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Effect of PD-1-deficiency on the development of CD8 T cell response for a minor histocompatibility antigen, H60

부조직 적합항원 H60 특이적 CD8 T 세포 반응 발달에서의 PD-1 결핍에 의한 영향 연구

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서울대학교 대학원

의과학과 의과학전공

김 주 현

ABSTRACT

Effect of PD-1-deficiency on the development of CD8 T cell response for a minor histocompatibility antigen, H60

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Activated CD8 T cells go through robust expansion and then a certain portion of the expanded CD8 T cells remain as memory cells after antigen is cleared. It has been well known that help from concomittantly activated CD4 T cells is essential for generation of memory CD8 T cells and the CD8 T cells activated in the absence of CD4 help show defect in memory expansion when re-exposured to the same antigen. PD-1

(programmed cell death-1; CD279), which is an inhibitory receptor up-regulated on activated T cells, has been suggested to be implicated in the memory generation of CD8 T cells. In this study, using a minor histocompatibility antigen H60 as a model antigen, for which specific primary and memory CD8 T cell expansion is heavily dependent on CD4 help, the impact of the presence and absence of PD-1-expression on the development of antigen-specific CD8 T cell response was investigated. In the absence of PD-1 expression, the H60-specific CD8 T cells underwent higher degree of proliferation and showed enhanced functionality including cytotoxicity for antigen clearance, compared to those with PD-1 expression, even under help-deficient condition. However, PD-1-deficiency did not restore central memory cell generation of CD8 T cells under help-deficient condition. These results suggest that role of PD-1 is not directly related to programming of memory CD8 T cell generation, but rather to enhancing effector function of activated CD8 T cells. The results from this study will provide insight into development of CD8 T cell response and memory generation.

Keywords: CD4 help, Memory CD8 T cell, PD-1, T cell differentiation
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LIST OF ABBREVIATIONS

B6, C57BL/6

B6.CH60, H60 congenic mice

WT, Wild-type

KO, Knock-out

T_{CM}, Central memory T cell

PBL, Peripheral blood lymphocyte

MPEC, Memory precursor effector cell

SLEC, Short-lived effector cell

INTRODUCTION

The ability of helper CD4 T cells to promote the cytotoxic CD8 T cell response is a central feature of the adaptive immune response. Help provided by concomitantly activated CD4 T cells is required to drive for antigen-activated CD8 T cells to proliferate and differentiate into long-lived memory cells (central memory T cells [Tcm]) that are needed for persistent immunity(1). CD4 help is provided directly to CD8 T cells or indirectly through conditioning of APCs, and secretion of cytokines (IL-2, IL-12, IL-15 and IL-21) and cell to cell-interaction via co-stimulatory molecules (CD40/CD40L and CD27/CD70) are known to mediate the CD4 help(2, 3). Nonetheless, the cellular mechanism underlying long-term CD8 T cell memory impairment after help-deficient priming remains to be elucidated.

Programmed cell death-1 (PD-1, CD279) is an inhibitory receptor induced in T cells by antigen stimulation and sustained PD-1 expression plays a key role in T cell dysfunction(4, 5). The engagement of PD-1 by PD-L1 or PD-L2, the ligand for PD-1, delivers inhibitory signals through activating phosphatases, resulting in dephosphorylation of key elements in

the T cell activation pathway. The dephosphorylation of these molecules leads to the inhibition of PI3K activity and downstream activation of Akt, which are important pathways in regulating proliferation, survival, and cytokine production of T cells(6).

During chronic viral infection and cancer, PD-1 expression leads to T cell dysfunction and exhaustion(7). Blockade of PD-1 signaling rescues exhausted T cells and is an effective treatment for chronic infections and cancer. Notably, *in vivo* PD-1-blockade results in durable tumor reduction or clearance in clinical study (8). Also, in the absence of PD-1 signaling, proliferation, cytokine production, and migration ability of antigen-specific CD8 T cells are restored in various persistent viral models (9-12). While majority of the studies regarding the role of PD-1 has been performed using chronic antigen models, the influence of PD-1-deficiency in CD8 T cell response against acute antigen model has been relatively unidentified. After acute infection with vaccinia virus, PD-1-deficient CD8 T cells were found to generate central memory CD8 T cells more efficiently, showing enhanced viral control after re-infection(13). Furthermore, less is known about the

role of PD-1 in the central memory CD8 T cell development under CD4 T cell help-deficient condition. S. Rameeza Allie et al. showed that a skewing of the memory population toward the central memory phenotype in the absence of PD-1 signaling (13). Shinichiro Fuse et al. showed that PD-1 blockade leads to rescue of the defective recall response of memory CD8 T cells generated in the absence of CD4 help during acute vaccinia virus infection(11). However, whether the generation of central memory CD8 T cells in the absence of both CD4 help and PD-1 signaling is enhanced still remains unclear. Especially, the influence of PD-1-deficiency on the generation of memory CD8 T cells specific for cellular antigens under help-deficient condition has never been addressed before.

To address this, a minor histocompatibility (H) antigen H60 was used as the model antigen. The H60-specific CD8 T cell response is dominant over the response for other minor H antigens under multiple antigen-disparity condition (14, 15). In this study, taking advantage of the fact that Hy-Dby locus at Y-chromosome provides H-2A^b-restricted CD4 epitope (NAGFNSNRANSSRSS; (16)), splenocytes originated from female or male H60 congenic mouse (B6.CH60) were used as

antigen sources without or with CD4-activating epitope, respectively, in inducing the specific CD8 T cell response in female C57BL/6 (B6) mice (17). Immunization of female B6 mice with male H60 congenic splenocytes (providing Hy-Dby-specific CD4 help) generates appropriate primary and secondary expansions of CD8 T cells specific for H60 (LTFNYRNL/H2-K^b), with their peak frequencies being in ranges of 7-15% and 40-50% in blood CD8 T cells, respectively. However, the immunization with female H60 congenic splenocytes (help-deficient condition) has never assisted the expansion (17). Recently, data from my laboratory (unpublished) showed that H60-specific CD8 T cells under CD4 help-deficient condition expressed surface markers representing the cells in effector memory (Tem; $CD62L^{lo}$ $CD44^{hi}$) and exhausted status (up-regulated PD-1).

In this study, changing the phenotype of PD-1-deficient CD8 T cells along the immune response against H60 under the CD4 help-deficient condition was examined. The results showed that the memory response of H60-specific CD8 T cells is partially restored in the absence of PD-1 signaling. In addition to enhance function as effector CD8 T cells, PD-$1^{-/-}$ H60-

specific CD8 T cells proliferated and migrate faster than wild type counterparts. But, the population of central memory CD8 T cells were not increased in PD-$1^{-/-}$ H60-specific CD8 responses. The results from this study propose that the PD-1 signaling is not directly relevant to CD62L expression as central memory cell, still contributed to fulfill the function of effective effector memory cells.

MATERIALS AND METHODS

1. Mouse

C57BL/6 (B6), B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ(CD45.1^{+}), B6.129S2-*Pdcd1*^{tm1Hon}/HonRbrc (*Pd-1*$^{-/-}$), B6-*Thy*$^{c-2J}$/J (B6.Albino), and B6.C-*H60*^{c}/DCR (B6.CH60) mice were from The Jackson Laboratory (Bar Harbor, ME, USA). Luciferase transgenic mouse (B6-Tg(CAG-effLuc); B6-Luc Tg) was previously described (18). TCR transgenic mouse lines of which T cells express TCR with specificity to H60-CD8 epitope/ H2-K^{b} were generated after microinjection of fertilized B6 egg with eukaryotic DNA fragments from pTα and pTβ cassett e plasmids containing TCRV$_{\alpha}$-J$_{\alpha}$ and TCRV$_{\beta}$-D$_{\beta}$-J$_{\beta}$ rearranged genomic sequences originated from H60-specific CTL clone #15 , respectively (19). Among the three founder lines showing positive selection on B6 background and negative selection on the B6.CH60 background (unpublished data), one of which TCR levels on peripheral blood CD8 T cells were comparable to those of wild type B6 T cells was selected and named J15. The J15 line was backcrossed to CD45.1^{+} or *Pd-1*$^{-/-}$ background. All the mice were maintained under specific pathogen-free conditions at the Center for Animal Resource

Development of Seoul National University College of Medicine in Korea and used for experiments at ages of 8 to 12 weeks, with approval of IACUC (Institutional Animal Care and Use Committee) of Seoul National University.

