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Dispensable role of chemokine receptors in migration of mycobacterial antigen-specific CD4$^+$ T cells into *Mycobacterium*-infected lung

Goro Matsuzaki[a,b,*], Masatoshi Yamasaki[a,1], Toshiki Tamura[c], Masayuki Umemura[a,b]

[a] *Molecular Microbiology Group, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa 903-0213, Japan*
[b] *Department of Host Defense, Graduate School of Medicine, University of the Ryukyus, Okinawa 903-0213, Japan*
[c] *Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo 189-0002, Japan*

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ABSTRACT

Mycobacterial antigen-specific CD4$^+$ Th1 cells have pivotal role in protective immunity against mycobacterial infections including pulmonary tuberculosis. In the course of the infection, Th1 cells differentiate in the lung-draining lymph nodes and migrate into the infected lung. Chemokine receptors on T cells are involved in T cell migration into the intestine and skin. However, role of chemokine receptors in the migration of CD4$^+$ T cells into the lung is not yet established. To address the issue, the role of chemokine receptors in T cell migration into the mycobacteria-infected lung was analyzed using mycobacterial Ag85B peptide 25-specific T cell receptor-transgenic (P25) CD4$^+$ T cells. The P25 T cells in the *Mycobacterium bovis* BCG-infected lung and lung-draining mediastinal lymph node expressed chemokine receptors CCR5, CCR6, CXCR3 and CXCR5 which bind chemokines expressed by the BCG-infected lung. To further analyze the role of the chemokine receptors in the migration of the BCG-primed P25 T cells into the lung or mediastinal lymph node, the P25 T cells were adoptively transferred into the BCG-infected wild type mice, and their migration into the lung was monitored. Unexpectedly, blocking of chemokine receptor function with pertussis toxin, a G-protein inhibitor, failed to suppress migration of the T cells into the infected lung although the treatment completely blocked migration of the mediastinal lymph node P25 T cells into the recipient lymph node. The results suggest that interaction of chemokine receptors on mycobacterial antigen-specific Th1 cells with chemokines is dispensable in their migration into the mycobacteria-infected lung.

1. Introduction

Mycobacterial antigen-specific CD4$^+$ T cells are pivotal in protection to mycobacterial infections including pulmonary tuberculosis (North and Jung, 2004; Flynn and Chan, 2001). In the course of pulmonary infection by *Mycobacterium tuberculosis*, lung dendritic cells phagocytose mycobacteria, migrate into the draining lymph node of the lung (the mediastinal lymph node in mice), and present the mycobacterial Ag to naïve mycobacterial Ag-specific T cells to induce their differentiation into Th1 cells (Khader et al., 2006; Wolf et al., 2008; Yahagi et al., 2010). The Th1 cells then egress from the lymph node to blood, then migrate into the infected lung to eliminate the bacteria. However, the exact mechanism of the mycobacterial Ag-specific CD4$^+$ T cell migration to the infected lung is not yet clarified. It is important to elucidate the mechanism to design effective vaccines which induce CD4$^+$ T cells that migrate into mycobacteria-infected lung effectively.

Interaction of chemokine receptors on T cells and chemokines has been reported to be important in T cell migration into not only in lymphoid organs but also in non-lymphoid sites of immune responses (Griffith et al., 2014). It has been reported that T cell migration into the gut is controlled by chemokine receptor CCR9 and its ligand CCL25 (Svensson et al., 2002; Mora et al., 2003), while T cell migration into the skin is controlled by interaction of CCR10 and CCL27 (Morales et al., 1999; Homey et al., 2002). Involvement of CCR4 in T cell migration into the lung was also reported in a respiratory allergy model (Mikhak et al., 2013). Furthermore, it has been reported that helper T cell subsets preferentially express certain chemokine receptors: Th1 cells express CCR1, CCR2, CCR5 and CXCR3, Th2 cells express CCR4

Abbreviations: AF, Alexa Fluor; APC, allophycocyanin; BCG, *Mycobacterium bovis* Bacille de Calmette et Guérin; P25, mycobacterial Ag85B peptide 25-specific TCR transgenic; PE, phycoerythrin; PPD, mycobacterial purified protein derivative; TCR, T cell receptor

* Corresponding author at: Center of Molecular Biosciences, Tropical Biosphere Research Center, University of the Ryukyus, Senbaru 1, Nishihara, Okinawa 903-0213, Japan.

E-mail address: matsuzak@comb.u-ryukyu.ac.jp (G. Matsuzaki).

[1] Present addresses: Masatoshi Yamasaki, Etiology Group, National Research Institute of Aquaculture, Mie 516-0193, Japan.

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and CCR8, Th17 cells express CCR6, and Tfh cells express CXCR5 (Griffith et al., 2014). Involvement of the chemokine receptors in T cell migration to non-lymphoid organs and the sites of inflammation have been reported (Griffith et al., 2014; Bromley et al., 2008).

Importance of chemokines and chemokine receptors in protective immunity to mycobacterial infections has been reported (Algood et al., 2003; Monin and Khader, 2014). In mouse infection models, mice deficient in various chemokine receptors including, CCR4, CCR6 and CXCR5 showed decreased protection (Stolberg et al., 2011; Freeman et al., 2006; Slight et al., 2013). Deficiencies of chemokines CCL5 and CXCL13, which are the ligands of CCR1/3/5 and CXCR5, respectively, are also showed to modify protection to mycobacterial infections (Slight et al., 2013; Vesosky et al., 2010). For all the results, it is not clarified which chemokine receptor is pivotal in migration of protective T cells into the infected lung.

To clarify chemokine receptors expressed by mycobacterial Ag-specific T cells and their importance in T cell migration into the infected lung, we analyzed CD4$^+$ T cells of mycobacterial Ag85B peptide 25-specific T cell receptor (TCR)-transgenic (P25) mice. When the P25 mice were intratracheally infected with *Mycobacterium bovis* Bacille de Calmette et Guérin (BCG), Th1 cells were detected from day 28 of the infection in the lung and the lung-draining mediastinal lymph node (Wolf et al., 2008; Yahagi et al., 2010). The in vivo-primed P25T cells expressed multiple chemokine receptors CCR4, CCR5, CCR6, CCR7, CXCR3, and CXCR5. However, the chemokine receptors were dispensable in T cell migration into the BCG-infected lung because inhibition of chemokine receptor function by pertussis toxin failed to block migration of the primed P25T cells into the infected lung in an adoptive transfer model.

