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Corresponding Author:	Meng Wang Institut Pasteur of Shanghai Chinese Academy of Sciences Shanghai, Shanghai CHINA
Corresponding Author's Institution:	Institut Pasteur of Shanghai Chinese Academy of Sciences
Corresponding Author E-Mail:	mwang@ips.ac.cn
Order of Authors:	Meng Wang
	Yuanyuan Huang
	Wangpeng Gu
	Haikun Wang
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1
2 **TITLE:**
3 Evaluation of T Follicular Helper Cells and Germinal Center Response during Influenza A Virus
4 Infection in Mice
5
6 **AUTHORS AND AFFILIATIONS:**
7 Meng Wang[1*], Yuanyuan Huang[1*], Wangpeng Gu[2,3], Haikun Wang[1]
8 [1]CAS Key Laboratory of Molecular Virology and Immunology, Institut Pasteur of Shanghai,
9 Chinese Academy of Sciences; University of Chinese Academy of Sciences, Shanghai, China
10 [2]School of Life Science, University of Science and Technology of China, Hefei, China
11 [3]State Key Laboratory of Cell Biology, CAS Center for Excellence in Molecular Cell Science,
12 Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, University of
13 Chinese Academy of Sciences, Shanghai, China
14 [*]These authors contributed equally to this work.
15
16 **Corresponding Author:**
17 Haikun Wang
18 hkwang@ips.ac.cn
19
20 **Email Addresses of Co-authors:**
21 Meng Wang (mwang@ips.ac.cn)
22 Yuanyuan Huang (yyhuang@ips.ac.cn)
23 Wangpeng Gu (guwangpeng327@hotmail.com)
24
25 **KEYWORDS:**
26 T follicular helper cells, germinal center, influenza A virus infection, Bcl6, tetramer, flow
27 cytometry, enzyme linked immunosorbent assay, immunofluorescence
28
29 **SUMMARY:**
30 This paper describes protocols of evaluating Tfh and GC B response in mouse model of influenza
31 virus infection.
32
33 **ABSTRACT:**
34 T follicular helper (Tfh) cells is an independent CD4$^+$ T cell subset specialized in providing help for
35 germinal center (GC) development and generation of high-affinity antibodies. In influenza virus
36 infection, robust Tfh and GC B cell responses are induced to facilitate effective virus eradication,
37 which confers a qualified mouse model for Tfh-associated study. In this article, we described
38 protocols in detection of basic Tfh-associated immune response during influenza virus infection
39 in mice. These protocols include: intranasal inoculation of influenza virus; flow cytometry staining
40 and analysis of polyclonal and antigen-specific Tfh cells, GC B cells and plasma cells;
41 immunofluorescence detection of GCs; enzyme-linked immunosorbent assay (ELISA) of influenza
42 virus-specific antibody in serum. These assays basically quantify the differentiation and function
43 of Tfh cells in influenza virus infection, thus providing help for studies in elucidating
44 differentiation mechanism and manipulation strategy.

INTRODUCTION:

In the recent decade, numerous studies have been focused on the newly identified CD4$^+$ T cell subset, Tfh cells, for its essential roles in germinal center (GC) B development. B cell lymphoma 6 (Bcl6), which is mainly considered as a gene repressor, is the lineage-defining factor of Tfh cells for the evidence that ectopic expression of Bcl6 is sufficient to drive Tfh differentiation while deficiency of Bcl6 results in vanished Tfh differentiation[1-3]. Unlike other CD4$^+$ T helper subsets performing their effector function by migration to the sites of inflammation, Tfh cells provide the B cells help mainly in the B cell follicular zone of spleen and lymph node. Co-stimulatory signals ICOS and CD40L, play significant roles in the interaction between Tfh and GC B cells. During Tfh differentiation, ICOS could transmit necessary signals from cognate B cells and also acts as receptor receiving migration signals from bystander B cells for B cell zone localization[4,5]. CD40L is a mediator of signals from Tfh cells for B cells proliferation and survival[6]. Another factor playing the similar roles as CD40L is the cytokine IL21, which is mainly secreted by Tfh cells. IL21 could directly regulate GC B cells development and production of high-affinity antibodies, but its role in Tfh differentiation is confusing[7,8]. PD-1 and CXCR5, which are now most frequently used in identifying Tfh cells in flow cytometry analysis, also plays significant roles in the differentiation and function of this subset. CXCR5 is the receptor of B cell follicular chemokine and mediates the localization of Tfh cells in the follicular[9]. PD-1 is now identified to have not only the follicular guidance function but also transmit critical signals in the process of GC B cells affinity maturation[10]. Based on these findings, evaluating the expression of these molecules could basically reflect the maturation and function of Tfh cells.

GC is an induced transient microanatomical structure in secondary lymphoid organs and highly dependent on Tfh cells, thus being a perfect readout to evaluate Tfh response. In GC, after receiving signals mediated by cytokines and co-stimulatory molecules, B cells are subject to class switch and somatic hypermutation to generate high-affinity antibodies[11]. Differential antibody class switches occur in differential cytokine niche, in which IL4 and IL21 induce IgG1 class switch while IFNγ induce IgG2 class switch[12]. Plasma cells are the producers of secreted antibodies and are terminally differentiated cells. Like Tfh cells, development of B cells in GC is associated with dynamic expression of many significant molecules. Based on the current study, GC B cells could be identified as B220$^+$PNA$^+$Fas$^+$ or B220$^+$GL7$^+$Fas$^+$ cells and plasma cells, compared to their precursors, downregulate expression of B220 and upregulate CD138 expression[13]. What is more, both characteristics could be detected in flow cytometry and immunofluorescence analysis, thus being appropriate evaluation of GC response.

Robust cellular and humoral response are induced in influenza virus infection, with Tfh and Th1 cells dominating CD4 T cells response[14], which makes it a perfect model for Tfh cells differentiation study. Influenza A/Puerto Rico/8/34 H1N1(PR8), which is commonly used mouse-adapted strain, is frequently used in this study[14-16]. Here, we describe some basic protocols of Tfh study-relevant assay in influenza virus infection: 1) intranasal inoculation of PR8 virus; 2) antigen-specific Tfh cells, GC and plasma B cells and IL21 detection with flow cytometry; 3) histological visualization of GC; 4) detection of antigen-specific antibody titer in serum with ELISA. These protocols could provide the necessary techniques for new researchers in Tfh-associated study.

89
90 **PROTOCOL:**
91 Animal experiments were approved by the Institutional Animal Care and Use Committee of
92 Institut Pasteur of Shanghai, China. All experiments were performed based on the Institutional
93 Animal Care and Use Committee-approved animal protocols.
94
95 NOTE: Virus infection of mice and isolation of organs should be performed under ABSL2 condition.
96
97 **1. Inoculation of PR8 influenza virus and recording of mice weight**
98
99 1.1. Prepare 8-week-old male C57BL/6 mice for infection at ABSL2 room.
100
101 NOTE: This protocol is also suitable in experiments with female mice.
102
103 1.2. **Dilution of PR8 virus**: take out the virus from the -80 °C freezer and incubate on ice until
104 it melts into liquid. Vortex the stock virus thoroughly and dilute the virus to 2 PFU/µL with sterile
105 phosphate-buffered saline (PBS, 135 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) in
106 a pre-chilled 1.5 mL tube.
107
108 1.3. **Mice anesthetization:** weigh each mouse and calculate the volume (4-fold (µL) the mouse
109 weight(g)) of sodium pentobarbital (2 mg/mL) to be used. Inject the calculated volume of sodium
110 pentobarbital intraperitoneally.
111
112 NOTE: This step is to make mice breathe steadily and peacefully, so that accurate titer of virus
113 could be inoculated intranasally. Too fast or slow heartbeat indicate inappropriate
114 anesthetization. In addition, the use of vet ointment is recommended to avoid eye dryness.
115
116 1.4. **Intranasal Inoculation**: vortex the diluted PR8 virus thoroughly. Pipet 10 µL and carefully
117 perform intranasal inoculation on one side drop by drop. After finishing inoculation of all mice in
118 one cage (maximum 5 mice) on this side, repeat inoculation on the other side (keep the breathing
119 of mouse peaceful and steady all through the inoculation). Infect each mouse with 40 PFU of PR8
120 virus in total.
121
122 1.5. Place the mice in sternal recumbency in warm cages for better revival.
123
124 1.6. Monitor the mouse weight daily for 10 days. (The infection day is recorded as Day 0).
125
126 **2. Isolation of lymphocytes from spleen and mediastinal lymph nodes (mLN)**
127
128 2.1. **Mouse euthanization**: Put the mice in a small chamber and euthanize the mice by
129 pumping into CO_2 peacefully from the bottom of the chamber. Take the mice out when they do
130 not move and perform cervical dislocation to ensure mice die completely. Dip the mice with 75%
131 ethanol and transfer to the biosafety hood.
132

2.2. Immobilize the mice with dissection needles onto the absorbent paper-covered dissection foam plate. Cut the skin along the abdominal midline and the hind legs with dissection scissors and stretch the skin with tweezers. Immobilize the stretched skin with dissection needles.

2.3. Prepare two 6 cm dishes for each mouse and keep them on the ice. Put the 70-µm cell strainer in each dish and add 5 mL of DMEM supplemented with 1% fetal bovine serum (DMEM (1% FBS)).

2.4. Spleen isolation: Cut the peritoneum to expose the abdominal cavity with dissection scissors. Take the spleen and put it in the prepared dish.

2.5. mLN isolation: Cut the diaphragma and the bottom of the cage rib to the vicinity of thymus. Pull the rib aside and pin it with dissection needles to expose the Thoracic cavity. Pull the lung aside to the right and use tweezers to take mLN, underneath the heart and near the ventral side of the trachea.

2.6. Put the mLN in the prepared dish.

2.7. Obtain the single cell suspensions: Mesh the spleen or LN gently with the plunger of a 3 mL syringe through the 70-µm cell strainer. Rinse the cell strainer with 1 mL of fresh DMEM (1% FBS). Resuspend the cell suspension and transfer to a 15ml centrifuge tube.