2. Adoptive transfer and immunization

CD8 T cells from J15 mice were purified from spleens and LNs by negative MACS (MACS; Miltenyl Biotec, Auburn, CA) and, after labeling with CFSE (5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester; eBioscience, San Diego, CA, USA), were adoptively transferred ($1-3 \times 10^6$) one day before priming. $2x10^7$ B6.CH60 cells ($2x10^7$) were injected i. p. to induce H60-specific CD8 T cell response.

3. Antibodies and Flow cytometry

Single cell suspensions were stained with antibodies or H60-tetramers (LTFNYRNL/H-$2K^b$) at 4℃ for 30 minutes in staining buffer (1xPBS containing 0.1% bovine serum albumin and 0.1% sodium azide). Intracellular staining was performed as previously described. Cells were run on FACSCalibur (BD Pharmingen, San Diego, CA) or LSRII-Green (BD Pharmingen)

and data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Antibodies used for flow cytometric analysis are as follows. Fluorescent-dye-conjugated antibodies against CD8, CD44, CD45.1, CD62L, CD127, mIgG, Granzyme B, IFN-γ and IL-2 were purchased from eBiosciences. Other antibodies were purchased from BD Pharmingen (anti-CD11a, -PD-1, and -KLRG1) and Biolegend (anti-CD69; San Diego, CA, USA).

4. *In vivo* bioluminescence imaging (BLI)

In vivo bioluminescence imaging (BLI) was performed using an IVIS 100 imaging system with a charge-coupled device (CCD) camera (Caliper Life Sciences, Hopkinton, MA, USA) as previously described (18).

5. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 5, GraphPad Software, San Diego, CA, USA). All error bars represent the SEM. P values were determined by Student's t-tests. (Two-tailed unpaired: *<0.05, **<0.01, ***<0.001)

RESULTS

Induction of tolerance of H60-specific CD8 T cell response by lack of CD4 help

First, to examine whether tolerance phenomenon observed with CD8 T cell response induced under help-deficient condition (20, 21) could be reproduced with H60 antigen model system, female B6 mice were primed with female H60 congenic (B6.CH60) splenocytes for induction of help-deficient primary response and ,40 days later, boosted with male H60 congenic splenocytes providing H60 CD8-epitope and Hy-Dby CD4-epitope in cognate fashion (helped boosting). PBLs from the primed and boosted mice were periodically checked for expansion of the H60-specific CD8 T cells by H60-tetramer-staining and subsequent flow cytometric analysis (Fig. 1A, 1B). Through the longitudinal analysis over the primary and secondary responses, CD8 T cells binding to H60-tetramer were not detect at significant levels in the blood of the help-deficient-and boosted group (Fig. 1C). Frequencies of H60-tetramer-binding cells peaked at 1-3% of peripheral blood and splenic CD8 T cells after the boost in the previous help-deficient group. This extremely low expansion of H60-specific

CD8 T cells in the help-deficient mice after the boost contrasted with vigorous re-expansion of those up to 40-50% in the helped mice, and verified establishment of H60-specific CD8 T cell tolerance during the primary help-deficient response.

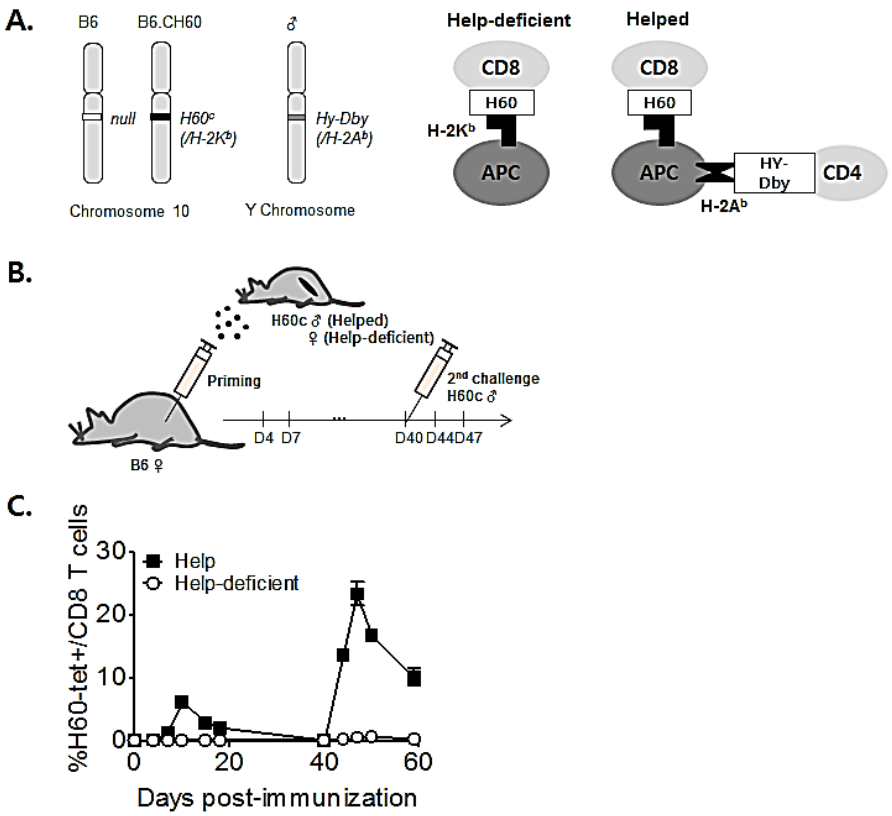

Figure 1. Impairment of memory expansion of H60-specific $CD8^+$ T cells activated in the absence of $CD4^+$ T cell-help.

(A) Diagrams of experimental systems using B6. CH60 mice. B6 mouse strain has null expression from H60 locus, while the B6. CH60 strain expresses *H60c* allele at the locus, which produces H-$2K^b$-restricted CD8-epitope to activate T cells of B6 mice. The *Hy-Dby* gene on the Y chromosome encodes the CD4-epitope presented by H-$2A^b$. Priming of female B6 mice with female or male cells from B6. CH60 mice induces helper-deficient of helped response for H60, respectively. (B) Female B6 mice were primed with female ($2x10^7$) B6. CH60 cells or male ($2x10^7$) B6.CH60 spleen cells, and then boosted with male

B6.CH60 spleen cells ($2x10^7$). Pooled PBLs from immunized mice were analyzed periodically using flow cytometry after staining with H60-tetramers, and anti-CD11a and anti-CD8 mAbs. (C) The frequencies of H60-tetramer-binding cells in blood $CD8^+$ T cells were then plotted. Data shown represent three independent experiments. (n=2~3/group/experiments)

Increased PD-1-positive population in the CD8 T cells primed under CD4 help-deficient condition

In previous study, it has been shown that B6. CH60 spleen cells injected under help-deficient condition persisted long in the primed female B6 mice, while those under helped condition have been cleared by day 14 post-immunization (unpublished data). This suggested that H60-specific CD8 T cells primed under help-deficient condition would be exhausted due to antigen-persistence, as has been shown by exhaustion of CD8 T cells in the chronically infected hosts (22). To examine this suggestion, the expression by the H60-specific CD8 T cells of PD-1, which is an immunoregulatory receptor known to be expressed highly on exhausted CD8 T cells (11), was investigated depending on the priming conditions. H60-specific CD8 T cells were enriched from the spleens of female B6 mice which had been primed with splenocytes from female (help-deficient) or male (helped) B6.CH60 mice via bead-based sorting after H60-tetramer-statining, and analyzed for expression of PD-1 by flow cytometry on day 28 post-priming, which is supposed to be in resting memory phase (Fig. 2A). The flow cytometric data showed that percentages of PD-1

expressing cells in the H60-tetramer-binding CD8 T cells were higher under help-deficient condition than helped condition (Fig. 2B) with higher mean fluorescence intensity (MFI) values (Fig. 2C). This high expression of PD-1 on the help-deficient CD8 T cells demonstrated a positive relationship between blunted recall responses of help-deficient CD8 T cells and the excessive up-regulation of PD-1 in memory phase.