2. Materials and methods

2.1. Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). B6-Ly5.1 mice, a congenic mice of C57BL/6 mice expressing CD45.1 allele instead of CD45.2 allele expressed by C57BL/6 mice, were bred at University of the Ryukyus. P25 TCR transgenic mice are TCR transgenic C57BL/6 mice which express mycobacterial Ag85B-specific H-2 A^b-restricted TCR from an Ag-85B-specific CD4$^+$ T cell hybridoma (Tamura et al., 2004). Female mice of 8–12 week-old were used in all the experiments. The animal experiments were done under approval of University of the Ryukyus Animal Experiment Committee and the Living Modified Organism Experiments Safety Committee of the University of the Ryukyus.

2.2. Microorganisms, infection and cell preparation

Mice were anesthetized by intraperitoneal inoculation of pentobarbital sodium (Somnopentyl, Kyoritsu, Tokyo, Japan) and intratracheally injected with 5×10^6 CFU of *M. bovis* BCG (Japan BCG Inc., Tokyo, Japan). On day 28 of the infection, mice were sacrificed, and injected with 5 ml of cold PBS from the right ventricle to perfuse the lung. The lung and mediastinal lymph node were then excised. The lung lymphocytes were prepared as described (Umemura et al., 2007). Briefly, the lung was minced, treated with collagenase, hyaluronidase and DNase for 30 min at 37°C, and passed through a stainless steel mesh to obtain a single cell suspension. The cells were suspended in 8 ml of 45% Percoll, overlaid on 4 ml 67.5% Percoll, and centrifuged at 800 *g* for 20 min at room temperature. The cells at interface of Percoll gradient were collected as the lymphocyte fraction. Lymph node cell suspension was prepared by pressing the lymph node between two glass slides.

Table 1
PCR primers.

gene name	sequence		Genebank
Ccl2	forward	AGCAGCAGGTGTCCCAAAGA	NM_011333.3
	reverse	GTGCTGAAGACCTTAGGGCAGA	
Ccl5	forward	GGAGTATTTCTACACCAGCAGCAAG	NM_013653.3
	reverse	GGCTAGGACTAGAGCAAGCAATGAC	
Ccl7	forward	CAATGCATCCACATGCTGCTA	NM_013654.3
	reverse	GACCCACTTCTGATGGGCTTC	
Ccl8	forward	GGACGCTAGCCTTCACTCCAA	NM_021443.3
	reverse	CAGCAGGTGACTGGAGCCTTA	
Ccl12	forward	CCACACTTCTATGCCTCCTGCTC	NM_011331.2
	reverse	CTGGCTGCTTGTGATTCTCCTGTA	
Ccl17	forward	TCAGTGGAGTGTTCCAGGGATG	NM_011332.3
	reverse	GGCGTCTCCAAATGCCTCA	
Ccl20	forward	GATCCAAAGCAGAACTGGGTGAA	NM_016960.2
	reverse	GGACAAGTCCACTGGGACACAA	
Ccl22	forward	CTGACGAGGACACATAACATCATGG	NM_009137.2
	reverse	CTTCACTAAACGTGATGGCAGAGG	
Cxcl9	forward	CCGAGGCACGATCCACTACA	NM_008599.4
	reverse	AGTCCGGATCTAGGCAGGTTTG	
Cxcl10	forward	ATCCGGAATCTAAGACCATCAAGAA	NM_021274.2
	reverse	GGACTAGCCATCCACTGGGTAAAG	
Cxcl13	forward	ACTCCACCTCCAGGCAGAATG	NM_018866.2
	reverse	AAGTTTGTGTAATGGGCTTCCAGA	
β-actin	forward	CATCCGTAAAGACCTCTATGCCAAC	NM_007393.3
	reverse	ATGGAGCCACCGATCCACA	

2.3. Cell culture

Naïve spleen cells were seeded in 24-well-plates at 5×10^6 cells/well, incubated for 90 min, and non-adherent cells were removed to obtain adherent cells as antigen-presenting cells. Lymphocytes were added into the wells at 2×10^6 cells/well with or without mycobacterial purified protein derivative (PPD, Japan BCG Inc.) at 5 μg/ml, and incubated at 37°C overnight. To detect intracellular IFN-γ, GolgiPlug (BD, Oxnard, CA) which contains Brefeldin A was added to the culture at the last 3 h of the culture. RPMI1640 medium (Wako, Osaka, Japan) with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO) was used in the cell culture.

2.4. Flow cytometry (FCM)

Cells were stained with phycoerythrin (PE)- or Alexa Fluor (AF) 700-conjugated anti-mouse CD4 mAb (BD and BioLegend, San Diego, CA), allophycocyanin (APC)-Cy7-conjugated anti-mouse CD45.2 mAb (BioLegend), and the following anti-mouse chemokine mAb: FITC-conjugated anti-CCR1 (R&D, Minneapolis, MN), APC-conjugated anti-CCR2 (BioLegend), AF 647-conjugated anti-CCR3 (BioLegend), PE-conjugated anti-CCR4 (BioLegend), AF 488-conjugated anti-CCR5 (BioLegend), PE/Cy7-conjugated anti-CCR6 (BioLegend), PE-conjugated anti-CCR7 (eBiosciences, San Diego, CA), FITC-conjugated anti-CCR9 (BioLegend), APC-conjugated anti-CCR10 (R&D), PerCP/Cy5-conjugated anti-CXCR3 (BioLegend), AF 647-conjugated anti-CXCR4 (BioLegend), PE/Dazzle-conjugated anti-CXCR5 (BioLegend), and FITC-conjugated anti-CXCR6 (R&D) mAb. To stain intracellular IFN-γ, cells cultured with GolgiPlug were surface stained, and then fixed, permeabilized and stained with AF 700-conjugated anti-IFN-γ mAb (BioLegend) using Cytofix/Cytoperm kit (BD) according to the manufacturer's instructions. The cells were analyzed with a FACSCanto flow cytometer with DIVA software (BD).

