의학석사 학위논문

Effect of IL-4 on the development and function of memory-like CD8 T cells in periphery

인터루킨-4가 말초에서 기억세포 유사 CD8 T 세포의 발달 및 기능에 미치는 영향

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서울대학교 대학원

의학과 중개의학전공

박 희 정

A thesis of the Degree of Master of Science

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Effect of IL-4 on the development and function of memory-like CD8 T cells in periphery

by

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A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Master of Science in Medicine (Translational Medicine) at Seoul National University College of Medicine

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인터루킨-4가 말초에서 기억세포 유사 CD8 T 세포의 발달 및 기능에 미치는 영향

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위 원 _____ (인)

ABSTRACT

Unlike conventional T cells that require activation process upon antigen encounter for acquisition of effector/memory function, innate CD8 T cells develop with a memory-like phenotype in the thymus and immediately respond on antigen stimulation like memory T cells. In these populations, NKT cells, T-T CD4 T cells, H2-H3 specific T cells, mucosal-associated invariant T (MAIT) cells, CD8$\alpha\alpha^{+}$ intraepithelial T cells, innate CD8 T cells expressing Eomesodermin (Eomes) are included. Eomes^{+} innate CD8 T cell has been recently identified in both mouse and human. Unlike other types of innate T cells, this type of cells requires IL-4 in the thymus for their development. These features are similar to that of virtual memory CD8 T cells and IL-4 induced memory-like CD8 T cells generated in peripheral tissues. However, the relationship between these three types of memory-like CD8 T cells has not been clearly documented. In the present study, IL-4 induced memory-like CD8 T cells generated in peripheral tissues was compared with innate CD8 T cell in the aspects of their phenotype and function. When IL-4/anti-IL-4 antibody complex (IL-4C) was injected into C57BL/mice every day for 7 days, Eomes^{hi}CXCR3^{+} CD8 T cell population was markedly increased in peripheral lymphoid organs and blood. These cells were generated from naïve CD8 T cells or accumulated via expansion of preexisting CD44^{hi}CXCR3^{low} CD8 T cells. Initially, majority of these CXCR3^{+} CD8 T cells express low level of CD44, followed by conversion to CD44^{hi} phenotype. This conversion was accelerated in IL-4 deficient mice,

and associated with acquisition of enhanced effector function. The phenotype and function of these CXCR3^{+} CD8 T cells was similar to innate CD8 T cells in CIITA-transgenic (CIITA^{Tg}) mice. After cessation of IL-4C treatment, Eomes expression level was gradually decreased in CXCR3^{+} CD8 T cells like innate CD8 T cells in CIITA^{Tg} mice. Finally, IL-4C treatment prevents the exhaustion of virus-specific CD8 T cells and progression to persistent viremia after lymphocytic choriomeningitis virus (LCMV) cone 13 (CL-13) infections. Similar finding has also been reported in CIITA^{Tg} mice. Taken together, this study demonstrate that IL-4-induced memory like CD8 T cells arising from peripheral tissue are very similar to innate CD8 T cells generated in thymus in the aspect of development process, phenotype, and function. Particularly, exogenous IL-4 treatments were able to provide an early defense mechanism like innate CD8 T cells.

Keywords: Innate CD8 T cells, Memory-like CD8 T cells, IL-4 complex, CXCR3, Lymphocytic choriomeningitis virus

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LIST OF ABBREVIATIONS

B6 : C57BL/6

CⅡTA : Major Histocompatibility Complex ClassⅡ transactivator

Eomes : Eomesodermin

IFN-r : Interferon-gamma

IL : Interleukin

PBS : Phosphate Buffer Saline

PLZF : promyelovytic leukemia zinc finger protein

TCR : T cell receptor

T-T : thymocyte-thymocyte

IL-4C : Interleukin-4 complex

GVHD : Graft-versus-host disease

CXCR3 : chemokine (C-X-C motif) receptor 3

LCMV : lymphocytic choriomeningitis virus

CL-13 : Clone 13

INTRODUCTION

Conventional T cells developed in thymus usually exhibit naïve phenotype when they exit into peripheral lymphoid tissue and require a sequential process of antigen encounter, activation, proliferation, and differentiation to achieve effector/memory phenotypes. However, some of T cells develop with a memory-like phenotype in the thymus and immediately respond on antigen stimulation. In this T cell population termed innate CD8 T cells, NKT cells, T-T CD4 (or T-CD4) T cells, H2-H3 specific T cells, mucosal-associated invariant T (MAIT) cells, CD8$\alpha\alpha^{+}$ intraepithelial T cells, innate CD8 T cells expressing Eomesodermin (Eomes) are included.[1-3]

NKT cells is a prototype of innate T cell in the aspect that are selected in the thymus by interaction with hematopoietic cells and express T cell receptors (TCRs) specific for non-classical MHC class Ib molecules.[1] Unlike conventional T cells that recognized self-antigenic peptides presented by MHC class I or II molecules, in cortical thymic epithelial cells for positive selection in the thymus, homotypic thymocyte-thymocyte (T-T) interaction is required in the generation NKT cells that express CD1d molecule-restricted TCR repertoire.[4] Another type of innate T cells, T-T CD4 T cells are also selected by T-T interaction.[5, 6] T-T CD4 T cells and NKT cells share several common characteristics. Their development is dependent on SLAM-SAP signaling pathway.[2, 7] They express promyelocytic leukemia zinc finger protein (PLZF) transcription factor, which is required for development

of both T-T CD4 T cells and NKT cells and their achievement of effector/memory function.[8-12] $PLZF^+$ T-T CD4 T cells and NKT cells produce IL-4. On the other hand, these two types of cells are distinct from each other in some characteristics. $PLZF^+$ T-T CD4 T cells are selected by MHC class II/self-antigenic peptide in thymocytes and thereby have diverse TCR repertoire, while CD1d-restricted NKT cells express a very limited TCR repertoire.[11, 12] MHC class II molecules are expressed in the thymocytes of human but not mouse.[13] Therefore, $PLZF^+$ T-T CD4 T cells were identified in CIITA-transgenic ($CIITA^{Tg}$) mice, which express MHC class II molecules in the thymocytes.[12]

$Eomes^+$ innate CD8 T cells had been initially found in $Itk^{-/-}$ mice.[14, 15] A large fraction of CD8 single positive (SP) thymocytes in mice deficient in the Tec kinase Itk expressed memory markers such as CD44 and CD122 and rapidly produce IFN-γ. Characteristically, these cells expressed high amount of the T box transcription factor, Eomes, and Eomes expression in these cells were correlated with their phenotype and function.[14] Subsequently, it was found a number of other mice deficient in T cell signaling molecules or transcription factors (such as *Klf2, Cbp or Id3*).[3] At first, these cells were considered to be selected on hematopoietic cells.[15], and their selection required SAP [16], similar to NKT cells. However, a few year later, it has been demonstrated that altered development of CD8 T cells in these gene-manipulated mice was not due to intrinsic deficiency of these genes but instead IL-4 produced by an expanded population of $PLZF^+$ T cells permitted the generation of $Eomes^+$ innate CD8 T cells.[17] In this aspect, these innate T

cells are also called IL-4-induced innate CD8 T cells.[18]. Eomes^{+} innate CD8 T cells were also identified in CIITA^{Tg} mice[19] and wild type BALB/c mice.[17] BALB/c mice have large number of PLZF^{+} NKT cells in their thymus, while there are small number of such cells in thymus of wild type C57BL/6 (B6) mice. Thus, PLZF^{+} NKT cells drives the generation of Eomes^{+} innate CD8 T cells in wild type BALB/c mice via IL-4 production.[17] In contrast, development of Eomes^{+} CD8 T cells in CIITA^{Tg} B6 mice is dependent on PLZF^{+} T-T CD4 T cells.[20] In human, fetal thymus have much abundant PLZF^{+} T-T CD4 T cells compared with NKT cells[12], and Eomes^{+} CD8 T cell population of innate phenotype was also found in human fetal thymus and spleen[20]. Similar to PLZF^{+} T-T CD4 T cells, Eomes+ innate CD8 T cells have diverse TCR repertoire[20].