2.8. Centrifuge the cell suspension at 350 x *g* for 6 min at 4 °C. Remove the supernatant and add 1 mL of DMEM (1% FBS).

2.9. Resuspend the cell pellet with a 1 mL-pipette thoroughly. Add 4 mL of DMEM (1% FBS) into the spleen cells suspension and keep them on ice for the following operations.

NOTE: It is necessary to resuspend the cell pellet with 1 mL of medium firstly, not 5 mL, for completely isolating single cells from pellet.

From this step onward, all the operations could be performed in the regular lab.

2.10. Spleen cell counting

2.10.1. Resuspend the cells by turning the tubes up and down for several times. Take 10 µL into 90 µL of red blood cell (RBC) lysis buffer (10 mM Tris-HCl pH 7.5, 155 mM NH_4Cl). Incubate at room temperature (RT) for 3 min and add 900 µL of cold PBS to stop the reaction.

2.10.2. Centrifuge at 400 x *g* for 6 min at 4 °C and remove the supernatant. Resuspend with 100 µL of cold PBS. Take 10 µL of cells into 10 µL of 0.4% w/v Typan Blue and take 10 µL out of the mixture for cell counting with the hemocytometer.

2.10.3. Calculation: Calculate cells as regular method. In brief, count cell numbers in two diagonal

corner squares on the hemocytometer and get N1, N2 for each corner square. The cell concentration of the 5-mL cell suspension should be calculated as (N1+N2)/2 x 10^4/mL.

3. Immunostaining of Polyclonal Tfh cells with PD-1 and CXCR5

3.1. Staining with biotin-anti-CXCR5 antibody

3.1.1. Resuspend the cell suspensions by turning the tube up and down. Take 2 x 10^6 cells into the FACS tube and add 2 mL of staining buffer (PBS (1% FBS, 1 mM EDTA)). Wash by vortexing on the vortex oscillation device.

3.1.2. Centrifuge at 350 x *g* for 6 min at 4 °C. Discard the supernatant by pulling out the liquid and dip the tube mouth on the absorbent paper twice.

3.1.3. Loosen the cell pellet with the residue liquid by tapping the bottom of tube. Put the tube in the tube holder on ice.

NOTE: The volume of residue liquid is approximately 25 µL.

3.1.4. Add 0.2 µL of anti-mouse CD16/CD32 (Fc-receptor blocker) for each tube. Vortex by tapping the tube bottom gently and incubated on ice for 10 min.

NOTE: Prepare antibody mixture for multiple samples by dilution with 5 µL of staining buffer for each tube. The recipe for mixture should be prepared by dilute (n/10+1) x 0.2 µL Fc-receptor blocker into (n/10+1) x 5 µL staining buffer and add 5.2 µL mixture into each tube.

3.1.5. Add 0.3 µL of biotin-anti mouse CXCR5 into the residue 30 µL of staining buffer for each tube and vortex by tapping the tube bottom.

NOTE: Prepare mixture as described in step 3.1.4.

3.1.6. Incubate on ice for 1 h with gently resuspending cells by tapping the tube at 30 min.

NOTE: Vortex at 30 min to avoid cell aggregates for better staining.

3.1.7. Add 2 mL of staining buffer and vortex on the vortex oscillation device. Centrifuge at 350 x *g* for 6 min at 4 °C and discard the supernatant as described in step 3.1.2. Vortex by tapping the tube and incubate on ice for subsequent staining.

3.2. Staining with other surface markers

3.2.1. Prepare antibody mixture (**Table 1**) as described in step 3.1.4.

3.2.2. Add antibody mixture into each tube. Vortex by tapping the tube bottom and incubate on

221 ice for 30 min.
222
223 3.2.3. Wash the cells with 2 mL of staining buffer. Centrifuge at 350 x *g* for 6 min at 4 °C.
224
225 3.2.4. Discard the supernatant and add 400 µL of staining buffer. Vortex the tube on the vortex
226 oscillation device and keep the tube in dark till flow cytometry analysis.
227
228 **4. Immunostaining of PR8 influenza virus NP-specific Tfh cells**
229
230 NOTE: This protocol of staining NP-specific Tfh cells is from previous studies[15,17].
231
232 4.1. Perform biotin-CXCR5 staining as described in step 3.1 except that the cell number taken
233 for staining is 3×10^6 for enough antigen-specific cells to be recorded in flow cytometry.
234
235 4.2. Add 0.3 µL of APC-conjugated-IAbNP311-325 MHC class II ($NP_{311-325}$) tetramer into the
236 tube from step 3.1.7. Prepare mixture for multiple samples as in step 3.1.4
237
238 NOTE: It is important to stain Tetramer before addition of anti-CD4 antibody as the binding
239 between CD4 and anti-CD4 antibody would interfere the optimal tetramer staining.
240
241 4.3. Resuspend the cell mixture by gently tapping the tube and incubate in dark at room RT
242 for 30 min.
243
244 NOTE: Cover a wet paper on the mouth of tubes to decrease evaporation
245
246 4.4. Add other surface markers mixture (**Table 1**) and continue incubation at RT for 30 min.
247
248 4.5. Wash and resuspend cells as described in steps 3.2.3 and 3.2.4.
249
250 **5. Immunostaining of Polyclonal Tfh cells with Bcl6**
251
252 5.1. Perform surface markers (**Table 2**) staining as described in section 3 except that the last
253 wash with 2 mL of PBS, instead of staining buffer.
254
255 5.2. Centrifuge at 350 x *g* for 6 min at 4 °C. Discard the supernatant and resuspend cell pellets
256 by gently tapping the tube bottom.
257
258 5.3. Add 300 µL of 3.7% formaldehyde solution (diluted from 37% formaldehyde with PBS)
259 into the tube for cell fixation. Vortex on the vortex oscillation device and incubate at RT for 15
260 min.
261
262 5.4. Add 2 mL of staining buffer for wash and centrifuge at 500 x *g* for 6 min at 4 °C. Discard
263 the supernatant and resuspend cells by gently tapping the tube.
264

5.5. Add 300 µL of 0.2% Triton-X 100 and resuspend cells by vortex on the vortex oscillation device. Incubate at RT for 15 min.

5.6. Add 2 mL of staining buffer for wash. Centrifuge at 500 x *g* for 6 min at 4 °C. Discard the supernatant and resuspend the cells by gently tapping the tube bottom.

5.7. Add 1.5 µL of PE-anti-Bcl6 antibody for each tube. Gently tap the tube bottom to resuspend the mixture and incubate at RT for 2 h with gently tapping the tube every 30 min.

NOTE: Cover a wet paper on the mouth of tubes to decrease mixture evaporation.

5.8. Add 2 mL of PBS supplemented with 0.01% Triton-X 100 into the tube. Vortex and centrifuge at 500 x *g* for 6 min at 4 °C.

5.9. Repeat washing as step 5.8. Resuspend the cells with 400 µL of staining buffer. Incubate the cells in dark on ice till the flow cytometry analysis.

6. Intracellular staining of IL21

6.1. Stimulating cells with PMA (phorbol 12-myristate 13-acetate) and ionomycin

6.1.1. Take 2 x 10^6 cells from spleen cells suspension and centrifuge at 350 x *g* for 6 min at 4 °C. Discard the supernatant and resuspend the cell pellet with 500 µL of complete T cell medium. Transfer the cells into the 24-well plate.

6.1.2. Add 20 nmol PMA and 2 µmol ionomycin into 500 µL of complete medium[18] and mix thoroughly by pipetting up and down.

6.1.3. Add solution prepared in step 6.2 into cells in the 24-well plate and mix by shaking the plate to stimulate cells. Set up the unstimulated control by adding 500 µL of complete T cell medium without addition of PMA and ionomycin into the cells. Incubate at 37 °C in a CO_2 cell incubator for 4 h.

6.1.4. Add 10 µmol BFA (Brefeldin A, dissolved with methanol) into each well to block the Golgi apparatus mediated protein transport. Put the plate back to the cell incubator and incubate for 2 h.

6.2. Performing cell surface marker staining

6.2.1. Resuspend the cells by gently pipetting up and down and transfer the cells into the FACS tube. Add 1 mL of staining buffer into the tube and centrifuge at 350 x *g* for 6 min at 4 °C.

6.2.2. Perform Fc receptor block staining as step 3.1.4.

309 6.2.3. Perform cell surface markers staining (**Table 3**) as described in steps 3.2.1-3.2.3 except
310 washing cells with 2 mL of PBS.
311
312 6.2.4. Centrifuge at 350 x *g* for 6 min at 4 °C. Discard the supernatant and resuspend cells by
313 tapping the tube bottom.
314
315 6.3. Add 0.2 µL of reagent from the Live/Dead Fixable Aqua Dead Cell staining kit and incubate
316 the tube in dark at RT for 10 min to perform the staining of dead cells.
317
318 6.4. Add 2 mL of PBS into the tube and vortex on the vortex oscillation device. Centrifuge at
319 350 x *g* for 6 min at 4 °C and discard the supernatant.
320
321 6.5. Perform the cell fixation as described in steps 5.3 and 5.4.
322
323 6.6. Add 300 µL of staining buffer to resuspend the cells and store the tubes in the 4 °C
324 refrigerator overnight. Centrifuge at 500 x *g* for 6 min at 4 °C to remove the supernatant.
325
326 NOTE: This step could be omitted and continue to step 6.7 directly following step 6.5.
327
328 6.7. Add 1 mL of saponin buffer (staining buffer supplemented with 0.2%(w/v) saponin) into
329 the tube and vortex on the vortex oscillation device. Incubate on ice for 20 min to perform cell
330 permeabilization.
331
332 6.8. Centrifuge at 500 x *g* for 6 min at 4 °C and discard supernatant.
333
334 6.9. Add 0.5 µL of human Fc-IL21 receptor into each tube. Prepare antibody mixture for
335 multiple as step 3.1.4 except that dilute antibody with saponin buffer instead of staining buffer.
336
337 6.10. Incubate at RT for 1 h with gently tapping the tube bottom to resuspend cells at 30 min.
338
339 6.11. Add 2 mL of saponin buffer to wash cells and centrifuge at 500 x *g* for 6 min. Discard the
340 supernatant and repeat washing once.
341
342 6.12. Add 0.1 µL of APC-anti-human Ig(H+L) into each tube. Prepare mixture for multiple
343 samples as step 3.1.4 except that dilute antibody with saponin buffer instead of staining buffer.
344
345 6.13. Incubate the samples on ice for 30 min and wash as step 6.11.
346
347 6.14. Resuspend the cells with 400 µL of staining buffer. Keep the sample in dark on ice till the
348 flow cytometry analysis.
349
350 **7. GC B and plasma cells staining**
351
352 7.1. Take the cells and perform anti-Fc-receptor antibody staining as steps from 3.1.1-3.1.4.

