Chapter 5

Monitoring the Interaction Between Dendritic Cells and T Cells In Vivo with LIPSTIC

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Abstract

Interactions between different cell types are key for immune function. Traditionally, interactions have been investigated in vivo by intravital two-photon microscopy, but the molecular characterization of the cells participating in a specific interaction is limited by the inability to retrieve the cells for downstream analysis. We recently developed an approach to label cells undergoing specific interactions in vivo, which we called LIPSTIC (Labeling Immune Partnership by Sortagging Intercellular Contacts). Here, we provide detailed instructions on how to track CD40-CD40L interactions between dendritic cells (DCs) and $CD4^+$ T cells using genetically engineered LIPSTIC mice. This protocol requires expertise in animal experimentation and multicolor flow cytometry. Once mouse crossing has been achieved, it takes 3 days or more to complete, depending on the kinetics of the interactions that the researcher wishes to investigate.

Key words Dendritic cells, Interactions, $CD4^+$ T cells, LIPSTIC

1 Introduction

Cell-cell interactions are essential for several biological processes. In the immune system, multiple functions rely on direct cell-cell contacts, including positive and negative selection of T lymphocytes in the thymus, presentation of antigen to T cells by antigen-presenting cells (APCs), killing of infected cells by cytotoxic $CD8^+$ T cells, and help to B cells provided by $CD4^+$ T cells for antibody affinity maturation. Despite such an essential role of direct cell-cell signaling in many immunological processes, studying cell-cell interactions in vivo remains a challenge.

To facilitate the study of cell-cell interactions in vivo, we recently developed a novel technology called LIPSTIC (Labeling Immune Partnerships by SorTagging Intercellular Contacts) [1, 2] that allows one to enzymatically label cell-cell interactions in vivo and subsequently retrieve interacting cells for downstream analysis.

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Here, we describe the LIPSTIC approach and provide technical details for its use in monitoring CD40-CD40L interactions between dendritic cells (DCs) and $CD4^+$ T cells in vivo using genetically engineered mice.

LIPSTIC is based on *S. aureus* enzyme Sortase A (SrtA), a transpeptidase capable of mediating the covalent ligation of a substrate containing the recognition sequence LPXTG (where X is any amino acids) to an N-terminal glycine residue. Upon recognition of the LPXTG motif, SrtA catalyzes the hydrolysis of the peptide bond between threonine and glycine and forms an acyl intermediate between the cysteine present on its catalytic pocket and the substrate threonine. The substrate can then be covalently ligated to an N-terminal glycine, thanks to the formation of a novel peptide bond [3]. For LIPSTIC, we genetically fused SrtA or a five-glycine (G5) tag to the extracellular portion of a ligand and receptor of interest. Upon ligand-receptor interaction, SrtA mediates the covalent ligation of a labeled substrate (e.g., a biotinylated or fluorescently labeled LPXTG peptide) to the G5-tagged molecule. After the interaction has occurred, the G5 expressing cell participating in interaction can be readily identified and retrieved by flow cytometry based on the presence on its surface of the covalently ligated label (Fig. 1) [1].

We employed the LIPSTIC approach to monitor CD40-CD40L interaction between DCs and $CD4^+$ T cells. In our published work [1], we generated two novel mouse lines: $Cd40^{G5}$, which constitutively expresses CD40 carrying a G5 tag at its extracellular portion, and $Cd40lg^{SrtA}$, which upon Cre recombination expresses CD40L fused to SrtA. Using these mice, it is possible to

