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Plasticity Characteristics of CXCR5+ CD4+ T Memory Cells and CXCR5- Non-TFH Memory Cells

Ching-En Lee
University Of Alabama At Birmingham

PLASTICITY CHARACTERISTIC OF CXCR5^{+} CD4^{+} T MEMORY CELLS AND CXCR5^{-} NON-TFH MEMORY CELLS

by

CHING-EN LEE

CHANDER RAMAN, COMMITTEE CHAIR
OLAF KUTSCH
STEFFANIE SABBAJ

A THESIS

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MULTIDISCIPLINARY BIOMEDICAL SCIENCE

ABSTRACT

CD4^{+} T memory cells are not only crucial for protecting tissues from reinfection and cancer, but also play active roles in various immune processes, including allergy, autoimmunity, graft rejection, and chronic inflammation (1). The preliminary data from Dr. Hui Hu's lab indicates that both CXCR5^{-} non-Tfh memory cells and CXCR5^{+} CD4^{+} T memory cells have plasticity, which is contrary to the previous finding that CXCR5^{-} non-Tfh memory cells are a committed population (2-6). However, the mechanisms that enable the CXCR5^{+} CD4^{+} T memory cells and CXCR5^{-} non-Tfh memory cells to differentiate into CXCR5^{-} and CXCR5^{+} effector cells are unclear. We hypothesize that their heterogeneous characteristics contribute to their abilities to differentiate into different effector cells during the recall responses. To test this hypothesis, we separated the CXCR5^{+} CD4^{+} T memory cells via CCR7 or T-B interaction and separated the CXCR5^{-} non-Tfh memory cells via Bcl6 expression. Subsequently, we compared their abilities to differentiate into CXCR5^{-} and CXCR5^{+} effector cells during the recall response. However, our results showed that there were no significant differences in the

ability to differentiate into $CXCR5^-$ and $CXCR5^+$ effector cells between the $CCR7^-$ and $CCR7^+$ $CXCR5^+$ $CD4^+$ T memory cells, between WT and μMT $CXCR5^+$ $CD4^+$ T memory cells, as well as between $Bcl6^-$ and $Bcl6^+$ $CXCR5^-$ non-Tfh memory cells. This finding did not support the hypothesis that the heterogeneous characteristics within $CXCR5^-$ non-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells significantly affect their abilities to differentiate into $CXCR5^-$ and $CXCR5^+$ effector cells during recall responses.

Keywords: $CXCR5^-$ non-Tfh memory cells, $CXCR5^+$ $CD4^+$ T memory cells, Plasticity, Heterogeneous, CCR7, Bcl6, T-B interaction.

DEDICATION

I wholeheartedly dedicate my profound gratitude to my mentor, Dr. Hui Hu, whose unwavering support throughout my master's degree has been invaluable. Dr. Hu's exemplary attitude and unwavering patience in the field of science have not only served as an inspiration but also continue to propel me forward.

I am equally indebted to every member of Dr. Hu's lab, as their collective efforts have not only honed my technical skills but have also provided immense emotional support during my master's journey. I am particularly grateful to senior member Fangming, whose willingness to review my work and assist me in self-discovery has been instrumental.

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Chapter 1

INTRODUCTION

$CXCR5^-$ non-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells

After pathogen clearance, 90% of $CD4^+$ T cells die and leave behind the long-lived memory cells(7-9). $CD4^+$ T memory cells induce a robust immune response because $CD4^+$ T memory cells generate a large antigen-specific population that can provide an efficient response upon reencountering the same antigen(7-9). Previous papers demonstrated the existence of two distinct subsets within $CD4^+$ T memory cells: $CXCR5^-$ non-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells(10-12). $CXCR5^+$ $CD4^+$ T memory cells have been shown to play a critical role in initiating a robust humoral immune response, as evidenced by their abilities to provide efficient B cell help(10, 11, 13). On the other hand, $CXCR5^-$ non-Tfh memory cells exhibit rapid cytokine production upon recall, indicating their involvement in the cellular response(14, 15). These findings highlight the functional difference within $CD4^+$ T memory cells and their specialized roles in immune responses.

Plasticity of $CD4^+$ T memory cells

Several studies indicate that only $CXCR5^+$ $CD4^+$ T memory cells display flexibility and that $CXCR5^-$ non-Tfh memory cells are a committed population during the recall response(2-6). For example, Pepper *et al.* showed that $CXCR5^-$ non-Tfh memory cells can only differentiate into $CXCR5^-$ effector cells, but $CXCR5^+$ $CD4^+$ T memory cells can differentiate into both $CXCR5^-$ and $CXCR5^+$ effector cells during the recall response(2). However, the preliminary data from Dr. Hu's lab indicates that approximately 40% of $CXCR5^-$ non-Tfh memory cells generated in the PR8 influenza infection model can differentiate into $CXCR5^+$ cells during the recall response. This result suggests that both $CXCR5^-$ non-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells generated in the PR8 influenza infection model have plasticity. The plasticity characteristic enables the memory cells to differentiate into effector cells that are required for humoral and cellular responses(16). Hence, to address the underlying mechanisms that enable $CXCR5^-$ non-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells to differentiate into $CXCR5^-$ and $CXCR5^+$ effector cells is important.

There are two potential mechanisms that can explain why memory cells have plasticity. One theory is that a single multi-potent memory cell could differentiate into different types of cells **(Fig. 1)**. Alternatively, a heterogeneous subpopulation within the

memory cell population could differentiate into specific effector cells. This study will examine whether the heterogeneity of the CXCR5^{+} CD4^{+} T memory cells and CXCR5^{-} non-Tfh memory cells contribute to their plasticity.

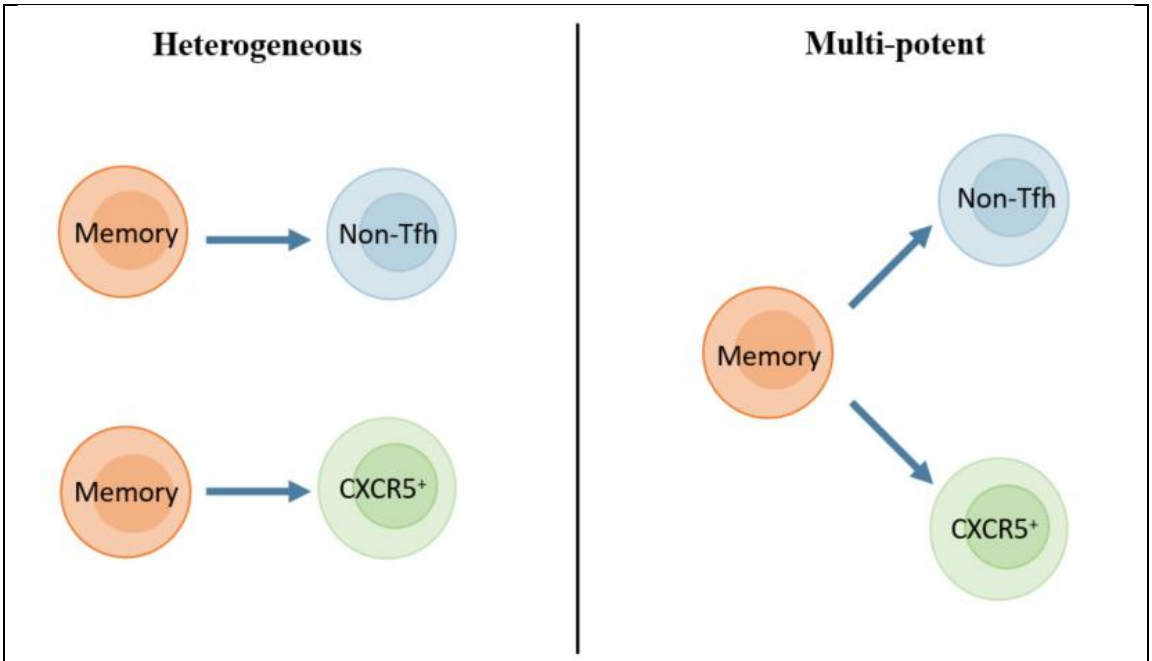

Figure 1: Schematic diagram of how heterogeneous and multi-potential characteristics contribute to the plasticity of memory cells

Heterogeneity of CD4^{+} T memory cells

Several papers stated that CD4^{+} memory cells are a heterogeneous population. Harrington *et. al* found that the interferon-*gamma* positive (IFN-γ^{+}) CD4^{+} T memory cells were able to quickly produce IFN-γ upon re-stimulation(15). In contrast, the IFN-γ^{-} CD4^{+} T memory cells displayed a lower ability to recall IFN-γ production. Other papers

also indicated that there is a specific $CD4^+$ T memory cells population in the LCMV and Listeria infection models that has higher expression of IFN-γ and other Th-1-related molecules during the recall response(17, 18).