353
354 7.2. Perform surface markers staining (**Table 4**) as steps from 3.1.5-3.1.7 except that the
355 incubation time is 30 min instead of 1 h.
356
357 7.3. Resuspend the cells with 400 µL of staining buffer. Keep in dark on ice till the flow
358 cytometry analysis.
359
360 **8. Isolation of serum from blood**
361
362 8.1. On day 14 post-infection (d.p.i 14), collect the blood from facial vein and incubate them
363 in a 4 °C refrigerator overnight.
364
365 NOTE: Perform blood collection at ABSL2 condition and from this step onward all the procedures
366 could be performed in the regular lab.
367
368 8.2. Centrifuge the blood at 400 x *g* for 10 min at 4 °C. Isolate the serum with the 200 µL
369 pipette carefully to avoid pollution of red cells. Divide into 3 vials for each sample and store them
370 at -80 °C.
371
372 **9. Assay of HA-specific antibody titer with ELISA**
373
374 9.1. Coat ELISA plates with 50 µL of 2 µg/mL HA protein solution per well and incubate them
375 in the 4 °C refrigerator overnight.
376
377 9.2. Wash three times with 200 µL of PBS-diluted 0.05% tween (PBST). Add 100 µL of PBST-
378 diluted 5% skimmed milk into each well and incubate at RT for 2 h to block the nonspecific
379 binding.
380
381 9.3. Serum dilution and incubation: Prepare 3% BSA in PBS as the dilution buffer. Dilute the
382 serum in dilution buffer as 1:50, 1:150, 1:450, ⋯⋯ to 1:36450 (3-fold serial dilution is
383 recommended). Add 50 µL of diluted serum to each well and incubate in the 4 °C refrigerator
384 overnight.
385
386 9.4. Discard the serum and quickly wash the wells once by adding 200 µL of PBST into each
387 well (shake it softly, then discard). Then slowly wash the plates on shaker with 200 µL of PBST
388 each time for 5 min, three times in total.
389
390 9.5. Add 100 µL of HRP-labeled detection antibody Ig, IgM, IgG1, IgG2b, IgG2c (1:5000, diluted
391 with PBST) and incubate at RT for 1 h. Wash the plates by PBST as described in step 9.4.
392
393 9.6. Take out equal volume of Buffer A and Buffer B (TMB) for at least 30 min at RT before use.
394 Mix A and B and add 100 µL of TMB into each well and incubate them for 10-30 min at RT by
395 shaking softly.
396

NOTE: This is a brief description of the TMB Substrate Reagent Set manual.

9.7. Pipette 100 µL of 2M H_2SO_4 into each well to terminate the reaction. Read the OD450 value through instrument.

9.8. Data Analysis: Get the final OD450 value by subtracting the background signal (OD450 value of empty well). Draw the curve corresponding to an antibody isotype of each sample with the dilution factor on the X axis and the OD450 value on the Y axis.

10. Histology

10.1. Isolate the spleens at d.p.i 10. Fix them in 3.7% formaldehyde solution for 1 h at RT. Discard the fixation buffer and wash with PBS for 5 min on the shaker for three times.

10.2. Dehydrate the spleens in PBS (10% sucrose) at 4 °C for 1 h and then dehydrate them in PBS (30% sucrose) at 4 °C with shaking softly until the spleens sink to the bottom of the 15 mL tube.

10.3. Take out the dehydrated spleens and dry completely. Embed them in optimum cutting temperature compound and cryosectioned.

10.4. Pre-chill the acetone at -20 °C. Incubate the tissue sections with pre-chilled acetone for 10 min. Wash the tissue with PBS for three times.

10.5. Permeabilize the tissue sections with PBS (0.2% Triton X-100) for 20 min and wash the them for three times with PBS.

10.6. Block the non-specific binding with PBS (10% normal goat serum) (block buffer) for 1 h at RT and wash the tissue sections with PBS once.

10.7. Block the non-specific binding with STREPTAVIDIN/BIOTIN blocking kit.

NOTE: Do not let the samples dry from this step onward.

10.8. Staining with primary antibody: Add block buffer-diluted biotin-PNA (25 µg/mL) and rat anti-mouse IgD (2.5 µg/mL) onto the tissue sections carefully. Incubate the tissue sections in the wet chamber in the 4 °C freezer overnight.

10.9. Quickly wash the tissue sections by PBST once. Quickly wash the tissue sections in the PBST with shaking slowly for 5 min. Repeat wash for three times.

10.10. Dilute Alexa Fluor 488-streptavidin (1:500) and Alexa Fluor 555-Goat-anti rat IgG (1:500) antibodies with block buffer and add them onto the tissue sections carefully.

441 10.11. Incubate at RT for 1 h.
442
443 10.12. Wash the tissue sections as step 10.8 and carefully mount the prolong solution. Cover the
444 tissue with coverslips carefully and keep them in dark at 4 °C until confocal analysis.
445
446 10.13. Analyze the magnitude of GC reaction by count the GC numbers per area size.
447
448 **REPRESENTATIVE RESULTS:**
449 **Characterization of mouse morbidity in influenza virus infection**
450 After influenza virus infection, mice will be less active and anorexic due to illness, which will be
451 reflected by severe weight loss, a commonly used symptom to monitor the mouse morbidity[19].
452 As shown in **Figure 1a**, PR8 virus-infected mice started to lose weight on day 6, reached to the
453 highest loss level on day 8 and returned to the initial level on day 10. As expected, weight loss
454 was not observed all through the period in PBS-treated control mice. For in vivo symptoms, virus
455 infection leads to robust lymphocytes expansion in the draining lymph node, mLN in this case.
456 Therefore, significantly larger size of mLNs were observed in PR8 virus-infected mice than in
457 control mice (**Figure 1b**). Taken together, these mice all showed expected symptoms and are
458 qualified for the subsequent Tfh-associated immune response study.
459
460 **Detection of Tfh differentiation and function-associated molecules**
461 To analyze Tfh differentiation, mice were sacrificed on day 5, 7,10 and 14 after infection and
462 mLNs or spleens were isolated for flow cytometry analysis. **Figure 2a** and **Figure 2b** show the Tfh
463 population gating strategy, with Tfh gated as PD-1^{hi} $CXCR5^{hi}$ cells and non-Tfh as PD-$1^{low}CXCR5^{low}$
464 cells. With this gating strategy, the kinetics of Tfh differentiation during influenza virus infection
465 were assayed. As shown in **Figure 2c,** Tfh differentiation initialized at day 5 and peaked at day 10.
466 So, we took samples of day 10 for further analysis. As shown in **Figure 3a**, robust Tfh cells were
467 induced in influenza virus-infected mice compared with control mice. To analyze Influenza virus-
468 specific Tfh cells, fluorochrome-labeled IAbNP311–325 MHC class II tetramers ($NP_{311-325}$) were
469 added in the polyclonal Tfh cells staining panel (**Table 1**). Both in mLNs and spleens, $NP_{311-325}$-
470 speciifc $CD4^+$ T cells were induced significantly compared with in control mice and $NP_{311-325}$-
471 speciifc Tfh cells could be analyzed by addition of PD-1 and CXCR5 into analysis (**Figure 3e**).
472 Because of essential roles of Bcl6 in Tfh differentiation, $Bcl6^+CXCR5^+$ could also be represented
473 as Tfh population. Consistently, Tfh cells identified with this strategy were also induced robustly
474 (**Figure 3b**). We further analyzed expression of Bcl6 in Tfh and non-Tfh cells. As shown in **Figure**
475 **3c**, higher expression of Bcl6 in Tfh cells than that in non-Tfh cells indicates successful Bcl6
476 staining. With similar strategy, ICOS, the other Tfh-associated molecules were also analyzed
477 (**Figure 3d**). Due to the specialized role of Tfh cells in providing B cells help, assay of IL21
478 expression, which is secreted mainly by Tfh cells and demonstrated to directly regulate B cells
479 survival and proliferation, could reveal Tfh cells function to some extent. As shown in (**Figure 3f)**,
480 intracellular staining of IL21 revealed that PRB infection induced significantly higher production
481 of this cytokine, with unstimulated cells as gating control. Taken together, these assays could
482 reflect basic information of Tfh differentiation and provide the insights into the B cell-help ability.
483
484 **Detection of GC B and plasma B cells development and influenza virus-specific antibodies in**

