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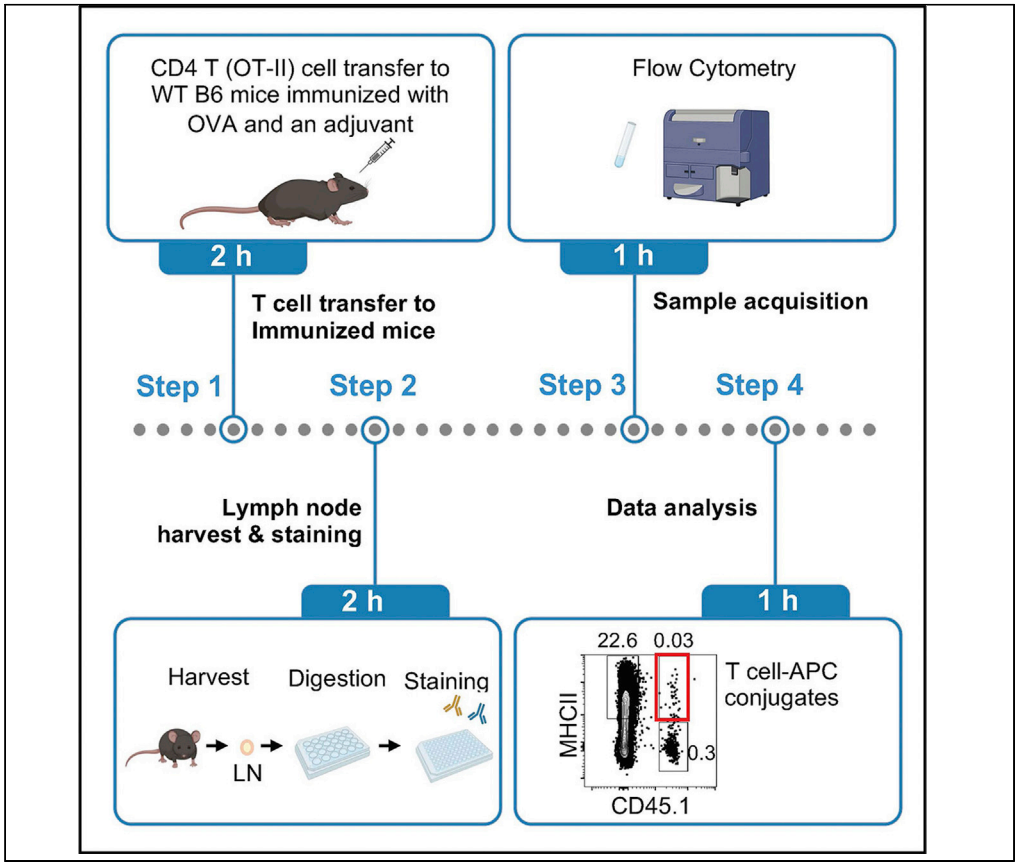
Protocol to quantify and characterize contact between T cells and antigen-presenting cells in the antigen-draining lymph nodes of mice using flow cytometry



Physical contact between T cells and antigen-presenting cells (APCs) is essential for priming antigen-specific T cells, but quantitating the antigen-dependent T cell-APC contact can be laborious. Here, we present a simple flow-cytometry-based protocol for quantitating T cell-APC contacts in the antigen-draining lymph node in mice immunized with ovalbumin (OVA). This protocol quantifies the contact between adoptively transferred OVA-specific TCR transgenic CD4T cells and dendritic cell (DC) subsets. This approach can be applied to other types of intercellular interactions between T cells and APCs.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A simple procedure to quantify antigen-dependent contacts between T cells and APCs

Identification of APC subsets interacting with T cells during T cell priming

Flow-cytometry-based approach allows to analyze multiple samples simultaneously

Steps for T cell transfer to immunized mice and lymph node harvesting

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Protocol to quantify and characterize contact between T cells and antigen-presenting cells in the antigen-draining lymph nodes of mice using flow cytometry

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SUMMARY

Physical contact between T cells and antigen-presenting cells (APCs) is essential for priming antigen-specific T cells, but quantitating the antigen-dependent T cell-APC contact can be laborious. Here, we present a simple flow-cytometry-based protocol for quantitating T cell-APC contacts in the antigen-draining lymph node in mice immunized with ovalbumin (OVA). This protocol quantifies the contact between adoptively transferred OVA-specific TCR transgenic CD4T (OT-II) cells and dendritic cell (DC) subsets. This approach can be applied to other types of intercellular interactions between T cells and APCs.

For complete details on the use and execution of this protocol, please refer to Tatsumi et al. (2021).[1]

BEFORE YOU BEGIN

Carefully choose cell type-specific markers

For analyzing the physical contact between APCs and T cells, this protocol relies on the detection of cellular conjugates by flow-cytometry. In order to clearly distinguish APC subsets from T cells in the T cell-APC conjugates, it is crucial that the markers used for defining the APC subset in the conjugate are not expressed by the T cell in the same conjugate. Before using this protocol, it is recommended to run a pilot experiment to verify the absence of APC subset markers on activated T cells. This can be confirmed, for instance, by examining the expression of those APC subset markers on the singlet CD4T cells in the same sample or on those stimulated *in vitro* with anti-CD3/CD28 antibodies.

Institutional permissions

This protocol requires tissues derived from mouse models. Ethical approvals must be obtained prior to starting this procedure. All mice used in this procedure were handled according to institutional guidelines under protocols approved by the Institutional Animal Care and Use Committee at Rutgers New Jersey Medical School.