In addition to the pathway of innate CD8 T cell generation in IL-4 rich intrathymic environment, similar cells also have been found in peripheral tissue of wild-type mice[21, 22]. Using MHC/peptide tetramers, a subpopulation of antigen-specific CD8 T cells bearing memory markers, such as CD44, CD122, Ly6C, in unimmunized mice[21]. Their presence even in germ-free mice supported that these cells acquired memory-like phenotype even in the absence of antigen stimulation. These antigen-inexperienced memory phenotype CD8 T cells has been called as virtual memory (VM) CD8 T cells[21-23]. These cells seem to arise via physiologic homeostatic mechanisms[21, 23]. Generation of VM CD8 T cells is dependent on endogenous IL-4. This population was expanded in Nedd4-family interaction protein 1 deficient mice in which peripheral CD4 T cell overproduce IL-4[24],

and reduced in IL-4 receptor deficient mice[22]. However, IL-4 receptor deficient mice still have substantial number of VM CD8 T cells[22].

Memory-like CD8 T population is also expanded in mice received IL-4/anti-IL-4 antibody complex (IL-4C)[25]. This IL-4 induces an innate CD8 T cell-like phenotype in peripheral CD8 T cells that includes elevated expression of CD44, CD122, CXCR3, and Eomes. However, the relationship between these three types in memory-like CD8 T cells (innate CD8 T cells, VM CD8 T cells and exogenous IL-4 induced memory-like CD8 T cells) has not been clearly documented. In the present study, IL-4 induced memory-like CD8 T cells was compared with innate CD8 T cell in the aspects of their phenotype and function.

MATERIALS AND METHODS

1. Mice

B6, BALB/c, IL-$4^{-/-}$ B6, OT-I B6, and CD45.1^{+} B6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). B6 were thymectomized (removal thymus) at 6 weeks of age and kept until 8 weeks of age. The plck-$CIITA^{Tg}$ mice were generated in Seoul National University College of Medicine[6]. All mice were bred and maintained under specific pathogen-free conditions in Bio Medical Center for Animal Resource Development at Seoul National University. All experiments were approved by the institutional animal care and use committee of the institute of Laboratory Animal Resource at Seoul National University, Korea.

2. Administration of IL-4 and anti-IL-4 antibody in vivo

According to a previous report [26], a mixture of 1.5 μg mouse IL-4 (Peprotech, Princeton, NJ, USA) plus 50 μg IL-4 antibody (11B11; Bio X Cell, West Lebanon, NH, USA) was intraperitoneally injected into mice every day. After 7 days of treatment, each lymphoid organ was analyzed.

For adoptive transfer, donor CD8 T cells were isolated from CD45.1 B6 mouse splenocytes using anti-CD8 microbeads by magnetic sorting (MACS; Miltenyi Biotec, Auburn, CA, USA). Next, these CD8 T cells were sorted into $CD44^{low}CXCR3^{-}$ and $CD44^{hi}CXCR3^{+}$ populations using a FACSAria (BD

Bioscience) and labeled by Cell Trace Violet (Life Tecnologies, Waltham, MA, USA). Each recipient B6 mouse received 3×10^6 sorted cells via lateral tail vein injection, followed by IL-4C treatment one day later.

3. Flow cytometric analysis.

The fluorochrome or -biotin-labeled monoclonal antibodies against the following antigens were purchased from BD Bioscience (San Jose, CA, USA), eBioscience(San Diego, CA, USA) and BioLegend (San Diego, CA, USA): CD8 (53-6.7), CD44 (IM7), CD62L (MEL-14), CD122 (TM-β1), CD124 (mIL4R-M1), CD127 (SB/199), CCR5 (7A4), CC57 (4B12), Sca-1 (D7), CXCR3 (CXCR3-173) and CD24 (M1/69). Cells from blood, thymus, spleen, lymph node, and bone marrow (BM) were resuspended in fluorescence-activated cell sorting buffer (phosphate buffered saline with 0.1% sodium azide and 0.2% bovine serum albumin). Cell surface markers were stained with fluorochrome-conjugated antibodies for 30min at 4°C and read by BD Calibur and BD LSR Fortessa (Becton Dickinson, Mountain View, CA, USA). The data were analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

For intracellular staining, prepared cells were resuspended in mixture of the fixation and permeabilization buffers from the Foxp3 staining buffer kit (eBioscience, San Diego, CA, USA). Then, intracellular staining was performed using the antibodies against mouse T-bet (eBio4B10), bcl2 (H-5) and Eomes (Dan11mag). Antibodies were purchased from BD Bioscience

(San Deigo, CA, USA).

4. Intracellular cytokine assay

For T cells stimulation, CD8 T cells isolated from mouse spleens were incubated with 50 ng/ml phorbol myristate acetate (PMA) and 1.5μM ionomycin (Sigma-Aldrich, St Louis, MO, USA) for 4 hours incubation in 37°C CO_2 incubator, followed by additional incubation in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich, St Louis, MO, USA) for 2 hours. Cultured cells were surface-stained with anti-CD8, anti-CXCR3, followed by fixation, permeabilization and intracellular cytokine staining with anti-IFN-γ (XMG1.2). Stained samples were read by BD LSRFortessa.

5. Graft versus host disease (GVHD) model

Recipient BALB/c mice were exposed to 800 rad of total body irradiation from a [^{137}Cs] source divided into two split dose 4h apart. The mice were rested for 4-24 hours before receiving BM cells. Donor cells were prepared from spleens of B6 mice injected with IL-4 and anti-IL-4 complex for 7d. Using anti-CD8 microbeads, CD8 T cells were selected by magnetic sorting (MACS; Miltenyi Biotec, Auburn, CA, USA). Next, these CD8 T cells were sorted into $CD44^{low}CXCR3^{+}$ and $CD44^{hi}CXCR3^{+}$ populations using a FACSAria (BD Bioscience) and labeled by Cell Trace violet (Life Tecnologies, Waltham, MA, USA). Each recipient mouse received 2×10^5

sorted cells mixed with 3×10^6 T-cell depleted BM cells from wild type B6 mice via lateral tail vein injection. The splenocytes were analyzed 8 weeks later

6. Virus infection

B6 mice were infected with 5 x 10^5 plaque forming units of LCMV CL-13 via intravenous infection. Virus specific CD8 T cells were detected using H-2D^b tetramers-GP33-41 or GP276-286 peptide complex. For cytokine analysis, splenocytes were restimulated by GP33-41 peptide and cultured with Golgi plug/Golgi stop for 5 hours. Titration of virus was determined by plaque assay as previously described [27]. Undetectable samples were given a half of each detection limit. The experiments were performed in the specific pathogen-free facility of the Yonsei Laboratory Animal Research Center at Yonsei University

7. Statistical analyses

All data were analyzed by a two way ANOVA using the GraphPad Prism software (GraphPad Software, CA, USA). Bar graph indicates the mean ± SD.