485 **serum**
486 The main function of Tfh cells is to provide B cell help in GCs, in which antibody class switch and
487 affinity maturation occurs. So, GC B development could indirectly reflect differentiation and
488 function of Tfh cells. GC B cells could be gated as $B220^+PNA^+Fas^+$ cells (**Figure 2d**). Through this
489 gating strategy, we assayed the kinetics of GC B cell response and found that GC B response
490 started at day 10 and continue to increase at day 14 (**Figure 2e**). Comparison between PR8 virus-
491 infected and control mice showed robust GC B were induced both in mLN and spleen after
492 influenza virus infection (**Figure 4a**), which is consistent with the induced Tfh differentiation in
493 PRB virus-infected mice. In addition, immunofluorescence staining with IgD and PNA provides
494 visualized images indicating induced GC reaction (green areas) in PR8 virus-infected mice (**Figure
495 4d**). Plasma cells, identified as $IgD^{low}CD138^+$ cells (**Figure 2c**), are also generated in PR8 virus-
496 infected mice (**Figure 3b**). Previous studies have identified that IFNY and IL21 could be secreted
497 from both Th1 and Tfh cells in virus infection and induce IgG2 and IgG1 class switch, respectively[20].
498 **Figure 4c** depicts the generation of influenza virus-specific antibody by ELISA assay of HA-specific
499 IgM, total IgG, IgG1, IgG2b and IgG2C. Together, all of these assays reflect the Tfh-associated B
500 cell responses in influenza virus infection.
501
502 **FIGURE AND TABLE LEGENDS:**
503 **Figure 1: Characterization of mouse morbidity.** 8-week-old male mice were infected with 40 PFU
504 of PR8 influenza virus by intranasal inoculation. Mice were weighed daily for 10 days (**a**) and mLNs
505 were isolated on d.p.i 10 (**b**). The error bars in (**a**) represent the mean ± SD. n = 4 mice per group.
506
507 **Figure 2. Gating strategy of Tfh cells and GC B cells.** (**a**) Lymphocytes are defined by FSC-A and
508 SSC-A, and cell singlets are gated with FSC-A, FSC-H and SSC-A, SSC-W. (**b**) After gating in $CD4^+$ T
509 cells, surface markers CD62L and CD44 are used to distinguish the naïve T cells ($CD44^{lo}CD62L^{hi}$)
510 and activated T cells ($CD44^{hi}CD62L^{lo}$). Polyclonal Tfh cells can be gated from activated T cells as
511 $PD\text{-}1^{hi}$ $CXCR5^{hi}$population, conversely, non-Tfh cells as $PD\text{-}1^{low}CXCR5^{low}$. PR8 virus-specific Tfh
512 cells are defined as $CD4^+CD44^+$ $NP_{311\text{-}325}$ $tetramer^+PD\text{-}1^{hi}$ $CXCR5^{hi}$ cells. (**c**,**e**) Kinetics of Tfh
513 frequency in activated cells (**c**) and GC B frequency in $B220^+$ cells (**e**). (**d**) GC B cells are gated as
514 $B220^+$ PNA^+FAS^+ cells, and plasma cells are IgD^-CD138^+ cells.
515
516 **Figure 3. Analysis of Tfh differentiation in PR8 virus-infected mice.** Mice were sacrificed on d.p.i
517 10 and mLNs and spleens were isolated for Tfh differentiation analysis. (**a**) Tfh percentage in
518 mLNs and spleens in PR8 virus-infected mice and PBS-treated mice (upper panel). The statistics
519 of Tfh cells (lower panel). (**b**) The intracellular staining of $Bcl6^+CXCR5^+$ cells (upper panel). The
520 statistics of $Bcl6^+CXCR5^+$ cells (lower panel). (**c**) Bcl6 and (**d**) ICOS expression in Tfh (line-red) and
521 non-Tfh cells (solid-gray). (**e**) Gating of $NP_{311\text{-}325}$-specific $CD4^+$ T cells in mLNs and spleens of PR8
522 virus-infected and PBS-treated mice (left panel). The percentage of PR8 virus specific Tfh cells in
523 mLNs and spleens (middle panel). “Isotype” indicates staining with irrelevant tetramer control.
524 The statistics of $NP_{311\text{-}325}$-specific $CD4^+$ T cells (right panel). (**f**) Intracellular staining of IL-21 in
525 spleens from PR8 virus-infected and PBS-treated mice, the unstimulated shown as control (left).
526 The statistics of IL-21 staining (right). **P < 0.01, ***P < 0.001 and **** P < 0.0001 (two-tailed
527 Student’s t-test). The error bars represent the mean ± SD. n = 3 mice per group.
528

529 **Figure 4. Analysis of GC B cell-associated response in PR8 virus-infected mice.** Mice were
530 sacrificed on d.p.i 10 and the mLNs and spleens were isolated for analysis. (**a**) The percentage of
531 GC B cells (upper panel). The statistics of GC B cells (lower panel). (**b**) The percentage of plasma
532 cells (upper panel). The statistics of plasma cells (lower panel). (**c**) Quantification of PR8 virus HA-
533 specific Ig, IgM, IgG1, IgG2b and IgG2c in the serum (d.p.i 14) of PR8 virus-infected mice and PBS-
534 treated mice. (**d**) Confocal microscopy of B cell follicles (IgD^+, Red) and GCs (PNA^+, Green) in the
535 spleen samples of PR8 virus-infected mice and PBS-treated mice (d.p.i 10). *P < 0.5, **P < 0.01,
536 and ***P < 0.001 (two-tailed Student's t-test). The error bars represent the mean ± SD. n = 3 mice
537 per group.
538
539 **Table 1. Surface marker (except for CXCR5) antibodies panel for staining Tfh cells (PD-**
540 **$1^{hi}CXCR5^{hi}$).**
541
542 **Table 2: Surface marker antibodies (except for CXCR5) panel for staining Bcl6 in Tfh cells.**
543
544 **Table 3: Surface marker antibodies panel for intracellular staining of IL21.**
545
546 **Table 4: Surface marker antibodies panel for staining GC B and plasma B cells**
547
548 **DISCUSSION:**
549 Due to specialized roles in providing B-cell help for generating high-affinity antibodies, Tfh cells
550 have been extensively studied in the mechanisms of differentiation and manipulation to provide
551 new strategies for vaccine design. Influenza virus infection induced vigorous Tfh and GC B cells
552 response, thus being an appropriate model for this field of research. In this article, we describe
553 protocols of influenza virus infection by intranasal inoculation, evaluation of Tfh-associated
554 response by flow cytometry, immunofluorescence and ELISA. These assays will facilitate
555 detection of Tfh differentiation, GC B development and influenza virus-specific antibodies and
556 help researchers explore and identify new crucial molecules in the immune response.
557
558 In studies with influenza infection mice models, weight loss is a commonly used indicator of
559 mouse morbidity. The expected weight change kinetics in influenza-infected mice is as described
560 in **Figure 1a**, which reflects the appropriate immune response induced in the mice. However,
561 abnormal cases would regularly occur, in which the mice lose their weight or do not show any
562 weight decline all through the observation period. According to our experiences, these mice
563 would mostly bear abnormal lower or higher immune response, thus disrupting the experiment
564 results. To avoid such variations, mice used in the experiment should be sex and age-matched to
565 guarantee the similar responsive ability to virus. Consistent virus titer infected by each mouse is
566 also important[21]. The virus titer used in this protocol is 40 PFU. However, the virus titer to induce
567 appropriate weight change kinetics in each lab could be variable due to the inconsistency in virus
568 titer evaluation procedure and mouse strains used in the experiment. So, titration of virus titer
569 for infection is necessary before immune response-relevant study.
570
571 In this protocol, we identified Tfh cells with frequently used markers PD-1, CXCR5 and the
572 essential transcription factor Bcl6. Although both PD-$1^{hi}CXCR5^{hi}$ and $Bcl6^+CXCR5^+$ cells could be

573 denoted as Tfh cells, they represent different population and do not have the precursor-progeny
574 relationship based on the fact that not all the PD-1^{hi}CXCR5^{hi} cells are $Bcl6^{+}$ and not all the
575 $Bcl6^{+}$CXCR5^{hi} cells are PD-1^{hi}CXCR5^{hi}. This phenotype could be explained by the heterogeneity of
576 Bcl6 expression in Tfh cells[22]. ICOS, a critical molecule for both Tfh differentiation and migration
577 should also be included in analysis of Tfh differentiation. In addition, other function-associated
578 co-stimulatory molecules, such as OX40 and CD40L should also be detected for their expression
579 level, though not contained in this protocol. IL21 and IL4 are both Tfh-secreted cytokines playing
580 roles in inducing IgG1 class switch. Protocols of detecting IL21 expression is described in this
581 paper. However, due to the difficulty in detection of IL4 in Tfh cells, IL4 GFP reporter mice were
582 used in previous studies[23]. In this protocol, we also used fluorochrome-labeled NP tetramers to
583 detect $NP_{311-325}$-specific Tfh cells. Nevertheless, the limit in the amount of $NP_{311-325}$-specific Tfh
584 cells confers the difficulty in further analysis. Therefore, adoptive transfer experiment of
585 influenza hemagglutinin specific-TCR transgenic (Tg) $CD4^{+}$ (TS-1) T cells, which could be isolated
586 from TS-1 mice, is an alternative strategy in solving this problem[24].
587
588 Here, we identified GC B as $B220^{+}PNA^{+}Fas^{+}$ cells in flow cytometry staining. An alternative
589 markers combination strategy to define GC B as $GL7^{hi}Fas^{hi}$ cells is also used in other papers[14,16].
590 We also use immunofluorescence to visualize GCs with combination of anti-IgD and PNA. Herein
591 addition of CD3 antibody could help visualize Tfh cells, thus enabling study of the interaction
592 between these two cell types[10].
593
594 Differentiation of Tfh cells is a multistage and multifactorial process, additional assay of other
595 significant molecules at multiple time point is necessary to elucidate more detailed mechanism
596 in Tfh differentiation. In addition, parameters detected here is also commonly used in other
597 models[18]. Therefore, besides in influenza virus infection, protocols described here, especially the
598 immunostaining part, could also provide instructions in Tfh-associated study with other models.
599
600 **ACKNOWLEDGMENTS:**
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604 National Key R&D Program of China (2016YFA0502202), the National Natural Science Foundation
605 of China (31570886).
606
607 **DISCLOSURES:**
608 The authors have nothing to disclose.
609
610 **REFERENCES:**
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612 follicular helper cell differentiation. *Science.* **325** (5943), 1006-1010, (2009).
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665