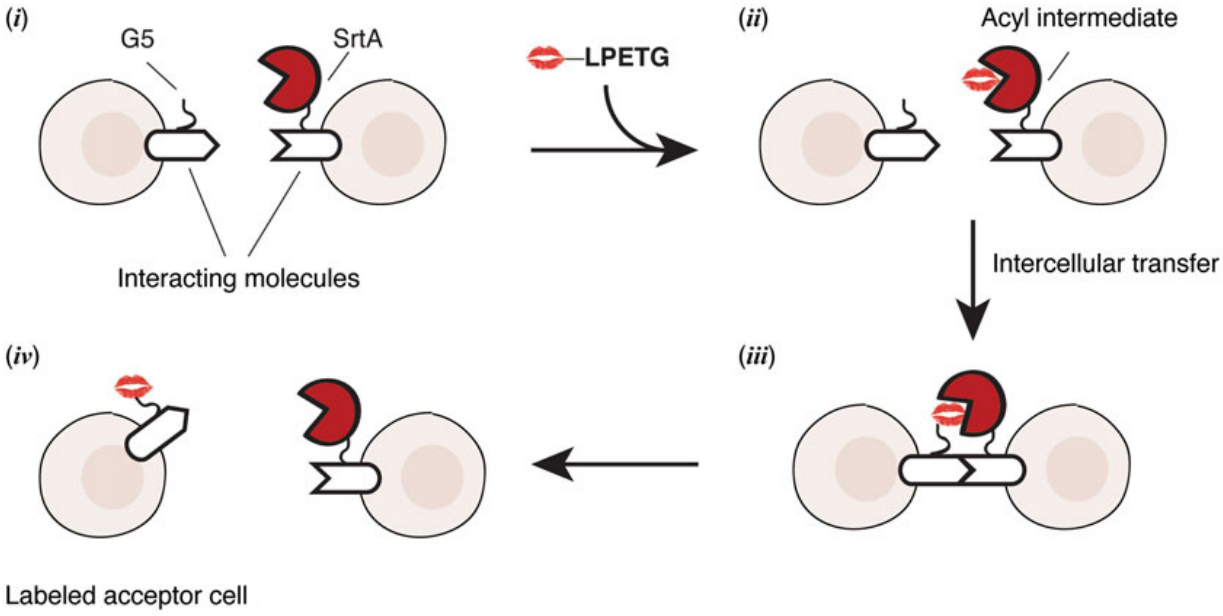

Fig. 1 LIPSTIC labeling to track cell-cell interactions. Schematic representation of the LIPSTIC approach

perform in vivo LIPSTIC labeling during the course of the immune response elicited by subcutaneous protein immunization or by transfer of antigen-pulsed DCs. Our published observations indicate that CD40-CD40L interactions between dendritic cells and $CD4^+$ T cells take place in two different modalities during the initial phases of the immune response: an early phase (at ~12 h post T cell transfer), when T cells label exclusively DCs that carry the cognate antigenic peptide, and a late phase (at ~48 h after T cell transfer), when non-cognate contacts between T cells and bystander DCs can also be observed.

In the following protocol, we provide details on how to track the interactions between DCs and antigen-specific $CD4^+$ T cells in the context of an immune response induced by immunization with ovalbumin (OVA) as model antigen. For this purpose, this protocol is divided into four experimental sections: (i) the adoptive transfer of OVA-specific *Cd40lg*$^{SrtA/SrtA}$ CD45.1/1 CD4-Cre$^+$ OT-II $CD4^+$ T cells into *Cd40*$^{G5/G5}$ hosts, (ii) the induction of an immune response in *Cd40*$^{G5/G5}$ hosts by immunization with OVA, (iii) the injection of SrtA substrate to achieve in vivo LIPSTIC labeling, and (iv) the analysis of LIPSTIC-labeled cells by flow cytometry. A schematic representation of the experimental design is provided in Fig. 2a.