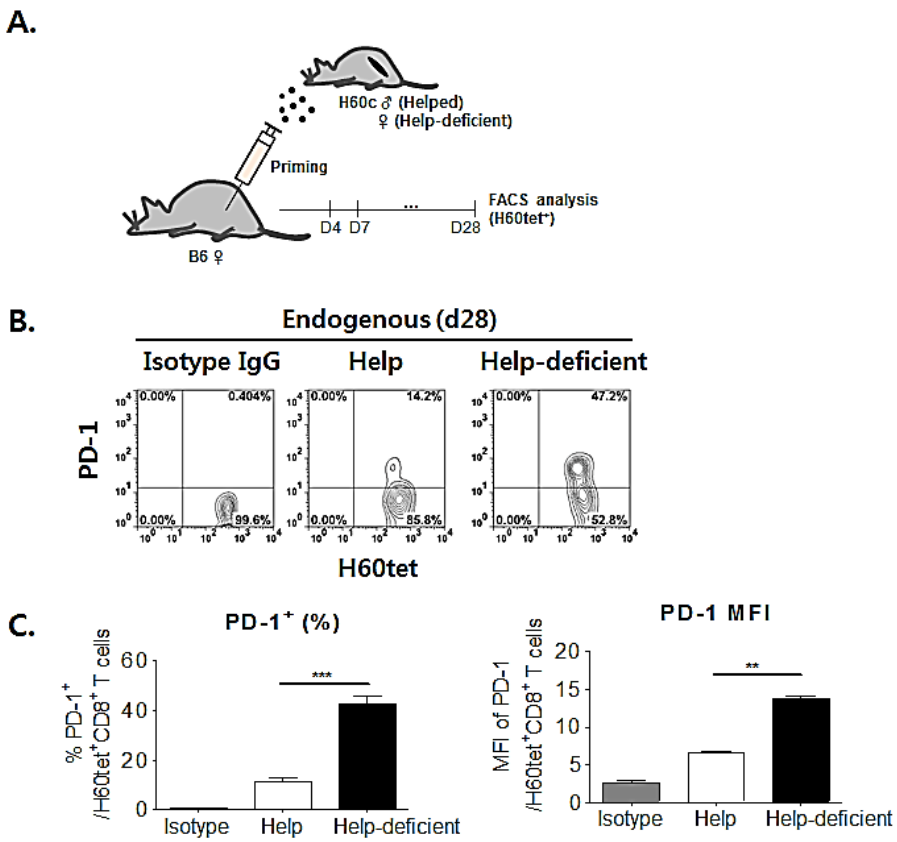

Figure 2. Significantly increased PD-1 expression of Ag-specific CD8 T cell under the CD4 help-deficient condition.

(A) Female B6 mice were primed with female (help-deficient) or male (help) $2x10^7$ B6.CH60 cells and sacrificed on day 28 post-priming for flow cytometric analysis. Representative flow cytometric data are shown after $CD8^+$ and $H60tet^+$ gating. (B) Percentage of PD-1^+ cells among the H60-specific CD8 population and (C) Fluorescence intensity per cell of PD-1 staining in the H60-specific CD8 populations are plotted. Data shown (B-C) represent three independent experiments (n=2~3/group/experiments) $^{**}P<0.01$. $^{***}P<0.001$.

Impaired memory response under the help-deficient condition was restored in the absence of PD-1 expression.

Blockade of PD-1 signaling has been known to restore proliferative and functional activity of exhausted CD8 T cells in chronic infection models (10, 23).

To verify that PD-1-mediated exhaustion phenotype of CD8 T cells was related to the impaired memory response primed under CD4 help-deficient condition in H60 model, whether help-deficient H60-specific CD8 T cells would be rescued from tolerance in the absence of PD-1-mediated signaling, showing memory expansion after antigen-re-encounter, was checked. Female PD-1-deficient ($PD\text{-}1^{-/-}$) mice were primed with male or female H60 congenic splenocytes, boosted 40-50 days after the priming, and longitudinally tracked via flow cytometry of the PBLs with H60-tetramer staining to detect H60-specific CD8 T cells in the peripheral blood (Fig. 3A). Data from the flow cytometry showed that frequencies of H60-tetramer-binding CD8 T cells in help-deficient $PD\text{-}1^{-/-}$ mice, which were primed with female H60 congenic splenocytes, were negligible during the tolerizing primary response but were increased up to 6.56% on day 7 post-boosting (an average

7.8-fold increase; Fig. 3B). In contrast, those of H60-tetramer-positive CD8 T cells in the blood of helped WT and PD-$1^{-/-}$ mice were not different and fold increases of the frequencies during the secondary response relative to the primary peak were similar in both groups (Fig. 3C). These data indicated the rescue from tolerance of the help-deficient H60-specific CD8 T cells in the PD-$1^{-/-}$ mice.

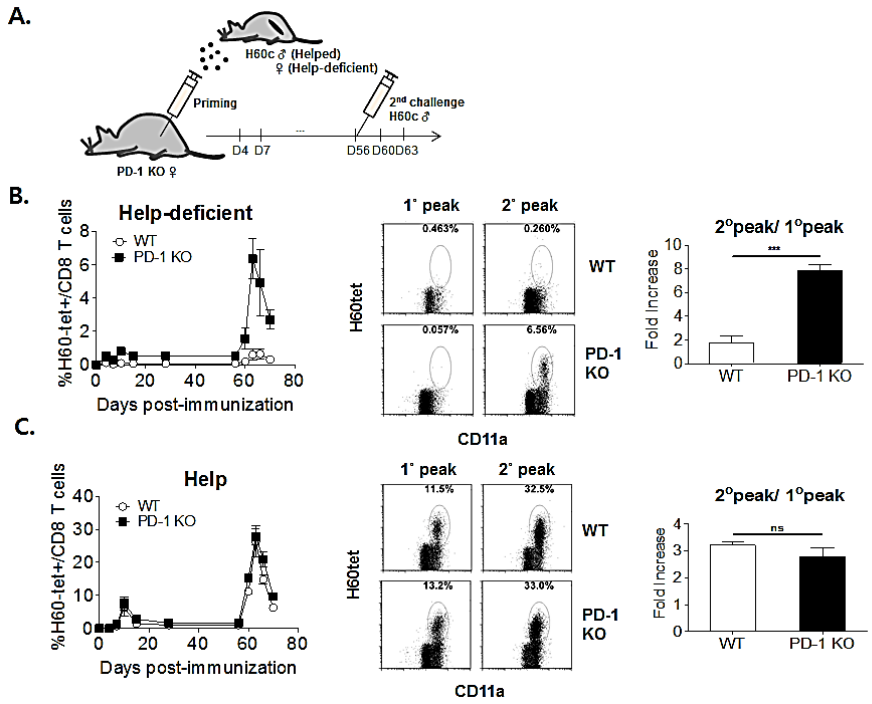

Figure 3. Memory response under the help-deficient condition was restored in the absence of PD-1 expression.

