



Assessing In Vivo T Cell-Dependent Antigen-Specific Antibody Responses

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Abstract

Antibodies produced by plasma cells are a major arm of adaptive immunity. Germinal center reactions that include germinal center B cells and follicular T cells are fundamental players for antibody production, particularly antigen specific antibodies. Here we describe multiple methods that we and others have developed to analyze the production of antigen-specific antibodies in mice, with protocols for assessing antibody affinity and antibody isotype. The detection of antigen-specific IgE in serum using a traditional enzyme-linked immunosorbent assay (ELISA) method is often problematic due to much higher amounts of IgG. Thus we provide a specialized protocol for the detection of antigen-specific IgE in serum using ELISA.

Key words Antigen, Antibody, ELISA, IgE, IgA, Allergy, Affinity

1 Introduction

A major function of the adaptive immune response is to produce highly specific antibodies (Abs) that bind to antigen (Ag) with high affinity and help eliminate pathogens and foreign substances. Long-lived memory B cells and plasma cells are generated from germinal center (GC) responses, which can then produce high-affinity Abs against pathogens [1, 2]. Follicular T helper (TFH) and regulatory (TFR) cells are two key classes of CD4⁺ T cells found in GCs [3–6]. The primary role of TFH cells is to help B cells form into GCs and to mediate the selection of high-affinity antibodies during an infection. TFH cells are required to produce high-affinity protective Abs; however, the overproduction of TFH cells can also lead to autoimmunity as nonspecific TFH cell help can override tolerance mechanisms and leads to the production of self-reactive Abs [7–9]. Ab responses are also controlled by T follicular regulatory (TFR) cells, which typically develop from regulatory T cells (Tregs) [3, 6, 10–14]. TFR cells, like TFH cells, localize to the GC due to CXCR5 expression and are dependent upon the transcriptional repressor protein Bcl6 for their development. In

contrast to TFH cells, TFR cells also express the canonical Treg master regulatory transcription factor FOXP3 [3, 10–14]. TFR cells are commonly observed to act as suppressor of the GC response [3, 10–12], but this is very dependent upon the model system used to study GCs and Ab responses [6]. A TFR-deficient mouse model where the *Bcl6* gene is specifically deleted in FOXP3+ T cells has been used by us and others to show that TFR cells can have a helper function in the GC and Ab response [6, 13, 15–19]. Recently, we have found TFR cells unexpectedly play an essential role in IgE production and anaphylaxis, as well as the GC reaction, using multiple different genetically altered mouse models [19]. Here, we describe three detailed methods (sheep red blood cell immunization, airway challenge, and gut immunization) that we have used to induce Ag-specific Ab production in mice. We also describe ELISA protocols to detect high-affinity Ag-specific Abs of different isotypes, and a specialized ELISA protocol that we used to identify Ag-specific IgE.

2 Materials

1. 96-well immuno plates for ELISA.
2. Phosphate buffered saline (PBS).
3. 1.5% NaHCO₃, H₂O.
4. Disposable plastic feeding needle for oral gavage.
5. 3 cc syringe.
6. Coating buffer: 0.1 M carbonate buffer, pH 9.5, 8.40 g NaHCO₃, 3.56 g Na₂CO₃, H₂O (alternatively PBS alone can be used as coating buffer).
7. Cholera toxin 100 µg/ml, PBS.
8. Peanut protein extract (defatted) 10 mg/ml, PBS.
9. House dust mite (HDM) extract (“D. Pteronyssinus Lyophilized”).
10. Haptenated proteins: commercially available ovalbumin (Ova) or bovine serum albumin (BSA), conjugated with nitrophenyl (NP) at different densities (NP7-BSA, NP16-Ova, NP20-Ova and NP40-BSA).
11. Sheep red blood cells (SRBC).
12. Wash buffer: 0.05% Tween-20, PBS.
13. Blocking buffer: 1% BSA, PBS.
14. Biotinylation kit: commercially available.
15. Capture and detection antibodies for ELISA (Table 1).