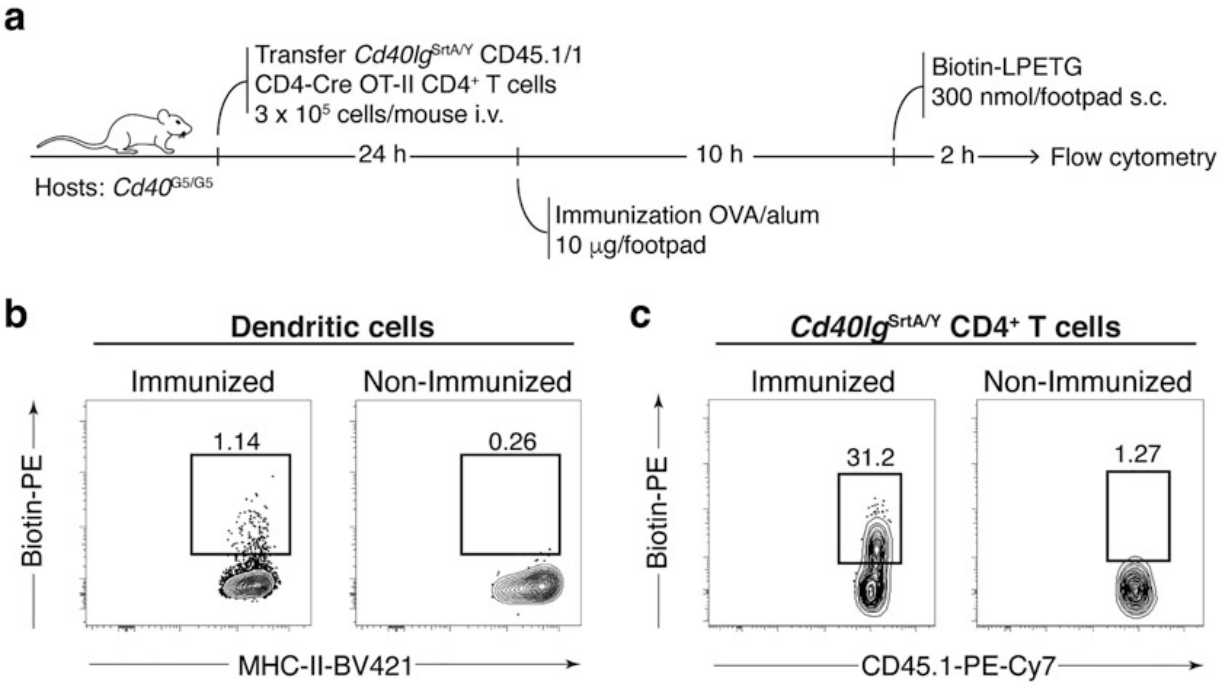

Fig. 2 LIPSTIC labeling of CD40-CD40L interactions between T cells and dendritic cells in the popliteal lymph node. (**a**) Experimental design for (**b**), (**c**). CD45.1 is encoded by *Ptprc*^{a}; homozygotes are indicated as CD45.1/1. (**b**) Flow cytometric analysis of popliteal LN cells showing biotin labeling of endogenous dendritic cells at 12 h post immunization (left) or nonimmunized control (right). (**c**) Flow cytometric analysis of popliteal LN cells showing biotin labeling of adoptively transferred *Cd40lg*$^{SrtA/Y}$ CD45.1/1 CD4-Cre OT-II $CD4^+$ T cells at 12 h post immunization (left) or nonimmunized control (right)

2 Materials

2.1 Mouse Strains and Animal Procedures

1. *Cd40*$^{G5/G5}$ and *Cd40lg*$^{SrtA/SrtA}$ CD45.1/1 CD4-Cre OT-II mice (*see* **Note 1**).
2. Rodent anesthesia system with isoflurane.
3. CO_2 euthanasia chamber (only if this euthanasia method is approved by your institution's animal ethics committee).
4. Scissors and forceps for spleen and lymph node harvesting.
5. 1 mL insulin syringes with 28-G 1/2 in. needle.

2.2 Isolation and Transfer of Naïve CD4$^+$ T Cells

1. PBE buffer: PBS, 0.5% bovine serum albumin (BSA), 2 mM EDTA.
2. Ammonium-chloride-potassium (ACK) lysis buffer for red blood cell lysis.
3. 70 μm strainers.
4. 3 mL syringes.
5. 50 mL Falcon tubes.
6. Naïve CD4$^+$ T cell isolation kit (Miltenyi Biotec, *see* **Note 2**).
7. Hemocytometer.