2.5. Cell transfer

Lymphocytes were prepared from the lung or mediastinal lymph node of the P25 mice (CD45.2$^+$ strain) on day 28 of intratracheal *M. bovis* BCG infection. The lymphocytes (5×10^6 lung lymphocytes or 5×10^7 mediastinal lymph node cells in 200 μl) were i.v. transferred

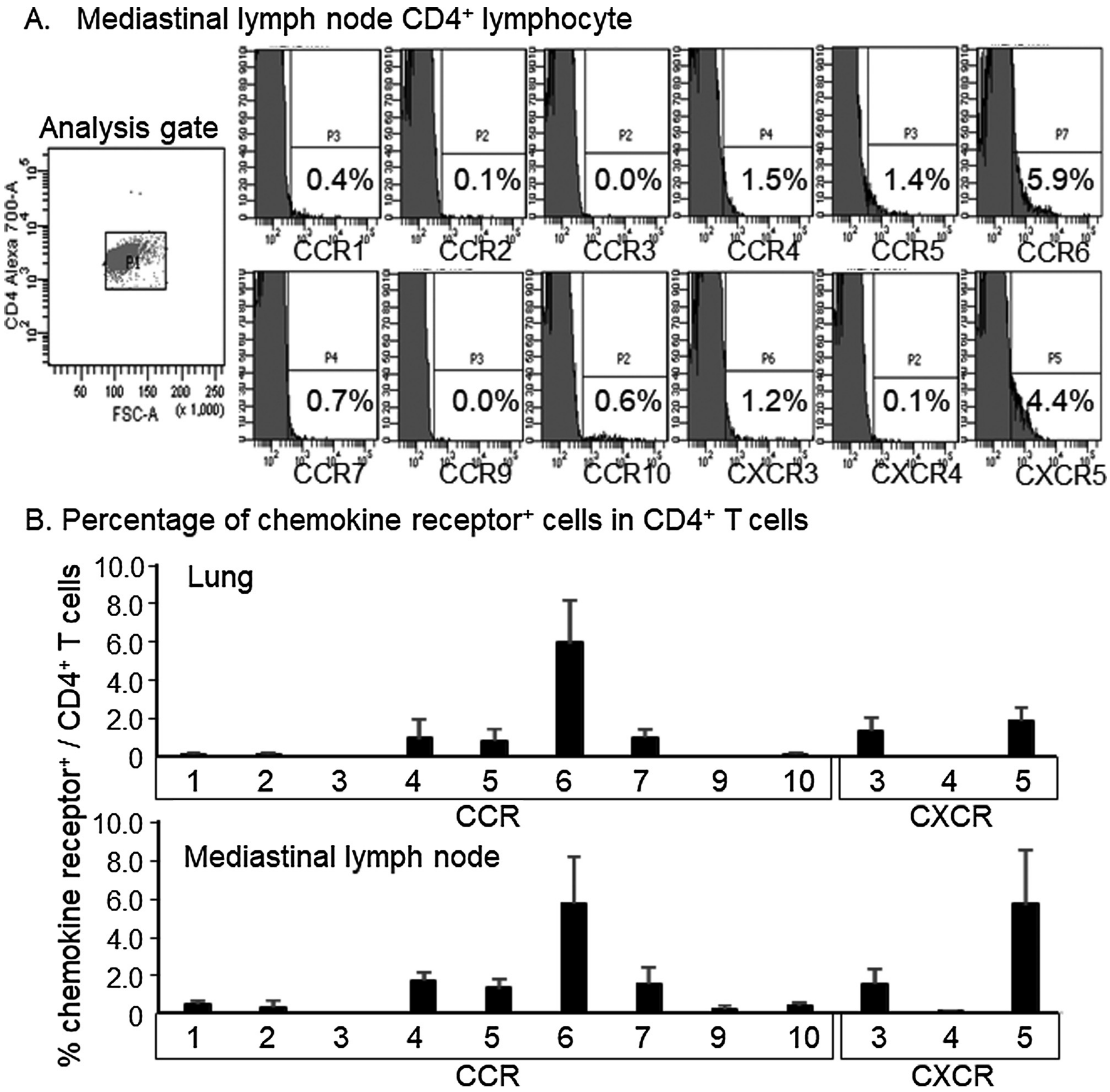

Fig. 1. Chemokine receptor expression by freshly isolated lung lymphocytes and mediastinal lymph node cells from the BCG-infected P25 mice. P25 mice were intratracheally infected with *M. bovis* BCG, and lymphocytes were prepared from the lung and mediastinal lymph node on day 28 of the infection. (A) A representative FCM profile of analysis gate on CD4$^+$ lymphocytes and histograms of chemokine receptor expression. A set of data of mediastinal lymph node cells are demonstrated. (B) Summary of a representative data from five independent experiments with on chemokine receptor expression by the lung lymphocytes and mediastinal lymph node cells from *M. bovis* BCG-infected P25 mice (n = 3).

into the B6-Ly5.1 mice (CD45.1$^+$ strain) which were intratracheally infected with *M. bovis* BCG 28 days before. To block chemokine receptor function, the lymphocytes were incubated with 100 ng/ml of pertussis toxin (BioAcademia, Osaka, Japan) for 3 h. The cells were then collected and i.v-transferred with 400 ng of pertussis toxin. Lymphocytes in the lung and mediastinal lymph node were prepared from the recipient B6-Ly5.1 mice 18 h after the transfer, stained with anti-CD45.2 and anti-CD4 mAb to detect donor-derived CD4$^+$ T cells, and analyzed by FCM.

2.6. Quantitative (q)PCR analysis

Expression of chemokines in the lung of naïve or BCG-infected mice were analyzed by qPCR analysis. RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Carlsbad, CA), reverse transcribed with a reverse transcriptase (SuperScript VILO cDNA Synthesis Kit, Thermo Fisher Scientific), and amplified with Taq polymerase premixed with SYBR Green using the Step One Realtime PCR System (Thermo Fisher Scientific). The qPCR was normalized and data were analyzed as described previously (Umemura et al., 2007). The primers used are shown in Table 1.

2.7. Statistics

To compare data of two groups, Student`s *t* test was applied. To compare more than three groups, one-way analysis of variance was performed followed by Tukey-Kramer multiple comparison test. All the statistical analyses were carried out using the GraphPad InStat software