Figure 1

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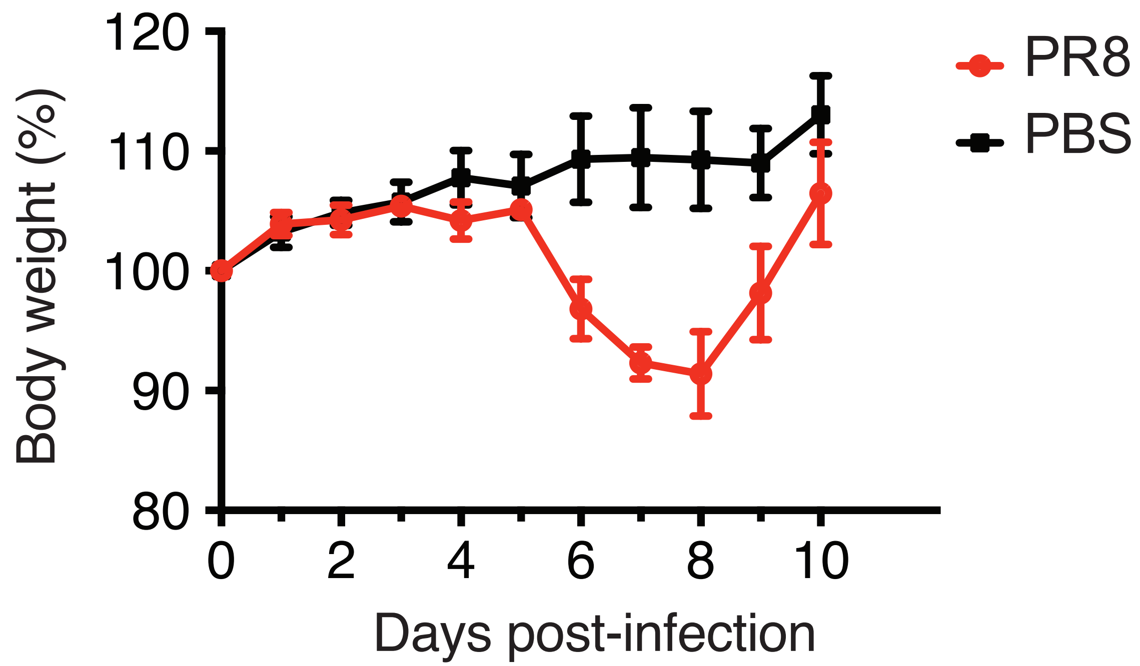
Figure 1



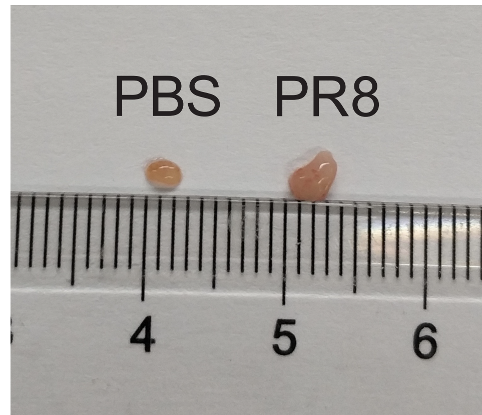


Figure 2

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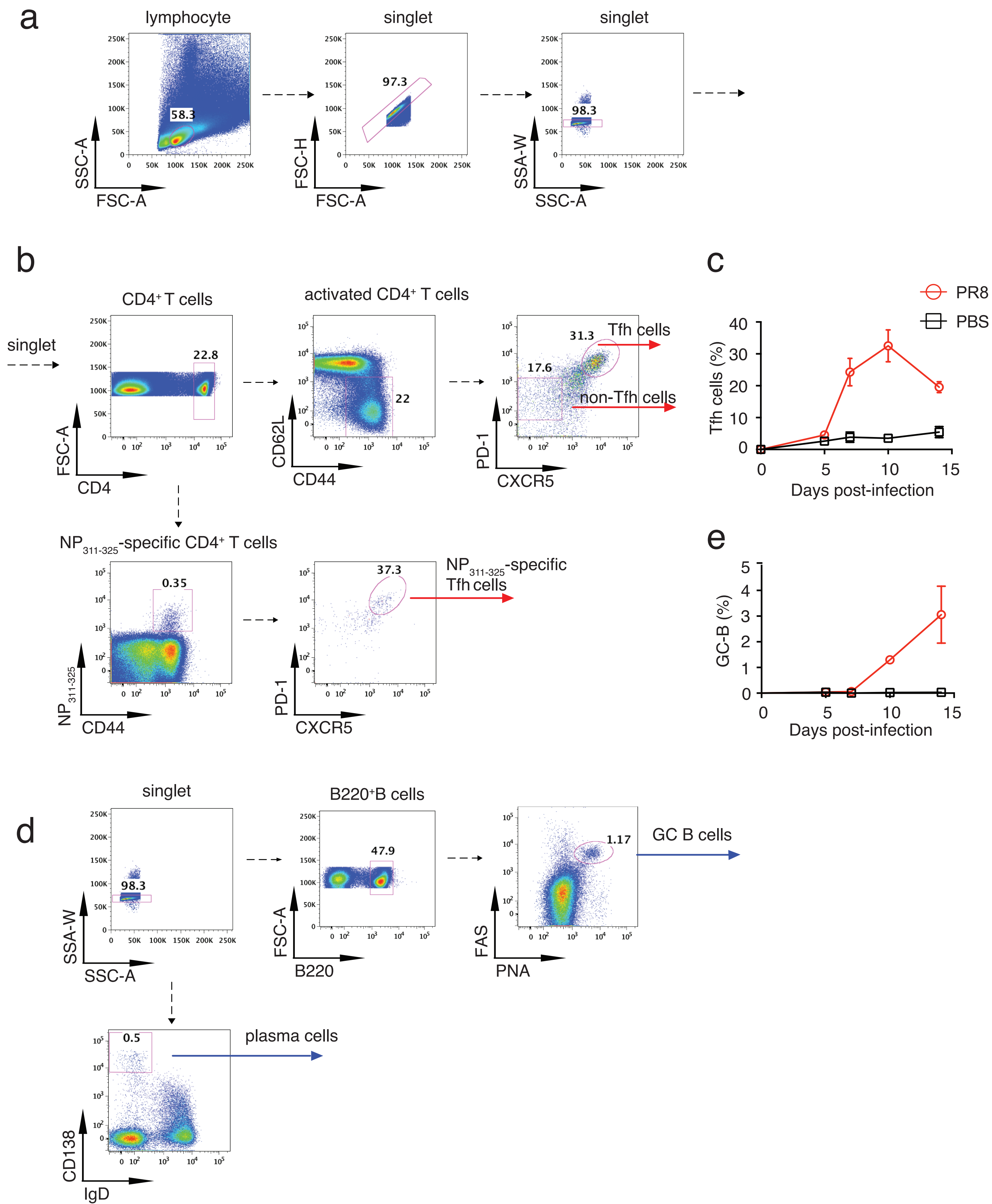

Figure 3

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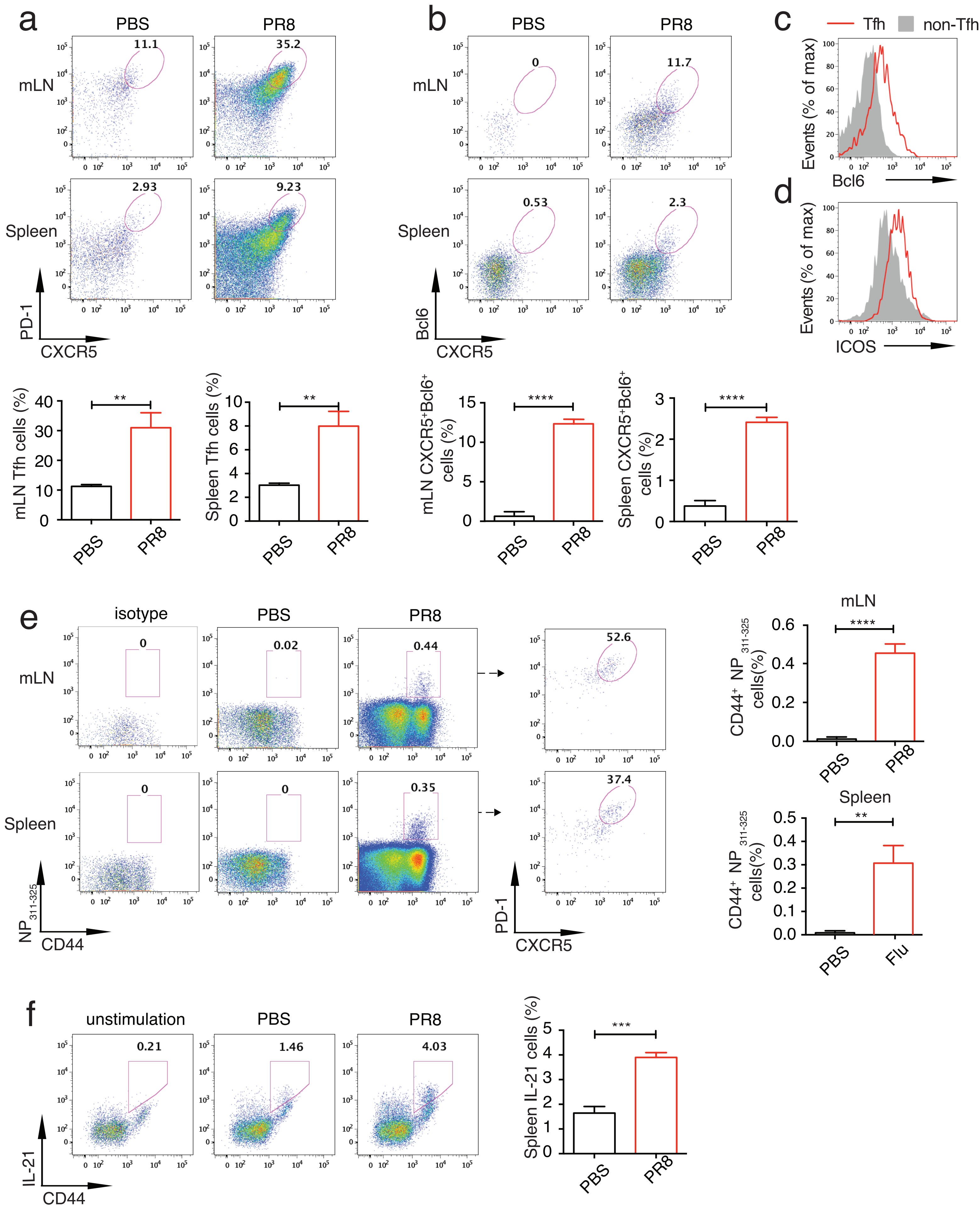