Immunization of mice

Timing: 30 s/mouse

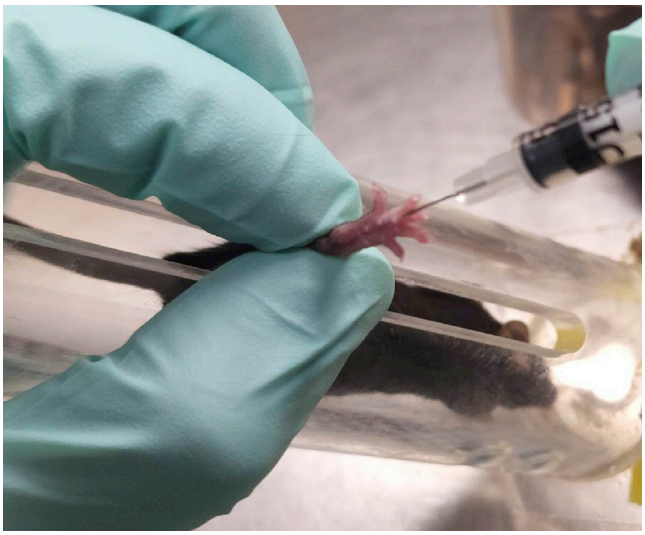

Figure 1. Footpad immunization

Place the animal in a restraint tube with one hind foot pulled out of the slit, and inject the antigen between the index and middle fingers using a 27–30 gauge needle forming a small bleb at the injection site.

1. One day before OT-II cell transfer, immunize the mice in the right hind footpad with 5 μg of low endotoxin ovalbumin (OVA) together with 50 μg of papain or 10 μL of Freund's complete adjuvant (FCA) in a total of 20 μL injection volume per footpad (Figure 1).

OT-II cell transfer

Timing: 2 h

2. Isolate CD4T cells from naive OVA-specific T cell receptor (TCR)-transgenic OT-II [B6.Cg-Tg(TcraTcrb)425Cbn/J] mice.

Note: We use OT-II mice on the congenic CD45.1 (B6.SJL-PtprcaPep3b/BoyJ) background (CD45.1;OT-II mice) or CD45.1;OT-II mice crossed to the Nur77-GFP reporter strain [C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J] (CD45.1;Nur77-GFP;OT-II mice) as donors.

⚠ CRITICAL: All procedures must be performed under an aseptic condition.

Alternatives: TCR transgenic CD4T or CD8T cells other than OT-II cells can be used when mice are immunized with the corresponding cognate antigen or infected with the corresponding pathogen. The congenic marker (CD45.1) and the activation reporter (Nur77-GFP, optional) should be chosen based on the host genotype so that those markers and reporters should only be expressed by the donor cells. Other possible markers for distinguishing the donor cells from the host cells include Thy1.1, caboxyfluorescein succinimidyl ester (CFSE) and Cell Tracer Violet (CTV). We have verified both CFSE and CTV work for detecting the OT-II-APC conjugates.

a. Euthanize the donor mouse and collect the spleen and all subcutaneous lymph nodes (LNs) into a 100 μm cell strainer placed in a 5 cm petri dish with 2 mL of ice-cold dissection medium (RPMI1640 with 10% FBS).
b. Mince the spleen and LNs into ~1 mm pieces with fine scissors.
    i. Mush the minced pieces by grinding them against the bottom of the 100 μm cell strainer with a sterile plunger of a 3 mL syringe.

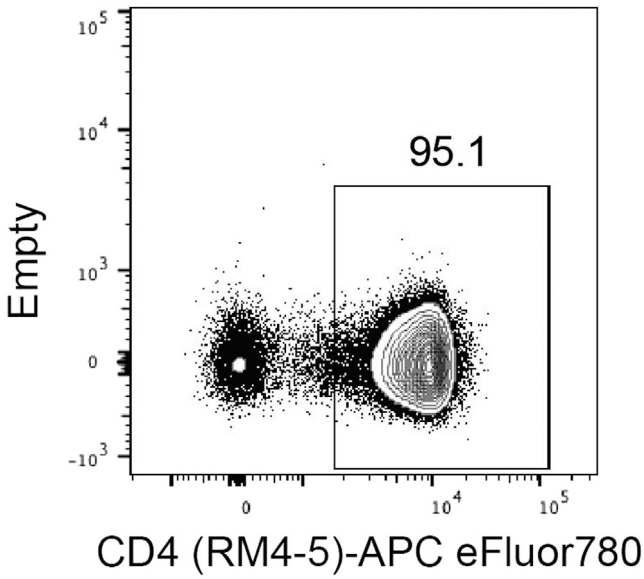

Figure 2. Purity of CD4$^+$ OT-II cells after magnetic sorting

Representative flow cytometric plots of cells stained with anti-CD4 antibody (Clone:RM4-5) after magnetic sorting using MojoSort™ Mouse CD4T Cell Isolation Kit (BioLegend Cat. No. 480033).

ii. Add 2 mL of digestion media containing 5 mg/mL collagenase D in RPMI1640 with 10% FBS (final concentration 2.5 mg/mL Collagenase D).
iii. Place the petri dish in a 5% CO_2 incubator and incubate at 37°C for 30 min.

Note: Enzymatic digestion with Collagenase D increases the cell isolation yield while keeping the majority of cell surface marker epitopes intact.

c. Isolate CD4T cell with the Mouse CD4T Cell Isolation Kit (BioLegend Cat#: 480033 or StemCell Technologies Cat#: 19852) according to the manufacturer's Protocol.
d. Wash and suspend the isolated OT-II cells in ice-cold phosphate-buffered saline (PBS) at 1×10^7 cells/mL.

Note: We typically obtain approximately 1×10^8 total spleen and lymph node cells from a naive OT-II mouse, of which $1.5–2.0 \times 10^7$ cells are isolated as CD4$^+$ OT-II cells with 90%–95% purity by using the kit from either BioLegend or STEMCELL Technologies (Figure 2).

3. Under light anesthesia with isoflurane, retro-orbitally transfer 5×10^6 cells of the donor OT-II cells into wild-type (CD45.2) C57BL/6N recipient mice immunized with OVA and an adjuvant 1 day earlier (Figure 3).

Note: Animals must be checked to ensure that bleeding (if any) has stopped prior to returning the animals to their cage.

Prepare reagents and buffers

Timing: 0.5–1 h

4. Prepare reagents and buffers as descried in the materials and equipment section.

Figure 3. Retro-orbital transfer of OT-II cells

(A) Lightly anesthetize the mouse with isoflurane.