2.3 Immunization

1. Imject Alum.
2. 1 mg/mL Ovalbumin solution (*see* **Note 3**).
3. PBS.
4. Rotator or shaker.

2.4 In Vivo LIPSTIC Labeling and Analysis of LIPSTIC-Labeled Cells by Flow Cytometry

1. 20 mM Biotin-LPETGS peptide in PBS (*see* **Note 4**). Dissolve lyophilized peptide in PBS to a final concentration of 20 mM. Sterilize solution by filtration trough a 0.22 μm filter. Aliquot solution and store at −80 °C for long-term storage. For in vivo labeling, prepare a 2.5 mM solution in PBS.
2. Collagenase digestion buffer: RPMI 2% fetal bovine serum (FBS), 20 mM HEPES, 400 U/mL type IV collagenase.
3. Plastic pestle for 1.5 mL microfuge tubes.
4. 1.5 mL microfuge tubes.
5. PBE buffer.
6. Antibodies and viability dyes for the analysis of interacting DCs and CD4$^+$ T cells: CD16/32, CD11c, CD11b, MHC II, Biotin, XCR1, B220, NK1.1, CD3, Zombie NIR fixable viability dye (*see* Table 1 and **Note 5**).

Table 1
Antibodies for DCs and CD4$^+$ T cell characterization by flow cytometry

Marker	Conjugation	Clone	Dilution	Manufacturer	Cat. N.
CD16/32	/	2.4G2	1 μg/mL	Bio X Cell	101242
CD11c	APC	N418	1:800	Biolegend	117310
CD11b	BV711	M1/70	1:800	Biolegend	101242
MHC-II	BV421	M5/114.15.2	1:400	Biolegend	107632
Biotin	PE		1:11	Miltenyi Biotec	130-090-756
XCR1	PerCP/Cy5.5	ZET	1:200	Biolegend	148208
CD45.1	PE/Cy7	A20	1:200	Biolegend	110730
B220	BV785	RA3-6B2	1:400	Biolegend	103246
NK1.1	BV785	PK136	1:400	Biolegend	108749
CD3	BV785	17A2	1:400	Biolegend	100355
Zombie	NIR	/	1:1000	Biolegend	423106

7. Flow cytometers equipped with lasers and emission filters suitable for the analysis of cells stained with the dyes listed in the antibody panel (*see* **Note 6**).
8. Cytofix (BD Bioscience).
9. FlowJo software for flow cytometry data analysis.

3 Methods

3.1 Adoptive Transfer of Ovalbumin-Specific Naïve Cd40lg$^{SrtA/SrtA}$ CD45.1/1 CD4-Cre OT-II CD4$^+$ T Cells into Cd40$^{G5/G5}$ Hosts (See Note 7)

1. Euthanize *Cd40lg*$^{SrtA/SrtA}$ CD45.1/1 CD4-Cre OT-II mice using the method approved by your institution's animal ethics committee.
2. Harvest the spleen using scissor and forceps.
3. Mesh the spleen through 70 μm strainer into 50 mL falcon tube using the plunger of a 3 mL syringe.
4. Rinse the strainer with PBE buffer and spin cells at 300 × *g* for 5 min.
5. Remove the supernatant and lyse the red blood cells by adding 1 mL of ACK lysis buffer per spleen. Incubate for 3–5 min at room temperature (RT). Stop the lysis by adding 4 mL of PBE buffer. Filter the cell suspension through 70 μm strainer.
6. Spin the cells at 300 × *g* for 5 min.
7. Perform the isolation of CD4$^+$ T cells using Miltenyi Biotec CD4$^+$ T cell isolation kit. Follow exactly the Miltenyi Biotec CD4$^+$ T cell isolation kit protocol.

8. Using the hemocytometer, count the number of $CD4^+$ T cells retrieved. The typical yield is 8–10 × 10^6 cells/spleen.
9. Carefully resuspend cells in PBS at a final concentration of 3 × 10^6 cells/mL.
10. Using an insulin syringe, inject 3 × 10^5 naïve (100 μL) $CD4^+$ T cells into *Cd40*$^{G5/G5}$ mouse intravenously using the method approved by your institution's animal ethics committee.

