 (diluted at 1:200 in Perm/Wash buffer) for HDM assays, or anti-IgG1 PE for NP-OVA assays, and incubate for 20 min.
8. Wash $2 \times$ with FACS buffer.
9. Analyze on suitable flow cytometer using representative gating strategies shown in Fig. 3 (*see Note 16*).

a. HDM (using FoxP3 reporter)



b. NP-OVA (using FoxP3 reporter)



c. NP-OVA (using GITR expression)

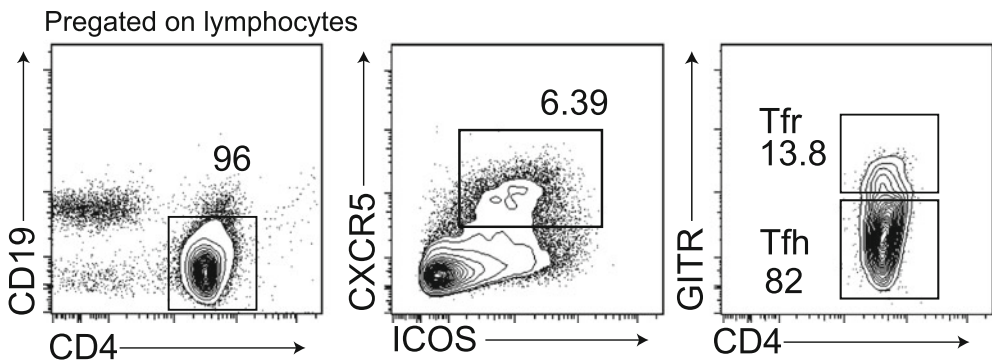


Fig. 1 Representative gating strategies to sort Tfh and Tfr cells for functional assays. **(a)** Sorting Tfh and Tfr cells from HDM-challenged mice. *Foxp3*^{GFP} mice were intranasally challenged with HDM on days 0, 2, 4, and

3.7 IgE Elisa to Assess B Cell Function

1. Coat MaxiSorp plates with anti-mouse IgE or anti-mouse Ig overnight at 4 °C.
2. Wash 6 times with 0.05% Triton-X100 in PBS.
3. Block with 1% BSA in PBS for 1 h at 37 °C.
4. Dilute the cell culture supernatant (previously collected) 1:10 for IgE, 1:50 for IgG in 1% BSA in PBS and add 95 µl to MaxiSorp plates.
5. Perform serial dilutions of IgE and/or IgG standard starting at 500 ng/ml on the same plate as samples. Incubate for 45 min at room temperature.
6. Wash 6× times with 0.05% Triton in PBS.
7. Add anti-mouse IgE AP or anti-mouse IgG AP diluted 1:1000 in 1% BSA to wells. Incubate for 45 min at room temperature.
8. Wash 6× with 0.05% Triton-X100 in PBS.
9. Add phosphatase substrate diluted in DEA buffer.
10. After color develops, measure absorbance at 405 nm on a suitable plate reader and calculate concentration of IgE and/or IgG (Fig. 4).

4 Notes

1. HDM is an efficient antigen causing lung inflammation as well as the differentiation of Tfh and Tfr cells in the draining lymph node. HDM also allows for the differentiation of Tfh13 cells. The potency of HDM can vary widely between lots. Each lot should be tested for in vivo activity.
2. The yield of Tfh and Tfr cells varies depending on age, antigen, and loss during preparation. The typical yield when using an HDM model is about 10,000 TFH and about 5000 TFR cells per mouse. If using an NP-OVA/CFA vaccine, the yield is about 80,000 TFH and about 25,000 TFR cells per mouse.
3. Using a FoxP3 reporter strain is optimal for sorting Tfh and Tfr cells. However, this is not available in some settings (for instance when a knockout is being compared to a WT strain). GITR positivity can be used as a surrogate to FoxP3 to sort TFR cells as in Fig. 1c. However, extreme care must be taken to ensure strict gating strategies to validate purity.

Fig. 1 (continued) 6 and mLN was harvested on day 7 according to protocol. Plots are pregated on singlets and lymphocytes. **(b)** Sorting Tfh and Tfr cells from NP-OVA vaccinated mice. *Foxp3*^{GFP} mice were given an NP-OVA/CFA vaccine on day 0 and dLNs were harvested on day 7 according to protocol. **(c)** Sorting Tfh and Tfr cells from NP-OVA vaccinated mice. WT mice were given an NP-OVA/CFA vaccine on day 0 and dLNs were harvested on day 7 according to protocol. Plots are pregated on singlets and lymphocytes