(B) Hold the mouse by its head to cause slight bulge of the eye. Use the gloved index finger of the non-dominant hand to draw back the skin above the eye and the thumb to draw back the skin below the eye.

(C) Insert the 27–30 gauge needle at approximately a 30°–45° angle to the center of the retro-orbital sinus and slowly administer cell suspension. Minor bleeding from the retro-orbital space may occur, but the animals must be checked to ensure bleeding (if any) has stopped prior to returning the animals to their cage.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD4-APC eFluor780 (clone RM4-5) (dilution 1:300)	Invitrogen	Cat#: 47-0042-82
Anti-mouse CD4-BV395 (clone GK1.5) (dilution 1:300)	BD Biosciences	Cat#: 563790
Anti-mouse/human CD45R/B220-Biotin (clone RA3-6B2) (dilution 1:300)	BioLegend	Cat#: 103204
Anti-mouse/human CD45R/B220-FITC (clone RA3-6B2) (dilution 1:300)	BioLegend	Cat#: 103206
Anti-mouse CD69-Pacific Blue (clone H1.2F3) (dilution 1:300)	BioLegend	Cat#: 104523
Anti-mouse I-A/I-E (MHCII)-Alexa Fluor 700 (clone M5/114.15.2) (dilution 1:300)	BioLegend	Cat#: 107622
Anti-mouse I-A/I-E (MHCII)- Allophycocyanin (clone M5/114.15.2) (dilution 1:300)	BioLegend	Cat#: 107613
Anti-mouse CD11c-PE-Cy7 (clone N418) (dilution 1:300)	BioLegend	Cat#: 117318
Anti-mouse CD326 (EpCAM)-PerCP-Cy5.5 (clone G8.8) (dilution 1:300)	BioLegend	Cat#: 118220
Anti-mouse CD301b-PE-Dazzle 594 (clone URA1) (dilution 1:300)	BioLegend	Cat#: 146816
Anti-mouse/human CD207 (Langerin)-PE (clone 4C7) (dilution 1:300)	BioLegend	Cat#: 144203
Anti-mouse CD45.1-APC (clone A20) (dilution 1:300)	BioLegend	Cat#: 110714
Anti-mouse CD45.1-BV510 (clone A20) (dilution 1:300)	BioLegend	Cat#: 110741
Anti-mouse CD45.1-APC/Fire750 (clone A20) (dilution 1:300)	BioLegend	Cat#: 110752
Anti-mouse CD16/32 (clone 2.4G2) (dilution 1:50)	BD Biosciences	Cat#: 553142
Normal rat serum (dilution 1:50)	Sigma	Cat#: R9759

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
RPMI1640	Corning	Cat#: 10-040-CV
Penicillin-streptomycin	Fisher Scientific	Cat#: 15-140-122
Fetal bovine serum	LDP	Cat#: FBS-02
BSA	AmericanBio	Cat#: 15-140-122
PBS	Sigma	Cat#: D8537
Ovalbumin (OVA), low endotoxin	Worthington	Cat#: LS003059
Papain	Sigma	Cat#: P4762
Freund's adjuvant complete	Sigma	Cat#: F5881
Collagenase D	Sigma	Cat#: 11088882001
Zombie Aqua Fixable Viability Kit	BioLegend	Cat#: 423012
Zombie UV Fixable Viability Kit	BioLegend	Cat#: 423108
Streptavidin-BV650	BioLegend	Cat#: 405232
Critical commercial assays		
EasySep™ Mouse CD4+ T Cell Isolation Kit	StemCell Technologies	Cat#: 19852
MojoSort™ Mouse CD4T Cell Isolation Kit	BioLegend	Cat#: 480033
Experimental models: Organisms/strains		
Mouse: C57BL/6N (B6) (6–12-week-old male or female)	Charles River Laboratory	Stock No: 027
Mouse: CD45.1 (B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ) (6–12-week-old male or female)	Charles River Laboratory	Stock No: 564
Mouse: OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J) (6–12-week-old male or female)	The Jackson Laboratory	Stock No: 004194
Mouse: Nur77^{GFP} (C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J) (6–12-week-old male or female)	The Jackson Laboratory	Stock No: 016617
Software and algorithms		
FlowJo (version 9.3.2 and 10.5.0)	Tree Star	N/A
Prism v 7.0c	GraphPad	N/A
Other		
BD LSRII	BD Biosciences	N/A
Attune NxT	Thermo Fisher Scientific	N/A

MATERIALS AND EQUIPMENT

Dissection medium (RPMI 10% FBS)

Reagent	Final concentration	Amount
RPMI1640	–	500 mL
FBS	10%	55 mL
Penicillin-Streptomycin (100 ×)	1 ×	5.6 mL

Dissection medium can be prepared in advance and stored at 4°C until use.

Collagenase D solution

Reagent	Final concentration	Amount
Collagenase D	0.25 g/mL	2.5 g
PBS	–	10 mL

Collagenase D can be prepared in advance and stored at −20°C until use.

FACS buffer w/o EDTA

Reagent	Final concentration	Amount
BSA	1%	5 g
Sodium azide	0.05%	0.25 g
PBS	–	500 mL

FACS buffer w/o EDTA can be prepared in advance and stored at 4°C for up to 3 months.

Fc block solution		
Reagent	Final concentration	Amount
anti-CD16/CD32 (2.4G2)	20 μg/mL	2 μL
Rat serum	2%	2 μL
PBS	–	100 μL
Fc block solution is made fresh each time and should be kept at 4°C in the dark.		

Zombie-UV/Aqua live/dead staining solution		
Reagent	Final dilution	Amount
Zombie-UV/Aqua	1:100	1 μL
PBS	–	100 μL
Zombie-UV/Aqua live/dead staining solution is made fresh each time and should be kept at 4°C in the dark.		

⚠ CRITICAL: DO NOT add EDTA to any of the buffers used in this protocol, as it can potentially disrupt the cellular conjugates formed *in vivo*.

