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A novel mouse model for tracking the fate of CXCR5-expressing T cells

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ABSTRACT

The germinal center (GC) reaction, a critical process in the humoral immune response, requires follicular helper T (Tfh) cells. Tfh cells express the master transcription factor Bcl6 and chemokine receptor CXCR5, which enable them to migrate from the T cell zone to B cell follicles and interact with GC B cells. However, CXCR5 is downregulated when Tfh cells become memory cells. Therefore, it is difficult to track Tfh cells continuously *in vivo*. In this study, we generated a mouse strain, *Cxcr5*^{CreERT2}*R26*^{Tomato}, in which the fluorescent protein tdTomato is inducibly expressed in CXCR5^{+} cells by tamoxifen administration. After the oral administration of tamoxifen, most Tfh cells in Peyer's patches (PP) from *Cxcr5*^{CreERT2}*R26*^{Tomato} mice were tdTomato^{+}. To track antigen-specific Tfh cells *in vivo*, OVA-specific OT-II T cells derived from *Cxcr5*^{CreERT2}*R26*^{Tomato} mice were transferred to wild-type mice, and the recipient mice were immunized with OVA followed by tamoxifen administration. CXCR5^{+} T cells became tdTomato^{+} and were mainly located in B cell follicles and GC areas 8 days after immunization. Four weeks after immunization, tdTomato^{+} OT-II T cells migrated from B cell follicles to the T-B border area and T cell zone after CXCR5 downregulation and CCR7 upregulation. These results indicate that *Cxcr5*^{CreERT2}*R26*^{Tomato} mice are a useful tool for studying the cell fate of differentiated Tfh cells *in vivo* and therefore have implications for the development of therapeutic strategies for infectious and autoimmune diseases.

1. Introduction

Follicular helper T (Tfh) cells are a specialized helper T cell subset. They provide essential help to B cells in the germinal center (GC), where Tfh cells control immunoglobulin class switching and somatic hypermutation. Tfh cells migrate to B cell follicles and GCs via the up-regulation of the chemokine receptor CXCR5 and down-regulation of CCR7. Tfh cells also express the co-stimulatory molecules PD-1 and ICOS and a master transcription factor for Tfh differentiation, Bcl6, and these molecules are important for their migration to B cell follicles and for the initiation and maintenance of B cell GC responses [1].

Tfh cells are able to develop into and be maintained as memory T cells [2–9]. Isolated effector Tfh cells are able to survive in naïve recipient mice for long periods of time [5,9]. In the memory phase, a substantial fraction of Tfh cells re-express CCR7 with the reciprocal down-regulation of CXCR5 and display the phenotype of central memory cells [2,6,7]. These Tfh-derived memory cells retain a low level of CXCR5 expression and are predominantly localized within the T cell zone in close proximity to B cell follicles [2,3,9]. The long-lived Tfh-derived memory cells can rapidly respond to antigen re-challenge and differentiate into effector Tfh cells, which contribute substantially to secondary antibody responses [2–9]. These observations suggest that Tfh memory cells are critical for efficient antibody responses.

Although CXCR5 has been used as a cell surface marker of Tfh cells, its expression is down-regulated in the memory phase [2,3,6–9]. Thus, it is difficult to distinguish Tfh-derived memory cells from other T helper cell subsets. In this study, we developed a new transgenic strain in which a tamoxifen-inducible Cre recombinase–estrogen receptor 2 fusion protein (CreERT2) is expressed from the *Cxcr5* promoter. Crossing these mice with inducible Rosa26-tdTomato reporter mice [10] allowed us to track the fate of

Abbreviations: GC, Germinal center; Tfh, Follicular helper T; OVA, Ovalbumin; CXC-R5, C-X-C chemokine receptor type 5; CCR7, C-C chemokine receptor type 7; ICOS, Inducible costimulator; PD-1, Programmed cell death 1; Bcl6, B-cell lymphoma 6; PP, Peyer's patches.