Table 1
ELISA capture and biotinylated secondary antibodies

Anti-mouse	Clone or other info	Final concentration or dilution factor	Company
IgE	R35-72	2 µg/ml	BD Pharmingen
IgE	LO-ME-3	5 µg/ml	BIO-RAD
Biotinylated-IgG1	A85-1	1:250	BD Pharmingen
Biotinylated-IgG2b	R12-3	1:250	BD Pharmingen
Biotinylated-IgG2a	R19-15	1:250	BD Pharmingen
IgG2c (HRP-conjugated)	Heavy chain specific	1:250	Southern Biotech
IgA (HRP-conjugated)	Heavy chain specific	1:1000	Sigma-Aldrich
IgM (HRP-conjugated)	Heavy chain specific	1:1000	Sigma-Aldrich
Pan IgG (HRP-conjugated)	Reactive with all IgG isotypes	1:1000	Sigma-Aldrich

16. Streptavidin-poly-conjugated-horse radish peroxidase (SA-HRP).
17. Substrate solution: commercially available 3,3',5,5'-Tetramethylbenzidine (TMB) Substrate Kit.
18. Stop solution: 2 M H₂SO₄.

3 Methods

3.1 Food Allergy or Gut (Intra-gastric or i. g.) Immunization Protocol

1. Deprive experimental mice of food chow for 2 h (allow access to drinking water). *See Note 1.*
2. After the 2 h food deprivation, oral gavage each mouse with 300 µl NaHCO₃ (sodium bicarbonate solution—1.5% NaHCO₃ in PBS). This will neutralize stomach acid and improve the stability of the introduced Ag. *See Notes 2 and 3.* The gavage is done without anesthesia using disposable plastic “feeding needles”.
3. Challenge mice with antigen half hour after bicarbonate feeding. Crude peanut protein is used in our food allergy model, but other protein Ags can be used such as ovalbumin. Ag stocks at 10 mg/ml are made in PBS and kept at –20 °C. Cholera toxin stocks of 100 µg/ml are made in PBS and kept at –20 °C. Gavage each mouse with 200 µl peanut-cholera toxin (PCT) solution (freshly mixed from 100 µl peanut stock and 100 µl cholera toxin stock, vortex thoroughly before feeding; 1 mg peanut and 10 µg cholera toxin for each mouse). Gavage feeding as in **step 2**, use new needles for each type of Ag. *See Note 3.*

4. After gavage feeding, keep mice starved for another half hour before putting back the mouse chow and transferring mice back to their normal housing racks.
5. Mice are primed twice, 1 week apart from each other. At various times after the second priming, mice are sacrificed for multiple-purposed analysis on desired days post challenge. We have typically analyzed the mice 1–4 weeks after the second priming, as that is when Ag-specific IgE is highest.
6. As an alternative, mice are primed eight times, once per week, 1 week apart from each other. At various times after the eighth priming, mice are sacrificed for multiple-purposed analysis. This 8-prime system produces very strong peanut-specific Abs (especially PN-IgA and PN-IgE).

3.2 Immunization with Sheep Red Blood Cells (SRBC)

1. Thoroughly mix SRBC stock solution before use. Take 2 ml from 100% SRBC stock (or 15 ml from 10% diluted SRBC) for 15–20 mice immunization in 50 ml tube and add PBS to 50 ml. *See Note 4.*
2. Wash the cells five times with sterile PBS by centrifuging the tube 5 min in $2000\text{--}2200 \times g$ 4 °C and discard the supernatant.
3. After the final wash, add PBS to make the volume at 1 ml. Take cells for counting and concentration determination.
4. Dilute SRBC cells in PBS at the concentration of 5×10^9 cells/ml.
5. Immunize each mouse with 200 μl SRBC through i.p. (1×10^9 /mouse).
6. On desired days post immunization, collect mouse blood and analyze serum SRBC-specific antibody responses (immune cell characterizations can be done in spleen or whole blood).

3.3 Airway Challenge (Allergic Airway Disease) Model

1. Mice are anesthetized using Isoflurane per IACUC requirements.
2. 25 μg model antigen (house dust mite (HDM) extract is most commonly used; alternatively peanut flour or *Aspergillus* fungal extract) in 20 μl PBS is dispensed to each mouse via i.n. using a pipette.
3. Put mice on a warm pad for full recovery and put back to cages afterwards.
4. Repeat the intranasal immunization with an institutionally approved routine (e.g. immunize every other day for 3 weeks).
5. Harvest blood and tissues for further antibody or cellular analysis on desired days post i.n. immunization.

3.4 Mouse Bleeding and Serum Preparation

1. Mouse blood is collected from the submandibular vein without anesthesia.
2. Restrain animals manually with a firm grip.
3. The submandibular vein (facial vein) is lightly punctured using a sterile lancet or needle (18G) to obtain blood, which is dripped into 1.7 mL tubes.
4. Following collection of blood, hold mouse by the tail upside down to ensure hemostasis and bleeding has stopped. If bleeding has stopped, release mouse back to cage.
5. Collected blood is stored at room temperature for at least 1 h before centrifuging at $10,000 \times g$ for 10 min.
6. After centrifuge, serum at the top of the tube is transferred into a new tube and stored at -20°C (or -80°C for long term storage) until further analysis.