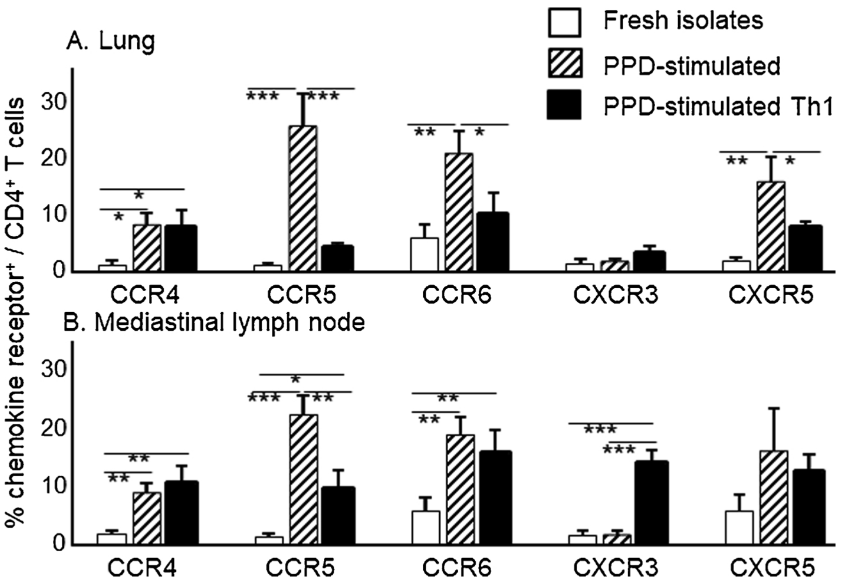

Fig. 2. Chemokine receptor expression of lung lymphocytes and mediastinal lymph node cells before and after in vitro Ag stimulation. The lung lymphocytes and mediastinal lymph node cells from the *M. bovis* BCG-infected P25 mice (n = 3) were stimulated with PPD, and total or IFN-γ$^+$-gated (Th1) CD4$^+$ T cells were analyzed by FCM on their expression of chemokine receptors. *$P < 0.05$, **$P < 0.001$, ***$P < 0.001$ compared to fresh isolates.

(GraphPad Software, La Jolla, CA). A P value of < 0.05 was considered to indicate a significant difference.

3. Results

3.1. Repertoire of chemokine receptors expressed by mycobacterial Ag-specific CD4$^+$ T cells in the lung and lung-draining lymph node of mice with pulmonary M. bovis BCG infecton

Although the role of chemokine receptors in T cell migration is established in the gut and skin, their role in T cell migration into the lung is yet to be elucidated. We therefore analyzed chemokine receptors expressed by mycobacterial Ag-specific CD4$^+$ T cells. The P25 transgenic mice with mycobacterial antigen-specific TCR transgene expressed on CD4$^+$ T cells were pulmonary infected with *M. bovis* BCG, and chemokine receptor expression on the CD4$^+$ T cells was analyzed on day 28 of the infection when the CD4$^+$ P25 T cells differentiate into Th1 cells and migrate into the infected lung (Slight et al., 2013; Vesosky et al., 2010). We analyzed expression of chemokine receptors which have been reported to be expressed by CD4$^+$ T cells (Griffith et al., 2014; Bromley et al., 2008). A representative FCM data of chemokine receptor expression is shown in Fig. 1A, and summarized in Fig. 1B. Freshly isolated CD4$^+$ P25T cells in the infected lung expressed CCR4, CCR5, CCR6, CCR7, CXCR3, and CXCR5 although only less than 10% of the cells expressed each chemokine receptor (Fig. 1). Freshly isolated CD4$^+$ P25 T cells of the lung-draining mediastinal lymph node showed similar chemokine receptor repertoire (Fig. 1).

It has been reported that chemokine receptor expression is down-regulated after interaction with their ligands, and the expression is recovered by in vitro culture of the cells in the absence of the chemokines (Neel et al., 2005; Meiser et al., 2008). We therefore reasoned that in vitro culture of freshly isolated T cells with antigen and antigen-presenting cells not only stimulate Ag-specific T cells but also restore chemokine receptor expression. In the next experiments, we cultured the lymphocytes from the lung and mediastinal lymph node of the BCG-infected P25 mice with PPD which contains Ag85B, and then re-evaluated expression of the chemokine receptors on the CD4$^+$ P25 T cells. We excluded CCR7 in the analyses because CCR7 functions as a receptor that mediate migration of T cells into lymphoid organs but not into the non-lymphoid organs including the lung (Griffith et al., 2014; Bromley et al., 2008). Significant increase was observed on CCR4, CCR5, CCR6 and CXCR5 expression in the lung lymphocytes, and CCR4, CCR5 and CCR6 expression in the mediastinal lymph node cells after the culture. We previously reported that IFN-γ$^+$ Th1 cells represents approximately 5% and 1.5% of the lung and mediastinal lymph node CD4$^+$ P25 T cells, respectively, after the culture with PPD (Yahagi et al., 2010). When analysis gate was set on the IFN-γ$^+$ Th1 cells in the cultured cells, the percentage of CCR4$^+$ cells in the lung lymphocytes and the percentages of CCR4$^+$, CCR5$^+$, CCR6$^+$ and CXCR3$^+$ cells in the mediastinal lymph node cells were significantly higher than those of the fresh isolates (Fig. 2). Therefore CCR4, CCR5, CCR6, CXCR3 and CXCR5 are the candidates of chemokine receptors involved in T cell migration from the mediastinal lymph node into the lung in the course of mycobacterial infection.

3.2. Co-expression of multiple chemokine receptors by mycobacteria-specific CD4$^+$ T cells

Since expression of chemokine receptors on a cell is not exclusive, a mycobacterial antigen-specific T cell could express multiple chemokine receptors. To address the possibility, antigen-stimulated CD4$^+$ P25T cells from the lung and mediastinal lymph node were analyzed on the expression of multiple chemokine receptors. As shown in Fig. 3, CCR6$^+$ cells represents approximately 25% of the P25T cells, and nearly 90% of the CCR6$^+$ P25 T cells co-express CCR5 in both organs. Among the CCR5$^+$CCR6$^+$ P25T cells, 40–50% of the cells co-express CXCR5. This means that approximately 10% of the P25 T cells are CCR5$^+$CCR6$^+$CXCR5$^+$ cells. Furthermore, 20–30% of the CCR5$^+$CCR6$^+$ P25 T cells also expressed CCR4. Therefore, multiple chemokines could affect migration of T cells in the BCG-infected lung.