Figure 4

Figure 4

Click here to access/download;Figure;Figure 4 GC-B Plasma Antibody.pdf

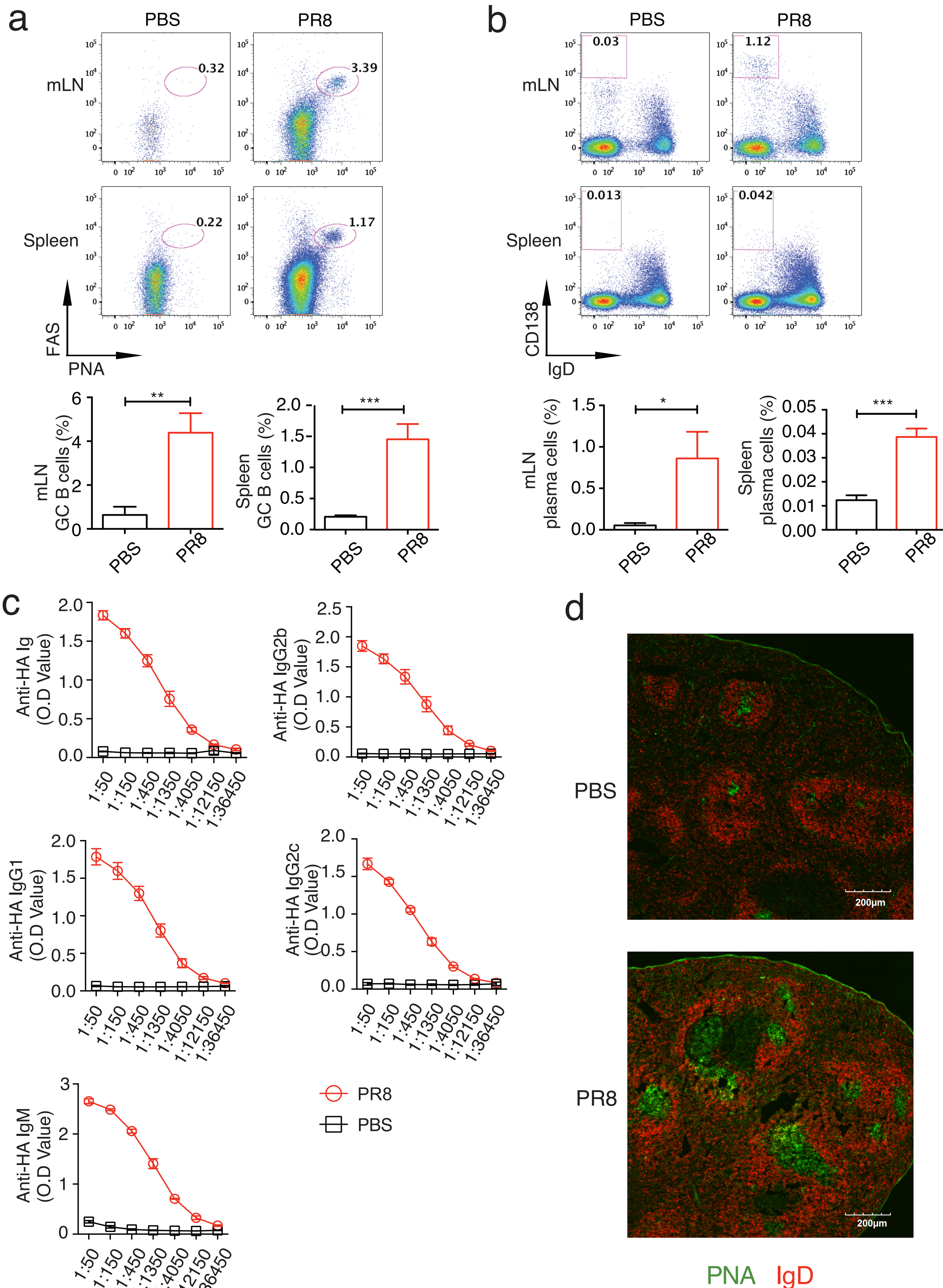

Table 1

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surface marker	fluorochrome	clone	volume per sample(ul)
CD4	Percp-eFluor 710	GK1.5	0.2
CD44	eVolve 605	IM7	0.2
CD62L	FITC	MEL-14	0.2
ICOS	BV421	7E.17G9	0.2
PD1	PE/Cy7	29F.1A12	0.3
Streptavidin	PE		0.2

Click here to access/download;Table;Table 2.xlsx

surface marker	fluorochrome	clone	volume per sample(ul)
CD4	Percp-eFluor 710	GK1.5	0.2
CD44	FITC	IM7	0.2
PD1	PE/Cy7	29F.1A12	0.3
Streptavidin	BV421		0.5

 Click here to access/download;Table;Table 3.xlsx

surface marker	fluorochrome	clone	volume per sample(ul)
CD4	Percp-eFluor 710	GK1.5	0.2
CD44	FITC	IM7	0.2

 Click here to access/download;Table;Table 4.xlsx

surface marker	fluorochrome	clone	volume per sample(ul)
B220	APC	RA3-6B2	0.2
IgD	eFluor 450	11-26c	0.2
CD95	PE/Cy7	Jo2	0.3
PNA	FITC		0.3
CD138	PE	281-2	0.2

 Click here to access/download;Table of Materials;Table of materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Description
Immunostaining of Tfh cells ,NP-specific Tfh cells and Bcl-6			
37% formaldehyde	Sigma	F1635	
Anti-CD16/32 mouse	Thermo Fisher Scientific	14-0161-86	
APC-conjugated-IAbNP311-325 MHC class II tetramer	NIH		
Bcl-6 PE	Biolegend	358504	clone:7D1
Biotin-CXCR5	Thermo Fisher Scientific	13-7185-82	clone: SPRCL5
CD4 Percp-eFluor 710	Thermo Fisher Scientific	46-0041-82	clone:GK1.5
CD44 eVolve 605	Thermo Fisher Scientifi	83-0441-42	clone:IM7
CD44 FITC	Thermo Fisher Scientifi	11-0441-82	clone:IM7
CD62L FITC	BD Pharmingen	553150	clone:MEL-14
ICOS BV421	Biolegend	564070	clone:7E.17G9
PD1 PE/Cy7	Biolegend	135216	clone:29F.1A12
Streptavidin BV421	BD Pharmingen	563259	
Streptavidin PE	BD Pharmingen	554081	
Intracelluar staining of IL21			
37% formaldehyde	Sigma	F1635	
anti-human IgG	Jackson ImmunoResearch Laboratories	109-605-098	
Brefeldin A	Sigma	B6542	
human FCc IL-21 receptor	R&D System		
ionomycin	Sigma	I0634	
Live/Dead Fixable Aqua Dead Cell staining kit	Thermo Fisher Scientific	L34966	

PMA	Sigma	P1585	
Saponin	MP	102855	
GC B and plasma cells staining			
B220 APC	Thermo Fisher Scientific	17-0452-81	clone:RA3-6B2
CD138 PE	BD Pharmingen	561070	clone:281-2
CD95 (FAS) PE/Cy7	BD Pharmingen	557653	clone:Jo2
IgD eFluor 450	Thermo Fisher Scientific	48-5993-82	clone:11-26c
PNA FITC	Sigma	L7381	
Assay of HA-specific antibody titer with ELISA			
PR8-HA	Sino Biological	11684-V08H	
BSA	SSBC		
Goat anti mouse Ig (SBA Clonotyping System-HRP)	SouthernBiotech	5300-05	
Goat anti mouse IgM(SBA Clonotyping System-HRP)			
Goat anti mouse IgG1(SBA Clonotyping System-HRP)			
Goat anti mouse IgG2b(SBA Clonotyping System-HRP)			
Goat anti mouse IgG2c(SBA Clonotyping System-HRP)			
TMB Substrate Reagent Set	BD Pharmingen	555214	
Histology			
Alexa Fluor 555-Goat-anti rat IgG	Life Technology	A21434	
anti-mouse IgD	Biolegend	405702	
biotinylated PNA	Vector laboratories	B-1075	
dilute Alexa Fluor 488-streptavidin	Life Technology	S11223	
normal goat serum	SouthernBiotech	0060-01	
Pro-long gold antifade reagent	Thermo Fisher Scientific	P3630	
STREPTAVIDIN/BIOTIN blocking kit	Vector laboratories	SP-2002	

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Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response: The spelling and grammatical errors have been corrected.

Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:

1) 1.1: Mention animal strain.

Response: The animal strain we used in the experiment is C57BL/6 and has been indicated in the revised manuscript.

2) 1.3: Do you perform toe pinch to test depth of anesthesia?

Response: Actually we didn't perform toe pinch to test depth of anesthesia. In this experiment we observe the heart beat of mice to judge whether anesthetization is appropriate.

• Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

4) Notes cannot be filmed and should be excluded from highlighting.

Response: The protocols for filming have been highlighted according to the rules above.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the

following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol

Response: Discussion has been written according to the rules above.

• Tables: Table 1–4 appear to be missing from the submission.

Response: Tables have been submitted.

• References: Please spell out journal names.

Response: We inserted the reference using the **JoVE EndNote style file**.

• Please define all abbreviations at first use.
Response: All abbreviations have been defined at first use.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Response: All the data are original and haven't been published.

Reviewer #1

Line 90: it would be helpful if the authors would include a description of the kinetics of the immune response in this model and explain why they would look at day 10 for the T cell response and 14 for the AB.
The authors should also indicate at what stage infection risk or BSL2 concerns are mitigated.

Response: We added the kinetics of Tfh and GC B response in the manuscript (Fig.2c and Fig.2e). **Fig.2c** shows that T cell response peaked at day 10 and Fig.2e shows GC B reaction continued to increase at day 14. We reasoned the influenza virus-specific antibody titer should also peak at day 14.

It has been indicated that at which steps procedures infection risks or BSL2 concerns could be mitigated (lane 208, lane 474).

98: what strain of mice is being used? Disease severity can vary between strains, so this is crucial information. The authors should indicate if female mice have the same magnitude of response in this strain.

Response: The mouse strain we used is C57BL/6 and the female mice have the same magnitude of response, all of which have been added in the revised protocol.