3.2 Immunization of Cd40$^{G5/G5}$ Hosts (See Note 8)

In general, alum should be one third of the final immunization mix. Here, we provide details for a 600 μL of immunization mix.

1. Vortex alum extensively.
2. Mix 200 μL of alum with 240 μL of ovalbumin (OVA) (1 mg/mL) and 160 μL of PBS.
3. Incubate the immunization mix at 4 °C on the rotator or shaker for at least 30 min.
4. For immunization of popliteal lymph nodes, using an insulin syringe inject 25 μL (10 μg of OVA) of immunization mix subcutaneously into the hind footpad of a *Cd40*$^{G5/G5}$ mouse.

3.3 Injection of SrtA Substrate to Achieve In Vivo LIPSTIC Labeling (See Note 9)

In order to capture solely antigen-specific T cell-DC interactions labeling needs to be performed within 12 h of immunization [1].

1. Briefly anesthetize the mice with isoflurane before each injection.
2. Inject Biotin-LPETG substrate subcutaneously into the hind footpad (20 μL of 2.5 mM substrate solution in PBS, equivalent of 50 nmol of substrate).
3. Repeat **steps 1** and **2** for a total of six times with 20 min interval between injections. Wait 40 min after the last injection before euthanasia.

3.4 Analysis of LIPSTIC-Labeled Cells by Flow Cytometry

1. Euthanize mice using the method approved by your institution's animal ethics committee.
2. Using forceps, carefully harvest popliteal lymph nodes and place them in 1.5 mL microfuge tube containing 200 μL of collagenase digestion buffer.
3. Incubate for 30 min at 37 °C.
4. Using a plastic pestle, gently macerate the LN against the wall of a 1.5 mL microfuge tube (*see* **Note 10**).
5. Add 600 μL of PBE buffer.
6. Pass the digested lymph node through a 70 μm strainer into a 1.1 mL microtube.
7. Spin cells down at 300 × *g* for 5 min. Remove supernatant.

8. Resuspend cells in 100 µL of PBE buffer with 1 µg/mL of anti-CD16/32 antibody for 5 min at room temperature.
9. Without washing, add 100 µL of 2× antibody solution in PBE buffer, for a final volume of 200 µL. Incubate at 4 °C for 15 min.
10. Add 800 µL of PBS. Spin cells down at 300 × *g* for 5 min. Remove supernatant.
11. Stain cells in PBS with Zombie fixable viability dye according to the manufacturer's instructions.
12. Fix cells with Cytofix (BD Biosciences) according to the manufacturer's instructions. If cells will be sorted for subsequent RNA extraction or functional assays, omit the fixation step.
13. Acquire samples using a flow cytometer equipped with appropriate lasers. Collect 1–2 million events per sample.
14. Analyze data using FlowJo software. To identify classical endogenous DCs, apply the following gating strategy (Fig. 3a, b):
    - Exclude doublets based on FSC and SSC parameters (area A, height H, width W).