3.3. Repertoire of chemokines expressed in the BCG-infected lung

To clarify which chemokines are expressed in the BCG-infected lung to bind to the chemokine receptors expressed by the mycobacterial antigen-specific CD4$^+$ T cells in the lung and mediastinal lymph node, we next analyzed gene expression of ligands for CCR4 (*Ccl17*, *Ccl22*), CCR5 (*Ccl5*, *Ccl8*), CCR6 (*Ccl20*), CXCR3 (*Cxcl9*, *Cxcl10*), and CXCR5 (*Cxcl13)* in the lung of BCG-infected mice. As shown in Fig. 4, pulmonary BCG infection induced expression of *Ccl5/Ccl8*, *Ccl20*, *Cxcl9/Cxcl10*, and *Cxcl13* which binds to CCR5, CCR6, CXCR3 and CXCR5, respectively. Similar gene expression profile was observed in the mediastinal lymph node of the infected mice. However, expression of CCR4 ligands *Ccl17* and *Ccl22* were not induced. Therefore, CCR5, CCR6, CXCR3 and CXCR5 expressed on the CD4$^+$ T cells are candidates of chemokine receptors which are involved in T cell migration into the

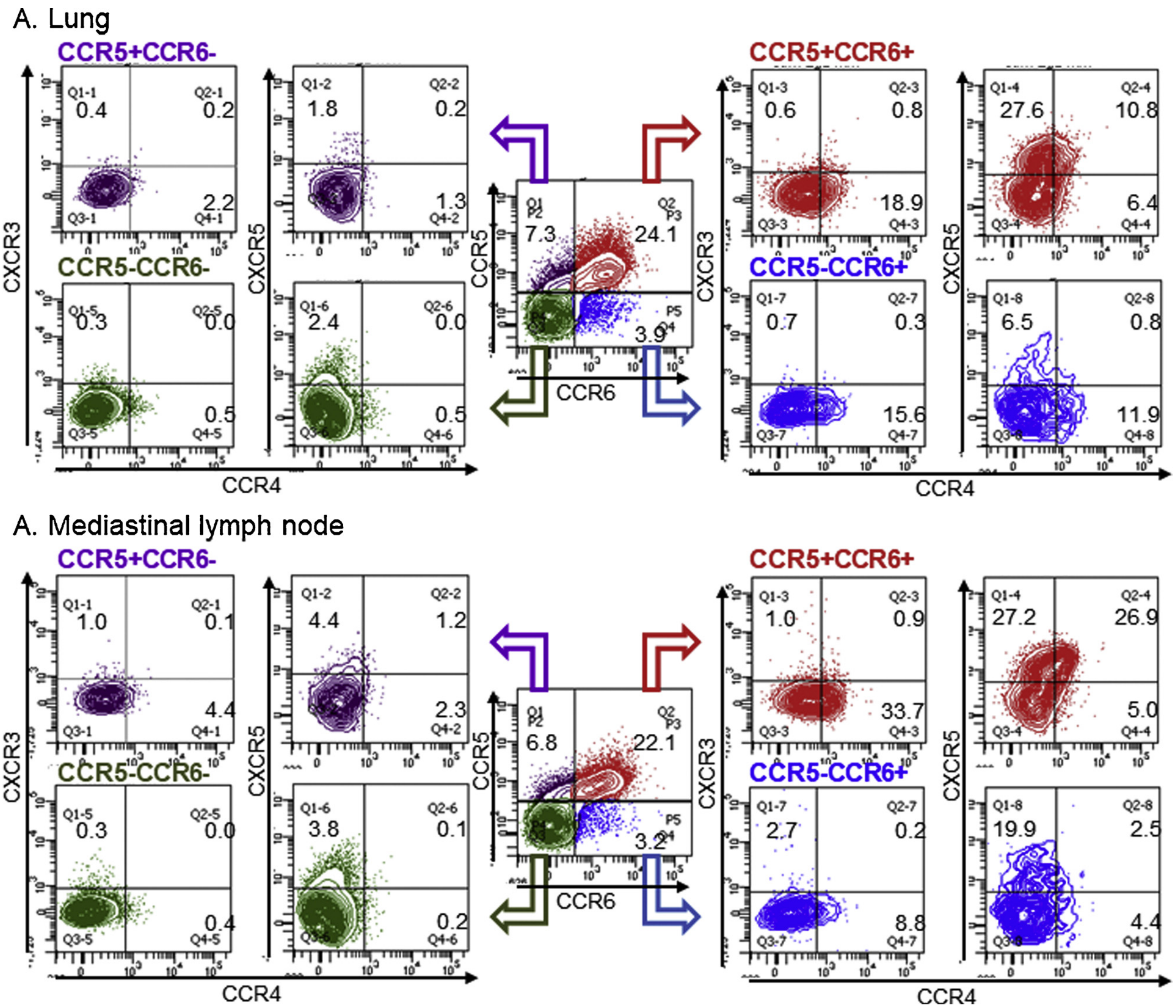

Fig. 3. Cells expressing multiple chemokine receptors are frequent in mycobacterial Ag-specific CD4$^+$ T cells. The lung lymphocytes (A) and mediastinal lymph node cells (B) stimulated with PPD were stained with anti-CD4, CCR4, CCR5. CCR6, CXCR3 and CXCR5 mAb, and analyzed by FCM. Contour plot of CCR5 and CCR6 expression on CD4$^+$ T cells (center of panels A and B) were gated on each quadrants (CCR5 + CCR6-, CCR5 + CCR6 +, CR5-CCR6- and CCR5-CCR6 +), and the profiles of CCR4/CXCR3 or CCR4/CXCR5 expression on each quadrant were shown. A representative data from repeated experiments with more than three samples are demonstrated.

mycobacteria-infected lung.

3.4. Blocking of chemokine receptor function failed to suppress mycobacterial Ag-specific CD4$^+$ T cell migration into the BCG-infected lung

To analyze involvement of chemokines and chemokine receptors expressed on T cells in T cell migration into the infected lung, the P25T cells from the lung of the BCG-infected P25 mice were adoptively transferred into the BCG-infected B6-Ly5.1 mice, and the donor T cells in the host lung were detected by staining with anti-CD45.2 mAb which were expressed on the donor P25T cells but not on the recipient B6-Ly5.1 mouse-derived T cells (Fig. 5A). Approximately 0.1% of the lung lymphocytes were the donor-derived CD4$^+$ P25 T cells after the transfer (Fig. 5B). The donor-derived T cells expressed CCR4, CCR5, CCR6, CXCR3 and CXCR5 (Fig. 5C) and the expression pattern was similar to the PPD-stimulated lung CD4$^+$ P25T cells (Fig. 2A).