100: there are several incorrect words throughout the entire protocol. For example, the authors use refrigerator instead of freezer.

Response: the words have been corrected

104 the authors later mention this in the discussion, but it would be appropriate to give the rationale here why mice are being anesthetized. In addition, the authors should indicate why they pick this specific injectable (which is not necessarily easily approved by animal care committees around the globe due to its variability in efficacy and risk of overdosing compared to other injectables (they should make a case that injectables are preferred over inhaled anesthetics).

Response: we agree with the reviewer that the rationale of anesthetization should be mentioned in the protocol section and it has been added in the note following step 1.3. With regard to reagents for anesthetization, inhaled anesthetics is indeed a better choice for its greater safety and easier control of anesthetization depth. However, there is not special equipment for treatment of inhaled anesthetics in our institute. So we have to choose injectable reagent for anesthetization. Actually pentobarbital is a commonly used injectable reagent[1], the amount of which for appropriate anesthetization has been studied in previous paper and titrated in our lab so that the risk of overdosing could be avoided.

117: presumably the mice are placed in a warm cage

Response: It's true that the mice for infection are placed in the warm cage and we have modified the description.

123: the authors should indicate that euthanasia should be performed according the institutional animal care committee and should include a secondary method of euthanasia. The current description does not fall under allowable practices at many institutes

Response: All the protocols have been approved by the institutional animal care and use committee and we have mentioned this in the beginning of this section. In brief,

the euthanization protocol we described here includes CO_2 euthanization and a second physical euthanization (cervical dislocation). This protocol has also been applied in the other institute [2].

128: proper language would include dissection board, dissection needles etc.

Response: Words have been corrected.

132-141: part is missing. Presumably the organs go in a tube or dish and not on ice.

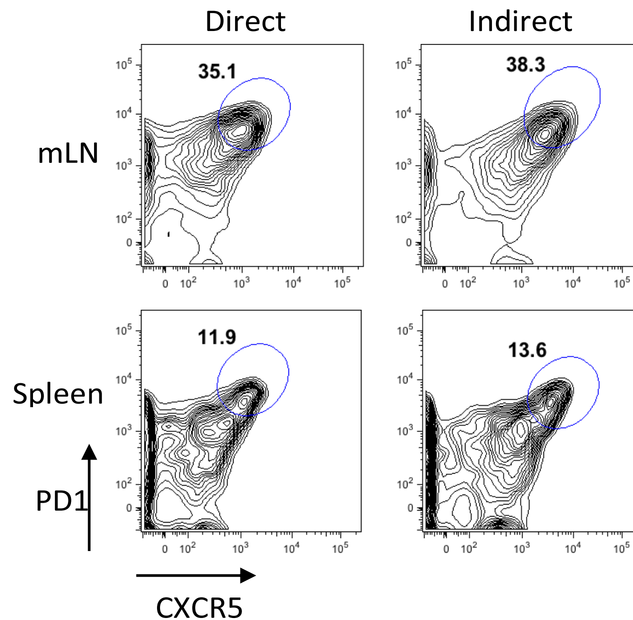
Response: The missing part has been added (lane 186-194).

156: counting point 2.10 should be a sub point from 2.9. counting calculations should be provided.

Response: We agree with the reviewer that counting point 2.10 should be a sub point from 2.9 and the edition has been made in the revised protocol. In addition, the calculation method has been added in the step 2.10.3 in the revised protocol.

166> it is completely unclear why biotinylated Ab are preferred over directly conjugated Ab. This makes the protocol more complex, results in increased background in biotin-high animals (such as those on a high fat diet or on a NOD like background)
Viability staining should be added to this section.

Response: Actually biotinylated antibody is commonly used for CXCR5 staining. We compared the Tfh staining using directly conjugated anti-CXCR5 antibody (eBioscience; Clone: SPRCL5) and biotinylated antibody with the same sample. as shown in **Res Fig.1**. Combined with PD1, staining with biotinylated CXCR5 generated more distinguished Tfh population than directly conjugated antibody.
Indeed, we have ever tried including viability staining in Tfh analysis. We found that viability staining didn’t affect the analysis of Tfh frequency as lymphocyte gating in the first step exclude almost all the dead cells(**Fig.2a**). So we don’t think viability staining is necessary in this section.



Res Fig 1. Tfh population staining with directly conjugated or biotinylated antibody. mLN and spleen cells from influenza virus-infected mice were stained for Tfh population with PD1 and CXCR5 antibody. “Direct” indicates using directly conjugated PE-anti-CXCR5 antibody and “Indirect” indicates using biotinylated anti-CXCR5 antibody, followed by stained with PE-streptavidin. FACS plots are gated from $CD4^{+}CD44^{hi}CD62L^{low}$ activated cells. Numbers indicate the frequency of Tfh cells in activated cells.

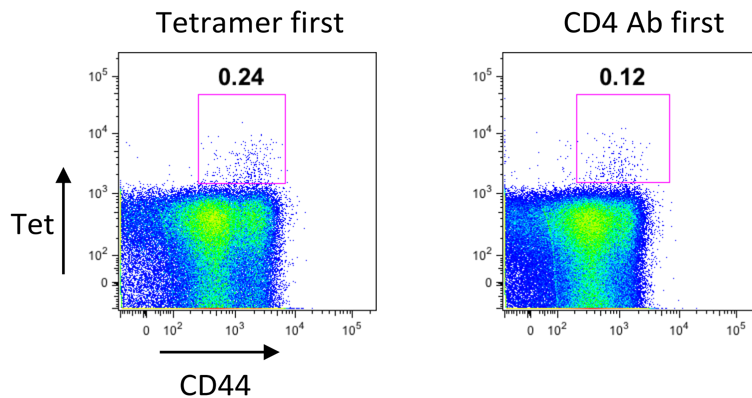
168: staining buffer is presumably PBS, indications on magnesium and calcium should be added.

Response: Indeed, staining buffer is mainly PBS and the recipe of PBS has been indicated (lane 111).

212: the authors indicate that this protocol is different from published protocols. They should explain what the advantage of this protocol is over the published ones. An irrelevant tetramer control should be included as negative control. The authors should indicate that specific CD4 Ab might interfere with the optimal binding. The authors should refer to mechanism that improve tetramer binding, such as other multimeric structures or inclusion of specific protein kinase inhibitors.

Response: Actually, the tetramer staining we described is just a more detailed edition than that in published paper. Staining results with an irrelevant tetramer control have been shown in **Fig.3e**.
We agree with the reviewer that CD4 Ab could interfere with tetramer staining. Thus tetramer was stained firstly before staining CD4 (lane 335). This strategy works in

avoiding the interference of optimal tetramer binding. As shown in Res Fig 2, we stained tetramer with two strategies: The first (Left, tetramer first) is to add tetramer first, as what we describe in the manuscript; the other (Right, CD4 Ab first) is to add CD4 Ab first. We could see that tetramer frequency stained with the first strategy is 2-fold of the frequency stained with the second strategy in the same sample. We agree with the reviewer that such strategy should be indicated in the protocol and note has been added following step 4.2.



Res Fig 2. Tetramer staining with two different strategies. mLN samples from influenza virus-infected mice were stained for tetramer with two different strategies. "Tetramer first" (Left) indicates staining of tetramer is before addition of CD4 Ab. "CD4 Ab first" indicates staining of CD4 is before staining tetramer. FACS plots are gated from $CD4^+$ T cells. Numbers indicate the frequency of $Tetramer^+$ cells in $CD4^+$ T cells.

239. The authors use in-house made buffers. It is likely that readers will use kits. A note should be included that kits should be appropriate for nuclear/transcription factor staining and not just for general intracellular staining.

Response: We agree that the kits should be appropriate for nuclear/transcription factor staining, especially for Bcl6[3,4]. However, we don't use kits for Bcl6 staining in our lab and don't know exactly the staining results. So we don't think such note should be added here.

262. the intracellular staining is very complex by using human Fc-IL21 R followed by anti-human Ig(H+L). Why is this better than directly conjugated ab?

Response: Actually we don't know why human Fc-IL21 R is better than directly conjugated ab. Besides IL21-reporter mice, it seems to be a common strategy to detect the production of IL21 in this field.

355. what type of milk is added?

Response: It is skimmed milk that is used in this step and has been specified in the revised protocol (lane 486).

367. the dilution factor should be replaced by the concentration.

Response: As the concentration of HA-specific antibody in serum is unknown, the concentration of diluted serum is also unknown. Actually the aim of this experiment is to compare the concentration of antibody in two samples, so it is not necessary to assay the accurate concentration in each sample.

370. what is an equal volume of balance.

Response: Actually this is a description of the manual from the TMB Substrate Reagent Set and we have added this information below “step 9.6” as a note.

374. analysis information should be provided (wavelength, subtraction of background wavelength, reference to a calculation method).

Response: Analysis method has been added in the step 9.8.

377. Information of isotype controls is missing, dilution of Ab should be replaced by concentration, rationale for use of goat serum should be given (can this be replaced by cow/horse/donkey/rabbit). Organization and word choice are not great: example quickly wash the tissue with shaking slowly for 5 min. analysis is missing. information on how to analyze these type of data sets is missing.

Response: We didn’t use isotype controls in this experiment. Instead, in negative controls we directly add fluorochrome-labeld secondary antibody without staining the primary antibodies (not shown in manuscript).
The dilution of antibody has been replaced with concentration.
As the fluorochrome-labeld secondary antibody is generated from goat serum. So to avoid non-specific signals, block buffer should also use goat serum.
The information of how to recognize GC has been provided in **Fig.4d**. In addition, we add the method of evaluating GC B response in step 10.12.

Reviewer #2:

1) The methods are focused on mice and I think this needs to be reflected in the title and abstract as these methods are not applicable for the evaluation of Tfh cells in humans.

   Response: We agree with the reviewer and have indicated that this protocol is only applicable in mouse models in the title and abstract.

2) The strain of mice needs to be specific in the methods. I am guessing it is C57B6 mice, but it is important to clarify as infection severity may vary in other mice. Additionally, the I-Ab NP tetramer will only work in mice with the I-Ab allele.