(A) Female PD-1-deficient or WT B6 mice were challenged according to the tolerizing and boosting schedules. (B) Longitudinal flow cytometric analysis with PBL pooled from female PD-1 KO or WT B6 mice primed with female ($2x10^7$) B6.CH60 cells and boosted with male B6.CH60 cells ($2x10^7$). Pooled PBLs from three immunized mice were periodically checked with flow cytometry after staining with H60-tetramer, CD11a, and CD8, and the frequencies of H60-tetramer-binding cells in blood CD8 T cells were plotted. Flow cytometric data shown represent primary (day 10 post-immunization) and secondary (day 7 post-boosting) peak ones. The fold increases of secondary peak frequencies relative to primary peak ones

are plotted. (C) Female PD-1 KO or WT B6 mice challenged according to the helped priming and boosting schedule. Plots of frequencies of H60-tetramer-binding CD8 T cells, representative flow cytometric data and fold increases in peak frequencies are shown. Data shown (B-C) represent three independent experiments (n=2~3/group/experiments) ***P<0.001.

PD-1-deficient CD8 T cells proliferated and migrated to inflamed sites in fast kinetics.

Numerous studies showed that the absence of PD-1 signaling enhanced the responsiveness of Ag-specific CD8 T cells such as proliferation, cytokine production and control of viral load (9-12). To verify whether the restored memory expansion of help-deficient CD8 T cells in PD-$1^{-/-}$ mice was related to phenotypic change of PD-$1^{-/-}$ CD8 T cells was investigated along the response development.

CD45.2^{+} CD8 T cells isolated from the PD-$1^{-/-}$ J15 mice or WT J15 mice were labeled with CFSE prior to adoptive transfer into CD45.1^{+} B6 mice which were, then, challenged against H60 under help-deficient or helped condition(to check the additional effect of PD-$1^{-/-}$ in the conventional response). The spleens and peritoneum lavarge were harvested from the adoptive hosts at day 5 after the challenges and CD45.2^{+} CD8 T cells in the organs were analyzed for proliferation and cytokine production via flow cytometry after gating on CD45.1 negative CD8 T cells (Fig. 4A).

First, whether PD-$1^{-/-}$ H60-specific CD8 T cells primed under helped or help-deficient condition went through faster

proliferation after immunization was checked. According to CFSE-dilution peak profiles, WT and PD-$1^{-/-}$ J15 CD8 T cells have passed several cycles of cell division on day 5 post-priming in all conditions except for naïve host (Fig. 4B). Cell division occurred significantly in advance in the PD-$1^{-/-}$ J15 CD8 T cells, compared with WT J15 CD8 T cells (Fig. 4C). In addition, CFSE-profiles of help-deficient WT J15 CD8 T cells were in discontinuous patterns, help-deficient PD-$1^{-/-}$ J15 CD8 T cells exhibited accumulation of more than 5 times divided cells within the spleen.

However, numbers of splenic PD-$1^{-/-}$ J15 CD8 T cells were not significantly different from those of WT counterparts in all conditions, although PD-$1^{-/-}$ CD8 T cells had more divisions (Fig. 4D). These data implied that PD-$1^{-/-}$ J15 CD8 T cells would proliferate faster and migrate earlier to inflamed site, and therefore, total numbers of PD-$1^{-/-}$ J15 CD8 T cells in the spleens did not increase, in spite of faster cell divisions. To check this, the numbers of WT or PD-$1^{-/-}$ J15 CD8 T cells present in the peritoneum was analyzed (Fig. 4F). The results showed that numbers of peritoneal PD-$1^{-/-}$ J15 CD8 T cells under helped or help-deficient conditions were significantly

increased than WT J15 CD8 T cells under both conditions. These results from the analyses of CFSE-profiles and cell numbers in spleen and peritoneum demonstrated that the proliferation and migration of CD8 T cells were accelerated in the absence of PD-1 signaling.

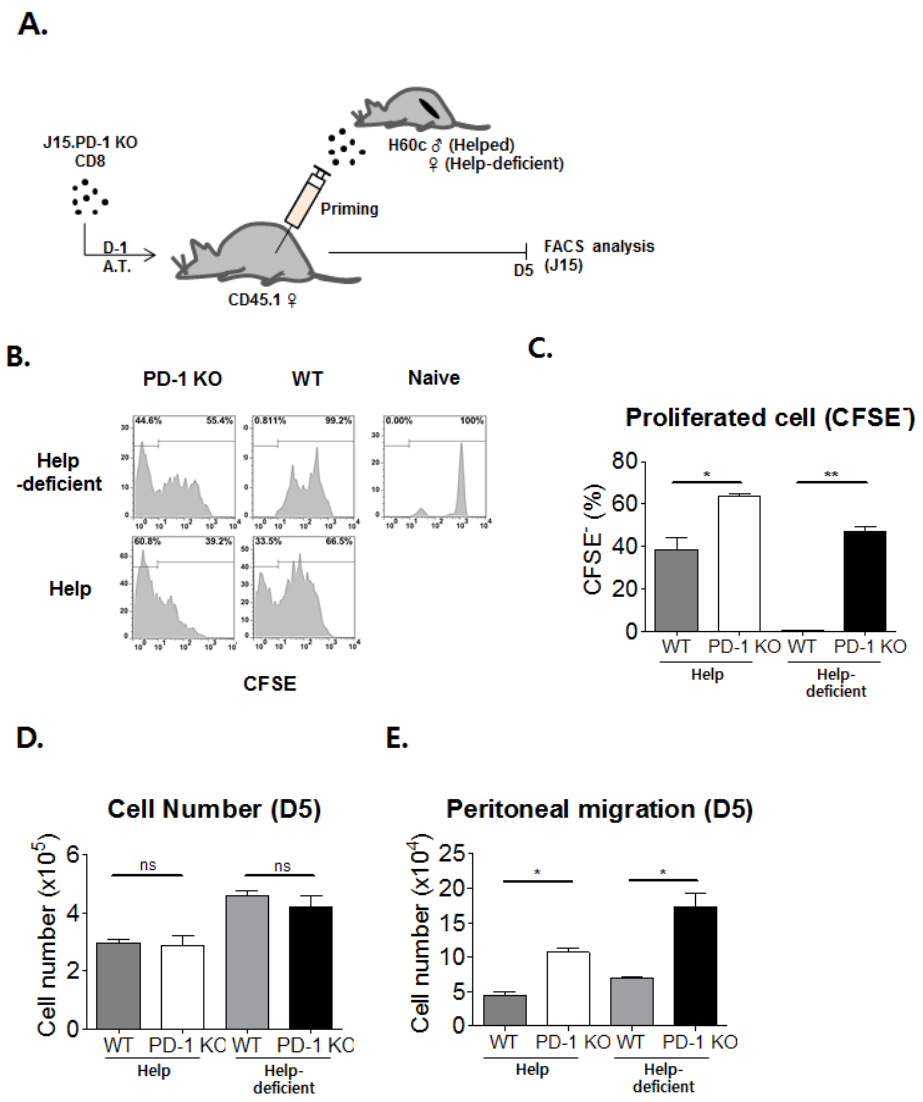

Figure 4. Earlier proliferation and migration of PD-1 deficient Ag-specific CD8 T cells.