STEP-BY-STEP METHOD DETAILS

The overall workflow for this protocol is shown in Figure 4.

Sample collection and enzymatic digestion

Timing: 1 h (for step 5)

This part details how to prepare cell suspensions from resected murine LNs.

1. Before euthanizing the mice, prepare LN digestion plates by placing a ~1 × 1 cm^2 piece of 100 μm nylon mesh at the bottom of each well of 24-well plates.
   a. Add 200 μL of dissection medium (RPMI1640 with 10% FBS) per well.
   b. Prepare as many wells as needed so that each well receives one LN of the mice. Place the plate on ice while collecting the LNs.
2. Euthanize the mice 3 or 24 h after the OT-II cell transfer and collect the popliteal draining LNs (Figure 5) on top of the nylon mesh in the well of a digestion plate.

   Note: Analysis on different time points allows examining whether the DC subsets binding to T cells changes over time during the process of priming.

3. Mechanically disrupt the LN.
   a. Cut the LN into tiny pieces with fine scissors.
   b. Gently mush it by grinding the LN pieces against the nylon mesh with a plunger of 1 mL syringe.
   c. Wash the scissors and the plunger with 800 μL of RPMI1640 with 10% FBS in the well, so that the total media volume in the well is 1 mL.
4. Digest LNs. Add 10 μL per well 0.25 g/mL Collagenase D (final concentration 2.5 mg/mL in RPMI1640) and incubate the plate in a 5% CO2 incubator at 37°C for 30 min.
5. Collect the digested LN cell suspension by gently pipetting up and down the media in the well with a 1 mL micropipette so that any remnants of the LN tissue will detach from the nylon mesh.
6. Place a ~1 × 1 cm^2 piece of nylon mesh on top of a 1.5 mL tube and pass the entire digested cell suspension (approximately 1 mL) through the mesh.
7. Take 10 μL for cell counting. Collect the remaining cells in the LN digestion plate by washing the well with ~0.6 mL ice-cold PBS and also pass the wash through the nylon mesh and combine with the rest of the cells.

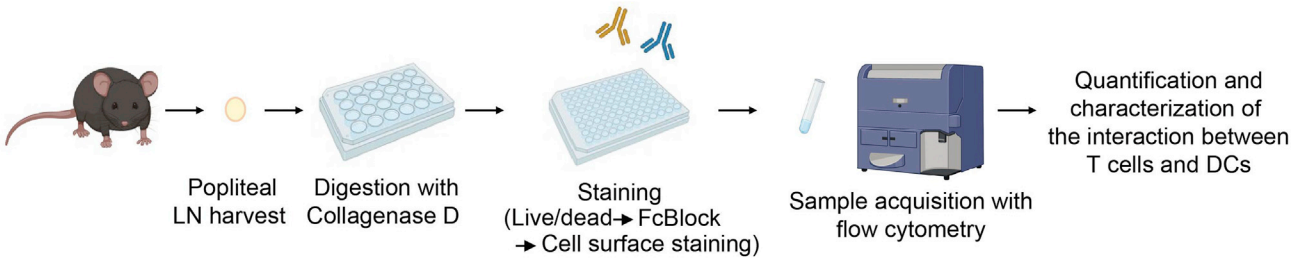

Figure 4. Operational workflow of this protocol

Note: Keep the cell suspensions on ice until pelleting.

8. Centrifuge the tubes at 500 × *g* for 5 min at 4°C. Remove supernatants and resuspend the cells in 200 μL of RPMI1640 with 10% FBS by gentle pipetting. Transfer the cell suspensions to 96-well U-bottom plates.
9. Centrifuge the plates at 850 × *g* for 2 min at 4°C, then dump the supernatant into the sink or a waste container.
10. Wash cells.
    a. Gently vortex the plate to loosen the cell pellet.
    b. Wash the cells by adding 200 μL/well EDTA-free PBS by a multi-channel pipette.
    c. Centrifuge the plates at 850 × *g* for 2 min at 4°C.
    d. Dump the supernatant into the sink or a waste container.

Staining the cells and acquisition on the flow cytometer

Timing: 2 h (for step 15)

This section describes how to stain dead cells with live/dead staining dye, stain cell surface markers with antibodies, and acquire samples by flow cytometry.

11. Stain dead cells.
    a. Loosen the cell pellets by gentle vortexing and add 20 μL/well dead cell staining dye (Zombie-UV, or Zombie-Aqua, diluted 1:100 in PBS).
    b. Suspend the cells by gently vortexing the plate.
    c. Incubate the plate on ice for 15–30 min.
    d. Wash the cells as in step 10.

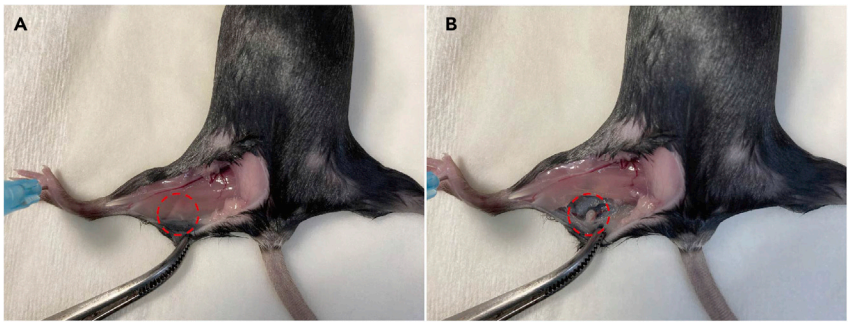

Figure 5. Location of popliteal lymph nodes of mice

(A and B) There is one popliteal lymph node localized within the fat tissue surrounding the nerves and major vessels of the popliteal fossa in each hind leg. Before (A) and after (B) removing the fat around the lymph node.