RESULTS

Exogenous IL-4 induced accumulation of CXCR3^{+} CD8 T cells.

To compare the phenotype and function of Eomes^{+} innate CD8 T cells and IL-4 induced memory-like T cells arising from peripheral tissues, CIITA^{Tg} mice were used. CIITA (MHC class II transactivator) is the master regulator for MHC class II expression[28], thereby CIITA expression driven by proximal promoter of the *lck* gene induces MHC class II expression in thymocytes and mature T cells in CIITA^{Tg} mice, allowing MHC class II-dependent T-T interaction for the generation of T-T CD4 T cells[6]. In these mice, some of T-T CD4 T cells express PLZF molecules, and PLZF^{+} CD4 T cells induce the development of Eomes^{+} innate CD8 T cells via IL-4 production[12]. Eomes^{+} innate CD8 T cells characteristically exhibit CD44^{hi}CXCR3^{+} phenotype [20]. Substantial fraction of CD44^{hi} memory CD8 T cells in spleen and lymph nodes of wild type mice also express CXCR3, and rare population of CD44^{low}CXCR3^{+} CD8 T cells was also detected. To obtain the IL-4 induced memory-like T cells, B6 mice were injected with IL-4C for 7 days, and CD8 T cells were isolated from the thymus, spleens and lymph nodes of the mice one day later. These complex was used because recombinant IL-4 has a very short half-life in vivo and its association with the anti-IL-4 antibody increase both biodisponibility and half-life of IL-4 [25, 29], and thus this complex have more effect than free IL-4 at the same dose in vivo [30]. In accordance with previous report [25], IL-4 treatment accumulated mainly CD44^{low}CXCR3^{+} population (Fig. 1). Of note, IL-4C treatment also

expanded $CXCR3^+$ CD8 SP population in the thymus (Fig. 1), but IL-4-induced accumulation of $CXCR3^+$ CD8 T cells in peripheral lymphoid organs was also observed in thymectomized mice, indicating that peripheral accumulation of these cells were not resulted from emigration from thymus (Fig. 2).

Next, other memory markers were examined, and it was found that both $CD44^{hi}CXCR3^+$ and $CD44^{low}CXCR3^+$ populations from IL-4C treated mice had higher expression of CD124 (IL-4 receptor α subunit) and Eomes and lower expression of Sca-1 compared with that of $Eomes^+$ innate CD8 T cells of $CIITA^{Tg}$ mice (Fig. 3A). Functionally, $CD44^{hi}CXCR3^{hi}$ populations from wild-type and IL-4C treated mice were similar to $Eomes^+$ innate CD8 T cells. When they were stimulated with PMA and ionomycin, majority of cells immediately produced IFN-γ, while $CD44^{low}CXCR3^+$ CD8 T cells showed lower IFN-γ production capacity (Fig. 3B).

IL-4C treatment effect was also repeated in monoclonal TCR system. For this experiment, $CD45.1^+$ OT-I CD8 T cells that have ovalbumin-specific monoclonal TCR were used and treated by IL-4C injection every day for 7 days. As shown in Fig. 4, both $CD44^{hi}CXCR3^+$ and $CD44^{low}CXCR3^+$ OT-I cells were markedly increased in the spleen of IL-4C treated recipients.

Next, it was investigated whether IL-4C-induced memory-like CD8 T cells were arisen from naïve T cells and/or accumulated via expansion of preexisting $CD44^{hi}CXCR3^+$ cells, most of which has been known to be virtual memory T cells [24]. For this, B6 mice received $CD44^{low}CXCR3^-$ (naïve) or

$CD44^{hi}CXCR3^{+}$ CD8 T cells were treated with IL-4C. Donor cells were isolated from CD45.1+ B6 spleens and labeled with Cell Trace violet prior to adoptive transfer. In these B6 mice, IL-4C treatment induced the proliferation (CTV^{low}) of both $CD44^{low}CXCR3^{-}$ and $CD44^{hi}CXCR3^{+}$ CD8 T cells, and, consistent with a previous report [25], drived the differentiation of naïve CD8 T cells into both $CD44^{low}CXCR3^{+}$ and $CD44^{hi}CXCR3^{+}$ cells (Fig. 5).

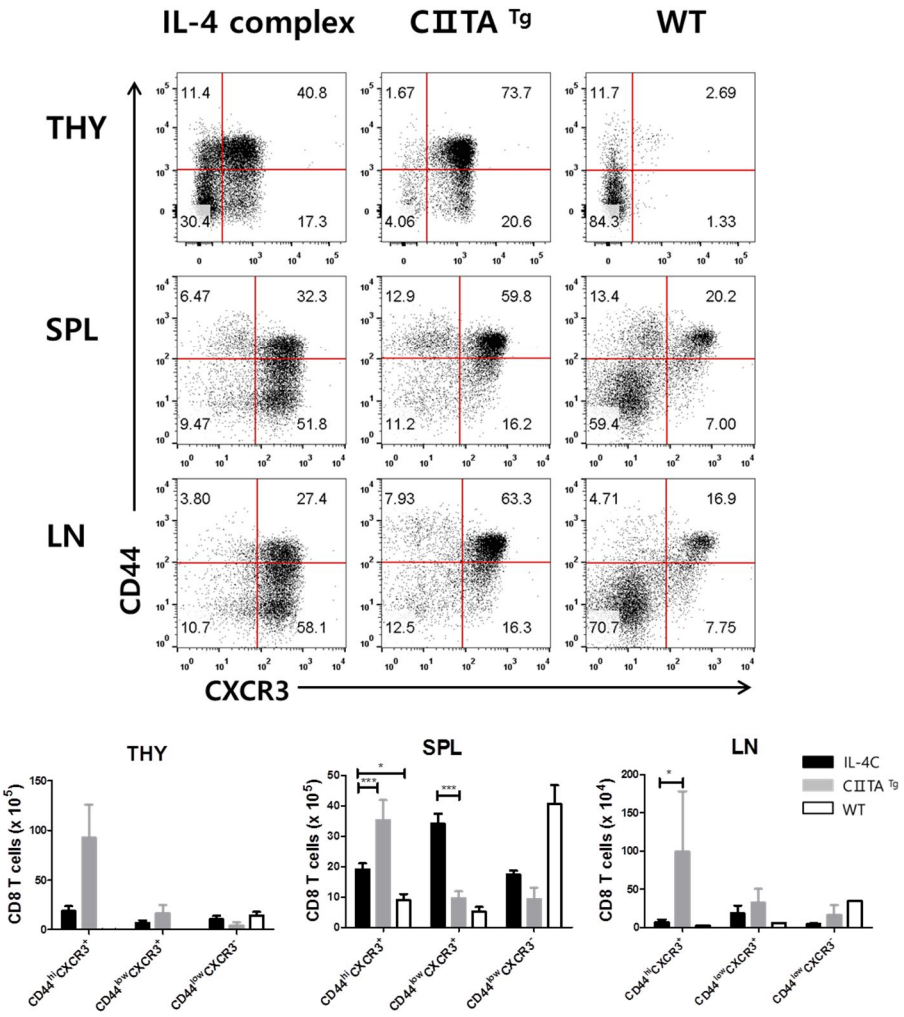

Figure 1. Generation of memory-like CD8 T population by IL-4 and anti-IL-4 antibody complex injection. Wild-type B6 mice were injected with IL-4 and anti-IL-4 antibody complex (IL-4C) every day for 7 days. At day 8, mice were sacrificed and the expression of CD44 and CXCR3 on the CD8 T cells isolated from thymus, spleen and lymph node were compared with those of wild type (WT) and CIITA-transgenic ($CIITA^{Tg}$) mice. Representative flow cytometry data (upper panel) and summarized graph (n=3, low panel) from

two independent are shown. Numbers in the plots indicate the percentages of cells in each quadrant. The bars indicate means ± SD. *p<0.05,***p<0.001.