(A) CFSE-labeled J15 CD8 T cells or *Pd-1*$^{-/-}$ J15 CD8 T cells were adoptively transferred into female CD45.1^{+} mice, which were then primed under helped or help-deficient condition (immunized with male or female B6.CH60 cells, respectively) and sacrificed on day 5 post-priming for flow cytometric

analysis. All data are analyzed after $CD8^+$ $CD45.1^-$ gating (**B**) Profiles of CFSE-dilution peaks among H60-specific CD8 T cells. (**C**) Bar graphs indicate percent proliferated cells ($CFSE^-$) analyzed by flow cytometry. (**D**) Number of splenic H60-specific CD8 T cells and (**E**) migrated peritoneal H60-specific CD8 T cells are plotted. Data shown (A-D) represent three independent experiments (n=2~3/group/experiments)* $P<0.05$, **$P<0.01$.

Enhanced IFN-γ production from Ag-specific CD8 T cells in the absence of PD-1 signaling.

Next, cytokine secretion by the Ag-specific PD-$1^{-/-}$CD8 T cells was tested. Spleen cells on day 5 in each group were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin and stained for production of IFN-γ, IL-2 and granzyme B. Intracellular cytokine staining for IFN-γ production showed that the frequency of IFN-γ-secreting J15 CD8 T cells was increased significantly in the absence of PD-1-signaling (Fig. 5A, B). The significant increase in the IL-2 and granzyme B production per cell basis in PD-$1^{-/-}$ J15 CD8 T cells compared with WT J15 CD8 T cells was not observed. Therefore, these data demonstrated that H60-specific CD8 T cells as effector were functionally enhanced in the absence of PD-1 signaling.

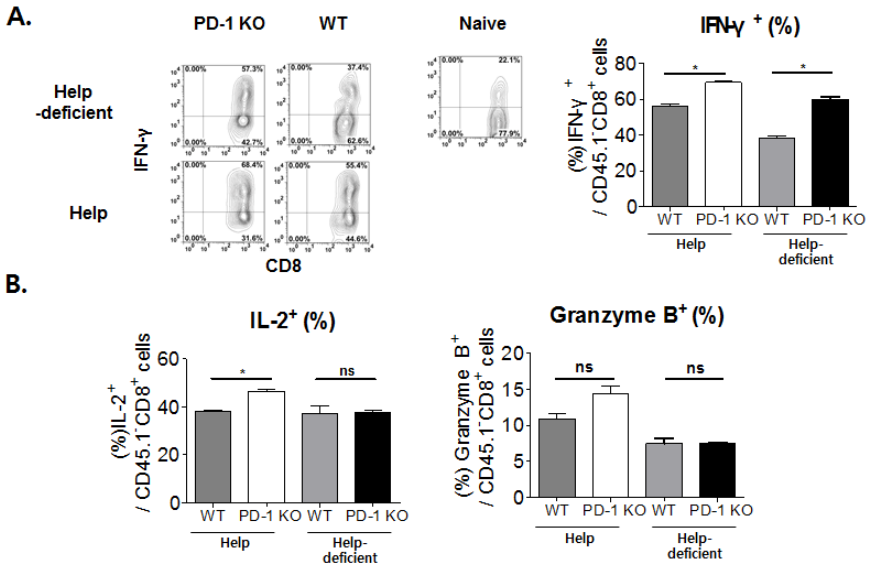

Figure 5. Enhanced secretion of IFN-γ of PD-1 deficient Ag-specific CD8 T cells.

(A) IFN-γ secretion of PD-1 KO or WT H60-specific CD8 T cells under helped or help-deficient condition. IFN-γ^+ frequencies among the H60-specific CD8 T cells are plotted. (B) IL-2 and granzyme B secreting H60-specific CD8 T cells are shown. Data shown (A-B) represent three independent experiments (n=2~3/group/experiments)* P<0.05.

Skewing toward memory precursor effector cells in the absence of PD-1 signaling.

The fate of effector CD8 T cells were subdivided to a population with a greater capacity for survival, which re-express the IL-7R (termed memory precursor effector cells [MPECs], terminally differentiated effector cell population with low capacity to survive long (termed short-lived effector cells [SLECs], which frequently express KLRG-1 (24-27). To examine whether PD-1 would play a role in the effector fate determination, PD-$1^{-/-}$ J15 CD8 T cells harvested on days 14 (contraction phase) after immunization were phenotyped for their expression of CD127/KLRG-1 (Fig. 6A).

First, I examined the frequency and number of MPECs in the spleens of adoptively hosts transferred with WT or PD-$1^{-/-}$ J15 CD8 T cells 14 days after priming under the help-deficient condition, and found that the frequency and number of $CD127^{hi}$ CD8 T cells significantly increased in the absence of PD-1 signaling compared with WT J15 CD8 T cells (Fig. 6B). However, there was no significant increase of frequency in KLRG1 expression by PD-$1^{-/-}$ J15 CD8 T cells. In addition, PD-$1^{-/-}$ J15 CD8 T cells primed under the helped condition

had similar frequencies of MPEC and SLEC compared with WT counterparts (Fig. 6C). However, the cell number of SLEC in the absence of PD-1 signaling slightly increased, because the total cell number of PD-$1^{-/-}$ J15 CD8 T cells increased.

Altogether, these data demonstrated that effector fate of PD-1 deficient H60-specific CD8 T cells were skewed toward MPEC (up-regulated CD127) under the help-deficient condition, suggesting the relevance of PD-1 signaling to make the memory precursor effector CD8 T cells.

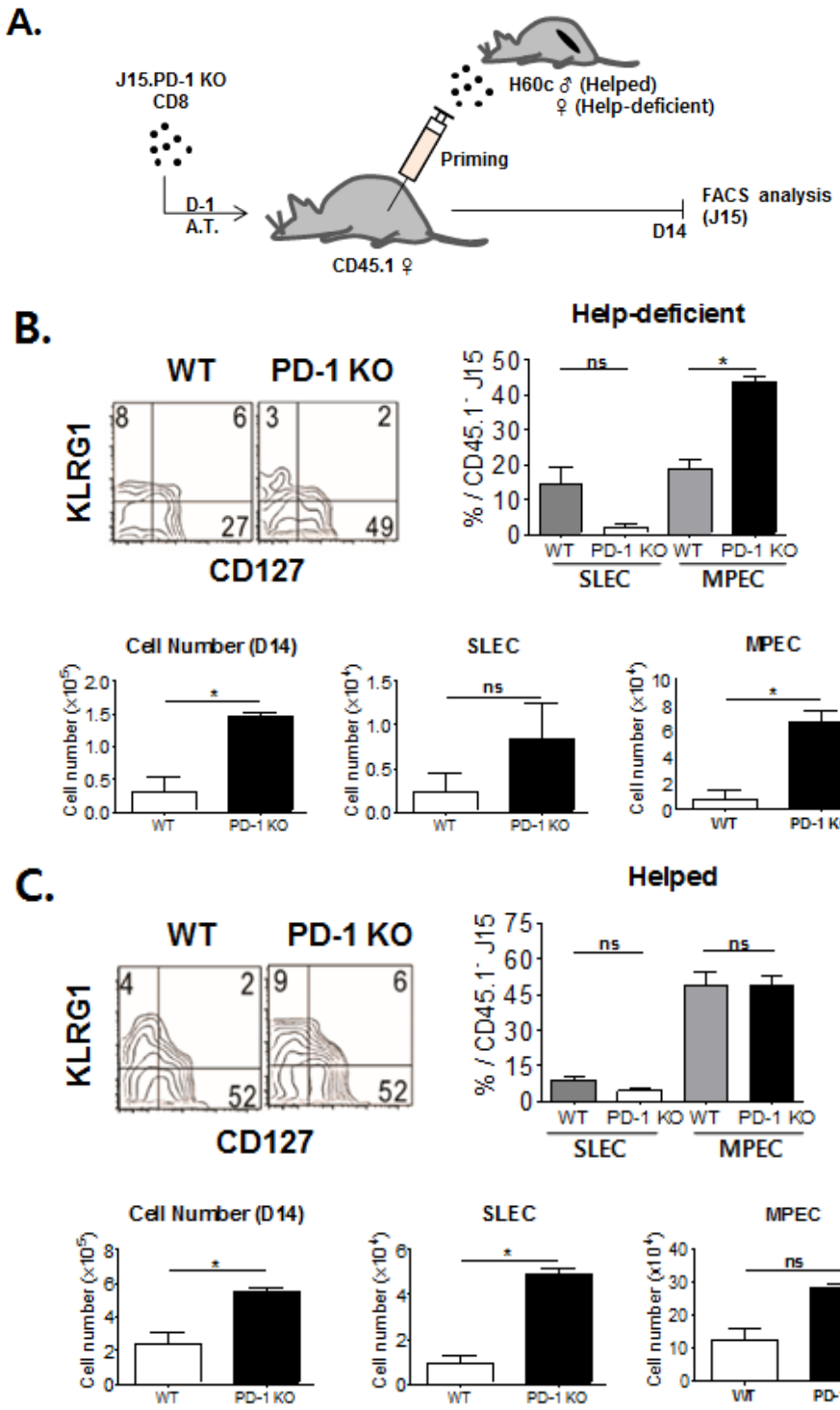
A.
J15.PD-1 KO
CD8
D-1
A.T.
H60c ♂ (Helped)
♀ (Help-deficient)
Priming
CD45.1 ♀
D14
FACS analysis
(J15)
B.
Help-deficient
WT
PD-1 KO
KLRG1
CD127
8
6
27
3
2
49
% / CD45.1- J15
ns
*
WT
PD-1 KO
WT
PD-1 KO
SLEC
MPEC
Cell Number (D14)
SLEC
MPEC
Cell number (×10⁵)
Cell number (×10⁴)
C.
Helped
WT
PD-1 KO
KLRG1
CD127
4
2
52
9
6
52
% / CD45.1- J15
ns
ns
SLEC
MPEC
Cell Number (D14)
SLEC
MPEC