Moreover, some papers also specifically indicates that the heterogeneity exists in the $CXCR5^-$ non-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells and showed that the different populations in the $CXCR5^-$ non-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells have different abilities to become non-Tfh, $CXCR5^+$ and GC-Tfh cells(4, 5). King *et al.* indicated that $CXCR5^+$ $CD4^+$ T memory cells can be divided into two populations via Folate receptor 4 (FR4), and they showed that only the $FR4^+$ population can differentiate into germinal center T follicular helper (GC-Tfh) cells during the recall response(5). Shaw *et al.* also demonstrated that the $Id3^+$ $CXCR5^-$ non-Tfh memory cells have a better ability to differentiate into $CXCR5^+$ cells compared to the $Id3^-$ $CXCR5^-$ non-Tfh memory cells(4).

These findings collectively highlight the heterogeneity present within $CXCR5^-$ non-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells, indicating that memory cells are not a homogeneous population. Understanding the functional diversity within these

populations is crucial for unraveling the complex dynamics of immune memory and optimizing immune responses.

C-C chemokine receptor type 7 (CCR7)

CCR7 is a chemokine receptor involved in guiding T cells from the bloodstream into lymph nodes and positioning them within the T cell zone of secondary lymphoid organs(19-21). Moreover, in $CD4^+$ T memory cells, CCR7 has been found to be a key marker that distinguishes two distinct populations(22). The $CCR7^+$ $CD4^+$ T memory cells cannot immediately produce the IFN-γ and Interleukin 4 (IL4) during the recall response, while $CCR7^-$ $CD4^+$ memory cells have the ability to recall the effector function(22). Furthermore, $CCR7^+$ cells have greater proliferative potential during the recall response(1, 23). These findings highlight the differential response of $CCR7^+$ and $CCR7^-$ $CD4^+$ T memory cells in recall response.

T and B cells interaction (T-B interaction)

During infection, T cells are initially primed by antigen-presenting cells (APCs) within the T cell zone of secondary lymphoid organs. Following activation, a subset of T cells upregulates the chemokine receptor CXCR5 and downregulates CCR7. This

dynamic expression of CXCR5 and CCR7 leads to the migration of T cells from the T cell zone to the B cell follicles, where they play a crucial role in supporting B cell responses and germinal center formation. This migration also allows the T cells to interact with B cells, which is a fundamental process in supporting the humoral response.

Several papers have shown that T-B interaction is critical for the humoral response. It not only triggers the migration of T cells to enter the B cell follicle but is also required for the differentiation of germinal center follicular helper T (GC-Tfh) cells, which can induce isotype switching, somatic hypermutation, and affinity maturation of the B cells(24-29). Moreover, T-B interaction is important for the generation of long-lived memory cells that provide long-term protection against a wide range of pathogens(24, 30-33). For example, Lund *et al.* showed that $CD4^+$ T memory cells differentiating in B-cell deficiency mice were unable to migrate to the infection site during the recall response(24).

The μMT mice are a specific strain of genetically engineered mice in which the μ chain of the immunoglobulin (Ig) molecule has been removed(34). This genetic alteration leads to the absence of mature B cells and significantly impairs the humoral immune response. The μMT mouse model has been extensively used to investigate the role of B cells in a variety of diseases and conditions such as viral and bacterial infections,

allergies, and autoimmune diseases(35, 36). Additionally, μMT mice have been used to study the interactions between B cells and other immune cells(25).

B-cell lymphoma 6 (Bcl6)

Bcl6 is a transcription factor that is primarily expressed in B cells and plays a critical role in regulating their differentiation and function. However, recent studies have also shown that Bcl6 is expressed in some subsets of $CD4^+$ T cells known as GC-Tfh and $CXCR5^+$ cells which play a key role in the development of humoral immune responses(37-42). Bcl6 promotes the expression of CXCR5 on activated $CD4^+$ T cells, and the higher CXCR5 expression in turn promotes the activated $CD4^+$ T cells to migrate to the B cell follicles and provide help to B cells during the germinal center reaction(43). On the other hand, studies also found that Bcl6 prevents the differentiation of the non-Tfh cells. Yu *et al.* showed that Bcl6 suppresses the expression of T-bet, a critical transcriptional factor for the Th1, and the production of IFN-γ, which is highly expressed by the Th1 cells(42). Moreover, Bcl6 also represses the expression of B lymphocyte-induced maturation protein-1 (Blimp-1), which is important for the differentiation of non-Tfh cells(38). Overall, Bcl6 is the transcriptional factor that triggers the T cell to become a $CXCR5^+$ cell and prevents the non-Tfh cell differentiation.

The fate of the Bcl6-expressing cells are difficult to track due to the intracellular localization of Bcl6, requiring nuclear and cell membrane permeabilization(44, 45). The Bcl6 reporter mouse that simultaneously expresses Bcl6 and Tdtomato allows for the identification of $Bcl6^+$ cells without fixation(46). The use of a T2A sequence between the Bcl6 and Tdtomato ensures the intact function of Bcl6(47). This unique tool enables us to discriminate cells populations by Bcl6 expression and to perform the adoptive transfer experiments to track these different populations during the recall response.

Hypothesis

We hypothesize that heterogeneous characteristics of the $CXCR5^+$ $CD4^+$ T memory cells and $CXCR5^-$ non-Tfh memory cells contribute to their abilities to differentiate into different effector cells during the recall responses. This study uses different markers and models to divide the $CXCR5^-$ non-Tfh memory cells or $CXCR5^+$ $CD4^+$ T memory cells into different populations and to test their abilities to differentiate into $CXCR5^-$ and $CXCR5^+$ effector cells during the recall response. Our goal is to understand whether the $CXCR5^-$ non-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells are a heterogeneous population and test whether the heterogeneity of $CXCR5^+$ $CD4^+$ T memory cells and

CXCR5$^-$ non-Tfh memory cells significantly affects their abilities to differentiate into CXCR5$^-$ and CXCR5$^+$ effector cells during recall responses.

Chapter 2

MATERIALS & METHODS

Mice

CD45.2^{+} C57BL/6, CD45.1^{+} C57BL/6, OT-II, CD45.1^{+} SMARTA, and μMT mice were purchased from Jackson Laboratories. Bcl6-Tdto reporter mice were generated at the Faculty of Pharmaceutical Sciences, Tokyo University of Science(46). OT-II mice were bred with CD45.1^{+} C57BL/6 congenic mice to generate CD45.1^{+}CD45.2^{+}OT-II^{Tg}. Bcl6-Tdto reporter mice were bred with CD45.1^{+} C57BL/6 congenic mice and OT-II mice to generate CD45.1^{+}CD45.2^{+}OT-II^{Tg} Bcl6-Tdto reporter mice. Experiments were conducted using age- and sex-matched male and female mice at 6-12 weeks of age. All animals were maintained in specific pathogen-free barrier facilities and were used in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham (Birmingham, Alabama).