3.5 ELISA for Ag-Specific Antibodies (Total IgG, IgG Subtypes or IgA), Using Ovalbumin as an Example

1. Coat ELISA plates with 100 μL /well of Ova protein diluted in PBS at 20 $\mu\text{g}/\text{mL}$. Seal plate and incubate overnight at 4°C .
2. After overnight coating plate, wash plates five times with wash buffer (0.05% Tween-20 in PBS). Block plates with 200 μL /well of blocking buffer (1% BSA in PBS) and incubate at room temperature for 1 h.
3. Dilute samples (serum or plasma at desired dilution factors, 1:5, 1:15, 1:45, 1:135, 1:405, and 1:1215) with blocking buffer (1% BSA in PBS).
4. After blocking, wash plates five times with wash buffer. Add all diluted samples at 100 μL /well into plates. Seal plate and incubate at room temperature for 2 h.
5. Right after 2 h sample incubation, dilute secondary HRP conjugated antibodies specific for isotype of interest (Table 1) with blocking buffer. If using biotinylated secondary antibodies, dilute SA-HRP 1:1000 along with the biotinylated secondary antibodies.
6. After sample incubation, wash plates five times with wash buffer. Add diluted secondary HRP conjugated antibodies at 100 μL /well, seal plate and incubate at room temperature for 1 h.
7. After Streptavidin-poly-HRP incubation, wash plates five times with wash buffer. Prepare Substrate Solution (1:1 mix of A and B solutions in TMB substrate kit). Add 100 μL /well of working Substrate Solution and incubate at room temperature for around 15 min in the dark. Add 50 μL /well of stop solution (2 M H_2SO_4) when plate color is developed and ready for reading. Read absorbance at 450 nm asap after the addition of stop solution.
8. OD is measured at 450 nm.

3.6 Antigen Challenge for Antibody Affinity Tests

For an Ab affinity test, NP19-Ova would be used either as a substitute for model Ags (peanut extract or HDM), or seeded into the model Ag of choice at a weight ratio of 1:10. An 8 priming system is required to get strong NP-specific IgE responses. If NP19-Ova is not commercially available, NP16-Ova or NP20-Ova also works fine.

3.7 ELISA Protocol for Measuring Ab Affinity with Hapten-Specific Antibodies (Total IgG, IgG subtypes, or IgA), Using NP as an Example

1. Coat ELISA plates with 100 µl/well of NP7-BSA (for detecting high-affinity Ab) or NP40-BSA (for low-affinity Ab) diluted in PBS at 20 µg/ml. Seal plate and incubate overnight at 4 °C.
2. After overnight coating plate, wash plates five times with wash buffer (0.05% Tween-20 in PBS). Block plates with 200 µl/well of blocking buffer (1% BSA in PBS) and incubate at room temperature for 1 h.
3. Dilute samples (serum or plasma at desired dilution factors, e.g. 1:5, 1:15, 1:45, 1:135, 1:405, and 1: 1215) with blocking buffer (1% BSA in PBS).
4. After blocking, wash plates five times with wash buffer. Add all diluted samples at 100 µl/well into plates. Seal plate and incubate at room temperature for 2 h.
5. Right after 2 h sample incubation, dilute secondary HRP conjugated antibodies specific for isotype of interest (Table 1) with blocking buffer. If using biotinylated secondary antibodies, dilute SA-HRP 1:1000 along with the biotinylated secondary antibodies.
6. After sample incubation, wash plates five times with wash buffer. Add diluted secondary HRP conjugated antibodies at 100 µl/well, seal plate, and incubate at room temperature for 1 h.
7. After Streptavidin-poly-HRP incubation, wash plates five times with wash buffer. Prepare Substrate Solution (1:1 mix of A and B solutions in TMB substrate kit). Add 100 µl/well of working Substrate Solution and incubate at room temperature for around 15 min in the dark. Add 50 µl/well of stop solution (2 M H₂SO₄) when plate color is developed and ready for reading. Read absorbance at 450 nm asap after the addition of stop solution.
8. OD is measured at 450 nm.
9. At the optimal point on the serum dilution curve, the ratio of the OD for the low-hapten conjugate (e.g. NP7-BSA) to the OD for the high-hapten conjugate (e.g. NP40-BSA) will give a relative measure of affinity. The higher this ratio is, the stronger the affinity.
10. A more detailed discussion on the use of NP-conjugated Ags to measure Ab affinity has been published by Heise and Klein [20].