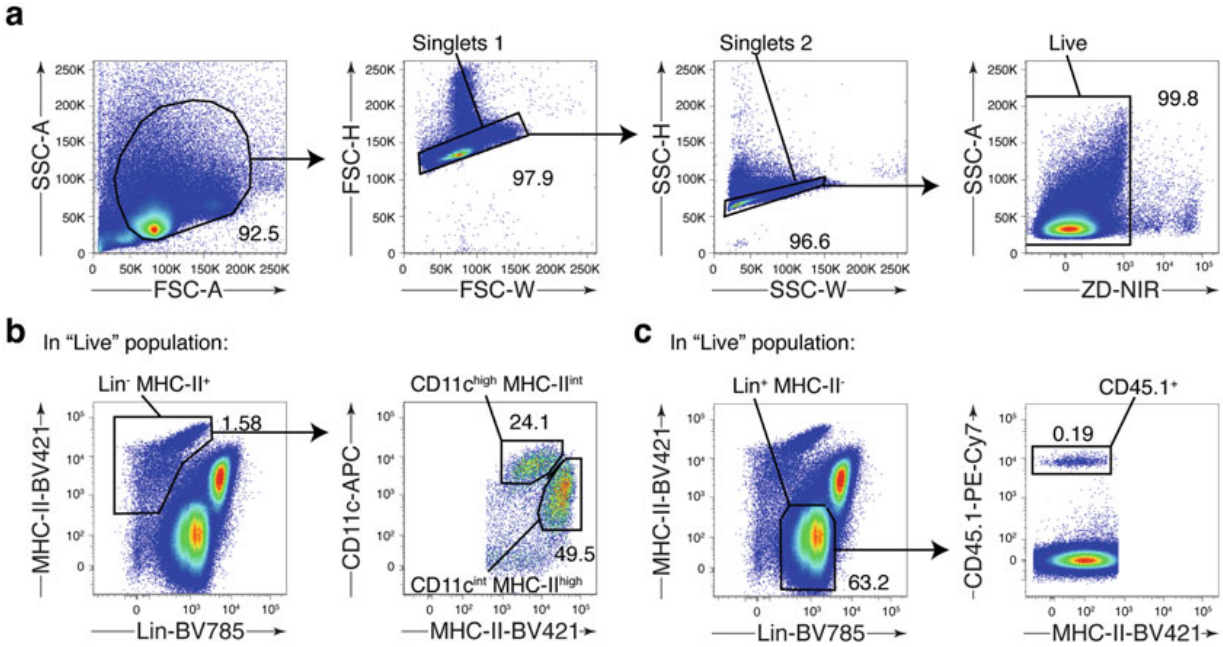

Fig. 3 Gating strategy for the analysis of endogenous dendritic cells and adoptively transferred T cells. (**a**) Gating strategy to exclude doublets and dead cells. ZD: Zombie viability dye. (**b**) In the "Live" gate, conventional DCs are identified as Lin^- ($B220^-$, $NK1.1^-$, $CD3^-$) $MHC\text{-}II^+$. In the mouse lymph node, two populations of conventional DCs with differential expression of CD11c and MHC-II can be identified: $CD11c^{high}MHC\text{-}II^{int}$ and $CD11c^{high}MHC\text{-}II^{int}$. (**c**) In the "Live" gate, adoptively transferred T cells are identified as Lin^+ $MHC\text{-}II^-$ $CD45.1^+$

- Exclude dead cells based on Zombie Dye (ZD) fluorescence intensity. Live cells are ZD^-.
- Gate on Lin^- ($B220^-$, $CD3^-$ and $NK1.1^-$)$MHC\text{-}II^+$ cells.
- Based on this gating strategy in the mouse lymph node two populations of conventional DCs with different expression of CD11c and MHC-II can be identified: $CD11c^{high}$ $MHC\text{-}II^{int}$ and $CD11c^{int}$ $MHC\text{-}II^{high}$.
- In the identified DC populations, analyze biotin fluoresce signal as well as CD11b and XCR1 to characterize labeled DCs.

To identify adoptively transferred CD45.1/1 *Cd40lg*$^{SrtA/SrtA}$ CD4-Cre OT-II T cells apply the following gating strategy (Fig. 3a, c):

- Exclude doublets based on FSC and SSC parameters (area A, height H, width W).
- Exclude dead cells based on Zombie Dye (ZD) fluorescence intensity. Live cells are ZD^-.
- Gate on $MHC\text{-}II^-$ Lin^+ cells.
- Gate on $CD45.1^+$ cells. This population corresponds to adoptively transferred T cells.

