A DISSERATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Constitutive type I IFN regulates phenotypic and functional heterogeneity of naïve CD8$^+$ T cells based on self-reactivity

자가반응성에 기반한 항존적 제 1 유형 인터페론의 naïve CD8$^+$ T 세포 표현형 및 기능의 다양성 조절

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Abstract

In a steady-state, naïve $CD8^+$ T cells have been defined as a homogeneous population through low and high expression of CD44 and CD62L, respectively. However, recent studies have demonstrated that $CD5^{hi}$ naïve $CD8^+$ T cells with a number of surface molecules being differently expressed consisted of heterogenic population. In the present study focused on Ly6C that is specifically expressed only on $CD5^{hi}$ naïve $CD8^+$ T cells at steady-state mice. The Ly6C induction in $CD8^+$ T cells is known to be increased by type I interferon (IFN). However, it needs to be further revealed how precisely generation of $Ly6C^+$ naïve $CD8^+$ T cells are regulated at molecular level in the extra-thymic environment at steady-state, and whether or not self-reactivity is involved in the generation of $Ly6C^+$ naïve $CD8^+$ T cells. Furthermore, it also remained to be uncovered whether the type I IFN induces not only the generation of $Ly6C^+$ naïve $CD8^+$ T cells but also functional features, such as clonal expansion and differentiation capacity, in acute viral infection.

The results showed that constitutive type I IFN induced generation of $Ly6C^+$ naïve $CD8^+$ T cells in steady-state mice, in which the generation was enhanced by self-T cell receptor (TCR) engagement. The effect of constitutive type I IFN was most prominent for the naïve $CD8^+$ T cell with higher intrinsic self-reactivity than lower counterpart, which is positively correlated to the expression level of CD5. Hence the greater heterogeneity has seen in $CD5^{hi}$ cells in the present study hinges on their particular attribute, namely heightened

responsiveness to cytokines, especially type I IFN, and to high affinity of TCR contact with self-peptides.

The results further suggested that the constitutive type I IFN signal influences not only the induction of Ly6C^{+} naïve CD8^{+} T cells but also their effector function-related genetic landscape (*T-bet*, *Eomes*, *IL-18Rap*, and *CCL5*) and pro-inflammatory cytokine production, especial to IFN-γ. Furthermore, Ly6C^{+} naïve CD8^{+} T cells favored to be differentiated into short-lived effector cell (SLEC) while CD5^{lo} naïve CD8^{+} T cells favor memory precursor effector cell (MPEC) in lymphocytic choriomeningitis virus (LCMV) infection model. Same with the effector precursor differentiation, CD5^{lo} naïve CD8^{+} T cells have generated more memory CD8^{+} T cells than Ly6C^{-} or Ly6C^{+} naïve CD8^{+} T cells. Furthermore, by temporally blocking of interferon alpha receptor 1 (IFNAR1) in the steady-state mice, Ly6C^{+} naïve CD8^{+} T cells were increasingly differentiated into MPEC while reducing SLEC differentiation. It suggested that constitutive type I IFN exposed during steady-state can affect the fate decision of naïve CD8^{+} T cells between MPEC and SLEC in LCMV infection models.

Collectively, this study demonstrated that the effect of constitutive type I IFN on naïve CD8^{+} T cells is closely related to its self-reactivity and directly affects their phenotype and effector function. Also type I IFN affected differentiation fate of naïve CD8^{+} T cells between SLEC and MPEC upon LCMV infection model. At the best of my knowledge, this is the first to demonstrate that the differentiation fate in infection had been pre-determined within naïve T cell

phase dependent on type I IFN together with self-reactivity.

Keywords: Naïve $CD8^+$ T cell, Self-reactivity, Constitutive type I IFN, SLEC, MPEC, LCMV

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List of Abbreviations

BACH2	BTB domain and CNC homolog 2
Bak	Bcl2-antagonist/killer
Bcl2/6/X_L	B cell lymphoma 2/6/X_L
Bim (BCL2L11)	Bcl-2-like protein 11
Blimp-1 (*Prdm1*)	B lymphocyte-induced maturation protein-1 (*PR domain zinc finger protein 1*)
CD4/CD8SP	CD4/CD8 single-positive
CK2	Casein kinase 2
CXCL9	CXC-chemokine ligand 9
DAMP	Damage-associated molecular pattern
DN	Double-negative
Eomes	Eomesodermin
FOXO1	Forkhead box protein O 1
GAS	Gamma-interferon activated site
GSEA	Gene set enrichment assay
HDAC1/2	Histone deacetylase 1/2
ID2/3	DNA-binding protein inhibitor 2/3
IFN	Interferon
IFNAR1/2	Interferon alpha receptor 1/2
IFNGR	Interferon gamma receptor
IL	Interleukin
IRF1/9	Interferon regulatory factor 1/9
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3