3.8 Antigen-Specific Mouse IgE ELISA Protocol

The principle here is to capture all IgE Abs first on the plate and thus remove competing IgG and IgA Abs from the serum, then add labeled Ag to the wells to detect the level of Ag-specific IgE. This is a more sensitive and accurate method to detect Ag-specific IgE. *See Note 5.*

1. Coat ELISA plates with 50 μ l/well of Anti-mouse IgE capture antibody [e.g. Clone LO-ME-3 (Table 1)] diluted in PBS at 5 μ g/ml. Seal plate and incubate overnight at 4 °C.
2. After overnight coating plate, wash plates five times with wash buffer (0.05% Tween-20 in PBS). Block plates with 200 μ l/well of blocking buffer (1% BSA in PBS) and incubate at room temperature for 1 h.
3. Dilute samples (serum or plasma at desired dilution factors, 1:5, 1:15, 1:45, 1:135, 1:405, and 1: 1215) with blocking buffer (1% BSA in PBS).
4. After blocking, wash plates five times with wash buffer. Add all diluted samples at 100 μ l/well into plates. Seal plate and incubate at room temperature for 2 h.
5. Right after 2 h sample incubation, dilute biotinylated peanut extract 1:2000 with blocking buffer. (Biotinylated peanut protein is readily generated with commercially available biotinylation kits).
6. After sample incubation, wash plates five times with wash buffer. Add diluted biotinylated peanut extract at 100 μ l/well, seal plate, and incubate at room temperature for 1 h.
7. After biotinylated peanut extract buffer incubation, dilute Streptavidin-Poly-HRP (1:5000) with blocking buffer. Wash plates five times with wash buffer and add 100 μ l/well of diluted Streptavidin-poly-HRP. Seal plate and incubate at room temperature for 30–45 min.
8. After Streptavidin-poly-HRP incubation, wash plates five times with wash buffer. Prepare Substrate Solution (1:1 mix of A and B solutions in TMB substrate kit). Add 100 μ l/well of working Substrate Solution and incubate at room temperature for around 15 min in the dark. Add 50 μ l/well of stop solution (2 M H₂SO₄) when plate color is developed and ready for reading. Read absorbance at 450 nm asap after the addition of stop solution.
9. OD is measured at 450 nm.

3.9 Preparation of SRBC Membrane Antigens for ELISA

1. All procedures are carried out at 4 °C. Take 5 ml SRBC from the initial 100% SRBC stock.
2. Wash SRBC with PBS and centrifuge at $13,000 \times g$ for 10 min.
3. Aspirate off the suspension, resuspend the cell pellet in 0.05 M Tris-HCl with 0.1 mM EDTA (pH 7.6), vortex thoroughly, and then centrifuge again.

4. Resuspend the cell pellet well in the same buffer and centrifuge again. This process is repeated seven to ten times to remove all visible traces of hemoglobin.
5. Before the last centrifugation, the pellet is resuspended and passed through a 40 μm cell strainer. After the final wash, the pellets from 5 ml SRBC are resuspended in 500 μl 0.1% SDS and stored at -20°C .

4 Notes

1. In the food allergy model, food deprivation is necessary to ensure that each mouse gets a similar concentration of food allergen for digestion. Chow simply can be removed from the cages, or ideally the mice can be placed in a fresh clean cage to prevent coprophagy of preexisting feces.
2. Before gavaging animals with food allergen plus cholera toxin, the gavage with NaHCO_3 solution helps ensure the best effect of cholera toxin as an adjuvant and also prevents excessive degradation of the Ags in the stomach prior to reaching the small intestine.
3. Some training is required to insert the feeding needle into the esophagus without going down the trachea or hurting the mouse. The mouse's head needs to be in the proper position for this technique to be successful [21]. Practicing this technique initially on dead mice and then nonexperimental mice is important to make sure the gavage can be done efficiently and with minimal trauma to the mouse.
4. SRBC immunization produces the strongest in vivo responses when used fresh or within a week of obtaining the SRBC stock. SRBCs over a month old can still induce a response but lose effectiveness.
5. We have tried many different protocols for detecting antigen-specific IgE. The ELISA protocol presented in Subheading 3.8, using biotinylated peanut Ag, works best and has given the most stable and reliable readout for IgE in the various types of in vivo models we describe here.

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