Quantifying in vivo recruitment of naïve CTL towards individual dendritic cells by standard immunofluorescence histology

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Method Article

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Abstract

Introduction

Dendritic cells \(DCs) attract naïve cytotoxic CD8+ T lymphocytes \(CTL) by chemokines, in order to activate them for immune defense against viruses and tumors. Here we describe a protocol for quantifying such recruitment towards individual DCs in secondary lymphatics by standard immunofluorescence histology. We used reporter mice expressing green fluorescent protein under a chemokine promoter and adoptively transferred into them CTL labeled with a red fluorochrome. Tissue sections from these animals were stained with a third color identifying anatomical regions, in our case the splenic B cell zone, which encircles the T cell zone in which DCs and CTL are located. The number of CTL in that zone and their location adjacent to chemokine-producing CTL can be determined by computer-assisted digital analysis.

Reagents

- Alpha murine B220-PE antibody \(e-bioscience, Cat. No. 12-0452) - Far Red DDAO-SE \(Invitrogen, Cat. No. C34553) - Magnetic beads \(Alpha murine CD8 MicroBeads) \(Miltenyi, Cat. No. 130-049-401) - Mounting medium \(Immu-Mount) \(Thermo Scientific, Cat. No. 9990402) - Tissue-Tek® \(Satura, Cat. No. 4141) - Blocking buffer - Dissolve 1% BSA \(PAA, Cat. No. K45-001) in PBS \(wt/vol). Store at 4°C. - MACS buffer - Dissolve 0,1% BSA and 2 mM EDTA \(Roth, Cat. No. 8043.2) in PBS \(wt/vol). Store at 4°C. - Paraformaldehyde - Dissolve 40 mg of PFA \(Sigma, Cat. No. 6148) in 200 ml PBS and incubate at 60°C until PFA is completely solved. Set pH to 7.4. Store at -20°C and further dilute to 4% \(wt/vol) in PBS before usage. CAUTION PFA is toxic. Avoid direct contact.

Equipment

- Cell F software \(Olympus) - Cover slip \(Menzel Gläser) - Dako Pen \(Dako Cytomation GmbH) - MACS columns and separator \(Miltenyi) - Microtome/Cryostat \(Leica) - Microscope \(IX71, Olympus), 10x objective

Procedure

Isolation and labeling of CTL \(day 1) 1. Isolate spleens from CTL-donor mice and generate a single cell suspension by extruding the spleen through a steel sieve with the plunger of a 2 ml syringe and filtering through a Nylon sieve \(100 µm). 2. Centrifuge cell suspension at 300 g for 6 mins, 4°C. 3. Resuspend cells in 600 µl of MACS buffer, add 20 µl of alpha murine CD8 MicroBeads per spleen and incubate for 15 mins at 4°C. 4. Centrifuge at 300 g for 6 mins, 4°C. 5. In the meantime, embed MACS columns \(1 column per 2-3 spleens) in magnetic field and equilibrate with 3 ml of MACS buffer. 6. Resuspend cells in 3 ml of cold MACS buffer, then pipette filtered cell suspension on column and wash with 3 ml MACS buffer. 7. Remove MACS columns from magnetic field and rinse with 4 ml MACS buffer. Capture passage \(=

positive fraction of cells) in a 15 ml tube. 8. Centrifuge at 300 g for 6 mins, 4°C. 9. Resuspend cells in 5 ml of PBS in a 15 ml tube and add 2.5 µl of FarRed staining solution into the lid of the tube, and invert tube carefully. Incubate for 20 mins at 37 °C. CRITICAL STEP Due to potential cytotoxicity of the staining reagent, do not exceed incubation time of 20 mins. 10. Stop staining reaction by adding 7 ml of ice-cold PBS and centrifuge at 300 g for 6 mins, 4 °C. 11. Resuspend cells in PBS and adjust the cell number to 12.5 x 106 cells per ml of PBS. Inject 200 µl \(2.5 x 106 cells) intravenously into mice of favored genetic background. Administration of antigen and adjuvant \(day 2) 12. Inject antigen with or without adjuvant intravenously into mice 16 hrs after adoptive T cell transfer. Injection of antigen alone should be included as a control. Preparation of cryosections \(day 2) 13. After 10 hrs, isolate spleens from injected mice, rinse them briefly in ice-cold PBS, and embed them directly in Tissue-Tek®. 14. Store at -20°C over night. PAUSE POINT Tissue blocks can be stored at -20°C for up to a week or for longer time after transfer to -80°C. Immunofluorescence staining and quantification of cell numbers \(day 3) 15. Prepare 5 µm cryosections from frozen tissue blocks by using a microtome/cryostat at a working temperature of -18°C. CRITICAL STEP Discard at least two sections between individual slides to avoid staining of the same cells in different sections. 16. Transfer sections on microscope slides for further treatment. PAUSE POINT Dry sections for several hours. CAUTION Avoid extended exposure to light. 17. Fix sections with iced acetone for 10 mins, then air-dry them for some minutes. CRITICAL STEP Do not exceed fixation time of 10 mins. Otherwise, tissue integrity might be lost. The fixation might be performed at 4°C. 18. Apply blocking solution \(1% BSA in PBS) to samples for 1 h at room temperature. 19. Stain sections with alpha murine B220-PE antibody for 1 h at room temperature, then wash twice with PBS. 20. Cover sections with mounting medium \(Immu-Mount) and cover slides. PAUSE POINT Slices can be stored at room temperature for up to one week. 21. View sections and capture images with a fluorescence microscope. Determine number of labeled cells with appropriate software. We used an Olympus IX71, employing a 10x objective. T cell numbers were counted \(utilizing the "Touch count" mode) and areas analyzed \("Closed polygon") with Cell F software \(Olympus). Furthermore, if distinct labels for multiple cell types are used, intercellular contacts can be enumerated.