Adoptive transfer, infection, and immunization

OT-II T cells were purified from the spleens via using the Invitrogen Dynabeads CD4 positive isolation kit (Thermo Fisher Scientific) or mouse CD4 (L3T4) MicroBeads (Miltenyi Biotec) (>95% $CD44^{lo}CD62L^{hi}$). For the CCR7 $CXCR5^+$ T memory cells recall experiment, 3×10^4 purified $CD45.2^+$ and $CD45.1^+$ $CD45.2^+$OT-II cells were transferred intravenously into 5-7 SMARTA recipient mice followed by influenza virus infection. The OT-II $CCR7^+CXCR5^+$ T memory cells and OT-II $CCR7^+CXCR5^+$ T memory cells were sorted at day 21 post-infection (p.i.) from the spleens of recipient mice infected with PR8-OVA, mixed at 1:1 ratio if possible, and co-transferred intravenously into $CD45.1^+$ SMARTA recipient mice. This is followed by intranasal infection with PR8-OVA at day 3 post-transfer **(Fig. 2)**. The standardized recovery ratio was calculated using the equation indicated **(Fig. 3)**.

For μMT $CXCR5^+$ recall experiments, 10,000 $CXCR5^+$ cells were sorted at day 21 p.i. from the mediastinal lymph nodes (medLNs) of 3-5 CD45.1 μMT or $CD45.2^+$ C57BL/6 mice infected with PR8 and transferred intravenously into $CD45.2^+$ or $CD45.1^+$ $CD45.2^+$ C57BL/6 recipient mice. This is followed by intranasal infection with PR8 at day 7 post-transfer **(Fig. 4)**.

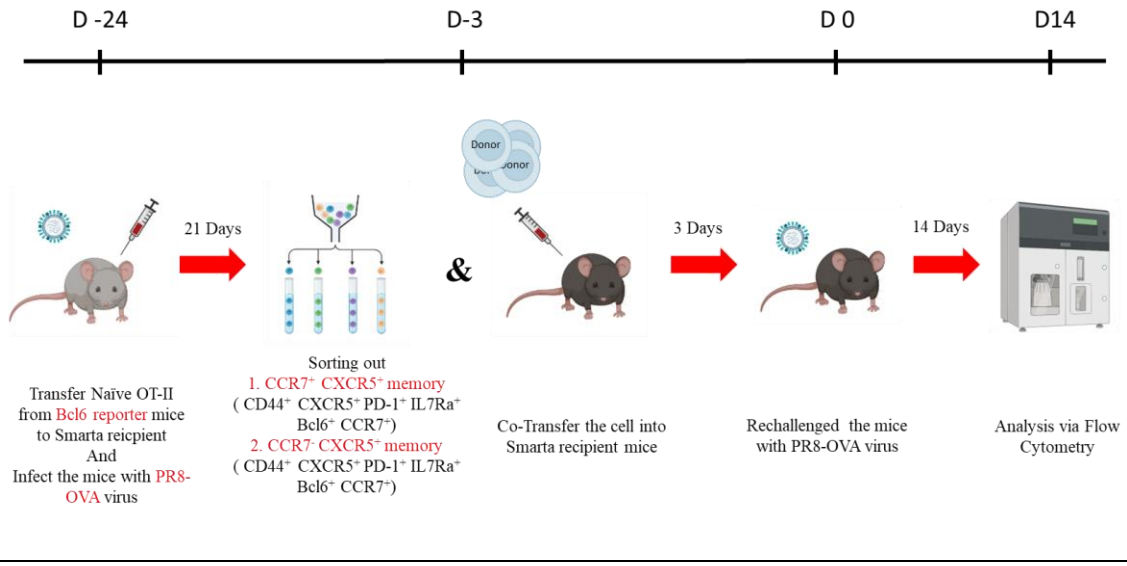

Figure. 2: Workflow for the CCR7 CXCR5⁺ CD4⁺ T memory recall experiment

$$\text{Normalized recover ratio} = \frac{\%\text{ of Population A in recall}}{\%\text{ of Population B in recall}} \div \frac{\text{Transfer Population A cells number}}{\text{Transfer Population B cells number}}$$

Figure. 3: Equation of the standardized recovery ratio

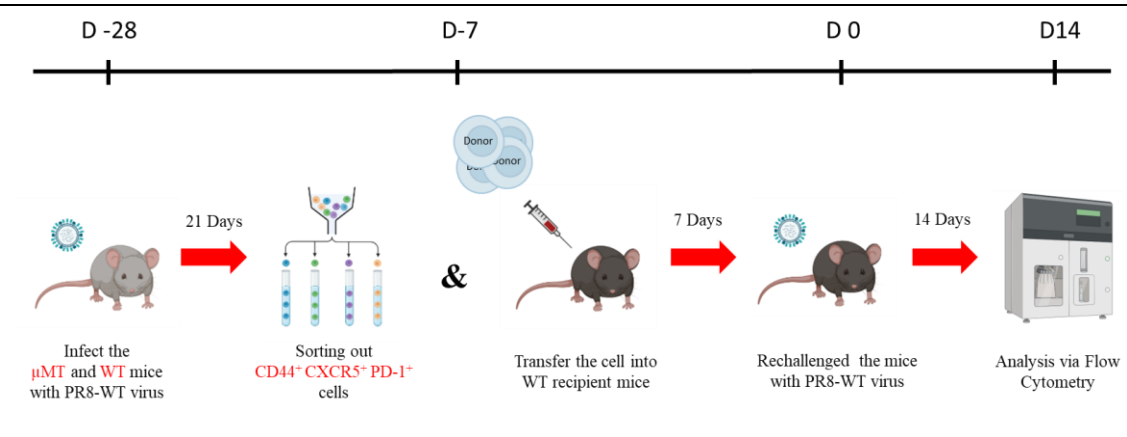

Figure. 4: Workflow for the µMT CXCR5⁺ CD4⁺ T memory recall experiment

For the Bcl6 CXCR5$^-$ non-Tfh memory cells recall experiment, 3 × 10^4 purified CD45.2$^+$ and CD45.1$^+$ CD45.2$^+$OT-II cells were isolated from Bcl6-Tdto reporter mice were transferred intravenously into 5-7 SMARTA recipient mice followed by influenza virus infection. OT-II Bcl6$^+$CXCR5$^-$ non-Tfh memory cells and OT-II Bcl6$^-$CXCR5$^-$ non-Tfh T memory cells were sorted at day 21 p.i. from the medLNs of recipient mice infected with PR8-OVA, mixed at 1:1 ratio if possible, and co-transferred intravenously into CD45.1$^+$ SMARTA recipient mice followed by intranasal infection with PR8-OVA at day 3 post-transfer **(Fig. 5)**.