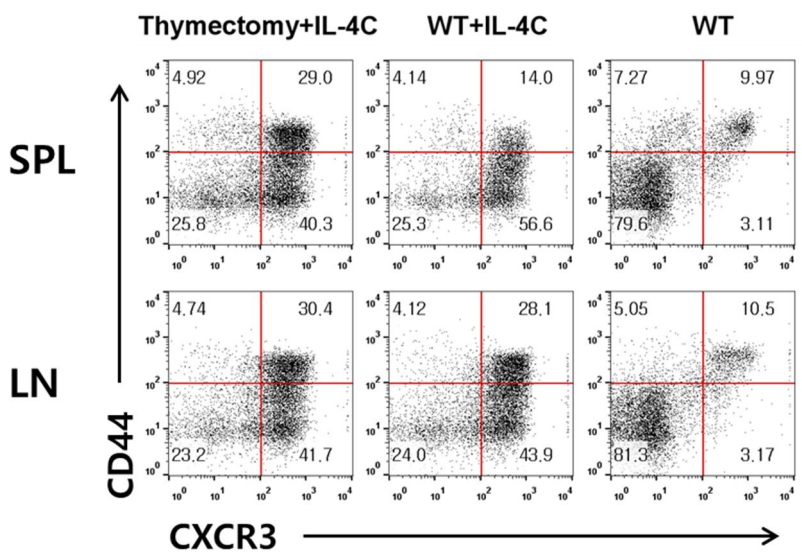

Figure 2. IL-4-induced accumulation of memory-like CD8 T cells in periphery were thymus- independent. Wild- type (WT) and thymectomized (removal of thymus) B6 were injected with IL-4 complex (IL-4) for 7d. CD8 T cells were isolated from spleen and lymph node and their expression of CD44 and CXCR3 were compared with those of untreated mice. Representative flow cytometry data from two independent experiments are shown. Numbers in the plots indicate the percentages of cells in each quadrant.

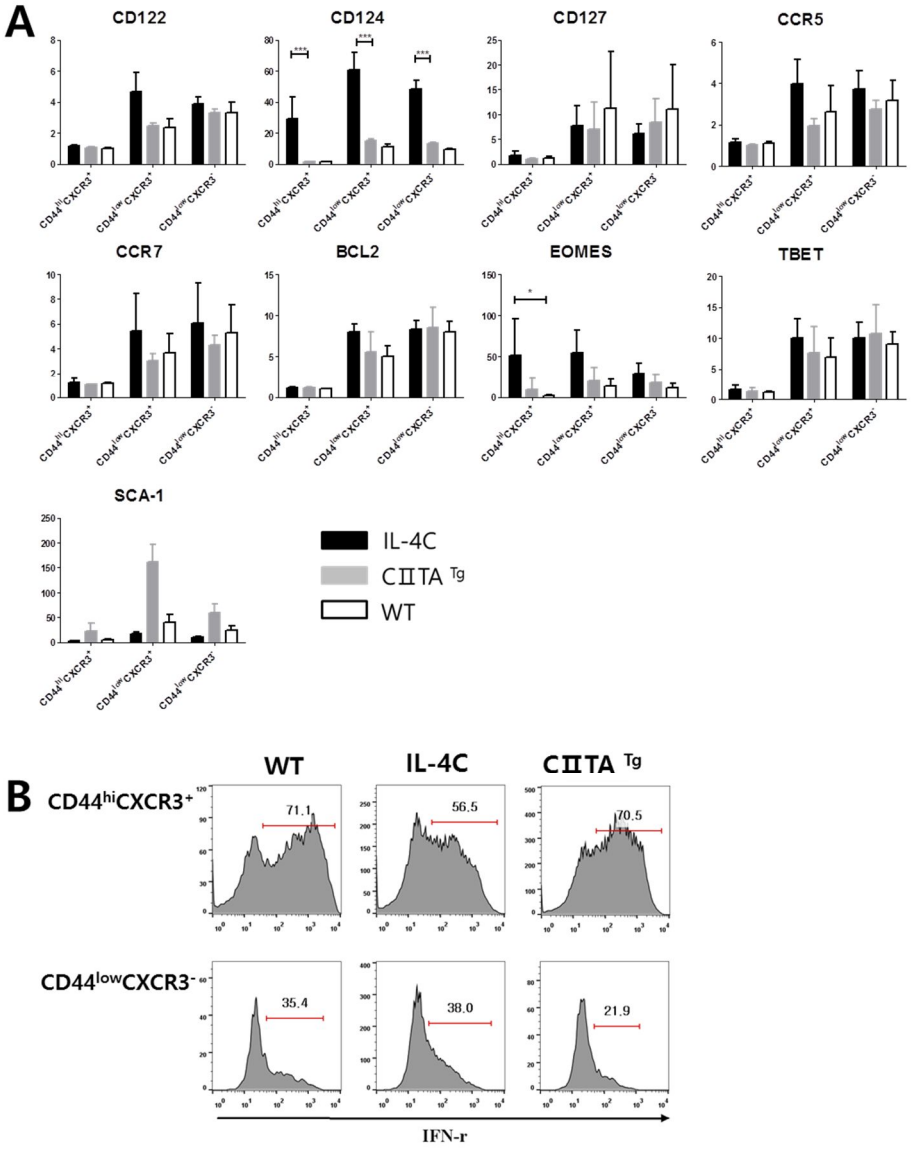

Figure 3. Phenotype and cytokine analysis of CD8 T cells in wild type, IL-4 complex treated and CIITA-transgenic mice. Splenic CD8 T cells were isolated from IL-4 complex (IL-4C)-treated mice and their expression of indicated markers (A) or IFN-γ production (B) were compared with those of untreated wild type (WT) and CIITA-transgenic ($CIITA^{Tg}$) mice. Summarized graph (n=3, A) and representative data from three independent experiments (B)

are shown. The bars indicate means ± SD. Numbers in the plots indicate the percentages of cells producing IFN-γ. *, $p < 0.05$, ***, $p < 0.001$

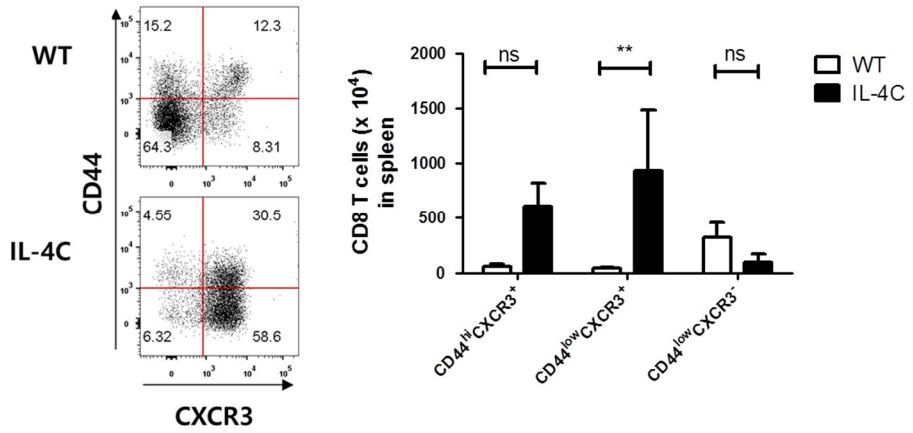

Figure 4. IL-4-induced accumulation of memory-like CD8 T cells in monoclonal TCR system. OT-1 TCR transgenic mice were treated with IL-4 complex for 7 days, and CD8 T cells were isolated from spleens and stained with ant-CD44 and anti-CXCR3 antibodies. Representative flow cytometry data (left panel) and summarized graph (n=3, right panel) are shown. Numbers in the plots indicate the percentages of cells in each quadrant. The bars indicate means ± SD. ns, nog significant; **, $p < 0.01$