Figure 6. The absence of PD-1 signaling increased the frequency and number of MPEC in Ag-specific CD8 T cells.

(A) Phenotypic analysis was extended to day 14. (B) Representative flow cytometric data and plots of numbers of SLEC and MPEC populations in the H60-specific CD8 T cells primed under the help-deficient condition were shown. (C) Representative flow cytometric data and plots of numbers of SLEC and MPEC populations in the H60-specific CD8 T cells primed under the helped condition were shown. Data shown (A-B) represent three independent experiments (n=2~3/group/experiments)* $P<0.05$.

Help-deficient CD8 T cells did not convert to central memory CD8 T cells in the absence of PD-1 signaling.

A skewing toward central memory T cells in late phase is a cardinal feature of enhanced recall response (28-30). In a mouse model of acute infection with vaccinia virus, S. Rameeza Allie et al. showed an increased frequency of Tcm(CD62L^{hi}CD44^{hi} CD8 T cell) and enhanced recall response after boosting in the absence of PD-1 signaling (13). Tcm has the ability to preferentially enter noninflamed lymph nodes, which requires expression of CD62L. Therefore, to determine whether the re-expansion of help-deficient H60-specific CD8 T cells in the PD-1-deficient mice was mediated through enhanced differentiation to Tcm generation, PD-1 deficient (PD-1$^{-/-}$ J15) CD8 T cells in the CD45.1^{+} adoptive hosts were phenotyped on day 39 post-priming under help-deficient condition(Fig. 7A). Numbers of PD-1$^{-/-}$ J15 CD8 T cells harvested from the spleen of help-deficient hosts were significantly higher than those of WT counterparts (Fig. 7B). However, phenotypically, PD-1$^{-/-}$ J15 CD8 T cells were similar with WT cells, in that majority of them (>80%) were in CD62L^{low}CD44^{hi} effector memory (Tem) state (Fig. 7C). One

exception was that proportions of CD69-positive cells were significantly reduced in the PD-$1^{-/-}$ J15 CD8 T cells. There was no specific increase of Tcm cell number in the absence of PD-1 (Fig. 7D). The bioluminescence imaging (BLI) analysis of the injected B6. CH60 cells (B6.CH60-Luc Tg) showed that the luminescence signal emanated from the allograft B6.CH60-Luc Tg cells has disappeared early in the help-deficient hosts that transferred with PD-$1^{-/-}$ J15 CD8 T cells than in those with WT J15 CD8 T cells(Fig. 7E), which demonstrated that antigen was cleared early in the absence of PD-1 in help-deficient H60-specific CD8 T cells.

Therefore, the reduction in proportion of $CD69^{+}$ cells could be attributed to the earlier rejection of the H60-allografts by the PD-$1^{-/-}$ J15 CD8 T cells in the help-deficient hosts than that by WT counterparts.

Altogether, these data demonstrated that even though the help-deficient cells were rescued from tolerance in the absence of PD-1 expression on their surface, the restored expansion of help-deficient CD8 T cells in the absence of PD-1 is not tightly associated with Tcm differentiation. This suggests that the capacity to re-expand and the differentiation

into CD62L^{hi} phenotype of Tcm cells may be independently regulated.