Table 1. Antibody panel for flow cytometry of OT-II cell-APC conjugates

Marker	Fluorochrome	Final dilution
CD45.1	Allophycocyanin	1:300
MHCII	Alexa Fluor (AF)700	1:300
CD69	Pacific Blue	1:300
CD11c	PE-Cy7	1:300
B220	FITC or Brilliant Violet (BV) 650 (when transferring Nur77-GFP OT-II cells)	1:300
CD207	PE	1:300
CD301b	PE Dazzle 594	1:300

Note: Although live/dead cell staining could be done together with Fc Block or cell surface marker staining step, some venders recommend buffers free of sodium azide and protein for fixable live/dead staining dye. We stain dead cells with Zombie-Aqua or Zombie-DAPI live/dead staining dye (BioLegend) diluted in PBS as a separate step.

12. Stain cell surface markers.
    a. Add 10 μL/well Fc block solution (see materials and equipment section).
    b. Suspend the cells by gentle vortexing.
    c. Incubate the plate on ice for 15 min.
    d. Without washing, add 10 μL/well cell surface staining Ab cocktails prepared in FACS buffer w/o EDTA as indicated in Table 1.
    e. Suspend the cells by gentle vortexing.
    f. Incubate the plate for another 15–30 min on ice.
    g. Wash the cells as in step 10.

Optional: To confirm that the OT-II-APC conjugates (defined as $CD45.1^+$ $MHCII^+$ double positive events with a high FSC/SSC ratio) are cellular conjugates formed *in vivo* rather than those formed *ex vivo* during the staining process, stain two sets of LN cells separately for CD45.1 and MHCII with different sets of fluorochromes (Table 2) as above in step 12, and then mix together and incubate for another 10 min at room temperature (20°C–25°C) (Figure 6).

13. Wash the cells as in step 10 and resuspend them in 200 μL PBS. Filter the cells by passing the cell suspension through a small piece of nylon mesh and collect them into a FACS tube (5 mL round-bottom polystyrene tube).

Note: Keep the samples on ice until loaded on a flow cytometer.

14. Define the gating strategy (see Figure 7 for an example).

Note: Refer to data analysis section for additional information on the gating strategy.

Table 2. Antibody panels for fluorochrome exchange

Marker	Fluorochrome set 1	Fluorochrome set 2	Final dilution
CD45.1	BV 510	APC/Fire750	1:300
MHCII	Allophycocyanin	AF700	1:300
CD69	Pacific Blue	Pacific Blue	1:300
CD11c	PE-Cy7	PE-Cy7	1:300
B220	FITC	FITC	1:300
CD207	PE	PE	1:300
CD301b	PE Dazzle 594	PE Dazzle 594	1:300

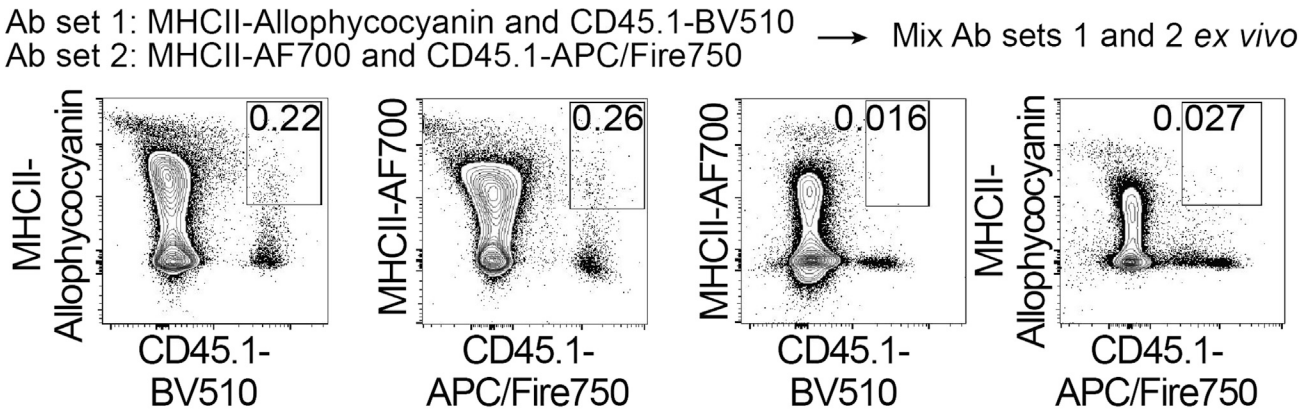

Figure 6. Detection of CD45.1$^+$ MHCII$^+$ OT-II-APC conjugates by fluorochrome exchange *in vitro*

Representative dot plots of CD45.1$^+$ MHCII$^+$ events in the fluorochrome exchange assay, where two sets of LN cells of OT-II-transferred mice are separately stained for CD45.1 and MHCII with different sets of fluorochromes (Ab set 1 or 2), mixed together *in vitro*, and incubated for another 10 min to allow fluorochrome exchange.

15. Acquire all of isolated LN cells (typically 1–2 × 10^6 cells from one popliteal dLN) while watching the flow rate.

Note: The optimal flow rate should be determined by the user for each experiment condition (Figure 8). We typically run at 5,000 events/s or less on the Attune NxT flow cytometer (Thermo Fisher Scientific).

EXPECTED OUTCOMES

In a typical C57BL/6N mouse one or two days after the immunization with OVA plus papain (3 h after the OT-II cell transfer), 1–2 × 10^6 cells are isolated from the draining popliteal LN. As shown in