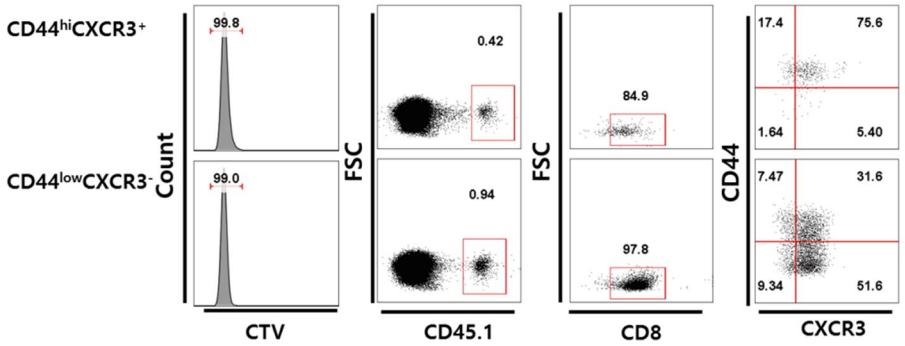

Figure 5. Generation of memory like CD8 T cells from naïve CD8 T cells and expansion of CD44^{hi}CXCR3^{+} CD8 T cells after IL-4C treatment. CD44^{hi}CXCR3^{+} (memory phenotype) and CD44^{lo}CXCR3^{-} (naïve) cells sorted from CD45.1 B6 splenocytes were labeled with Cell Trace Violet (CTV) and transferred into CD45.2 B6 mice via intravenous injection. These recipient mice were injected with IL-4 and anti-IL-4 antibody complex (IL-4C) and proliferation and phenotypic change of transferred CD8 T cells in spleen was analyzed. Numbers in the plots indicated the percentages of cells in each population or quadrant.

Maintenance and maturation of IL-4-induced CXCR3$^+$ CD8 T cells.

To follow how long CXCR3$^+$ CD8 T cells were able to be maintained after cessation of IL-4C treatment, peripheral blood mononuclear cells were repeatedly analyzed for about 1 month. In IL-4C treated wild type mice, total CXCR3$^+$ populations were slightly decreased for the follow-up period (Fig. 6A). However, Eomes expression level was gradually decreased in CXCR3$^+$ CD8 T cells (Fig. 6B). In contrast, CD44 expression levels gradually increased and eventually majority of CXCR3$^+$ CD8 T cells exhibited CD44^{hi} phenotype (Fig. 6C). This trend was more accelerated in IL-4-deficient mice (Fig. 6C). These suggest that CD44^{low}CXCR3$^+$ CD8 T cells might be a preparatory stage for maturation into CD44^{hi}CXCR3$^+$ memory-like CD8 T cells and endogenous IL-4 delay this maturation process.

Next question was whether CD44^{low}CXCR3$^+$ CD8 T cells are able to be converted to CD44^{hi} phenotype in response to TCR stimulation. To test this possibility, CD44^{low}CXCR3$^+$ or CD44^{hi}CXCR3$^+$ CD8 T cells isolated from spleens of IL-4C treated B6 mice were injected into sublethally irradiated BALB/c mice. For tracing, donor cells were labeled with Cell Trace violet prior adoptive transfer, and recovered on day 14 after adoptive transfer. Then, Cell Trace violet^{low} proliferated population was gated, and some of CD44^{hi}CXCR3$^+$ donor cells maintained their phenotype in terms of CD44 and CXCR3 expression, but CXCR3 expression was down regulated in the other cells (Fig. 7). In the case of CD44^{low}CXCR3$^+$ donor cells, CD44 expression level was up-regulated in almost all of them, and their phenotype was nearly

identical to that of activated $CD44^{hi}CXCR3^{+}$ donor cells. These data indicate that $CD44^{low}CXCR3^{+}$ CD8 T cells are also converted into $CD44^{hi}CXCR3^{+}$ CD8 T cells via TCR stimulation.

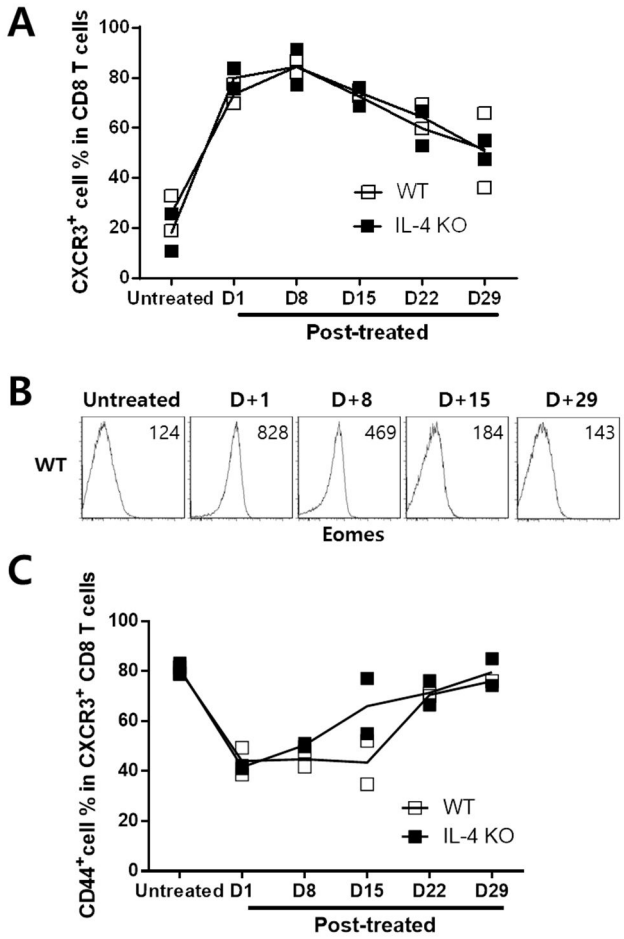

Figure 6. Endogenous IL-4 effect on maintenance of IL-4-induced memory-like CD8 T cells. Wild type (WT) and IL-4 knock-out (IL-4 KO) B6 mice were injected with IL-4 complex for 7 days, and percentage of $CXCR3^+$ CD8 T cell populatoin in peripheral blood (A) and expression level of Eomes (B) and CD44 (C) on $CXCR3^+$ CD8 T cells were analyzed by flow cytometry on day 1, 8, 15, 22, 29. . The numbers in histograms (B) indicate the mean fluorescent intensity of Eomes expression. Summarized data from two experiments are shown (A & B)