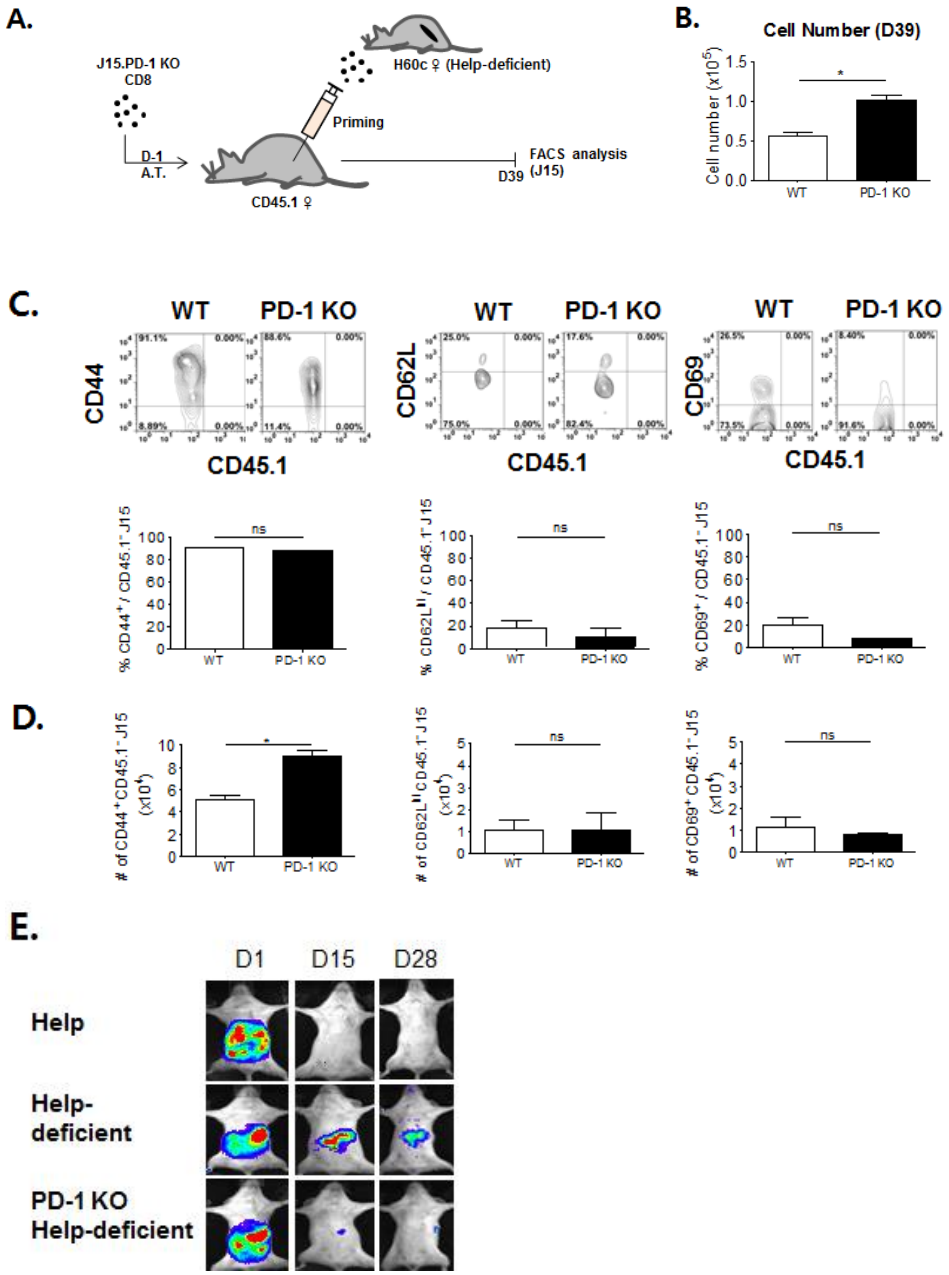

Figure 7. PD-1 signaling does not have the effects to differentiate to central memory CD8 T cells.

(A) PD-1 KO or WT J15 CD8 T cells ($CD45.1^-$) in the help-deficient adoptive hosts ($CD45.1^+$) were phenotyped on day 39 post-priming. (B) Plots of total splenic cell numbers of H60-

specific CD8 T cells on day 39 post help-deficient priming are shown. (**C**) Representative flow cytometry data and plots of percentages values corresponding to each population in the $CD45.1^{-}$ J15 CD8 T cells are shown. (**D**) The numbers of $CD44^{+}$, $CD62L^{hi}$ and $CD69^{+}$ of H60-specific CD8 T cells are plotted. (**E**) BLI analysis on the primed B6.CH60 Tg allografts. B6.Albino hosts adoptively transferred with WT or PD-1 KO J15 CD8 T cells were monitored periodically after helped or help-deficient priming with the B6.CH60-Luc Tg cells. Data shown (A-E) represent three independent experiments (n=2~3/group/experiments)* $P<0.05$.

DISCUSSION

The studies presented in this work demonstrate that the PD-1 is up-regulated on the Ag-specific CD8 T cells primed under the help-deficient condition and a proportion of Ag-specific CD8 T cells increase in the primary and memory phases of the CD8 T cell response for cell-based antigen, H60, in the absence of PD-1 signaling. Although PD-1-deficient H60-specific CD8 T cells showed enhanced functionality, such as proliferation, migration, cytokine production, and conversion to MPEC under help-deficient condition, the frequency of central memory CD8 T cells in the their response was not affected by the lack of PD-1 expression in CD8 T cells. They remained as effector memory T cells which contributed to restore of the recall expansion partially. This suggests that CD62L expression as the surface marker of central memory T cells is not directly related to the PD-1 signaling.

Numerous studies showed that the PD-1 blockade restored the function of exhausted CD8 T cells, such as proliferation, cytokine secretion, and viral control in memory phase (9-12). In line with this, enhanced proliferation, migration, and cytokine secretion of antigen-specific CD8 T cells were observed in the

absence of PD-1 signaling. Especially, two studies using acute hepatitis C and acute rabis encephalipis showed that PD-1 played a role in the dysfunction and migratory capacity of the CD8 T cells (31, 32). I hypothesized that enhancement of IFN-γ-secretion by PD-1-deficient CD8 T cells in peritoneum would increase recruitment of Ag-specific effector CD8 T cells, which would facilitate Ag-clearance. The result from Weiyi Peng et al.'s study, which showed that anti-PD-1 blockade could increase IFN-γ at the tumor site, and thereby increase chemokine-dependent trafficking of immune cells into malignant disease sites, supports this hypothesis (12).

Although several groups showed that granzyme B secretion by PD-1-deficient CD8 T cells was enhanced, enhancement of granzyme B secretion by PD-1-deficient H60-specific CD8 T cells on day 5 post-priming was not observed in the phenotyping analysis. According to previous data in my lab, vigorous granzyme B secretion by H60-specific CD8 T cells in H60 model was observed at day 7 post-priming.

The direct relationship between CD62L and PD-1 in chronic inflammation has not yet been identified. But, in acute vaccinia virus model, S. Rameeza Allie et al. showed cell-intrinsic role

for PD-1 signaling in affecting the quality of CD8 memory T cells via examining $CD62L^{hi}$ expression of Ag-specific CD8 T cells during acute viral infection. On the other hand, in chronic LCMV clone 13 model, Ha et al. showed that combinatorial therapeutic vaccination using anti-PD-L1-blocking antibody did not induce CD62L expression on LCMV-specific CD8 T cells but increase CD127 expression. This finding is similar to the result that H60-specific CD8 T cells under CD4 help-deficient condition skewed toward MPEC ($CD127^{hi}KLRG1^{low}$) in the absence of PD-1 signaling but remained as effector memory CD8 T cell phenotype ($CD44^{hi}$ $CD62L^{low}$) in memory phase. Therefore, PD-1-deficiency could not rescue the CD62L expression under the condition where antigen persisted.

Recently, TCR/CD28 signaling results in the activation of PI3K/Akt pathway, which can trigger the nuclear exclusion of FOXO1 driving the transcription of eomes, IL-7R, CCR7 and CD62L (33, 34). Thus, under CD4 help-deficient condition, antigen persistence results in the continuous activation and down-regulation of CD62L expression in antigen-specific CD8 T cells in the end. Although antigen clearance under CD4 help-deficient condition was expedited in the absence of PD-1

signaling, PD-1-deficient CD8 T cells were activated more continuously than helped ones because their number during priming was too low to eliminate the antigens. I guess that the absence of PD-1 signaling would not rescue the expression of CD62L in chronic infection. To prove this, further studies, such as examination of CD62L expression of H60-specific CD8 T cells on various day point post-priming and restore of CD62L expression on the PD-1-deficient H60-specific CD8 T cells after experimental antigen elimination, are required.

In conclusion, the results from this work showed that PD-1 signaling in Ag-specific CD8 T cells can modulate the rescue of memory response after secondary antigen challenge, although PD-1-deficient Ag-specific CD8 T cells still remained Tem phenotype under CD4 help-deficient. The absence of PD-1 signaling enhanced the capacity of proliferation, IFN-γ secretion, migration and eliminating antigens. PD-1 signaling itself did not have direct effect on differentiation of the central memory CD8 T cells in CD4 help-deficient condition. This information may be valuable especially in designing effective combinational vaccinations in settings where CD4 T cells are unavailable, such as in the context of chronic HIV infection.

REFERENCES

1. Bevan MJ. Helping the CD8(+) T-cell response. Nat Rev Immunol. 2004;4(8):595-602.

2. Ahlers JD, Belyakov IM. Molecular pathways regulating CD4(+) T cell differentiation, anergy and memory with implications for vaccines. Trends Mol Med. 2010;16(10):478-91.

3. Castellino F, Germain RN. Cooperation between CD4+ and CD8+ T cells: when, where, and how. Annu Rev Immunol. 2006;24:519-40.

4. Jin HT, Ahmed R, Okazaki T. Role of PD-1 in regulating T-cell immunity. Curr Top Microbiol Immunol. 2011;350:17-37.

5. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008;26:677-704.