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CXCR5$^+$ T cells including Tfh cells *in vivo*. These mice are a potentially useful tool for analyses of Tfh cell fate as well as their functions.

2. Materials and methods

2.1. Mice

To generate *Cxcr5*^{CreERT2} mice, the CRISPR/Cas9 system was used. The donor plasmid (pUC57-Cxcr5-P2A-CreERT2) contained the P2A sequence and CreERT2 between the 1.4-kb 5′-arm and the 1.4-kb 3′-arm. The CreERT2 fragment was obtained from pCAG-CreERT2 (plasmid #14797; Addgene, Cambridge, MA, USA). The 3′-end of the 5′-homology arm was the final coding sequence of *Cxcr5*. The 5′-end of the 3′-homology arm was the 5′-end of the *Cxcr5* 3′UTR. The pX330 plasmid (#42230; Addgene) was used as a CRISPR expression vector [11]. The CRISPR target sequence (5′-CGAGACTTCCGGGATCTAGAAGG-3′) was selected for integration of the P2A and CreERT2 sequence just before the stop codon of *Cxcr5*. The target sequence was inserted into pX330 (pX330-Cxcr5). The pX330-Cxcr5 vector and donor plasmid pUC57-Cxcr5-P2A-CreERT2 were co-injected into the pronuclei of fertilized oocytes obtained from C57BL/6J mice. *Cxcr5*^{CreERT2} mice were generated at the Laboratory Animal Resource Center, University of Tsukuba. Rosa26-tdTomato reporter mice and OT-II transgenic mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). C57BL/6J mice were purchased from Sankyo Labo Service (Tokyo, Japan). All mice used in this study were maintained under specific-pathogen-free conditions and animal care was in accordance with the guidelines of Tokyo University of Science.

2.2. Flow cytometry analysis

A flow cytometry analysis was performed using the following antibodies: Anti-CD4 (RM4-5), PD-1 (RMP1-30), B220 (RA3-6B2), CD45.1 (A20), ICOS (7E.17G9), CCR7 (4B12) (BioLegend, San Diego, CA, USA), CXCR5 (2G8), GL7 (GL7) (BD Biosciences, San Jose, CA, USA), and Fas (15A7) (eBioscience, San Diego, CA, USA). Data were collected using FACSCalibur (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

2.3. Tamoxifen treatment

The expression of tdTomato was induced by the oral administration of 2 mg of tamoxifen (Cayman Chemical, Ann Arbor, MI, USA) in corn oil (Wako, Osaka, Japan) once per day.

2.4. Cell preparation, T cell transfer, and immunization

CD4$^+$ T cells were isolated from mouse spleens using the Mojosort magnetic cell separation system (BioLegend). For T cell transfer experiments, 2×10^5 OT-II T cells were transferred to mice intravenously. The next day, mice were immunized intraperitoneally with OVA (100 μg) (Sigma Aldrich, St. Louis, MO, USA) in alum.

2.5. Immunofluorescent staining and fluorescence microscopy

For immunofluorescent staining, spleens were fixed in 4% (wt/vol) paraformaldehyde and equilibrated in 30% (wt/wt) sucrose. 6–7 μm sections were stained and mounted with Fluorescent mounting medium (Dako, Carpinteria, CA, USA). All histological analyses were performed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Monoclonal antibodies against B220, CD4 (BioLegend), and GL7 (BD Biosciences) were used.

2.6. Statistical analysis

Statistical analyses were performed using a two-tailed unpaired Student's *t*-test. Differences were significant when $p < 0.05$.