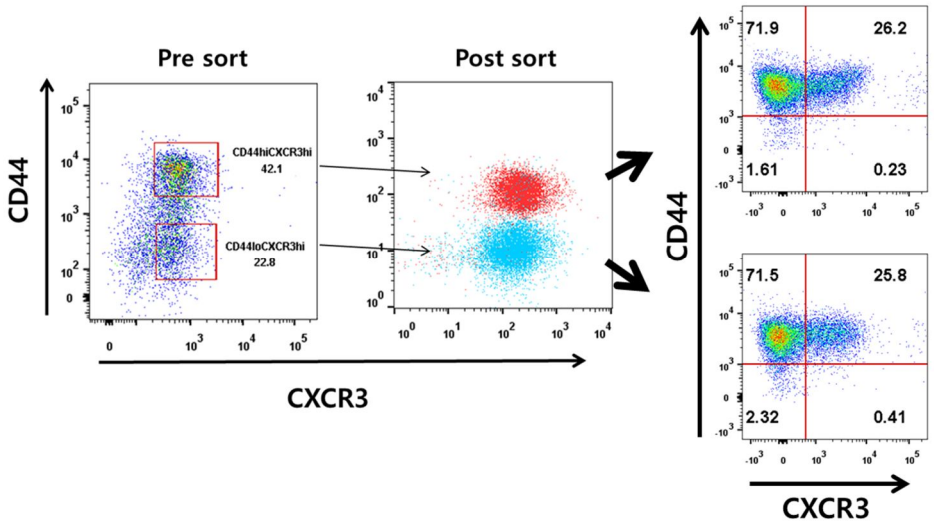

Figure 7. Conversion of IL-4 induced CD44^{low}CXCR3^{+} CD8 T cells into CD44^{hi} phenotype. (A) Splenic CD8 T cells from IL-4 complex treated B6 mice were sorted into CD44^{hi}CXCR3^{+} and CD44^{lo}CXCR3^{+} by FACS Aria and labeled with Cell Trace Violet (CTV). CTV-labeled 2×10^{5} cells of each population were mixed with 3×10^{6} T cell depleted BM cells from untreated B6 mice, and transferred into irradiated BALB/C mice intravenously. On day14 after cell transfer, splenocytes from recipient BALB/C mice was stained with antibodies against H-2Kb, CD8, CD44 and CXCR3, and the expression of CD44 and CXCR3 in CTV^{low} H-2Kb^{+} proliferated donor CD8 T cells. Numbers in the plots indicate the percentages of cells in each quadrant.

Control of chronic viral infection by IL-4C treatment

Recently, it has been demonstrated that IL-4 induced innate CD8 T cells generated in thymus provided effective defense mechanism against chronic virus infection [18]. So, it was assessed whether IL-4C treatment is also able to facilitate virus control via expansion of memory-like CD8 T cells. For this test, B6 mice were treated with IL-4C every days for 7 days, followed by lymphocytic choriomeningitis virus (LCMV) clone 13 (CL−13) inoculation. Then, virus-specific CD8 T cell frequency in spleens and lungs were measured using H-2D^{b} tetramer bound to GP33 or GP276 peptide of LCMV at 8, 15, 30 days post infection (DPI). It has been well known that virus specific CD8 T cells fail to sufficiently expand in CL-13 infected wild type mice and up-regulated expression of PD-1 inhibitory molecules in the virus-specific CD8 T cells cause functional exhaustion[31]. Consistent with this, virus-specific CD8 T cell frequency was low in control mice and PD-1 expression level on the surface of these CD8 T cells was consistently up-regulated, (Fig. 8). The exhausted function of virus specific CD8 T cells in control mice was confirmed by ex vivo restimulation assay. When splenocytes isolated from control mice were re-stimulated with viral peptides, only small fraction of tetramer-positive cells produced IFN-γ and TNF-α (Fig. 9). These indicate that virus was not efficiently controlled and chronic infection was established. In contrast, virus specific CD8 T cell frequency in IL-4C treated mice was much higher than those of control mice. Moreover, virus-specific CD8 T cells in IL-4C-treated mice showed much lower levels of PD-1

expression on their surface and very strong cytokine response compared with T cells of control mice (Fig. 8 and 9A). And, this enhanced antiviral CD8 T cell activity was associated with a rapid drop in serum virus titer of IL-4 treated mice (Fig 9B). Taken together, these indicate that IL-4C treatment had a beneficial effect on virus control.

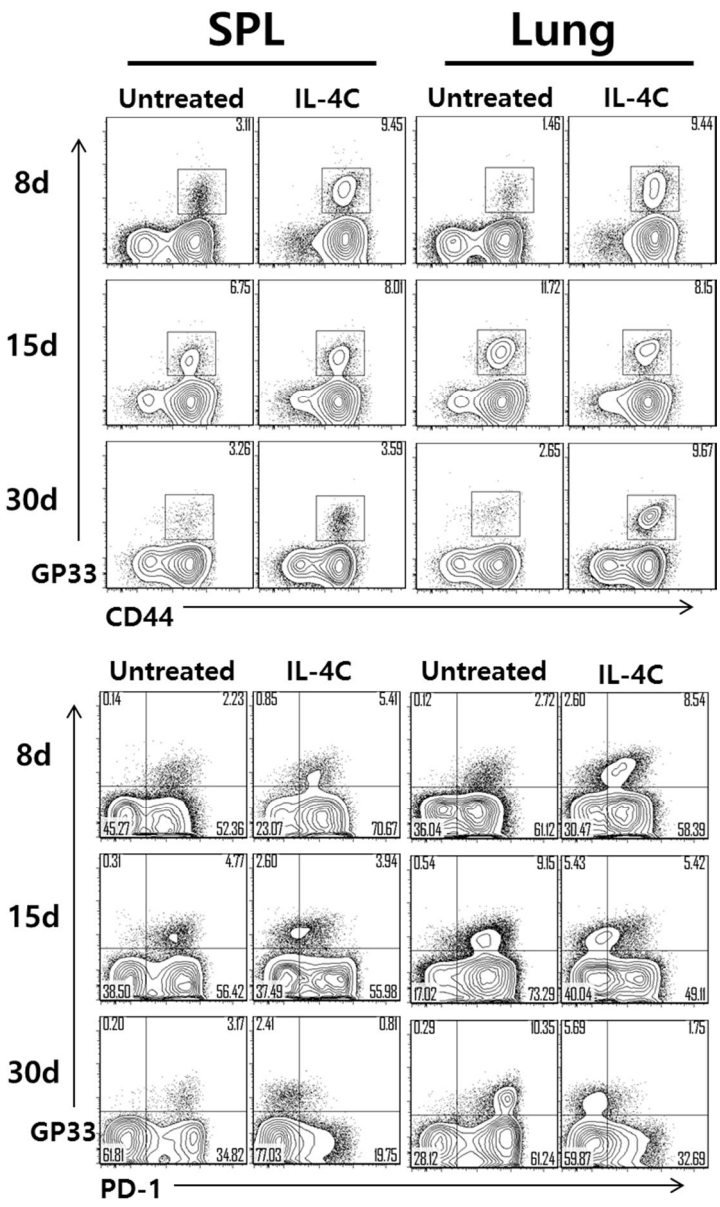

Figure 8. LCMV-specific CD8 T cell frequency and their PD-1 expression levels in IL-4 complex treated mice during chronic virus infection. Wild-type B6 mice were injected with IL-4 complex (IL-4C) for 7 d and infected with 5×10^5 PFU of LCMV CL-13 one day later. As untreated control mice, naïve B6 mice were infected with CL-13. Lymphocytes were isolated from

spleens and lungs and LCMV GP_{33-41} (GP33) peptide/H-2 Db tetramer-positive CD8 T cell frequency (upper panel) and PD-1 expression level in GP33-specific CD8 T cells (low panel) were analyzed by flow cytometry. Representative data from each group (n=2) are shown. Numbers in the plots indicate the percentages of cells in each quadrant.