6. Kamphorst AO, Ahmed R. Manipulating the PD-1 pathway to improve immunity. Curr Opin Immunol. 2013;25(3):381-8.

7. Francisco LM, Sage PT, Sharpe AH. The PD-1 pathway in tolerance and autoimmunity. Immunol Rev. 2010;236:219-42.

8. Brahmer JR, Drake CG, Wollner I, Powderly JD, Picus J, Sharfman WH, et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. J Clin Oncol. 2010;28(19):3167-75.

9. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature. 2006;439(7077):682-7.

10. Ha SJ, Mueller SN, Wherry EJ, Barber DL, Aubert RD, Sharpe AH, et al. Enhancing therapeutic vaccination by blocking PD-1-mediated inhibitory signals during chronic infection. J Exp Med. 2008;205(3):543-55.

11. Fuse S, Tsai CY, Molloy MJ, Allie SR, Zhang W, Yagita H, et al. Recall responses by helpless memory CD8+ T cells are restricted by the up-regulation of PD-1. J Immunol. 2009;182(7):4244-54.

12. Peng W, Liu C, Xu C, Lou Y, Chen J, Yang Y, et al. PD-1 blockade enhances T-cell migration to tumors by elevating IFN-gamma inducible chemokines. Cancer Res. 2012;72(20):5209-18.

13. Allie SR, Zhang W, Fuse S, Usherwood EJ. Programmed

death 1 regulates development of central memory CD8 T cells after acute viral infection. J Immunol. 2011;186(11):6280-6.

14. Choi EY, Yoshimura Y, Christianson GJ, Sproule TJ, Malarkannan S, Shastri N, et al. Quantitative analysis of the immune response to mouse non-MHC transplantation antigens in vivo: the H60 histocompatibility antigen dominates over all others. J Immunol. 2001;166(7):4370-9.

15. Choi EY, Christianson GJ, Yoshimura Y, Sproule TJ, Jung N, Joyce S, et al. Immunodominance of H60 is caused by an abnormally high precursor T cell pool directed against its unique minor histocompatibility antigen peptide. Immunity. 2002;17(5):593-603.

16. Scott D, Addey C, Ellis P, James E, Mitchell MJ, Saut N, et al. Dendritic cells permit identification of genes encoding MHC class II-restricted epitopes of transplantation antigens. Immunity. 2000;12(6):711-20.

17. Ryu SJ, Jung KM, Yoo HS, Kim TW, Kim S, Chang J, et al. Cognate CD4 help is essential for the reactivation and expansion of CD8 memory T cells directed against the hematopoietic cell-specific dominant minor histocompatibility antigen, H60. Blood. 2009;113(18):4273-80.

18. Song MG, Kang B, Jeon JY, Chang J, Lee S, Min CK, et al. In vivo imaging of differences in early donor cell proliferation in graft-versus-host disease hosts with different pre-conditioning doses. Mol Cells. 2012;33(1):79-86.

19. Jeon JY, Jung KM, Chang J, Choi EY. Characterization of CTL Clones Specific for Single Antigen, H60 Minor Histocompatibility Antigen. Immune Netw. 2011;11(2):100-6.

20. VanderVegt FP, Johnson LL. Induction of long-term H-Y-specific tolerance in female mice given male lymphoid cells while transiently depleted of CD4+ or CD8+ T cells. J Exp Med. 1993;177(6):1587-92.

21. Filatenkov AA, Jacovetty EL, Fischer UB, Curtsinger JM, Mescher MF, Ingulli E. CD4 T cell-dependent conditioning of dendritic cells to produce IL-12 results in CD8-mediated graft rejection and avoidance of tolerance. J Immunol. 2005;174(11):6909-17.

22. Wherry EJ, Blattman JN, Murali-Krishna K, van der Most R, Ahmed R. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J Virol. 2003;77(8):4911-27.

23. Blackburn SD, Shin H, Freeman GJ, Wherry EJ.

Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. Proc Natl Acad Sci U S A. 2008;105(39):15016-21.

24. Huster KM, Busch V, Schiemann M, Linkemann K, Kerksiek KM, Wagner H, et al. Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets. Proc Natl Acad Sci U S A. 2004;101(15):5610-5.

25. Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Antia R, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol. 2003;4(3):225-34.

26. Joshi NS, Cui W, Chandele A, Lee HK, Urso DR, Hagman J, et al. Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity. 2007;27(2):281-95.

27. Pham NL, Badovinac VP, Harty JT. A default pathway of memory CD8 T cell differentiation after dendritic cell immunization is deflected by encounter with inflammatory cytokines during antigen-driven proliferation. J Immunol. 2009;183(4):2337-48.

28. Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, et al. Central memory self/tumor-reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci U S A. 2005;102(27):9571-6.

29. Hinrichs CS, Gattinoni L, Restifo NP. Programming CD8+ T cells for effective immunotherapy. Curr Opin Immunol. 2006;18(3):363-70.

30. Stark FC, Gurnani K, Sad S, Krishnan L. Lack of functional selectin ligand interactions compromises long term tumor protection by CD8+ T cells. PLoS One. 2012;7(2):e32211.

31. Urbani S, Amadei B, Tola D, Massari M, Schivazappa S, Missale G, et al. PD-1 expression in acute hepatitis C virus (HCV) infection is associated with HCV-specific CD8 exhaustion. J Virol. 2006;80(22):11398-403.

32. Lafon M, Megret F, Meuth SG, Simon O, Velandia Romero ML, Lafage M, et al. Detrimental contribution of the immuno-inhibitor B7-H1 to rabies virus encephalitis. J Immunol. 2008;180(11):7506-15.

33. Kim EH, Suresh M. Role of PI3K/Akt signaling in

memory CD8 T cell differentiation. Front Immunol. 2013;4:20.

34. Vermijlen D, Braun MY, Marchant A. Do PI3-kinase mutations drive T cells insane? Cell Mol Immunol. Epub 2014 Mar 3; DOI: 10.1038/cmi.2014.11.

국문 초록

활성화된 CD8 T 세포는 활발한 클론 증식을 거치며, 항원이 제거된 이후 일부의 세포만이 기억 세포로 남게 된다. 항원특이적인 CD8 T 세포 반응은 CD4 T 세포의 도움에 의존적이며, 특히 CD4 T 세포의 도움이 있을 때에만 1 차 면역 반응뿐만 아니라 2 차 면역 기억 반응 또한 효과적으로 일어난다. PD-1 은 활성화된 CD8 T 세포에서 발현되는 억제성 수용체로서, 정상적인 CD8 T 세포의 기능 수행에 관련이 있는 것으로 알려져 있다. 본 연구에서는, 부조직적합항원 H60 를 이용하여 CD4 도움에 의존적인 1 차 면역반응 및 2 차 면역반응을 유도함으로써, PD-1 과 CD4 도움이 결핍된 CD8 T 세포 간의 연관성을 밝히고, PD-1 결핍 쥐에서 CD4 도움을 받지 않은 상태의 H60 항원 특이적인 기억 면역 반응이 회복되는 것을 확인하였다. 또한, PD-1 결핍의 H60 특이적인 CD8 T 세포는 항원 인지 후 빠른 분열 및 감염 장소로의 빠른 이동, 그리고 효과적인 싸이토카인 분비 및 항원 제거 능력을 가짐을 확인하였다. 하지만, PD-1 신호가 존재하지 않는 CD8 T 세포 자체가 중심 기억 세포로 분화하지 않았으며, 이 결과는 PD-1 신호 자체가 중심 기억 세포로 분화하는 데에 직접적으로 관여하기 보다는 간접적인 도움을 주는 것을 시사한다. 이러한 결과는 PD-1 신호 차단 시 항원 특이적 CD8 T 세포의 기억

세포로의 분화를 이해함으로써, 효과적인 백신 개발 연구에 기여할 수 있을 것이다.

주요어 : CD4 T 세포 도움, 기억 CD8 T 세포, PD-1, T 세포 분화

학 번 : 2012-21789