Response: It’s true that the mouse strain we used is C57BL/6 and the information has been indicated in protocol 1.1.

3) I couldn't find tables listed in lines 510-517. Please check

Response: We are sorry that tables are lost while submission and have been added in the revised edition.

4) RBC lysis buffer needs to be described in detail (source? If in-house, what are the ingredients?)

Response: The ingredient of RBC lysis buffer has been indicated in the revised edition (lane 214)

5) The clone of antibody would be useful, please include in tables.

Response: The clone of antibody has been included in tables.

Reviewer #3:

Major Concerns:

1. Figure 4 is missing from the manuscript.

Response: We are sorry for the mistake. Figure 5 is actually Figure 4.

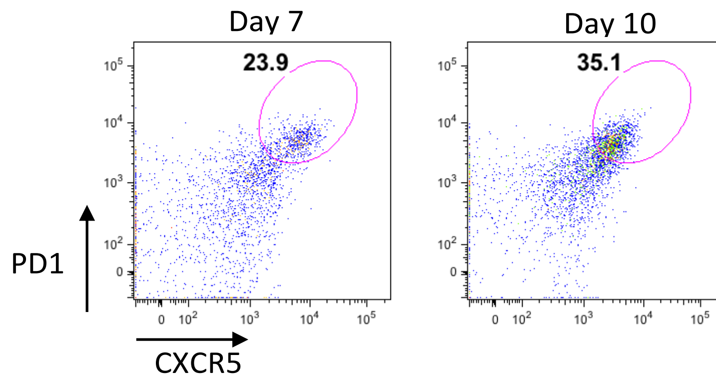
2. Figure 2b-c: showing a better separation for Tfh cells and plasma cells would be more convincing.

Response: We have renewed the FACS image, which shows better separation of Tfh cells (**Fig.2b**). As for plasma cells, actually we couldn’t find better figures. However, as plasma cells are all IgD^{low} cells, IgD^{hi} cells as negative gating controls for gating $CD138^{+}$ cells.

3. Figure 3a: separation of PD-1, CXCR5 double positives is not convincing.

Response: We checked our previous data and found that Tfh population in day 10 influenza virus-infected samples is exactly like what Fig 3a shows. Actually Day 7 Tfh cells is a more distinguished population than Day 10. **Res Fig.2** shows Tfh cell differentiation on day 7 and day 10. We could see that though Day 10 samples show worse separation of Tfh cells, Tfh frequency of Day 10 is much larger than that of Day

7 sample.



Res Fig 4. Tfh staining with Day 7 and Day 10 mLN cells. These cells are gated from $CD4^{+}CD44^{hi}CD62L^{low}$ activated cells. Numbers indicate the frequency of Tfh cells.

Minor Concerns:

1. A native English speaker should edit the manuscript.

Response: Thanks for the suggestion and the manuscript has been edited.

2. Please write "influenza virus" instead of influenza throughout the text.

Response: The correction has been made.

3. Line 48: first mention of Bcl6, please write "B cell lymphoma 6 (Bcl6)"

Response: The correction has been made.

4. Line 96: "PR8 influenza virus" instead of PR8.

Response: The correction has been made.

5. Line 111: 10 µl seems to be a very low volume. 25-30 µl is what most people use.

Response: Actually 10 µl is just for one side and the total volume is 20 µl.

6. Lines 156-158: I would mention that step 2.9 needs to be repeated if RBC lysis is not complete. It often happens.

Response: Actually, we didn't encounter such problem in the previous experiment. In this step, I think the key is the ratio of the lysis buffer volume to the cell volume, which is 9 in our protocol and sufficient for effective RBC lysis.

7. Line 352: I would add the volume: 100 µl HA protein at 1 mg/ml

Response: We have revised the description to "Coat ELISA plates with 50 µl 2µg/ml HA protein solution per well".

8. Lines 355: step 9.2: 3% BSA in PBS can be used as blocking buffer.

Response: We agree that 3% BSA in PBS could be used as blocking buffer. But with 5% skimmed milk in the PBST, we could also get reasonable result. As shown in Figure 4d, there is hardly background signal in the well of samples from PBS-treated mice.

9. Lines 358-361: adding an anti-HA monoclonal antibody as a positive control can be useful.

Response: The aim of this ELISA protocol is to compare the titer of HA-specific antibody in two samples. So it's a relative value. We agree with the reviewer that HA-specific antibody in this section would facilitate assaying a definite value of antibody titer. But it's not necessary in this kind of experiments.

Reviewer #4:

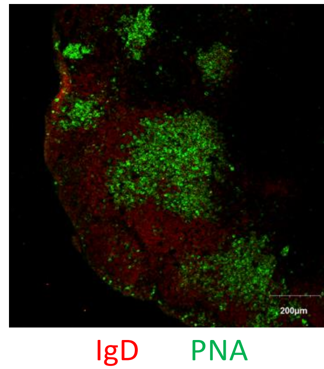
Major Concerns:

1. The mice infected with PR8 influenza virus model is well established. In general, the body weight loss is almost 20% in mice infected with PR8 influenza virus at day 8 and maintain at day 10. However, the body weight is decreased to 90% at day 8, and quickly recovered to the initial level at day 10. After the PR8 influenza infection, there are more leukocyte infiltration and severe tissue damage in the lung. It is better to show the Hematoxylin and eosin (H&E) staining images of lung sections in the mice infected with PR8 influenza virus.

Response: The weight change kinetics is affected by several factors, such as virus titer and mice strain. So it's normal the weight loss we observed is not similar with that in published papers. In addition, weight loss and enlarged mediastinal lymph node could be a good sign of influenza virus infection and the main part should be assay of Tfh-associated response. So we don't think HE staining of Lung sections is necessary in this protocol.

2. The GC B response induced by influenza virus is more robust in mLNs than in spleens, however, in figure 4 d, the confocal microscopy showed the B cell follicles and GCs in the spleen samples of PR8-infected mice and PBS treated mice. Moreover, the quality of confocal image is not well. You should perform the confocal immunofluorescence staining of GCs and B cell follicles in the mLNs clean and clearly.

Response: Actually, both spleen and mLN are appropriate for GC confocal analysis. However, mLNs in PBS-treated mice is too small for confocal analysis. So we just provide the confocal image of mLNs from influenza virus-infected mice here (**Res Fig 4**), not in the manuscript. Indeed, there was robust GC reaction in mLNs of influenza virus-infected mice.



Res Fig 5. Confocal image of GC B in influenza virus-infected mLN. On day 10 after infection, mLN from influenza virus-infected mice was isolated and stained with IgD (red) and PNA (green) for GC B detection.

3. In the protocol 3 "Immunostaining of Polyclonal Tfh cells with PD1 and CXCR5", the description is not clearly and mess up. Such as "3.1.5. Add 0.3 µl biotin-anti mouse CXCR5 for each tube and vortex by tapping the tube bottom." You can modify the sentence, such as "Resuspend cells in 100 µl of biotin-anti mouse CXCR5 antibody at 1:100 diluted in FACS buffer" might be better.

Response: As described in the “Note” following 3.1.3. The residue volume after we discard the washing buffer is about 25 µl. Then the volume would be 30 µl after Fc-receptor blocker (diluted in 5 µl staining buffer) was added. So the dilution factor is 1:100 when we add 0.3 µl antibody. But we agree with the reviewer that it will be more clear if we indicate the volume of residue staining buffer in the tube (lane 300).

4. This Article addresses the details for measuring TFH and GC B cells in PR8 influenza virus model. The authors described that the bodyweight of the mice infected with PR8 was decreased at the highest level on day 8. However, the TFH and GC B cells were analyzed at the day when the mice recovered in this article. It is better to show the kinetics of TFH and GC B cells responses during infection with PR8 influenza virus before picking an appropriate time point for analysis.

Response: We agree with the reviewer that showing immune response kinetics (Tfh and B cells) would be will be more helpful for better understanding this model. We added the kinetics in Fig.2c and Fig.2e.

Reference:

1 Gargiulo, S. *et al.* Mice anesthesia, analgesia, and care, Part I: anesthetic considerations in preclinical research. *ILAR J.* **53** (1), E55-69, doi:10.1093/ilar.53.1.55 (2012).

2 Rodriguez, L., Nogales, A., & Martinez-Sobrido, L. Influenza A Virus Studies in a Mouse Model of Infection. *J Vis Exp.* (127), doi:10.3791/55898 (2017).

3 Leavenworth, J.W., Verbinnen, B., Yin, J., Huang, H., & Cantor, H. A p85alpha-osteopontin axis couples the receptor ICOS to sustained Bcl-6 expression by follicular helper and regulatory T cells. *Nat Immunol.* **16** (1), 96-106, doi:10.1038/ni.3050 (2015).

4 Xu, L. *et al.* The transcription factor TCF-1 initiates the differentiation of T(FH) cells during acute viral infection. *Nat Immunol.* **16** (9), 991-999, doi:10.1038/ni.3229 (2015).

Rebuttal Letter-2nd revision

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thanks for the suggestion and we have checked the spelling and grammar issues.

2. Step 8.2: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Response: Correction has been made.

3. Please define all abbreviations before use, e.g., PBST, etc.

Response: Correction has been made.

4. Step 10.13: Please write this step in the imperative tense.

Response: The correction has been made.

5. Step 2.10.1: What is the temperature for incubation?

Response: The incubation temperature is room temperature, which has been indicated in the revised manuscript.

6. Please do not abbreviate journal titles for references.

Response: The references has been edited according to rules above.

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have thoroughly proofread the manuscript.

2. Step 10.13: Please write this step in the imperative tense.

Response: Correction has been made.

3. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

Response: Actually, we didn’ t use vet ointment in the anesthetization step. However, we agree it is better to perform this operation. So we add this in the note as a advice (lane 114).

6. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

Response: We have revised step 1.5 to include this information.

4. Discuss maintenance of sterile conditions during survival surgery.

5. For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

7. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

Response (4,5,7): Actually we didn’ t perform survival surgery in this experiment. I guess the misunderstanding is due to the operation of collecting blood from facial vein. Actually after doing this step, mice were euthanized for spleen or mLN isolation.