It has been reported that Th1 cells preferentially expressed CCR5 and CXCR3 (Griffith et al., 2014; Bromley et al., 2008). It is therefore expected that CCR5 and CXCR3 are involved in migration of CD4$^+$ P25 Th1 cells in the BCG-infected lung. However, preliminary experiments showed that anti-CXCR3 or anti-CCL5 (a ligand of CCR5) mAb treatment failed to suppress migration of the CD4$^+$ P25T cells into the BCG-infected lung (data not shown). Since multiple chemokine receptors were expressed by individual lung CD4$^+$ P25 T cell (Fig. 3), it is possible that blockage of single chemokine or chemokine receptor is compensated by other chemokine-chemokine receptor interaction. To verify involvement of chemokine receptors in migration of the CD4$^+$ P25 T cells into the BCG-infected lung, we therefore blocked all chemokine receptors by using pertussis toxin. Pertussis toxin produced by *Bordetella pertussis* inhibits heterotrimeric G-protein of Gi family which is indispensable in chemokine receptor function (Locht et al., 2011). Pertussis toxin treatment of the lung lymphocytes of the BCG-infected P25 mice before and during the adoptive transfer failed to suppress their migration into the lung and mediastinal lymph node because the ratio and absolute number of the donor CD45.2$^+$CD4$^+$ P25 T cells was not affected by the pertussis toxin treatment (Fig. 6A).This suggest that the migration of the CD4$^+$ P25T cells into the lung and mediastinal lymph node of the BCG-infected recipient mice does not depend on chemokine receptors.

It has been reported that resident memory T cells lose their ability to

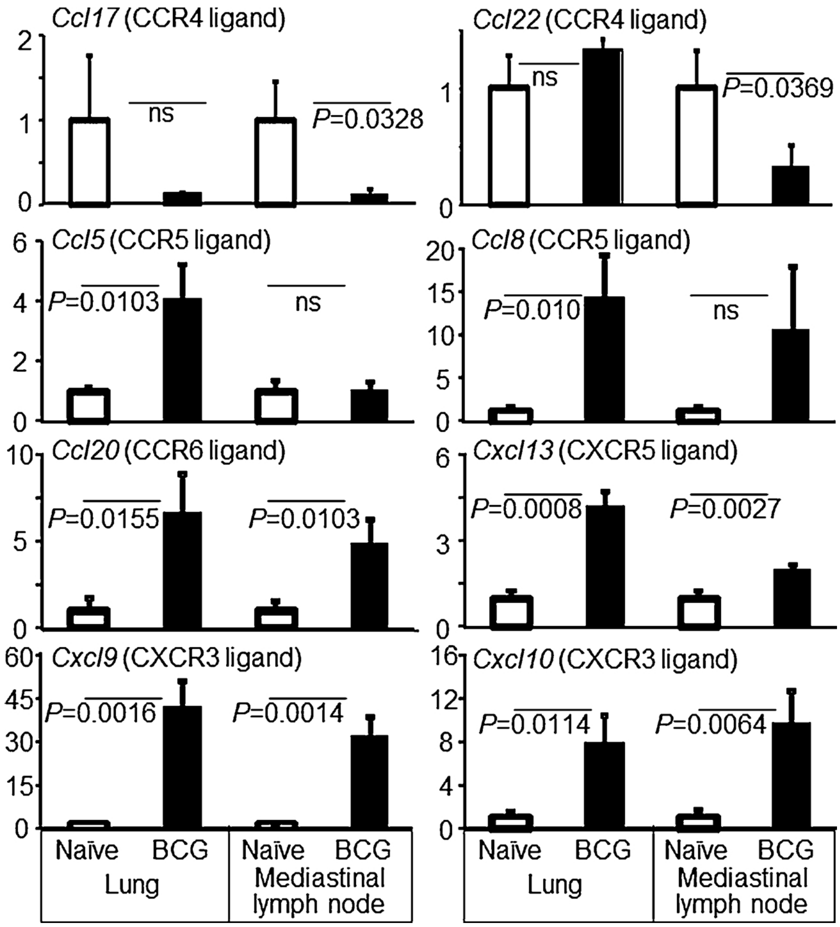

Fig. 4. Expression of chemokines which are the ligands of chemokine receptors expressed by the mycobacterial Ag-specific CD4$^+$ T cells in the *M. bovis* BCG-infected lung. Wild type C57BL/6 mice were infected with *M. bovis* BCG, and the infected lung and its draining mediastinal lymph node were analyzed for expression of chemokines. As negative control, the organs were prepared from the naïve C57BL/6 mice. Expression of chemokines which are the ligands of chemokine receptors expressed by the CD4$^+$ P25 T cells in the infected lung were analyzed by qPCR. The figure shows mean and SD of the data from the naïve and the BCG-infected mice (n = 3).

migrate into the organs of their residence (Iijima and Iwasaki, 2014), and the lung P25T cells could acquire characteristics of such resident memory T cells and failed to efficiently migrate into the lung and lymph node in chemokine-dependent manner. In contrast, mediastinal lymph node T cells might maintain their activity to migrate into the lung. Therefore, we next analyzed whether migration of the mediastinal lymph node T cells from the BCG-infected P25 mice were blocked by the pertussis toxin treatment. As shown in Fig. 6B, pertussis toxin completely blocked migration of the mediastinal lymph node P25T cells into the mediastinal lymph node of the recipient mice (Fig. 6B), which is in consistent to the requirement of CCR7 in recruitment of T cells into lymphoid organs (Griffith et al., 2014; Bromley et al., 2008). However, migration of the lymph node P25T cells into the BCG-infected lung was not blocked by the pertussis toxin treatment (Fig. 6B). These results demonstrate that chemokine receptor-mediated signaling is dispensable in migration of mycobacterial antigen-specific T cells into the mycobacteria-infected lung.

4. Discussion

In this report, we demonstrated that chemokine receptors are dispensable in migration of mycobacterial Ag-specific T cells in the *M. bovis* BCG-infected lung. It is generally accepted that interaction of chemokines and their receptors expressed on leukocytes is indispensable in migration of the leukocytes into the site of immune response (Johnston and Butcher, 2002). However, involvement of chemokine receptors in T cell migration into the infected lung is not well documented. In murine pulmonary mycobacterial infection model, we found that pertussis toxin which block function of chemokine receptors failed to suppress migration of primed mycobacterial antigen-specific CD4$^+$ T cells into the *M. bovis* BCG-infected lung although the T cells express multiple chemokine receptors which bind chemokines expressed by the infected lung. This is in contrast to CCR10-dependent T cell migration into the skin and CCR9-dependent T cell migration into the intestine (Svensson et al., 2002; Mora et al., 2003; Morales et al., 1999; Homey et al., 2002).