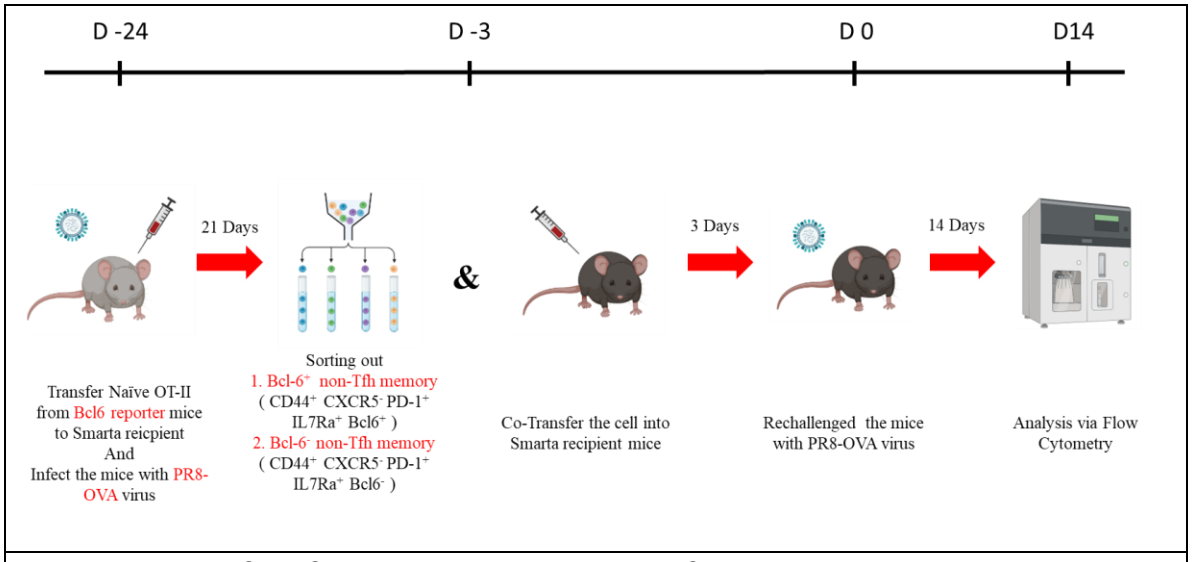

Figure. 5: Workflow for the Bcl6 CXCR5$^-$ non-Tfh memory recall experiment

For all influenza virus infection experiments, mice were immobilized with isoflurane and intranasally infected with mouse-adapted influenza virus A/Puerto

Rico/8/34 (PR8) at a dose of 15,000 virus forming units (v.f.u.) or PR8 virus expressing the OVA (323-339) epitope (PR8-OVA) at a dose of 200-1,000 v.f.u.

Cell preparation, staining and flow cytometry

MedLNs and spleens were mashed through the 70 μm filter to obtain single cell suspensions. Spleen samples were further incubated with ACK lysis buffer to remove red blood cells. Cells were counted using trypan blue staining, and 3×10^6 to 5×10^6 cells were suspended in 50 μl phosphate buffered saline (PBS) containing 2% bovine serum albumin and 2 mM EDTA (FACS buffer) for staining. Nonspecific antibody binding was blocked with anti-CD16/CD32 antibodies (Biolegend) in the FACS buffer for 10 minutes before staining. Dead cells were excluded using a Live/Dead Fixable Dead Cell staining kit or Fixable Viability Dye eFluor 780 (Invitrogen). Samples were analyzed or sorted using custom BD LSR II, BD FACSymphony or BD FACSAria instruments in the UAB Comprehensive Flow Cytometry Core Facilities. Flow cytometry results were analyzed using FlowJo software (v.10.7.1).

Statistics

Independent and paired two-tailed Student's *t*-tests and independent and repeated measure two-way ANOVAs were performed using GraphPad Prism software (v.8.2.1). All error bars represent standard deviation. For further details, see figure legends.

Chapter 3

RESULTS

$CCR7^-$ and $CCR7^+$ $CXCR5^+$ $CD4^+$ T Memory Cells Give Rise to $CXCR5^-$ and $CXCR5^+$ Cells during the Recall Response

Based on previous studies which demonstrate the differential responses of $CCR7^+$ $CD4^+$ T memory cells(22, 23), we hypothesized that $CXCR5^+$ $CD4^+$ T memory cells could be subdivided into two distinct populations based on CCR7 expression, and that $CCR7^+$ $CXCR5^+$ $CD4^+$ T memory cells that would exhibit a different ability to differentiate into $CXCR5^+$ cells during the recall response compared to $CCR7^-$ $CXCR5^+$ $CD4^+$ T memory cells.

To test if we could separate the $CXCR5^+$ $CD4^+$ T memory cells into $CCR7^-$ and $CCR7^+$ populations, we transferred naïve OT-II $CD4^+$ cells into SMARTA recipient mice followed by PR8-OVA infection. After waiting for 21 days, we harvested the spleens and performed staining for the indicated markers. Our results revealed that approximately 40% of $CXCR5^+$ cells were $CXCR5^+$ $CD4^+$ T memory cells ($CXCR5^+PD\text{-}1^+IL7\text{-}Ra^+$),

and around 50% of CXCR5^{+} CD4^{+} T memory cells in the spleen expressed CCR7 **(Fig. 6A)**.

Subsequently, we evaluated the recall response of CCR7^{+} and CCR7^{-} CXCR5^{+} CD4^{+} T memory cells with a specific focus on their capacity to differentiate into CXCR5^{-} and CXCR5^{+} effector cells. We transferred naïve OT-II cells into 5-7 SMARTA recipient mice followed by PR8-OVA infection. At day 21 post PR8-OVA infection, we sorted CCR7^{+} (CCR7^{+}CXCR5^{+}PD-1^{+}IL7Ra^{+}) and CCR7^{-} (CCR7^{-}CXCR5^{+}PD-1^{+}IL7Ra^{+}) CXCR5^{+} CD4^{+} T memory cells from spleens and co-transferred them into SMARTA recipient mice, which were subsequently infected with PR8-OVA after a resting period of 3 days. At day 14 p.i., we harvested the medLNs and lungs for flow cytometry analysis.

Upon reinfection, we observed that the CCR7^{-} to CCR7^{+} ratio before the transfer and after the recall is not significantly different. This may indicates that CCR7^{+} and CCR7^{-} CXCR5^{+} CD4^{+} T memory cells exhibit similar recovery abilities **(Fig. 6B, C)**. Furthermore, we tested their migration abilities since CCR7 is a homing marker for T cell migration to secondary lymphoid organs(23, 48). Our results indicated that the ratio of CCR7^{+} and CCR7^{-} CXCR5^{+} CD4^{+} T memory cells in the lungs was similar to their