ISRE	Interferon-stimulated response element sequence
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
KLRG1	Killer cell lectin-like receptor G 1
LCMV	Lymphocytic choriomeningitis virus
LEF1	Lymphoid enhancer binding factor 1
LM	Listeria monocytogenes
MHC class I	Major histocompatibility class I
MP	Memory phenotype
MPEC	Memory precursor effector cell
mTOR	Mammalian target of rapamycin
MX1	Myxovirus resistance 1
NCR	Natural cytotoxicity receptor
NK cell	Natural killer cell
OAS	Oligoadenylate synthase
PAMP	Pathogen-associated molecular pattern
pDC	Plasmacytoid dendritic cell
PKCβ/δ	Protein kinase C β/δ
PMA	Phorbol myristate acetate
RAG	Recombination-activating gene
SCID	Severe combined immunodeficiency
SH2	Src homology 2
SHP2	SH2 domain-containing protein tyrosine phosphatase 2
SIN3A	SIN3 transcription regulator homologue A
SLEC	Short-lived effector cell
SOCS1/3	Suppressors of cytokine signaling 1/3

STAT1/2/3/4/5	Signal transducer and activator of transcription 1/2/3/4/5
T-bet (*Tbx21*)	T-box transcription factor
TAP1	Transporter associated with antigen processing 1
TCF7	Transcription factor 7
TCR	T cell receptor
TEC	Thymic epithelial cell
TLR	Toll like receptor
TYK2	Tyrosine kinase 2
VM	Virtual memory

I. Literature review

1. Type I interferon

1.1. General feature of type I interferon

1.1.1. Discovery of interferon

It has been discovered that a meaningful soluble factor from culture supernatant from pieces of chorio-allantoic membrane treated with heat-inactivated influenza virus can inhibit propagation of live influenza virus, named as interferon [1]. Since its discovery, over 50 years, interferon (IFN) has been studied as one of the most well-known soluble factors for survival, proliferation and differentiation of various immune and non-immune cells *in vitro* and *in vivo*.

Basically, there are three types of IFN family, type I IFN, type II IFN (IFN-γ) and type III IFN (IFN-λ1, IFN-λ2, and IFN-λ3) [2] (Table 1). Especially, type I IFN consist of 14 functional IFN-α genes in mice (13 in human) and other subtypes of IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-τ, and IFN-ω. When those substances provoke to molecular signals through with the recognition by their specific receptors, they usually utilize JAK/STAT molecules for deliver the signals to down-stream pathways.

Table 1. Summary of three types of I IFN family.

Ligand	Alternative names	Receptor	Signal transducer
Type I IFN	IFN-α IFN-β IFN-δ IFN-ε IFN-κ IFN-τ IFN-ω	IFNαR1 IFNαR2	Jak1, Tyk2 STAT1, STAT2, STAT3 STAT4, STAT5
Type II IFN	IFN-γ	IFNγR1 IFNγR2	Jak1, Jak2 STAT1, STAT3, STAT5
Type III IFN	IFN-λ1 IFN-λ2 IFN-λ3	IL-28R1 IL-10R2	Jak1, Tyk2 STAT1, STAT2, STAT3 STAT5

1.1.2. Production of type I IFN

The concentration of type I IFN in general is dramatically increased upon infectious condition in blood stream as well as local tissues. It has been well-known that the highest amount of type I IFN under the infectious condition is produced by plasmacytoid dendritic cells (pDCs) through the stimulation of toll-like receptor (TLR) 7 and TLR9 [3, 4]. The pDC is arguably the strongest producer of type I IFN, but it is a limited fact only when the host has been infected or laid on the inflammatory condition [5, 6].

Recent studies have intensively suggested the importance of type I IFN, either constitutively produced or pre-existing, in uninfected host. Although it had been hard work to detect the exact concentration of constitutively produced type I IFN, recent studies have showed that basal level of type I IFN is evidently

produced in human and mice by measuring the mRNA level and suggesting developmental defect of thymus within IFNα receptor 1 knock out mice (IFNAR1 KO) [7-10]. Detection of type I IFN expression has been visualized by using IFN-β luciferase reporter mice [8]. Furthermore, the study showed that type I IFN is produced in not only lymphoid but also non-lymphoid organs, especially higher in the thymus than other lymphoid organs.