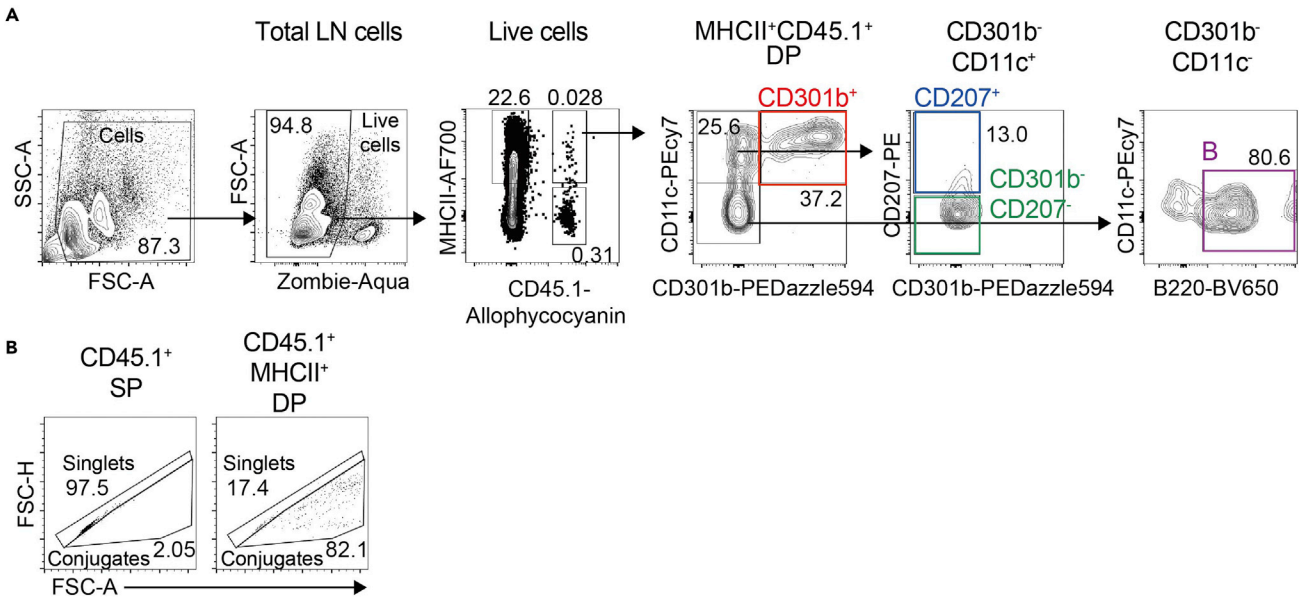

Figure 7. Gating strategy for detecting cellular conjugates between APCs and OT-II cells

(A) Representative dot plots of gating strategy used in this protocol are shown. Adapted from Tatsumi et al. (2021).[1] Reprinted with permission from AAAS.

(B) Representative forward scatter area (FSC-A) and height (FSC-H) of CD45.1 single positive (SP) or CD45.1$^+$ MHCII$^+$ double positive (DP) events 3 h after OT-II cell transfer.

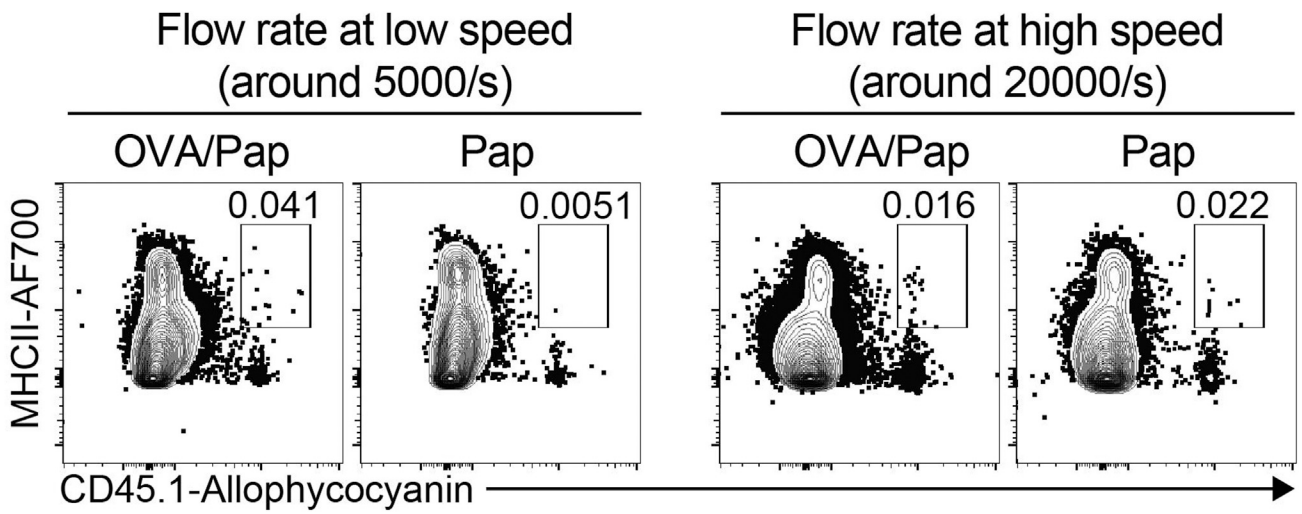

Figure 8. Increase in the false CD45.1$^+$MHCII$^+$ conjugates at high flow rate

Stained LN cells from mice immunized with OVA and papain (OVA/Pap) or papain alone (Pap) were analyzed at a low speed (around 5,000 events/s) or high (around 20,000 events/s) flow rate using Attune NxT flow cytometer (Thermo Fisher Scientific). When cells were acquired at a higher flow rate, CD45.1$^+$MHCII$^+$ "false conjugates" start to appear even in samples prepared from mice immunized without the cognate antigen (OVA), likely due to the increased frequency of multiple singlet cells passing through the detector simultaneously. Representative dot plots of CD45.1$^+$MHCII$^+$ are shown.

Figure 1 and in our previous publication,[1] the percentage of the CD45.1$^+$ MHCII$^+$ OT-II-APC conjugates among the total LN events ranges between 0.02%–0.03%. Approximately 25% of the CD45.1$^+$ MHCII$^+$ cellular conjugates contain CD301b$^+$ DCs when the mice are immunized with OVA plus papain (Figure 9).

Besides identifying the DC subset conjugated with OT-II cells, this protocol is useful for assessing the activation status of OT-II cells in the OT-II-APC conjugates. The Nur77-GFP reporter (a surrogate for TCR signaling) and CD69 are upregulated in most of the OT-II cells conjugated with any of the DC subsets as early as 3 h after the adoptive transfer, whereas these markers are expressed by only a half of the OT-II cells conjugated with B cells (Figure 10).