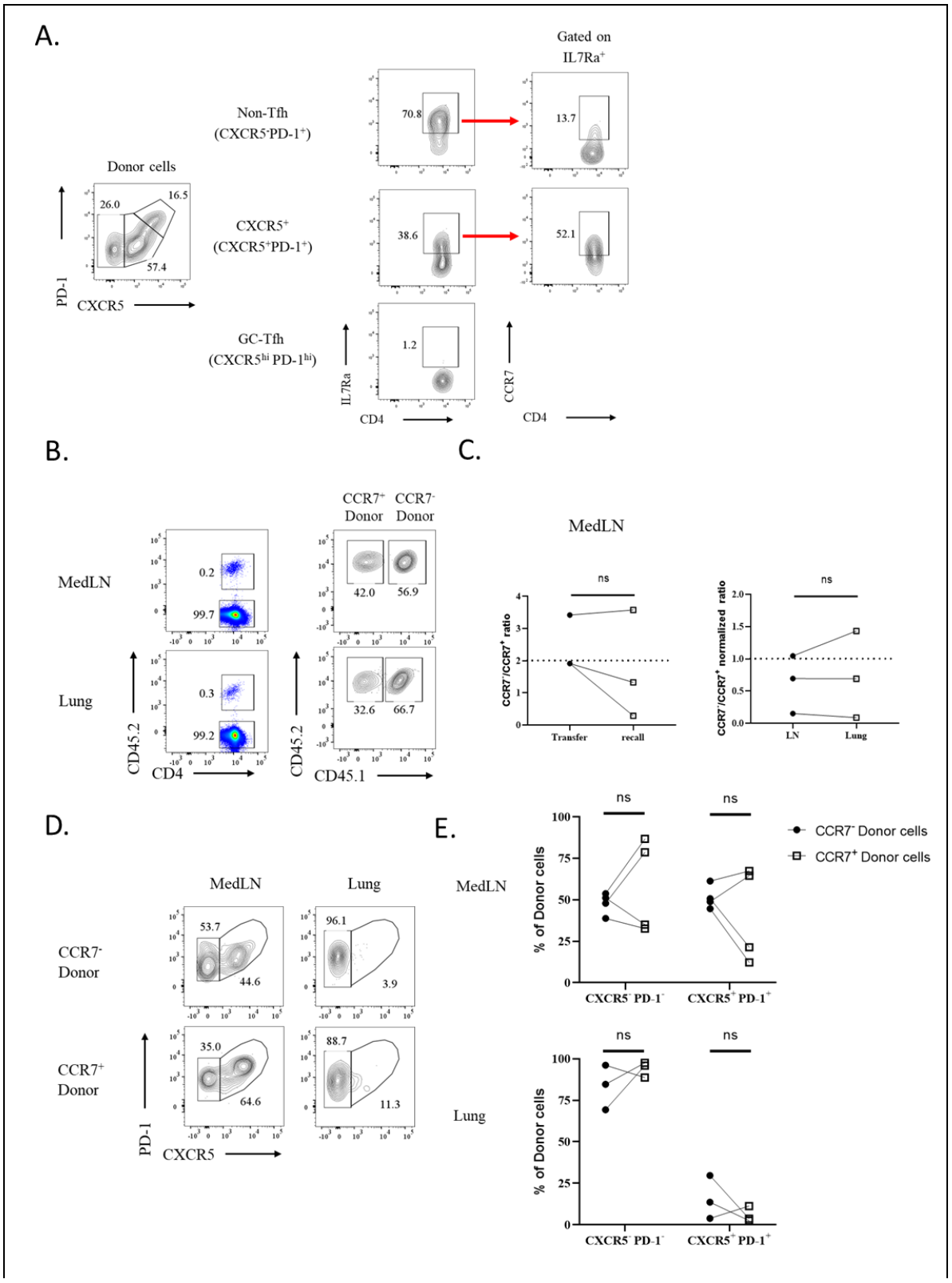
A.
Gated on IL7Ra+
Non-Tfh (CXCR5-PD-1+)
Donor cells
26.0
16.5
57.4
PD-1
CXCR5
CXCR5+ (CXCR5+PD-1+)
70.8
13.7
38.6
52.1
GC-Tfh (CXCR5hi PD-1hi)
1.2
IL7Ra
CD4
CCR7
CD4
B.
C.
CCR7+ Donor
CCR7- Donor
MedLN
Lung
0.2
99.7
0.3
99.2
42.0
56.9
32.6
66.7
CD45.2
CD4
CD45.2
CD45.1
MedLN
ns
CCR7-/CCR7+ ratio
Transfer
recall
CCR7-/CCR7+ normalized ratio
LN
Lung
D.
E.
MedLN
Lung
CCR7- Donor
CCR7+ Donor
53.7
44.6
96.1
3.9
35.0
64.6
88.7
11.3
PD-1
CXCR5
% of Donor cells
CXCR5- PD-1-
CXCR5+ PD-1+
CCR7- Donor cells
CCR7+ Donor cells

Figure 6. CCR7$^-$ and CCR7$^+$ CXCR5$^+$ CD4$^+$ T memory cells give rise to CXCR5$^-$ and CXCR5$^+$ cells during the recall response. A. CD45.2$^+$ OT-II CD4$^+$ T cells were transferred into the CD45.1$^+$ SMARTA recipient mice followed by intranasal infection with PR8-OVA. Donor CD4$^+$ T cells at day 21 p.i. in the spleen were analyzed. **B-E.** CD45.2$^+$CD45.1$^+$ and CD45.2$^+$ OT-II CD4$^+$ T cells were transferred into 5-7 CD45.1$^+$ SMARTA recipient mice followed by intranasal infection with PR8-OVA. CCR7$^+$CXCR5$^+$IL7Ra$^+$ and CCR7$^-$CXCR5$^+$IL7Ra$^+$ CD4$^+$ T cells at day 21 p.i. were sorted from the spleens and co-transferred into CD45.1$^+$ SMARTA recipient mice followed by intranasal infection with PR8-OVA after resting for 3 days. Donor CD4$^+$ T cells at day 14 p.i. in the medLNs and lungs are analyzed for **(B)** their recovery, **(C)** CCR7$^-$/CCR7$^+$ ratio before transfer and after the recall response in the medLNs, with quantification of indicated populations (n = 3). The circle symbol represents the CCR7$^-$/ CCR7$^+$ ratio of the cells sorted from 5-7 mice, while each square symbol represents the CCR7$^-$/CCR7$^+$ ratio of the donor cells in a single recipient mouse during the recall response. **(C)** Donor CD4$^+$ T cells are analyzed for the standardized ratio of the two donor cell populations after the recall response in the medLNs and lungs, with quantification of indicated populations (n = 3). The linked circle and square symbols represent the standardized ratio after the recall response in the medLNs and lungs, respectively, in the same recipient mouse during the recall response. **(D)** Donor CD4$^+$ T cells are analyzed for PD-1 and CXCR5 expression, **(E)** with quantification of indicated populations (n = 3). The linked circle and square symbols represent the CCR7$^-$ and CCR7$^+$ CXCR5$^+$ memory donor cells, respectively, in the same recipient mouse during the recall response. The presented data are pooled results of at least two independent experiments. **(C)** A two-tailed paired *t*-test and **(E)** a two-way ANOVA with Sidak's multiple comparisons test is employed for statistical analysis.

ratio in the medLNs, which suggested that effector cells that differentiated from CCR7$^-$ CXCR5$^+$ CD4$^+$ T memory cells did not preferentially migrate to the lungs **(Fig. 6C)**. Additionally, we found that their abilities to differentiate into CXCR5$^-$ and CXCR5$^+$ effector cells were comparable **(Fig. 6D, E)**.

Overall, our findings indicate that the heterogeneity within CXCR5$^+$ CD4$^+$ T memory cells based on CCR7 expression does not significantly impact their recall response.

$CXCR5^+$ $CD4^+$ T Memory Cells from µMT Mice Give Rise to $CXCR5^-$ and $CXCR5^+$ Cells during the Recall Response

Since the $CCR7^-$ and $CCR7^+$ $CXCR5^+$ $CD4^+$ T cells did not respond differently during the recall response, we planned to use a different method to separate the $CXCR5^+$ $CD4^+$ memory cells in two populations. As previous studies have shown that T-B interaction is critical for an ideal $CD4^+$ T memory cells formation(24, 30, 31), we hypothesized that T-B interaction would affect the ability of $CXCR5^+$ $CD4^+$ T memory cells to differentiate into $CXCR5^+$ and $CXCR5^-$ effector cells during the recall response. In our study, we utilized $CXCR5^+$ cells derived from µMT mice, which are B-cell-deficient mice, to simulate the absence of T-B cell interaction. While it has been observed in previous studies that $CD4^+$ T memory cell formation is impaired in µMT mice(30), our findings indicate that memory cells (IL7-Ra^+ cells) can still differentiate in µMT **(Fig. 7A)**. This allowed us to purify $CXCR5^+$ $CD4^+$ T memory cells from µMT mice for our recall experiments. At day 21 p.i., we sorted out $CXCR5^+$ $CD4^+$ T cells ($CD44^+CXCR5^+PD\text{-}1^+$) from µMT and WT mice. The $CXCR5^+$ $CD4^+$ T cells sorted from µMT and WT donor mice were separately transferred into WT recipient, and the memory response was induced via PR8-WT infection after resting for 7 days. Following the

reinfection, we observed that the recovery ability of the $CXCR5^+$ $CD4^+$ T memory cells from μMT and WT mice was similar **(Fig. 7B, C)**. However, we found that the $CXCR5^+$ $CD4^+$ T memory cell from the μMT mice had lower PD-1 and CD44 expression, which is the marker for the activation **(Fig. 7D, E)**. Because $CD4^+$ T cells that can be successfully activated upregulate these two markers, we gated on the $CD44^+$ and $PD\text{-}1^+$ population for further analysis. During the recall response, we found that both the $CXCR5^+$ $CD4^+$ T memory cells from μMT and WT mice had an ability to differentiate into non-Tfh, $CXCR5^+$,and GC-Tfh cells **(Fig. 7F)**. We further compared their abilities to differentiate into non-Tfh, $CXCR5^+$ and GC-Tfh cells during the recall response and found that the abilities of WT and μMT $CXCR5^+$ $CD4^+$ T memory cells to differentiate into non-Tfh, $CXCR5^+$ and GC-Tfh cells were similar **(Fig. 7G)**.