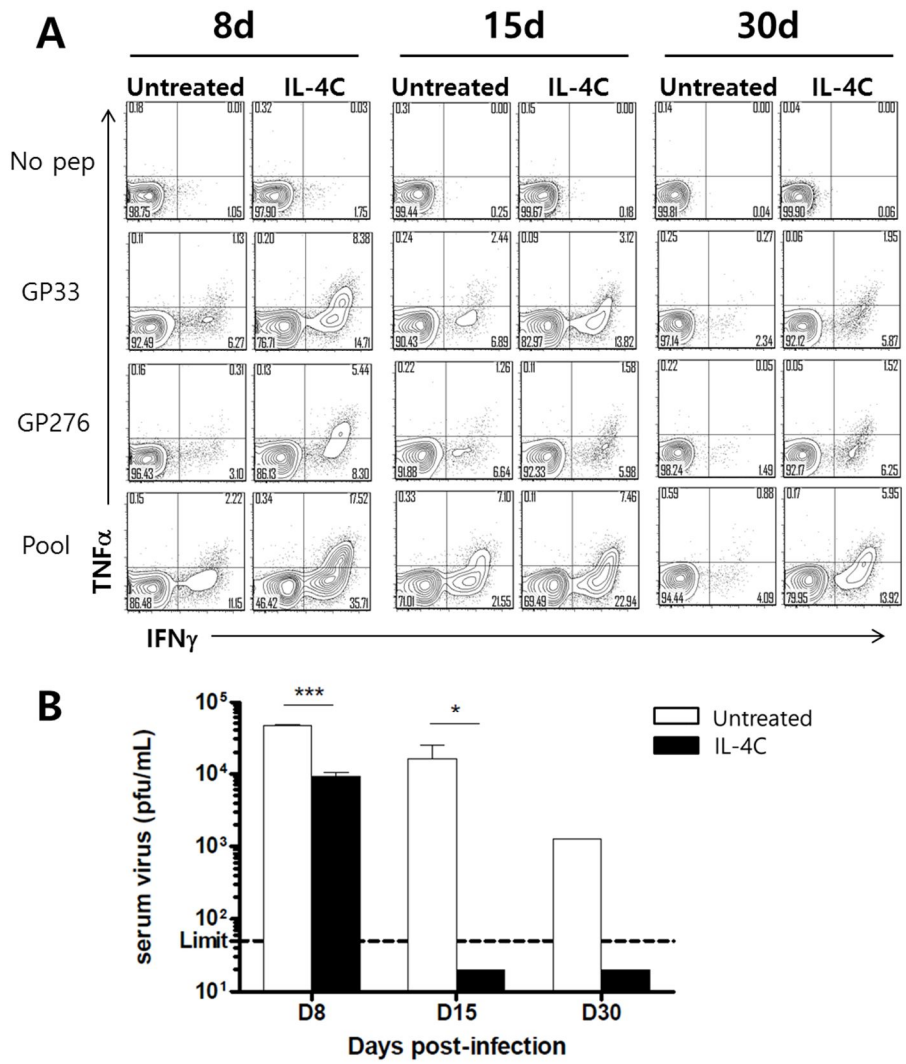

Figure 9. Virus control in IL-4 complex treated mice during LCMV CL-13 infection. (A) B6 mice were treated with IL-4 complex for 7 days, and splenocytes were isolated from control (untreated) or IL-4 complex treated (IL-4C) mice at day 8, 15 and 3 after LCMV CL-13 infection, They were ex vivo re-stimulated with LCMV GP_{33-41}(GP33), $GP_{276-286}$(GP276) or pooled peptides. Representative data from each group (n=2) are shown. Numbers in the plots indicate the percentages of cells in each quadrant. (B) Virus titers in

serum were measured from IL-4C treated and untreated mice after infection with LCMV CL-13. Dashed line means the virus detection limit. Undetectable groups were given a half of the limit. Summarized data ($n \leq 4$) are shown. The bars indicate means ± SD. $p<0.05$, ***$p<0.001$.

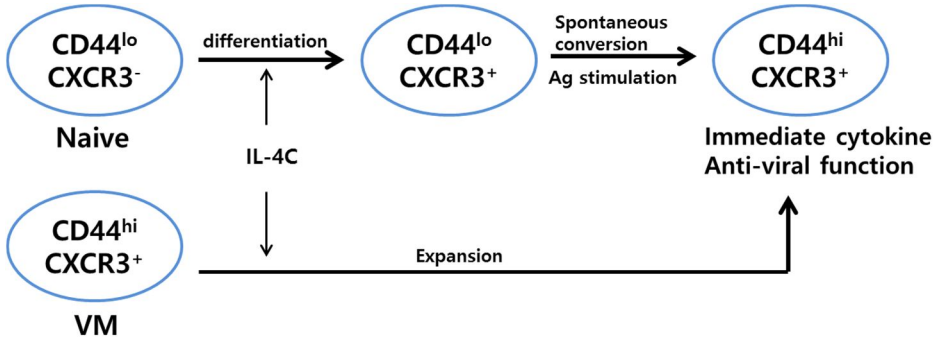

Figure 10. Diagram showing the development of memory-like CD8 T cell after IL-4 and anti-IL-4 antibody complex treatment. By injection of IL-4C, $CD44^{lo}CXCR3^{+}$ (preparatory stage) and $CD44^{hi}CXCR3^{+}$ (memory-like stage) CD8 T cells are generated from $CD44^{lo}CXCR3^{-}$ (naïve) CD8 T cells. In addition, IL-4C treatment might induce the expansion of preexisting $CD44^{lo}CXCR3^{+}$ and $CD44^{hi}CXCR3^{+}$ CD8 T cells. $CD44^{lo}CXCR3^{+}$ CD8 T cells are able to be converted to $CD44^{hi}CXCR3^{+}$ memory-like phenotype spontaneously after cessation of IL-4C treatment, or by T cells stimulation by specific antigens. IL-4C induced memory-like CD8 T cells immediately produce effector cytokines such as IFN-γ and exhibit anti-viral immunity.

DISCUSSION

IL-4 is a common γ chain cytokine that promote differentiation of naïve $CD4^+$ T cells into the Th2 subset and inhibit Th1 response[32, 33]. IL-4 is also able to regulate CD8 T cell development and function. Endogenous IL-4 stimulates CD8 T cell proliferation [26]. In the absence of IL-4, CD8 T cell response against malaria and Leishmania is defected [34, 35]. In thymus, IL-4 is required to the development of innate CD8 T cells [3, 17, 20]. In addition, exogenous IL-4 (IL-4/anti-IL-4 Ab) induces memory-like phenotype in peripheral CD8 T cells [25]. In the present study, exogenous IL-4 treatment induced up-regulation of Eomes, CD122, CD124 and CXCR3 in CD8 T cells. In accordance with the previous report [25], IL-4 was more potent in expansion of $CD44^{low}CXCR3^+$ population than that of $CD44^{hi}CXCR3^+$ CD8 T cells. This suggests that $CD44^{low}CXCR3^+$ population is most susceptible against IL-4 stimulation. In the wild type mice, $CD44^{low}CXCR3^+$ population was rare, compared with other population. However, this population had the highest CD124 (α chain of IL-4 receptor) expression, suggesting that highest IL-4 susceptibility of $CD44^{int/low}CXCR3^+$ population might be associated with its IL-4 receptor expression level. In the case of $CD44^{low}CXCR3^-$ naïve T cells, their cell number was not increased after IL-4C treatment despite IL-4 receptor expression in these cells. This might be due to conversion to memory-like CD8 T cells expressing increased levels of CXCR3 [25].