It has been reported that the T cells in the mycobacteria-infected lung express various chemokine receptors. Sakai et al. reported that murine Th1 cells in the parenchyma of *M. tuberculosis*-infected lung showed CXCR3$^{\text{high}}$ CX3CR1$^{\text{low}}$ phenotype, while those in the lung vasculature showed CXCR3$^{\text{low}}$ CX3CR1$^{\text{high}}$ phenotype, and the parenchymal Th1 cells preferentially migrate into the lung parenchyma after adoptive transfer (Sakai et al., 2014). In rhesus macaque, CXCR3$^+$ CX3CR1$^-$ CD4$^+$ Th1 cells were also detected in granulomas of the infected lung (Kauffman et al., 2018). In human, *M. tuberculosis*-specific CD4$^+$ Th1 cells were shown to express CXCR3 and CCR6 (Acosta-Rodriguez et al., 2007). All the results suggest that CXCR3 expression is associated with mycobacterial Ag-specific Th1 cells which migrate into parenchyma of the infected lung. However, our results suggest that T cell migration into the lung does not depend on the expression of chemokine receptors. It is still possible that certain chemokine receptors such as CXCR3 are involved in microscopic localization of the Th1 cells and determine protective function of the Th1 cells. Further analyses are required to address this issue.

Mice deficient in various chemokine receptors showed impaired immune response to mycobacterial pulmonary infections. CXCR3$^{-/-}$ mice showed decreased granuloma formation in the lung after *M. tuberculosis* infection (Seiler et al., 2003). CCR4$^{-/-}$ mice showed decreased mycobacterial antigen-induced granuloma formation in a PPD-beads model (Freeman et al., 2006). Mice deficient in CCL5, a ligand of CCR5, also showed decreased CD4$^+$ T cell number in the infected lung (Vesosky et al., 2010). In these reports, it is not clear whether

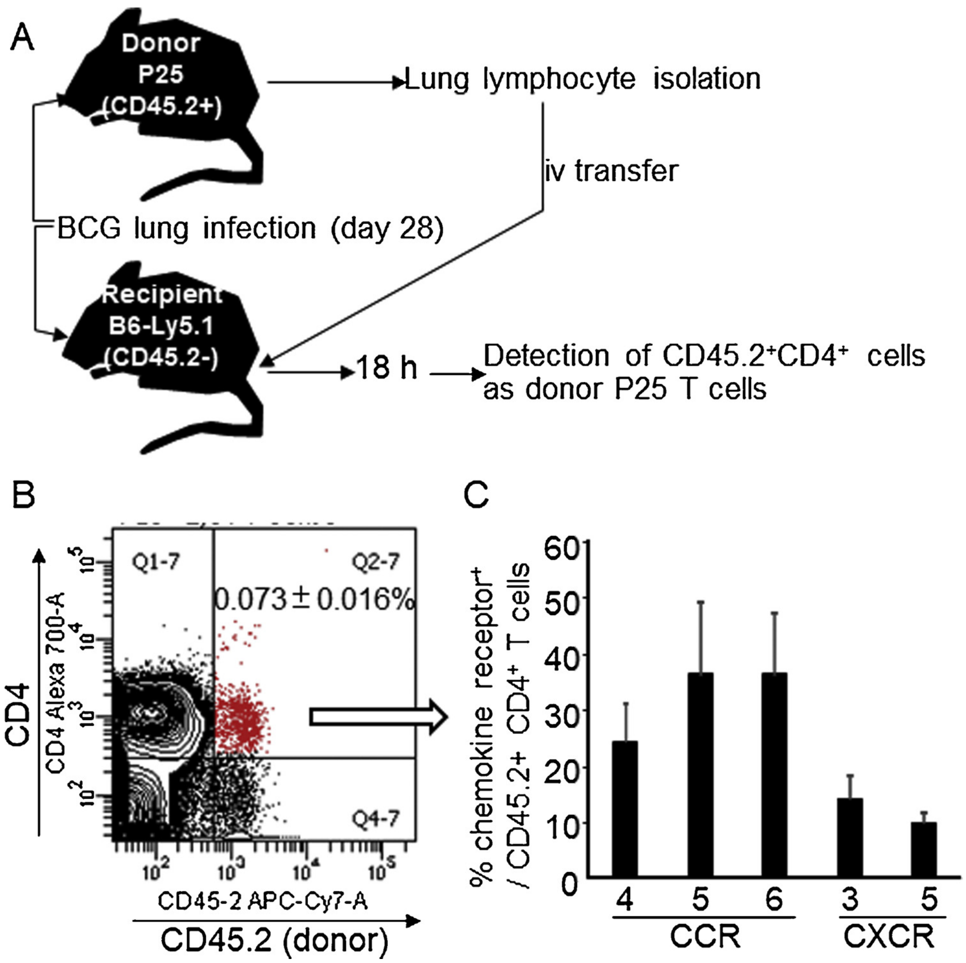

Fig. 5. Migration of mycobacterial Ag-specific $CD4^+$ T cells from the *M. bovis* BCG-infected lung into the lung of recipient wild type mice with pulmonary *M. bovis* BCG infection. P25 mice were infected with *M bovis* BCG in the lung, and the lung lymphocytes prepared on day 28 of the infection were adoptively transferred into the BCG-infected B6-Ly5.1 mice (A). The recipient mice were analyzed 18 h after the transfer. The donor derived T cells in the recipient lung were detected as $CD45.2^+CD4^+$ T cells (B). Chemokine receptor expression of the donor-derived T cells in three individual mice is summarized (C). The data demonstrated is a representative of five experiments.

deficiencies of the chemokine receptors or chemokines influence T cells directly or indirectly via induction of other leukocyte subsets such as macrophages and dendritic cells. Among chemokine receptor-expressing T cells, $CXCR5^+$ $CD4^+$ T cells were detected in the *M. tuberculosis*-infected lung, and $CXCR5^{-/-}$ mice showed decreased protection (Slight et al., 2013). Although the data clearly demonstrated involvement of CXCR5 on T cell-dependent protective immunity against *M. tuberculosis*, comparable number of mycobacterial antigen-specific Th1 cells were induced in the lung of $CXCR5^{-/-}$ and wild type mice after pulmonary *M. tuberculosis* infection (Slight et al., 2013). Therefore, the report on CXCR5 does not support importance of chemokine/chemokine receptor interaction in migration of mycobacterial antigen-specific T cells into the infected lung. This is consistent to our observations showing that chemokine receptor is dispensable in migration of mycobacterial antigen-specific $CD4^+$ T cells from the lung draining lymph node to the lung, or re-entry of the lung $CD4^+$ T cells into the lung.