Overall, our findings indicate that the heterogeneity within $CXCR5^+$ $CD4^+$ T memory cells based on T-B interaction does not significantly impact their recall response.

$Bcl6^-$ and $Bcl6^+$ $CXCR5^-$ Non-Tfh Memory Cells Give Rise to $CXCR5^-$ and $CXCR5^+$ Cells during the Recall Response

As previous studies have shown that Bcl6 can induce $CXCR5^+$ T cells

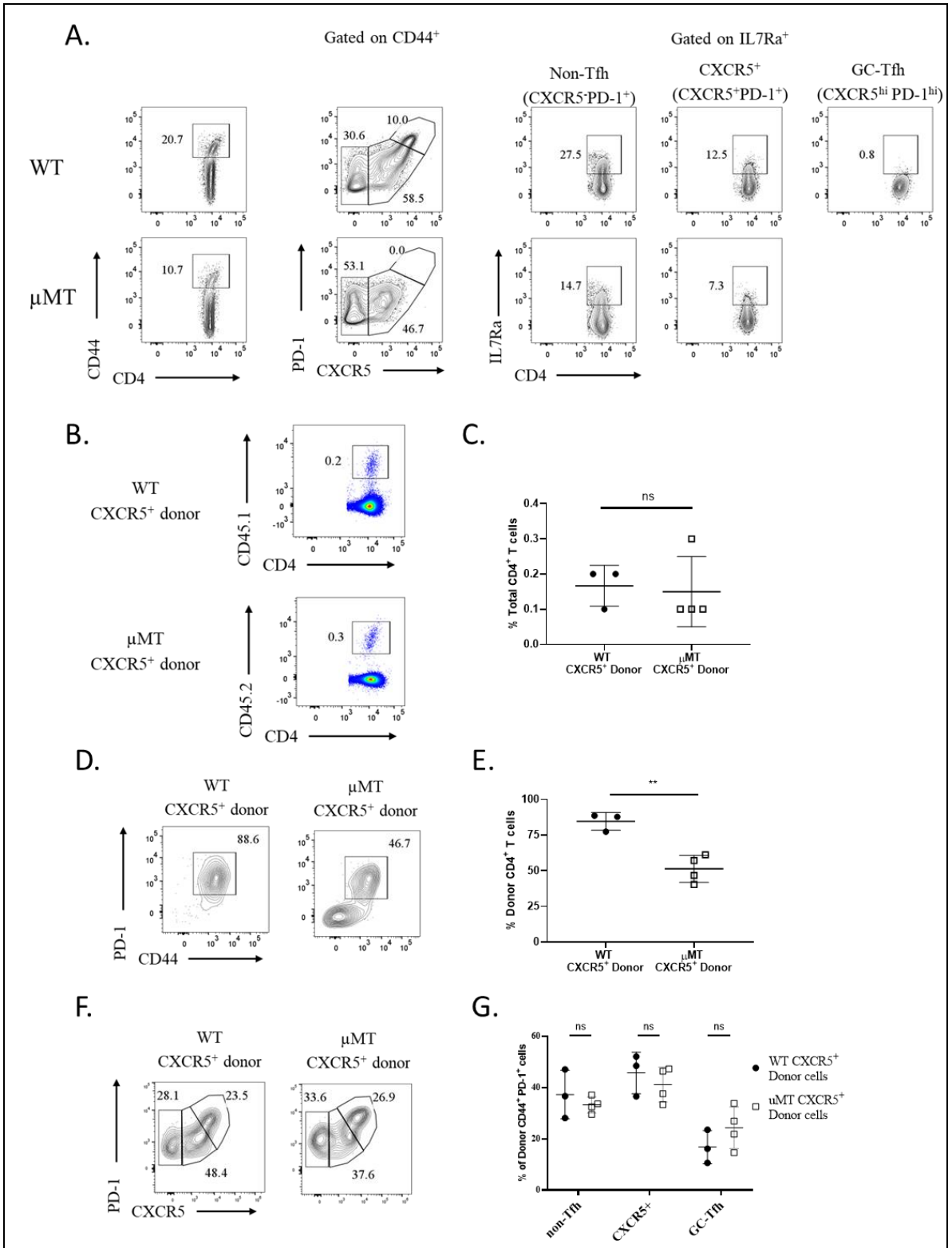
A.
Gated on CD44+
Gated on IL7Ra+
Non-Tfh (CXCR5-PD-1+)
CXCR5+ (CXCR5+PD-1+)
GC-Tfh (CXCR5hi PD-1hi)
WT
μMT
20.7
10.0
30.6
58.5
27.5
12.5
0.8
10.7
0.0
53.1
46.7
14.7
7.3
CD44
CD4
PD-1
CXCR5
IL7Ra
CD4
B.
WT CXCR5+ donor
μMT CXCR5+ donor
0.2
0.3
CD45.1
CD45.2
CD4
C.
ns
% Total CD4+ T cells
WT CXCR5+ Donor
μMT CXCR5+ Donor
D.
WT CXCR5+ donor
μMT CXCR5+ donor
88.6
46.7
PD-1
CD44
E.
**
% Donor CD4+ T cells
WT CXCR5+ Donor
μMT CXCR5+ Donor
F.
WT CXCR5+ donor
μMT CXCR5+ donor
28.1
23.5
48.4
33.6
26.9
37.6
PD-1
CXCR5
G.
ns
ns
ns
% of Donor CD44+PD-1+ cells
WT CXCR5+ Donor cells
uMT CXCR5+ Donor cells
non-Tfh
CXCR5+
GC-Tfh

Figure 7. CXCR5$^+$ CD4$^+$ T memory cells from µMT mice give rise to CXCR5$^+$ and CXCR5$^-$ cells during the recall response. A. C57BL/6 and µMT mice were intranasally infected with PR8. CD4$^+$ T cells at day 14 p.i. in the medLNs were analyzed. **B-G.** CD45.1$^+$CD45.2$^+$ C57BL/6 and CD45.2$^+$ µMT mice were intranasally infected with PR8. CD44$^+$CXCR5$^+$ CD4$^+$ T cells at day 21 p.i. were sorted and transferred into CD45.1$^+$ (for donor cells from µMT mice) or CD45.2$^+$ (for donor cells from C57BL/6 mice) C57BL/6 recipient mice followed by intranasal infection with PR8 after resting for 7 days. Donor cells at day 14 p.i. in the medLNs were analyzed for **(B)** their recovery, **(C)** with quantification of the indicated population (n=3-4), **(D)** PD-1 and CD44 staining, and **(E)** with quantification of indicated population (n=3-4). **(F)** The PD-1$^+$ CD44$^+$ donor cells were analyzed for PD-1 and CXCR5 staining, **(G)** with quantification of indicated population (n=3-4). The presented data are pooled results of at least two independent experiments, and each symbol represents donor cells in one recipient mouse. **(C&E)** A two-tailed unpaired *t*-test or **(G)** two-way ANOVA with Sidak's multiple comparisons test is employed for statistical analysis.

differentiation and prevent non-Tfh cell differentiation(40-42), we hypothesized that CXCR5$^-$ CD4$^+$ non-Tfh memory cells can be divided into two distinct populations via Bcl6, and that Bcl6$^+$ CXCR5$^-$ non-Tfh memory cells would exhibit a stronger ability to differentiate into CXCR5$^+$ cells during the recall response.

First, we tested whether we could separate CXCR5$^-$ non-Tfh memory cells into Bcl6$^-$ and Bcl6$^+$ populations using the Bcl6-Tdto reporter mice. Naïve OT-II CD4$^+$ T cells isolated from the Bcl6-Tdto reporter mice were transferred into SMARTA recipient mice followed by PR8-OVA infection. At day 21 p.i., the medLNs were harvested and stained for the markers indicated. We found that around 40% of the CXCR5$^-$ non-Tfh memory cells express Bcl6 **(Fig. 8A)**.