1.2. Regulation of type I IFN signaling

1.2.1. Initiation of type I IFN signals via JAK/STAT

The canonical signaling component of type I IFN is composed of IFNAR, signal transducer and activator of transcription 1 (STAT1), STAT2, IFN-regulatory factor 9 (IRF9), Janus kinase 1 (JAK1), and tyrosine kinase 2 (TYK2). All cells having these molecules in the cytoplasm of and play a role in binding to the heterodimers of IFNAR1 and IFNAR2. By the engagement of type I IFN to their receptors, activated JAK1 and TYK2 phosphorylates the IFNAR, followed by recruits and phosphorylates STAT protein. Then, the phosphorylated STAT proteins form dimer that acts as transcription factors to translocation into nuclear, and induce the activation of IFN-stimulated genes (ISGs) [11].

It has been uncovered that two predominant STAT complexes are formed in response to type I IFN, and that each can control distinct gene expression

program. The complex recruited with phosphorylated STAT1 (pSTAT1), pSTAT2, and IRF9, named interferon-stimulated gene factor 3 (ISGF3) [12] in cytoplasm translocate into nucleus and then bind to IFN-stimulated response element sequences (ISRE) to activate classical antiviral genes, such as *2′-5′-oligoadenylate synthase (OAS)* and *myxovirus resistance 1 (MX1)* [13, 14]. Whereas, homodimer complex of pSTAT1 binds to gamma-interferon activated site sequences (GASs) to provoke the expression of pro-inflammatory genes, such as *IRF1* and *CXC-chemokine ligand 9 (CXCL9)* [15, 16].

Another canonical signaling pathway for type I IFNs is the utilization of STAT3 homodimers. IFNα-activated STAT3 homodimers indirectly inhibit interferon stimulated genes (ISGs) by binding to transcriptional repressors, which are not well understood. On the other hand, IFNα-activated STAT3 homodimer can bind to co-repressor complex SIN3 transcription regulator homologue A (SIN3A) [17, 18]. The SIN3A which contains HDAC1 and HDAC2 suppresses induction of STAT3 target genes by promoting de-acetylation of STAT3 and histones [19]. Indeed, although STAT3 was sufficiently phosphorylated by the typical type I IFN signal, the reason for the lack of expression of STAT3 target genes had remained unknown. However, several studies, using SI3A knockdown system, showed to increase of STAT3 target genes suggesting that STAT3 can counterbalance type I IFN-induced STAT1 and ISGF3 function [19].

1.2.2. Suppression of type I IFN signaling

Suppression of type I IFN signaling plays an important role on the regulation of broad biological situations, including exacerbation of disease or lethality, both acute and chronic diseases, and toxicities [20]. The type I IFN signaling can be suppressed by downregulation of IFNAR and/or induction of negative regulators. The negative regulators as a part of negative feedback loop to suppress excessive type I IFN responses. The SOCS, the most well-defined negative regulators of type I IFN signaling, targets to tyrosine kinase activity of JAK to inhibit their function to phosphorylate IFNAR. Especially, the SOCS1 and SOCS3 that suppress phosphorylation of JAK1, TYK2, and STAT1 are the most potent negative regulator of type I IFN signaling [21]. Of these, SOCS1 has been known to inhibit type I IFN signaling more directly. Overexpression of the SOCS1 has shown inhibition of type I IFN-induced antiviral and anti-proliferative responses [21-23]. SOCS3 has been reported to inhibit IL-6 family signaling, but is also involved in suppressing type I IFN-mediated antiviral responses [24]. The downregulation of IFNAR can be induced by stimulation of interleukin (IL)-1, TLR4, immunoreceptor tyrosine-based activation motif (ITAM)-associated receptors, and oxidative and metabolic stress [25-27]. The best known internalization mechanism of IFNAR is induced by p38-mediated phosphorylation of IFNAR. Phosphorylation by p38 provokes CK2-mediated phosphorylation of IFNAR1, thereby increasing receptor internalization, ubiquitination and degradation [28]. On the other hand, recruitment of protein kinase C (PKC) β/δ or SH2 domain-containing protein

tyrosine phosphatase (SHP)2 to IFNAR also suppresses type I IFN signaling through dephosphorylation of signaling intermediate, such as JAK1 [29].

1.3. Type I IFN on T cell immunity

1.3.1. Constitutive expression of type I IFN

It has been suggested that tiny amount of type I IFN is constitutively maintained although host have never been infected with foreign antigen. These constitutive type I IFN has been postulated to be induced by on-going low-grade exposure to food antigen, commensal microbes, stimuli during tissue remodeling and damage, acting as important biological function of immune cells [30] (e.g. proliferation of hematopoietic stem cells [31, 32], development of NK and B cells [33, 34], macrophages function and homeostasis [35, 36]). In the field of T cell study, it is important to note that most studies have addressed the role of type I IFN in activating or activated, not naïve $CD8^+$ T cells.