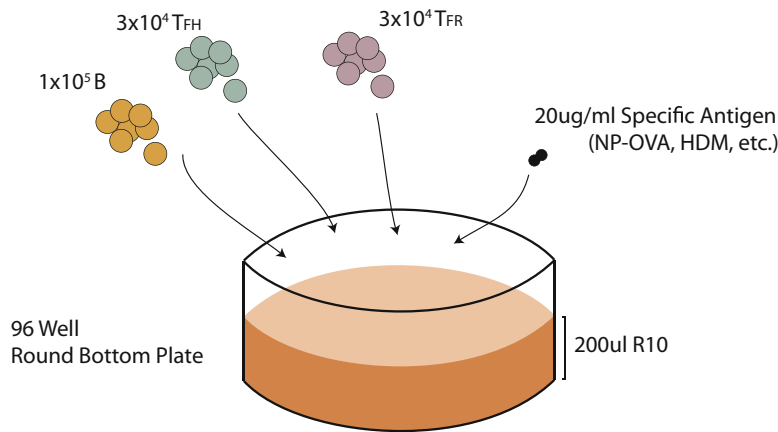
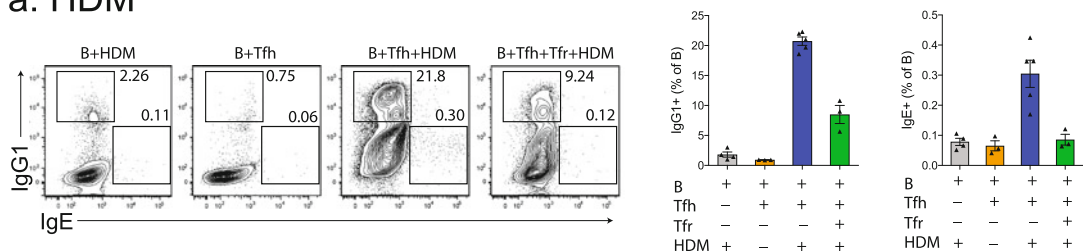


Fig. 2 Schematic of in vitro assay components. Specified components are added in combination to a 96-well U-bottom plate

a. HDM



b. NP-OVA

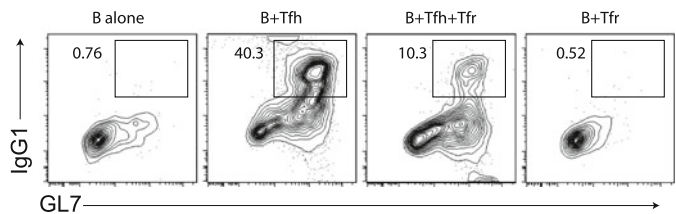


Fig. 3 Typical flow cytometric results from in vitro assays. **(a)** In vitro assay in which B, Tfh, and Tfr cells from dLNs of HDM-challenged mice were sorted and cultured with HDM for 6 days. (left) Assessment of IgG1 versus IgE class switching in B cells. Plots are pregated on CD19+CD4– cells. (right) Quantification of plots. **(b)** In vitro assay in which B, Tfh and Tfr cells from NP-OVA vaccinated mice were sorted and cultured with NP-OVA for 6 days. Plots are pregated on CD19+IA+CD4– cells

4. Round bottom plates facilitate cellular communication and give best results. However, other plates may be used depending on experimental goals. For instance, if imaging is the end readout, flat bottom plates or glass slides with dividers can be used.