After establishing that non-Tfh cells can be separated into $Bcl6^+$ and $Bcl6^-$ populations, we evaluated the recall response of $Bcl6^+$ and $Bcl6^-$ $CXCR5^-$ non-Tfh memory cells, with a particular focus on their respective capacities to differentiate into $CXCR5^-$ and $CXCR5^+$ effector cells. We transferred naïve OT-II cells into the SMARTA recipient mice followed by PR8-OVA infection. At day 21 p.i., we sorted out $Bcl6^-$ ($Bcl6^-CXCR5^-PD\text{-}1^+IL7Ra^+$) and $Bcl6^+$ ($Bcl6^+CXCR5^-PD\text{-}1^+IL7Ra^+$) $CXCR5^-$ non-Tfh memory cells. $Bcl6^-$ and $Bcl6^+$ $CXCR5^-$ non-Tfh memory cells were co-transferred into SMARTA recipient mice which were infected with PR8-OVA after resting for 3 days.

At day 14 p.i., we harvested the medLNs for flow cytometry analysis.

Following the rechallenge, we found that the recovery ability was similar between the $Bcl6^-$ and $Bcl6^+$ $CXCR5^-$ non-Tfh memory cells **(Fig. 8B, C)**. We further found that both $Bcl6^-$ and $Bcl6^+$ $CXCR5^-$ non-Tfh memory cells can differentiate into $CXCR5^-$ and $CXCR5^+$ effector cells in medLNs, and that the ability to differentiate into $CXCR5^+$ and $CXCR5^-$ effector cells were similar **(Fig. 8D, E)**.

Overall, our findings indicated that the heterogeneity within $CXCR5^-$ non-Tfh memory cells based on Bcl6 expression does not significantly impact their recall response.

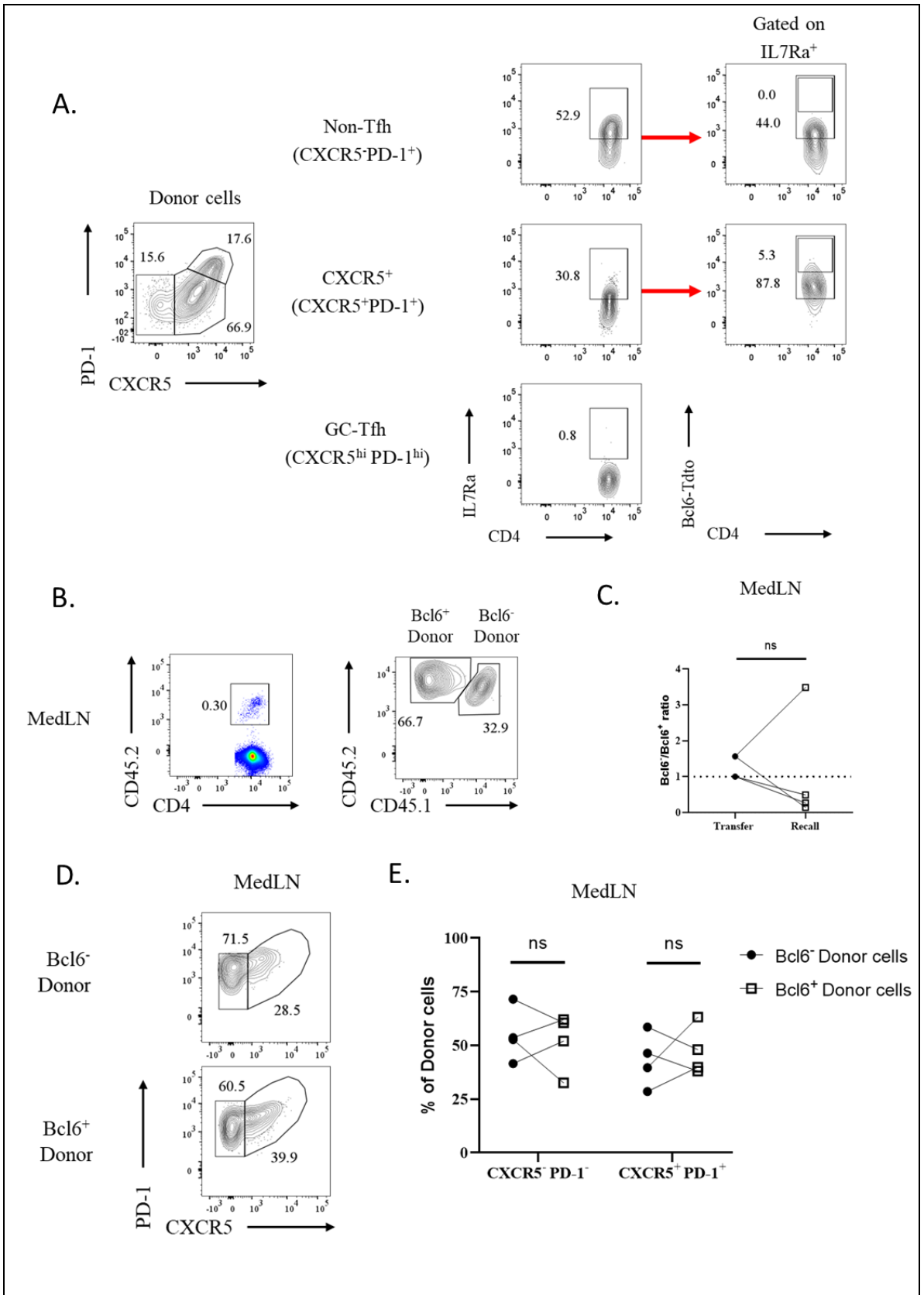
A.
Gated on IL7Ra+
Non-Tfh (CXCR5-PD-1+)
CXCR5+ (CXCR5+PD-1+)
GC-Tfh (CXCR5hi PD-1hi)
Donor cells
PD-1
CXCR5
IL7Ra
CD4
Bcl6-Tdto
CD4
52.9
0.0
44.0
30.8
5.3
87.8
0.8
15.6
17.6
66.9
B.
MedLN
CD45.2
CD4
Bcl6+ Donor
Bcl6- Donor
CD45.2
CD45.1
0.30
66.7
32.9
C.
MedLN
ns
Bcl6-/Bcl6+ ratio
Transfer
Recall
D.
MedLN
Bcl6- Donor
Bcl6+ Donor
PD-1
CXCR5
71.5
28.5
60.5
39.9
E.
MedLN
ns
ns
% of Donor cells
CXCR5- PD-1-
CXCR5+ PD-1+
Bcl6- Donor cells
Bcl6+ Donor cells

Figure 8. Bcl6$^-$ and Bcl6$^+$ CXCR5$^-$ non-Tfh memory cells give rise to CXCR5$^-$ and CXCR5$^+$ cells during the recall response. A. CD45.2$^+$ OT-II CD4$^+$ T cells from Bcl6-Tdto reporter mice were transferred into the CD45.1$^+$ SMARTA recipient mice followed by intranasal infection with PR8-OVA. Donor CD4+ T cells in the medLNs were analyzed at day 21 p.i.. **B-E.** CD45.2$^+$CD45.1$^+$ and CD45.2$^+$ OT-II CD4$^+$ T cells from Bcl6-Tdto reporter mice were transferred into the CD45.1$^+$ SMARTA recipient mice followed by intranasal infection with PR8-OVA. Bcl6$^-$CXCR5$^-$IL7Ra$^+$ and Bcl6$^+$CXCR5$^-$IL7Ra$^+$ non-Tfh memory cells at day 21 p.i. were sorted and co-transferred into CD45.1$^+$ SMARTA recipient mice followed by intranasal infection with PR8-OVA after resting for 3 days. Donor CD4$^+$ T cells at day 14 p.i. in the medLNs were analyzed for **(B)** their recovery and **(B)** the Bcl6$^-$/Bcl6$^+$ ratio before transfer and after the recall response in the medLNs, **(C)** with quantification of indicated populations (n = 4). The circle symbol represents the Bcl6$^-$/Bcl6$^+$ ratio of the cells sorted from 5-7 mice, while each square symbol represents the Bcl6$^-$/Bcl6$^+$ ratio of the donor cell in a single recipient mouse during the recall response. **(D)** Donor CD4$^+$ T cells were analyzed for PD-1 and CXCR5 expression **(E)** with quantification of indicated populations (n = 4). The linked circle and square symbols represent the Bcl6$^-$ and Bcl6$^+$ non-Tfh memory donor cells, respectively, in the same recipient mouse during the recall response. The presented data are pooled results of at least two independent experiments. **(C)** The two-tailed paired *t*-test and **(E)** a two-way ANOVA with Sidak's multiple comparisons test is employed for statistical analysis.