Even if chemokine-chemokine receptor interaction is dispensable in $CD4^+$ T cell migration into the infected lung, chemokine receptors may still be important in protective immunity against pulmonary mycobacterial infections. CXCR5 expression on the mycobacterial antigen-specific $CD4^+$ T cells has shown to be important in granuloma formation and protective immunity against pulmonary *M. tuberculosis* infection (Slight et al., 2013). Correlation of CXCR3 expression with $CD4^+$ T cell localization in parenchyma of *M. tuberculosis*-infected lung and lung granulomas has been reported (Sakai et al., 2014; Kauffman et al., 2018). These reports also support involvement of CXCR3 in $CD4^+$ T cell localization in granulomas. It has been reported that IL-17A-producing T cells enhance migration of protective $CD4^+$ T cells into the mycobacteria-infected lung and enhances protective immunity against pulmonary *M. tuberculosis* infection (Khader et al., 2007; Fukui et al., 2015). Since IL-17 A induces expression of various chemokines including CXCR3 ligands CXCL9, CXCL10 and CXCL11 (Khader et al., 2007), it is possible that the IL-17A-dependent enhancement of protective immunity against pulmonary tuberculosis depends on induction of chemokines which control localization of protective T cells in the parenchyma of the infected lung. Alternatively, chemokine-chemokine receptor interaction could be important in maintenance of protective Th1 cells in the lung. It was reported that resident memory Th1 cells in the vagina requires CCL5 for their retention (Iijima and Iwasaki, 2014) and similar chemokine-dependent retention of Th1 cells could occur in the mycobacteria-infected lung.

Chemokine-independent migration of T cells have also been reported in T cell populations other than activated $CD4^+$ T cells. Migration of naïve $CD8^+$ T cells into the lung was not suppressed but rather enhanced by pertussis toxin treatment (Harp and Onami, 2010). These data indicates that migration of T cells into the lung does not depend on chemokine-chemokine receptor interaction in general. Interestingly, the migration of the naïve T cells into the lung depends neither chemokines nor selectins (Harp and Onami, 2010) although both of the molecules are important in leukocyte migration in various organs (Johnston and Butcher, 2002). All these observations suggest that steps of T cell migration into the lung should be revised as a sequence which does not include chemokine-chemokine receptor interaction and selectins.

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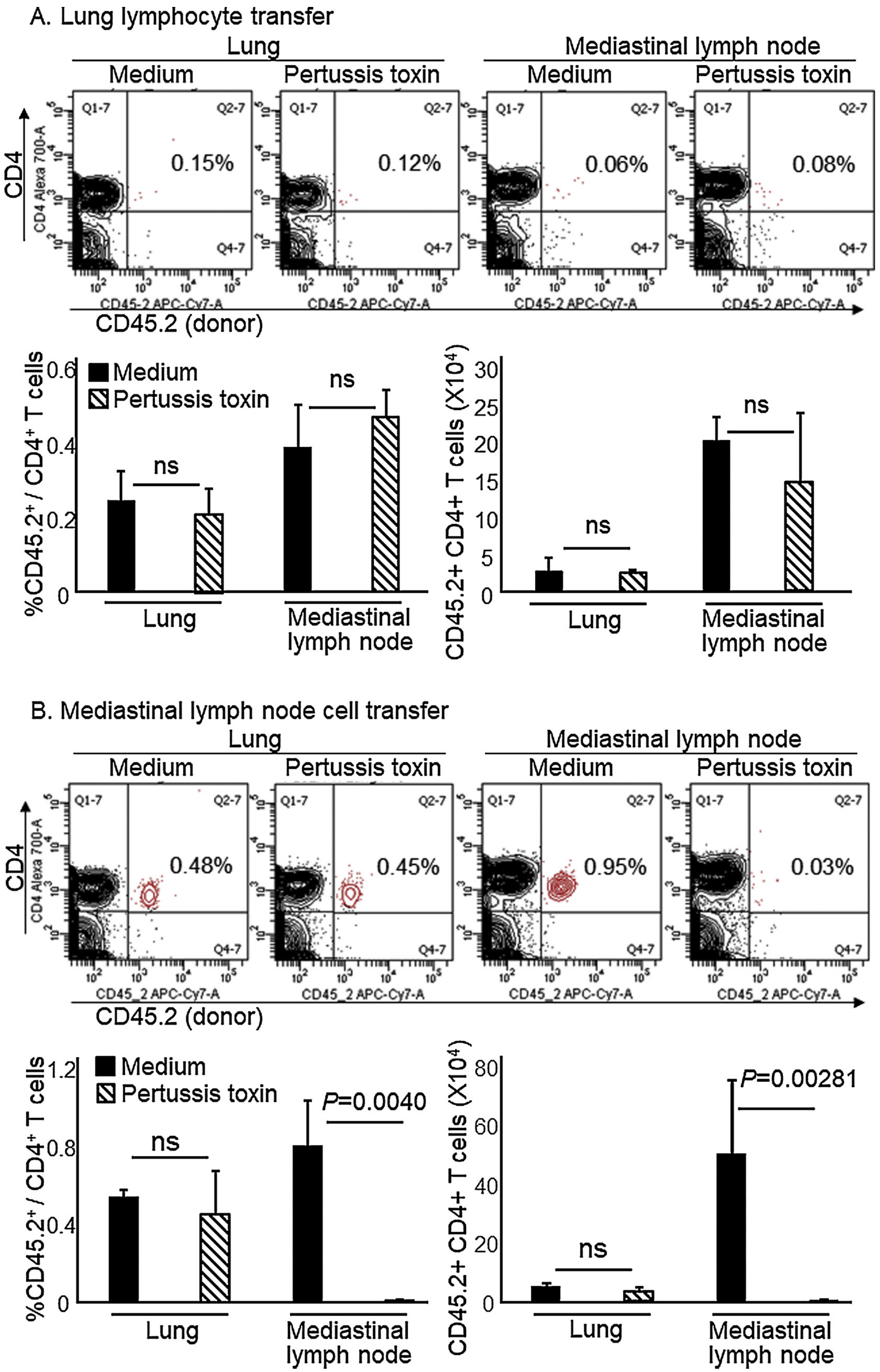

Fig. 6. Influence of pertussis toxin on the migration of the donor mycobacterial Ag-specific T cells into the *M. bovis* BCG-infected lung of the recipient mice. The lung lymphocytes (A) or mediastinal lymph node cells (B) from the *M. bovis* BCG-infected P25 mice on day 28 of the infection were treated or untreated with pertussis toxin, and then adoptively transferred to the BCG-infected B6-Ly5.1 mice, and the lung lymphocytes and mediastinal lymph node cells of the recipient mice were analyzed 18 h after the transfer. The donor-derived P25T cells were detected as $CD45.2^+CD4^+$ T cells, and their percentages in total $CD4^+$ T cells and absolute number were calculated (n = 3).

Conflict of interest

The authors declare no conflict of interest.

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