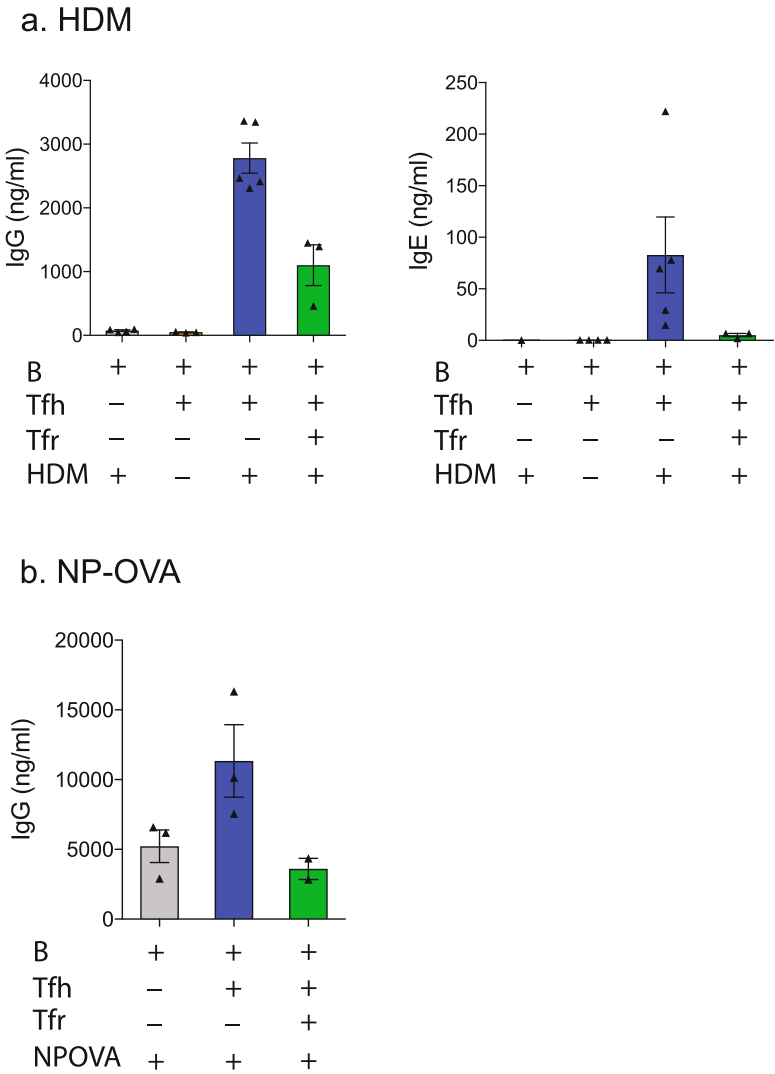


Fig. 4 Typical ELISA results from in vitro assays. **(a)** In vitro assay in which B, Tfh, and Tfr cells from dLNs of HDM-challenged mice were sorted and cultured with HDM for 6 days. (left) Assessment of IgG by ELISA. (right) Assessment of IgE by ELISA. **(b)** In vitro assay in which B, Tfh, and Tfr cells from NP-OVA vaccinated mice were sorted and cultured with NP-OVA for 6 days. Assessment of IgG by ELISA is shown

5. HDM or NP-OVA is added to the wells to activate Tfh cells to mediate the B cell functional assays. Anti-CD3 (clone 2c11) and anti-IgM F(ab')₂ can be used as an alternative to polyclonally activate T and B cells. Although polyclonal stimulation may activate cells more universally, specific antigen tends to give much more robust responses. However, responder frequency of Tfh (and B) cells may vary in different systems which will help dictate B cell responses. When piloting a new antigen

system, it is beneficial to include additional wells with anti-CD3/IgM to separate technical issues with cell sorting versus responder frequency.

6. IgE responses are only typically assessed with HDM systems. For HDM and NP-OVA systems, anti-mouse Ig is used to coat wells. Although anti-mouse Ig will bind IgE, a specific capture antibody for IgE is essential because of low abundance of IgE.
7. The primary readout in these assays is class switching and class switched antibody secretion. However, in some settings antigen-specific antibody is produced, which can be assessed. For instance, HDM-specific IgE can be assessed using an anti-HDM IgE kit following the manufacturer's protocol.
8. HDM challenge schedule can be altered depending on the experimental setup. Challenge every 2 days maximizes the total number of Tfh cells in the dLN at day 7. Extended challenges may enhance Tfh cell numbers, but may compromise the ability of Tfh (and Tfr) cells to expand and perform effector functions in vitro.
9. Time of harvest may be altered depending on experimental question (e.g. functionality of Tfh/Tfr cells in late GC responses). However the in vitro assays were optimized for harvest at the "peak" of Tfh/Tfr responses on day 7.
10. It is critical to keep cells and buffers cold from the time of harvest until plating and to perform steps quickly. Failure to keep cells cold, or delays during sort preparation, will lead to increased cell death and poor effector responses at the end of assays.
11. To sort GC B cells, GL7+Fas+ cells can be sorted.
12. Cell sorting using this protocol can also be used in adoptive transfer experiments to assess Tfh and Tfr function in vivo, or used in downstream genomics analysis.
13. In this protocol we culture an equal amount of Tfh and Tfr cells to assess Tfr suppression. The amount of Tfr cells can be decreased depending on experimental goals. For instance, if enhanced suppressive capacity is to be tested, then suboptimal amounts of Tfr cells (e.g. 1×10^4) may be used.
14. Including control wells without antigen is good practice. However, care must be taken in interpretation of the control well data. Since B cells originate from dLNs it is probable that some B cells may have antigen that can be presented in vitro.
15. Harvesting on day 6 maximizes the amount of secreted antibody while still allowing assessment of class switching by flow cytometry. Depending on experimental goals, cultures may be harvested earlier. For instance, harvesting on day 4 gives a more sensitive analysis of class switching by flow cytometry.

but secreted antibody will be very low. Harvesting the culture after the nutrients in the media are depleted will give erroneous results. Check for yellowing of culture media daily.

16. In addition to B cells, Tfh and/or Tfr cells may be analyzed for altered phenotype by flow cytometry. T cells can be pre-gated on IA-CD19-CD4+ cells, and Tfr cells identified as FoxP3+. However, it is important to note that some Tfr cells will down-regulate FoxP3 to become ex-Tfr cells in these assays and will be indistinguishable from Tfh cells [12]. Use of congenic markers may be utilized if this is a concern.

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