Chapter 4

DISCUSSION

Our attempt to separate $CXCR5^+$ $CD4^+$ T memory cells based on CCR7 expression, which is a marker for central memory cells, did not reveal significant differences between the two populations. This finding suggests that plasticity may not be a characteristic specific to central memory cells. Additionally, our results indicate that central memory cells do not show a preference for proliferation, which contradicts previous findings(1, 23). However, it is important to consider factors such as the low transfer number and low recovery rate in our experiment before drawing a definitive conclusion.

Although our results indicate that $CCR7^-$ and $CCR7^+$ $CXCR5^+$ $CD4^+$ T memory cells have similar potential for differentiation during the recall response, it is also important to consider that the adoptive transfer of cells into naïve mice may not fully replicate the dynamics observed in a realistic immune response. The normal localization of memory cells during transfer could be disrupted, potentially impairing our ability to detect differences between $CCR7^-$ and $CCR7^+$ $CXCR5^+$ $CD4^+$ T memory cells. Moreover, the transfer of $CCR7^-$ and $CCR7^+$ $CXCR5^+$ $CD4^+$ T cells into naïve recipient mice raises concerns about the maintenance of CCR7 expression in these cells. It is unclear whether

the transferred cells will retain their CCR7 expression in the new microenvironment. While studies such as those conducted by Jenkins *et al.* have suggested that effector cells that upregulate CCR7 expression do not downregulate it in certain models like the Listeria infection model(2), it remains unclear whether the same holds true for the influenza model used in our study. The possibility of CCR7 downregulation or alteration in expression levels in response to different microenvironments cannot be ruled out. Therefore, further investigations are required to determine whether CCR7 expression is maintained or potentially altered in the transferred $CXCR5^+$ $CD4^+$ T cells in our experimental setup. Overall, our result does not provide us with a clear understanding of whether the heterogeneity of $CXCR5^+$ $CD4^+$ T memory cells contributes to their plasticity.

Although previous studies have shown the critical role of T-B interactions in the formation of $CD4^+$ T memory cells, our μMT recall experiment indicated that T-B interactions may not be critical for $CXCR5^+$ $CD4^+$ T memory cell plasticity. However, it is unclear whether T-B interactions are necessary for the establishment of other functions of memory cells, such as B-cell-helping function. To address this, the experiment comparing the isotype switching, somatic hypermutation and affinity maturation of B cells after transferring of $CXCR5^+$ $CD4^+$ T memory cells with or without T-B interaction

can be conducted. Moreover, migration ability is another factor that could be investigated since Laud *et al.* found that the $CD4^+$ T memory cells differentiate in a B-cell-deficient model cannot migrate to the infection site(24).

Previous studies have shown that T-B interactions are required for the formation of GC-Tfh cells, which are critical for the humoral response. Although GC-Tfh cells are believed to become memory cells after the immune response(49-51), the lack of a GC-Tfh cell labeling system has impaired the ability of the researcher to track their fate. Because GC-Tfh memory cells and $CXCR5^+$ $CD4^+$ T memory cells without germinal center response experiment share similar features such as CXCR5, PD-1 and Bcl6 expression, the inability to track GC-Tfh fate has also hindered our ability to distinguish GC-Tfh memory cells from CXCR5+ memory cells that haven't experienced GC response(51, 52). The μMT mice, a B-cell-deficiency model, are unable to generate the GC-Tfh population. This has been confirmed by our preliminary data demonstrating the absence of GC-Tfh cells in μMT mice **(Fig. 7A)**. While the comparison of $CXCR5^+$ $CD4^+$ T memory cells from μMT and WT mice provides valuable insights into the potential function of GC-Tfh memory cells, it is crucial to acknowledge that our findings alone do not provide sufficient evidence to definitively conclude that GC-Tfh memory cells are not different from other $CXCR5^+$ $CD4^+$ T memory cells that lack germinal

center response. This is because it is unclear whether GC-Tfh memory cells formed on day 21 in PR8 influenza model. Overall, our results do not provide us with a clear understanding of whether the heterogeneity of $CXCR5^+$ $CD4^+$ T memory cells contributes to their plasticity.

Although previous studies have shown that $CXCR5^-$ non-Tfh memory cells are generally considered a committed population, we observed in our study that $CXCR5^-$ non-Tfh memory cells in the influenza model were able to differentiate into $CXCR5^+$ effector cells during the recall response, suggesting a degree of plasticity. This finding seems to contradict previous conclusions. The Degree and Committed model proposed by Ahmed *et al.* may provide a potential explanation for this inconsistency(16). According to this model, individual effector cells acquire transcriptional and epigenetic programming with varying degrees of polarity towards Tfh or non-Tfh lineages. The level of polarization determines their response upon encountering the antigen in the future. Highly polarized cells committed to the non-Tfh lineage would not be able to differentiate into $CXCR5^+$ effector cells during recall responses. It is worth noting that the non-Tfh cells purified from secondary lymphoid organs in the influenza model, which produces a localized infection, may exhibit less polarization compared to the LCMV model, which produces a systemic infection. This difference in polarization conditions

could potentially explain the observed plasticity in $CXCR5^-$ non-Tfh memory cells in our study.

Although our data reveal that $Bcl6^+$ non-Tfh memory cells do not differ from $Bcl6^-$ non-Tfh memory cells, there are still some important points to consider. We found that the $Bcl6^+$ non-Tfh memory cells exhibit similar recovery rates to the $Bcl6^-$ non-Tfh memory cells. This result contradicts the findings of Egawa *et al*, which reported that $Bcl6^+$ $CD4^+$ T cells exhibit a higher recovery rate compared to $Bcl6^-$ $CD4^+$ T cells(44). However, it is important to note that our data showed a large variation, which emphasizes the need for further experiments to validate and strengthen our findings. Increasing the number of experiments or increasing the number of transferred cells could help improve the statistical power and reliability of the results. Moreover, it is predicted that non-Tfh memory cells expressing Bcl6, a critical transcription factor for CXCR5 expression, would have a greater tendency to differentiate into $CXCR5^+$ effector cells during the recall response. Although our results do not align with this prediction, there are several points that should not be ignored. Firstly, it is important to determine whether the differential Bcl6 expression remains after transferring the cells into naïve mice and allowing them to become true memory cells. Previous studies have shown similar Bcl6 expression levels between non-Tfh memory cells and Tfh memory cells(51). Inability to

detect the Bcl6 difference between non-Tfh memory cells and Tfh memory cells raises doubts about the detectability of Bcl6 expression differences in $Bcl6^-$ and $Bcl6^+$ non-Tfh memory cells. However, it should be noticed that it would be worthwhile to investigate Bcl6 expression using Bcl6-Tdto reporter mice, as previous studies relied on antibody staining, which has lower sensitivity. Overall, our results do not provide us with a clear understanding of whether the heterogeneity of $CXCR5^-$ non-Tfh memory cells contributes to their plasticity.

Chapter 5

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