4 Notes

1. *Cd40*$^{G5/G5}$ and *Cd40lg*$^{SrtA/SrtA}$ CD4-Cre OT-II mice are available upon request from our laboratory. Mice carrying the CD4-Cre or OT-II TCR transgene are commercially available. To be able to reliably identify $LIPSTIC^+$ events, we advise to always include in the experiment control mice that receive *Cd40lg*$^{+/+}$ $CD4^+$ T cells instead of *Cd40lg*$^{SrtA/SrtA}$; these samples will provide a baseline that accounts for potential nonspecific effects such as nonenzymatic uptake of SrtA substrate, direct binding to the secondary reagent, or autofluorescence. Experimental procedures involving animals must be carried out according to all relevant institutional and governmental regulations.
2. We routinely use the naïve $CD4^+$ T cell isolation kit (Miltenyi Biotec) to isolate naïve $CD4^+$ T cells. Other strategies granting the isolation of $CD4^+$ T cells can also be employed.
3. To prepare an ovalbumin solution suitable for immunization, dissolve lyophilized ovalbumin in PBS to a final concentration of 1 mg/mL. Sterilize solution by filtration trough a 0.22 μm filter. Aliquot and store at −20 °C for long-term storage.

4. Biotin-LPETGS peptide can be purchased from the company of choice as custom synthesis specifying the following characteristics: Sequence: LPETGS; N-terminus modification: biotin-aminohexanoic acid; C-terminus modification: amide; purity: 95%.
5. We focused our analysis on the conventional DC compartment in mouse lymph nodes. We provide this flow cytometry panel for DC identification and subsetting as guideline, but other markers can be used depending on the needs of the investigator. The secondary reagent used to detect the SrtA substrate should be conjugated to a very bright fluorochrome such as phycoerythrin (PE), and we recommend anti-biotin antibody (Miltenyi Biotec) owning to its high specificity and low background compared to streptavidin conjugates. Finally, we use Zombie NIR fixable viability dye (Biolegend) to discriminate live cells from dead cells. To prepare it for use, dissolve the lyophilized dye in 100 ul of DMSO. Aliquot and store at −20 °C for up to 1 month. Other fixable and non-fixable viability dyes can be used.
6. We currently use BD LSR II, Fortessa, or Symphony for flow cytometric analysis. If instruments with the suitable set up are not available, antibody panel can be changed according to cytometer configuration.
7. In order to monitor an antigen-specific response, we found it useful to take advantage of adoptive transfer of naïve antigen-specific *Cd40lg*$^{SrtA/SrtA}$ CD4-Cre^{+} CD4^{+} T cells. This approach has two advantages: (i) It ensures that the interactions being followed are the result of the immune response of interest (and not of baseline immune interactions occurring at the same site), and (ii) it allows us to easily synchronize the T cell response and infer the T cell interaction mode (antigen-dependent vs independent) based on the time of the adoptive transfer. Conversely, the analysis of a response mediated by an endogenous, polyclonal CD4^{+} T cell response in mice carrying both LIPSTIC alleles (i.e., *Cd40*$^{G5/G5}$ *Cd40lg*$^{SrtA/SrtA}$ CD4-Cre^{+}) can also be informative but may be confounded by other ongoing responses that result in background labeling.
8. The procedure as described is tailored to monitor the response occurring in the popliteal lymph node upon footpad immunization with a soluble antigen adsorbed in alum but can be extended to responses induced by other stimuli (e.g., viral and bacterial infection and tumor progression) and to other anatomical sites.
9. To allow SrtA enzymatic reaction to occur in vivo, we inject a short SrtA substrate consisting of a biotin moiety linked to the N-terminus of the SrtA recognition motif LPETG. The low

molecular weight (~1 kDa) of this substrate leads to a short half-life in lymph or serum, which we compensate for by administering the substrate repeatedly (e.g., six times over a 2-h period in the case of footpad administration). We found biotin to be an excellent label for in vivo applications given its solubility and the availability of secondary detection reagents for flow cytometry. Nevertheless, different SrtA substrates can be designed to carry virtually any label of choice as for instance peptide tags (Flag-tag, Myc-tag, His-tag) or fluorophores. Since water solubility of the substrate is a key aspect to facilitate its delivery in vivo, we advise to carefully take into account solubility profile of the label of choice when designing novel SrtA substrate for in vivo administration.

10. Alternatively, the digested lymph node can be dissociated by passing it through a 19-gauge needle seven times.

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