After cessation of IL-4 injection, total $CXCR3^+$ population was only slightly decreased during 1 month. However, CD44 expression level in these

cells gradually increased and majority of $CXCR3^+$ CD8 T cells eventually exhibited $CD44^{hi}$ phenotype. Of note, this conversion of $CD44^{low}CXCR3^+$ cells to $CD44^{hi}CXCR3^+$ cells was accelerated in IL-4-deficient mice. CD44 expression level in T cells increase in diverse situations delivering TCR activation signals, such as positive selection of thymocytes, homeostatic proliferation of T cells in lymphopenic host, and activation by agonistic antigens. In the present study, GVHD model revealed that activation with alloantigen induced the differentiation of IL-4 induced $CD44^{low}CXCR3^+$ CD8 T cells into $CD44^{hi}CXCR3^+$ or $CD44^{hi}CXCR3^-$ effector/memory cells. In the absence of exogenous antigen stimulation, TCR activation by low affinity self-antigens seems to upregulate CD44 expression in $CXCR3^+$ CD8 T cells, and endogenous IL-4 would delay this conversion process. This conversion of $CD44^{low}CXCR3^+$ cells to $CD44^{hi}CXCR3^+$ cells was associated with acquisition of enhanced effector function. $CD44^{hi}CXCR3^+$ population produced much higher amount of IFN-γ than $CD44^{low}CXCR3^+$ cells. In this aspect, this step is a kind of maturation process.

When fetal thymic organ culture is treated with IL-4, it is sufficient to promote major aspects of innate CD8 T cell phenotype in CD8 SP thymocyte [20]. However, in vitro treatment of wild-type thymocytes with IL-4 was not able to upregulate CXCR3 expression. In the present study, most of splenic CD8 T cells of IL-4C treated mice have higher expression of CD124, compared with untreated CD8 T cells. This reflects that most of CD8 T cell received IL-4 induced stimulating signal. However, CXCR3 expression was

not still increased in some of CD8 T cells, even though these cells have higher expression of Eomes than naïve subset of untreated mice. As mentioned previous report [36], these suggest that other signals are required for a full innate CD8 T cell-like phenotype.

Eomes expression was markedly increased in CD8 T cells of IL-4C treated mice, particularly in $CXCR3^+$ populations. However, after IL-4C treatment was discontinued, Eomes expression level in these populations gradually decreased. Similarly, although $CIITA^{Tg}$ mice have abundant IL-4 producing $PLZF^+$ CD4 T cells and $Eomes^+CD44^{hi}CXCR3^+$ innate CD8 T cells in their thymus[20], $CD44^{hi}CXCR3^+$ CD8 T cells in spleen of these mice expressed only slightly higher level of Eomes, compared with $CD44^{hi}CXCR3^+$ population of wild-type naïve mice. Taken together, IL-4 is required for high level expression and maintenance of Eomes in both centrally IL-4 induced innate CD8 T cells and peripherally IL-4 induced memory-like CD8 T cells.

In the aspect of IFN-γ producing capacity upon in vitro stimulation, $CD44^{hi}CXCR3^+$ CD8 T cells of IL-4C treated mice was similar to innate CD8 T cells of $CIITA^{Tg}$ mice. Along with this, IL-4C treatment prevents the exhaustion of CD8 T cells after LCMV CL-13 infection. LCMV virus strain can be divided into Armstrong and CL-13. When mice was infected with Armstrong strain, CD8 T cells produce robust amount of cytokine such as IFN-γ and TNF-α and eliminated virus within 8 days post infection[27]. However, during CL-13 infection, CD8 T cells are defected in their

proliferation activity, cytokine production and cytotoxic function, and express upregulated inhibitory molecule PD-1[27, 37-39]. As a result, virus are not eliminated and persisted for 3 month [27]. In the present study, IL-4C treated mice contained a higher number of virus-specific CD8 T cells in spleens and lungs than untreated mice after CL-13 infection, and these cells was not exhausted in terms of cytokine production capacity and PD-1 expression levels. Also, serum virus titers rapidly decreased in IL-4C treated mice. Similar findings were reported in $CIITA^{Tg}$ mice, where innate CD8 T cells facilitated rapid control of viremia and induction of functional anti-viral T cell response during CL-13 infection.

Conclusively, this study demonstrated that IL-4-induced memory like CD8 T cells arising in peripheral tissue are very similar to innate CD8 T cells generated in thymus in the aspect of development process, phenotype, and function. These memory like CD8 T cells are able to be generated from naïve CD8 T cells and also accumulated via expansion of virtual memory CD8 T cells (Fig. 10). Functionally, IL-4C induced memory like CD8 T cells were able to provide an early defense mechanism against viral infection as like as innate CD8 T cells.

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국문 초록

선천 T 세포는 흉선내에서 기억세포와 비슷한 표현형을 발현하며 외부 항원에 대해 즉각 대응하여 사이토카인을 분비할 수 있다. 이러한 선천 T 세포에는 흉선 내에서 2 형 조직복합체를 발현하는 흉선세포끼리의 상호작용에 의해 생성되는 선천 T 세포가 포함되며 흉선내에서 분비되는 인터루킨-4 에 의해 Eomes 를 발현하는 선천 CD8 T 세포가 유도됨이 이전 연구에서 확인되었다. 흉선에서 유도된 이 세포는 가상 기억 T 세포 및 IL-4 복합체 처리를 통해 말초기관에서 유도되는 T 세포와도 비슷한 특징을 발현하지만 이들 사이의 관계에 대해서는 명확히 밝혀지지 않았다. 본 연구에서는 말초기관에서 인터루킨-4 처리를 통해 유도되는 T 세포와 흉선내의 선천 T 세포를 표현형 및 기능의 측면에서 비교하였다. 인터루킨-4 복합체를 처리하였을 때 CXCR3 양성 세포군이 크게 증가하였으며 이 세포는 미감작세포군으로부터 분화 및 이미 존재하고 있던 가상기억 T 세포의 증식에 의해 유도되었다. CD44 발현은 낮은군에서 CD44 가 높게 발현되는 군으로 전환되는 것을 확인하였다. 또한 CD124 (인터루킨-4 수용체 알파)와 Eomes 를 높게 발현하며 자극을 받았을 때 즉각적으로 인터페론 감마를 분비하였다. 마지막으로 림프구맥락수막염바이러스 (lymphocytic choriomeningitis virus)에 감염된 마우스 모델을 이용하여 인터루킨-4

복합체를 처리한 마우스군에서 훨씬 많은 항바이러스 CD8 T 세포가 관찰되고 PD-1 발현이 감소되었으며, 바이러스가 빠르게 제거됨을 확인하였다. 이와 비슷한 반응이 선천 T 세포를 갖는 CIITA 재조합마우스에서도 나타남을 이전의 연구를 통해 밝혀졌다. 또한 사이토카인의 분비가 증가함을 통해 인터루킨-4 복합체 투여가 바이러스 진행 예방 효과가 있음을 확인하였다.

주요어 : 선천 CD8 T 세포, 기억세포 유사 CD8 T 세포, 인터루킨-4 복합체, CXCR3, 림프구맥락수막염바이러스

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