The majority of the CD45.1$^+$ MHCII$^+$ events represent cellular conjugates formed *in vivo* in the dLN rather than those formed *ex vivo* during the staining process. This can be experimentally tested by splitting the LN cells into two samples and staining them with two different sets of fluorochromes for the CD45.1 and MHCII (e.g., sample 1: CD45.1-BV510 and MHCII- Allophycocyanin ; sample 2 CD45.1-APC/Fire750 and MHCII-AF700), and then mixing those samples together *in vitro* (see

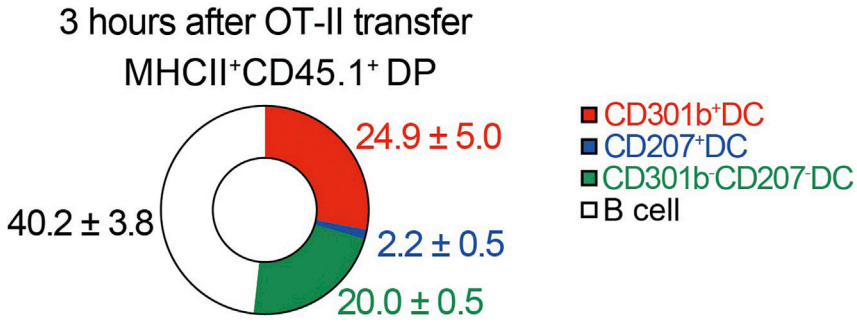

Figure 9. APC subset composition in the CD45.1$^+$ MHCII$^+$ OT-II-APC conjugates

CD301b$^+$ DCs, CD207$^+$ DCs, CD301b$^-$ CD207$^-$ DCs, and B220$^+$ CD11c$^-$ B cells were identified within the CD45.1$^+$ MHCII$^+$ DP events in Figure 1. Data represent mean ± SEM. Adapted from Tatsumi et al. (2021).[1] Reprinted with permission from AAAS.

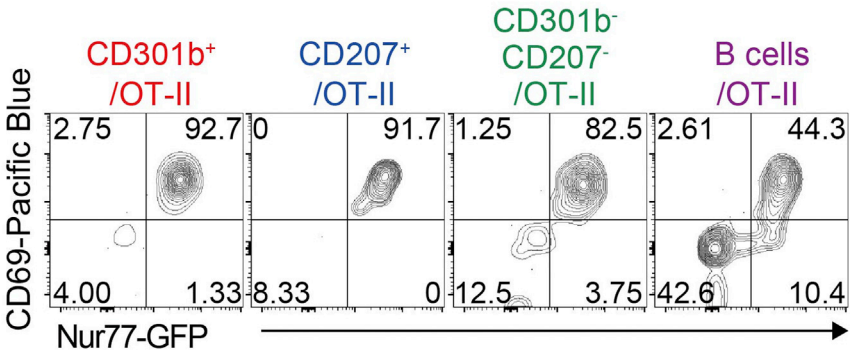

Figure 10. Expression of CD69 and Nur77-GFP reporter in OT-II cells conjugated with different APC subsets
Representative contour plots for the CD45.1$^+$ MHCII$^+$ events with the indicated APC subset markers in the dLNs 3 h after OT-II transfer. Adapted from Tatsumi et al. (2021).[1] Reprinted with permission from AAAS.

"optional" procedure in step 12). If the OT-II-APC conjugates are formed *in vitro*, there should be OT-II-APC conjugates formed between OT-II cells from the sample 1 and APCs from the sample 2 and *vice versa*, which would result in cell-cell conjugates represented as BV510$^+$AF700$^+$ and APC/Fire750$^+$Allophycocyanin$^+$ events ("fluorochrome exchange" between sample 1 and 2) in flow cytometry. However, only a minimal fluorochrome exchange is observed (Figure 6).

The majority of the CD45.1$^+$ MHCII$^+$ events detected in this protocol are antigen-dependent conjugates, as the double positive events are reduced when the recipient mice are immunized with papain alone compared to when immunized with OVA and papain (Figure 11). Moreover, the frequency of the cells conjugated with OT-II cells among CD301b$^+$ DCs is lower in the mice immunized with papain alone than in those immunized with OVA and papain, while no significant difference is observed in most of other APC subsets including B cells, contribute to a significant portion (approximately 40%) of the cellular conjugates observed (Figure 11B). The majority of the OT-II-B cell conjugates detected in this protocol thus seem to be antigen-independent. Accordingly, more than 50% of OT-II cells conjugated with B cells do not express Nur77 and CD69 even in the presence of OVA, as described in Figure 10.

QUANTIFICATION AND STATISTICAL ANALYSIS

Timing: 1 h

This part outlines how to identify and analyze the OT-II-APC conjugates in the flow cytometry data.

- To detect cellular conjugates, unlike commonly used approaches for flow cytometric data analysis where cellular conjugates (doublets) are excluded, all events are analyzed without excluding doublets. Representative data are shown in Figure 7.
- Among the total LN cells, the OT-II-APC conjugates are identified as CD45.1$^+$ MHCII$^+$ double positive events, as the OT-II cells express CD45.1 but not MHCII while host APCs express MHCII but not CD45.1. The gating strategy is presented in Figure 7. CD45.1$^+$ MHCII$^+$ events can be further characterized with additional markers for each APC subset such as CD11c, CD207, CD301b as well as B220 for B cells (Figure 7).
- For analyzing the data obtained from the optional procedure described in step 12, check the frequencies of conjugates formed between the cells from the two separate samples stained independently with two sets of antibodies shown in Table 2. The events showing "fluorochrome exchange" between these two samples (e.g., BV510$^+$ AF700$^+$ and APC/Fire750$^+$ Allophycocyanin$^+$) represent CD45.1$^+$ MHCII$^+$ conjugates formed *in vitro* during the staining process.