3. Results

3.1. Generation and characterization of *Cxcr5*^{CreERT2} reporter mice

To track CXCR5-expressing Tfh cells, we generated a knock-in mouse possessing a gene encoding a tamoxifen-inducible Cre recombinase–estrogen receptor 2 fusion protein (CreERT2) in the *Cxcr5* locus (*Cxcr5*^{CreERT2}; Fig. 1A). To monitor Cre reporter activity, we crossed the mice with conditional reporter mice in which Cre activity induces the expression of tdTomato from the ubiquitously active *Rosa26* locus (*Cxcr5*^{CreERT2}*R26*^{Tomato}; Fig. 1A). Thus, CXCR5-expressing cells permanently expressed tdTomato after tamoxifen treatment. To validate the reporter system, we analyzed CD4$^+$ T cells and B cells in Peyer's patches (PP), in which Tfh cells and GC B cells are continuously generated by external stimuli, such as the intestinal microbiota. Approximately 80% of CXCR5$^+$CD4$^+$ T cells expressed tdTomato in PP after tamoxifen administration (Fig. 1B). In the CXCR5^{hi}PD-1^{hi} population, approximately 90% of cells were tdTomato$^+$, whereas in the CXCR5^{lo}PD-1^{lo} population, only approximately 4% of cells were tdTomato$^+$ (Fig. 1C). Although most B cells expressed CXCR5, only approximately 25% of the Fas$^-$GL7$^-$ non-GC B cells expressed tdTomato (Fig. 1D). In contrast, the Fas$^+$GL7$^+$ GC B cell population contained about 80% tdTomato$^+$ cells (Fig. 1D). Lower labeling efficiency was observed among non-GC B cells than in GC B cells or CXCR5$^+$ T cells, probably owing to activation status of cells, which may affect tamoxifen-induced cre activity or chromatin status at the *Rosa26* locus. These data indicated that Tfh cells could be effectively labeled with tdTomato using *Cxcr5*^{CreERT2}*R26*^{Tomato} mice.

3.2. Fate mapping of antigen-specific Tfh cells

To track antigen-specific Tfh cells *in vivo*, we crossed *Cxcr5*^{CreERT2}*R26*^{Tomato} mice with Ly5.1 OT-II transgenic mice (OT-II *Cxcr5*^{CreERT2}*R26*^{Tomato}). CD4$^+$ T cells were isolated from mice and then transferred to wild-type B6 mice, followed by immunization with OVA in alum. The recipient mice were orally administered tamoxifen on days 4 and 6 after immunization and donor CD4$^+$ T cells in the spleen were analyzed (Fig. 2A). About 50% of CXCR5$^+$ donor OT-II T cells expressed tdTomato on day 5 and this percentage increased to about 80% on day 8 (Fig. 2B). CXCR5$^-$ tdTomato$^+$ OT-II T cells also gradually increased until day 8 (Fig. 2B), suggesting that CXCR5 expression was down-regulated in cells that previously expressed CXCR5. The expression of another Tfh cell marker, PD-1, was higher in tdTomato$^+$ OT-II T cells than in tdTomato$^-$ OT-II T cells from day 5 to day 8 (Fig. 2C). In contrast, CCR7 expression was lower in tdTomato$^+$ OT-II T cells than in tdTomato$^-$ OT-II T cells until day 8 (Fig. 2C). The expression of ICOS, which is considered a cell surface marker for Tfh cells, was comparable in tdTomato$^+$ and tdTomato$^-$ OT-II T cells in this system (Fig. 2C). tdTomato$^+$ OT-II T cells were still detected 28 days after immunization. Expression levels of CXCR5, PD-1, and ICOS were down-regulated and the expression of CCR7 was up-regulated in both tdTomato$^+$ and tdTomato$^-$ OT-II T cells on day 28 compared to its expression on day 8, and the expression levels became indistinguishable between tdTomato$^+$ and tdTomato$^-$ OT-II T cells, although CXCR5 expression was slightly higher in tdTomato$^+$ cells than in tdTomato$^-$ cells (Fig. 2C). These data indicated that antigen-specific Tfh cells could be tracked from the effector phase to the memory phase using this reporter mouse system.

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