Timing

3 days

Critical Steps

see procedure

Anticipated Results

Resulting immunofluorescence images should allow for enumeration of fluorescently labeled T cells per unit area of splenic DC/T cell zone. Fig. 1 shows recruitment of FarRed-labeled CTLs to the splenic DC-T cell zone which is encircled by blue B220+ B cell follicles. After challenge with the glycolipid alpha-galactosyl ceramide, NKT cells license DCs and CCL17 is induced, thereby facilitating recruitment of CTL

in heterozygous CCL17-eGFP knock-in mice4 \(Fig 1 left). In the absence of a functional CCL17 allele \(in homozygous CCL17-eGFP knock-in mice), the recruitment of CTL to the spleen is substantially reduced and DC-T cell contacts are not observed \(Fig 1 right).

References

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Figures

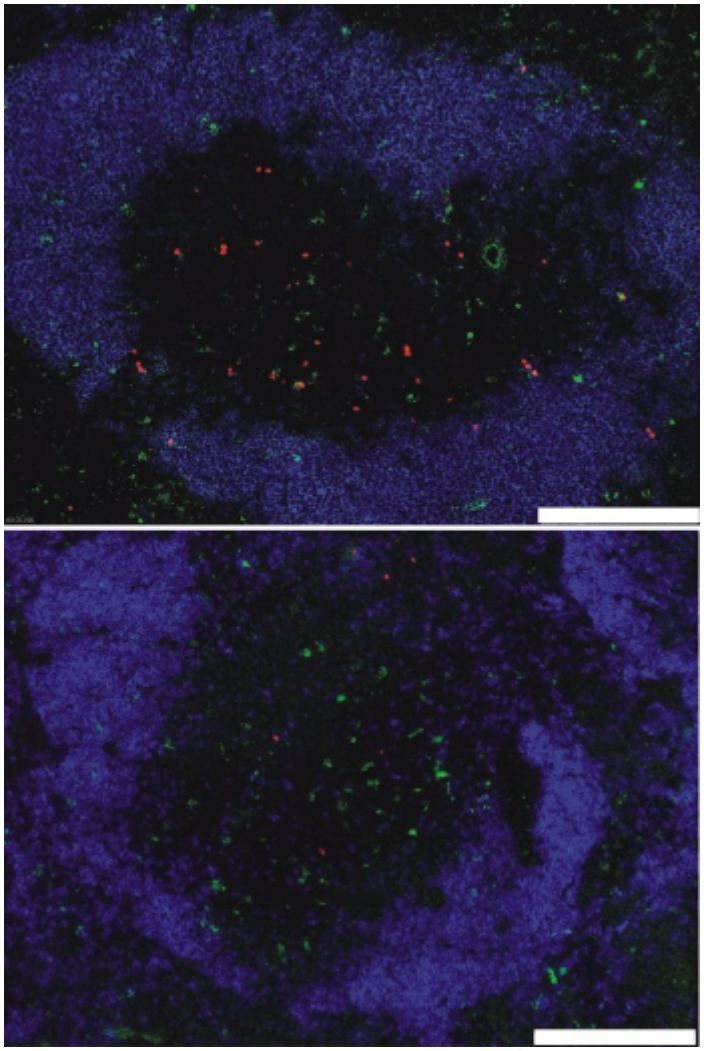

Figure 1

Immunofluorescence staining of spleen cryosections from CCL17-eGFP reporter mice (left) or CCL17-deficient mice (right) injected with 2.5 x 106 far-red fluorochrome labeled OT-I cells on day -1 and then injected with OVA + aGC on day 0. Spleens were isolated 10hrs after injection of antigen. White bars represent 200 um. Blue staining indicates B220+ cells, eGFP+ DCs appear in green, and OT-I cells are shown in red.