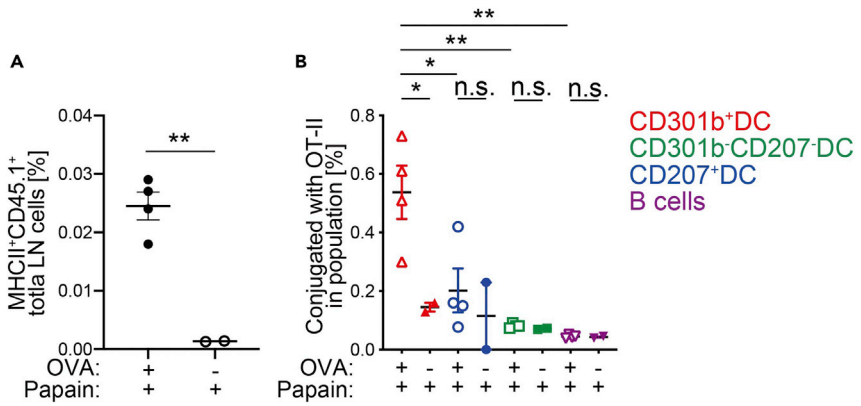

Figure 11. Antigen dependency of the formation of CD45.1$^+$ MHCII$^+$ APC-OT-II conjugates

(A and B) The frequency of the total CD45.1$^+$ MHCII$^+$ events (A) and the frequency of OT-II conjugated fraction within each antigen-presenting cell subset (B) 3 h after OT-II transfer were compared between mice immunized with papain alone compared to those immunized with OVA plus papain. Data are represented as mean ± SEM. *P < 0.05, **P < 0.01, and n.s., by two-tailed Student's t test. Adapted from Tatsumi et al. (2021).[1] Reprinted with permission from AAAS.

LIMITATIONS

Since this protocol was designed to detect the cellular conjugates between the adoptively transferred donor OT-II cells and the skin-derived migratory DC subsets of the host in the skin-draining LNs, the cell preparation procedures such as the enzymatic digestion and antibody panels are optimized for skin-resident DC subsets and OT-II CD4T cells. We have verified that the antigen-specific interaction between CD8T cells and DCs can be quantified by a similar approach using herpes simplex virus (HSV)-specific TCR transgenic CD8T cells in the model of HSV infection in mice (data not shown). In addition, a similar approach has also been used for analyzing the interaction between B cells and CD4T cells.[2] However, additional optimization may be required when working with other types of T cell-APC interactions.

While this flow cytometry-based protocol is useful for quantifying the OT-II-APC conjugates and identifying the APC subset actively interacting with OT-II cells, additional imaging-based approaches such as immunohistochemistry and imaging flow cytometry may be needed to fully verify and interpret the data. For instance, histological analyses in combination with this protocol provide better understanding of the intranodal localization of the OT-II-DC conjugates.[1] Moreover, the combination of this protocol with single cell RNA sequencing (scRNA-seq) offers a powerful tool for analyzing the molecular features of the interacting cells.[3,4]

TROUBLESHOOTING

Problem 1

Contamination of false CD45.1$^+$ MHCII$^+$ conjugates.

Potential solution

Keep the flow rate between 2,000–5,000/events when using Attune NxT (Thermo Fisher Scientific) in step 15. Users must optimize the flow rate depending on their machine's preference, as acquisition at high speed may increase the chance of detecting "false conjugates" from multiple singlet cells passing the detector simultaneously (Figure 8).

Another reason for the increase in false conjugates is due to contamination of dead cells. Dead cells have autofluorescence and can increase non-specific antibody binding. Exclusion of dead cells is required in step 11. We use Zombie-UV/Aqua Fixable Viability Kit (BioLegend) to separate live cells from dead cells.

Problem 2

Low or no detection of CD45.1$^+$MHCII$^+$ conjugates.

Potential solution

Low purity of the donor OT-II cells can result in low or no detection of CD45.1$^+$MHCII$^+$ conjugates. Highly purified (>90% CD4$^+$) OT-II cells should be used for the transfer. If the purity is low, the cells should be re-sorted by magnetic cell sorting.

Another potential reason for poor conjugate detection could be low numbers of donor CD4T cells for the transfer. Based on our experience, transferring at least 1 × 10^6 OT-II cells is recommended for obtaining clean data.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yosuke Kumamoto (yosuke.kumamoto@rutgers.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Row fcs files used in this study have not been deposited in a public repository but are available from the corresponding author on request.

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AUTHOR CONTRIBUTIONS

N.T. and Y.K. conceptualized and designed the study. N.T. and A.D.-P. performed experiments. N.T. analyzed the data. N.T. and Y.K. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Tatsumi, N., Codrington, A.L., El-Fenej, J., Phondge, V., and Kumamoto, Y. (2021). Effective CD4T cell priming requires repertoire scanning by CD301b(+) migratory cDC2 cells upon lymph node entry. Sci. Immunol. *6*, eabg0336. https://doi.org/10.1126/sciimmunol.abg0336.

2. Reinhardt, R.L., Liang, H.E., and Locksley, R.M. (2009). Cytokine-secreting follicular T cells shape the antibody repertoire. Nat. Immunol. *10*, 385–393. https://doi.org/10.1038/ni.1715.

3. Giladi, A., Cohen, M., Medaglia, C., Baran, Y., Li, B., Zada, M., Bost, P., Blecher-Gonen, R., Salame, T.M., Mayer, J.U., et al. (2020). Dissecting cellular crosstalk by sequencing physically interacting cells. Nat. Biotechnol. *38*, 629–637. https://doi.org/10.1038/s41587-020-0442-2.

4. Cohen, M., Giladi, A., Barboy, O., Hamon, P., Li, B., Zada, M., Gurevich-Shapiro, A., Beccaria, C.G., David, E., Maier, B.B., et al. (2022). The interaction of CD4(+) helper T cells with dendritic cells shapes the tumor microenvironment and immune checkpoint blockade response. Nat. Cancer *3*, 303–317. https://doi.org/10.1038/s43018-022-00338-5.