Recent studies have suggested T cells require constitutive type I IFN as regulatory factor during their development in thymus and functional modification in periphery. It has been reported that type I IFN signal deficient CD4 single-positive (CD4SP) and CD8 single-positive (CD8SP) thymocytes shows their phenotypic abnormality, reduction of absolute cell number, and diminished expression of STAT1 within their final step of maturation in thymus. It has suggested that constitutive type I IFN signaling is importantly involved

in maturation from thymocytes into naive T cells [10]. In addition, it has been shown that the phenotype, function and age-dependent expansion of $CD44^{hi}CD49d^{lo}$ virtual memory (VM) $CD8^+$ T cells are strongly affected in the absence of type I IFN signaling by eomesodermin (eomes)-dependent fashion [37].

Even though aforementioned studies suggested importance of constitutive type I IFN in certain kind of $CD8^+$ T cells and development of thymocytes, it was paradoxically unrevealed the role of constitutive type I IFN to functionality of peripheral T cells [10, 37, 38]. Thus, it remains yet to be unveiled whether the constitutive type I IFN exposed during the steady-state condition could affect functional modification of peripheral naïve T cells.

1.3.2. Robust activation of type I IFN by external stimulation

A high amount of type I IFN is rapidly produced upon viral or bacterial infection. It is a frontline of defense mechanism, activating innate immune cells including DCs, macrophages, and NK cells [39]. Although type I IFN can be produced in almost all cells by recognizing the pathogen associated molecule patterns (PAMPs) or damage associated molecule patterns (DAMPs) in virus or bacterial infection [40-42], the most common source of robust type I IFN production during virus or bacterial infection has been known as TLR7- and TLR9-stimulated pDCs [43, 44].

Robust concentration of type I IFN produced in inflammatory condition affects various immune cells, as mentioned earlier. T cells are directly or indirectly affected by strong type I IFN stimulation. It has been shown that the clonal expansion and granzyme B production of effector $CD8^+$ T cells are reduced in $IFNAR1^{-/-}$ mice infected with lymphocytic choriomeningitis virus (LCMV) [45]. It has been also reported the clonal expansion of $CD8^+$ T cells in mice with LCMV infection is accompanied by high phosphorylation of STAT4, not STAT1, by robust type I IFN [46]. And it has been confirmed that the high phosphorylation of STAT4 was seen only in the effector state of antigen-specific $CD8^+$ T cells, and, interestingly, it was not observed in IFNAR1 deficient mice [47].

Together with the clonal expansion, the other direct role of robust type I IFN in LCMV infection is to protect the proliferating effector $CD8^+$ T cells against death by activated NK cells [48, 49]. NK cells has been well-reported to show strong cytolytic function against physiologically stressed cells such as tumor cells and virus-infected cells. The increased expression level of MHC class I and natural cytotoxicity receptor (NCR) ligand by robust type I IFN in LCMV infection plays an important role in selecting target cells for NK cells to attack [50]. It was reported that IFNAR1 deficient effector $CD8^+$ T cells cannot increase the expression of MHC class I and NCR ligand, consequently killed by NK cells [48, 49].

Together with the direct effect of robust type I IFN, it has been reported to affect T cells indirectly by controlling the function of DCs [51-53]. DC is one of the

most essential and well-studied innate immune cell that are directly required to $CD8^+$ T cells for priming and antigen-presentation in infection situation [54]. A study has reported that robust type I IFN produced early in LCMV infection directly enhances the cross-priming ability of DCs, making $CD8^+$ T cells possess more potent cytotoxicity. They also have suggested that this robust ability of type I IFN could improve cytotoxic capacity of $CD8^+$ T cells without the help of $CD4^+$ helper T cells, and IFN-β administration alone was sufficient without the need for LCMV infection [55]. The other study has shown that the expression of both IL-15 and the IL-15 receptor α-chain are increased by stimulation of poly(I:C) or IFN-α/β in DCs, and then co-stimulatory molecule and IFN-γ production in the DCs were increased by the autologous IL-15 and IFN-α/β. Consequently, the enhanced ability of DC induced to stronger clonal expansion of $CD8^+$ T cells [51].

2. $CD8^+$ T cells

2.1. General characteristics

2.1.1. Developmental stage in thymus

$CD8^+$ T cells are developed at the thymus after migrating as T cell precursors generated in the bone marrow. Then, $CD8^+$ T cells underwent a series of preprogrammed process, called β-selection followed by the positive selection and negative selection [52]. In the first step of $CD8^+$ T cell development,

double-negative (DN) thymocytes expressing incomplete TCR (pre-TCR) received pre-TCR signaling for rearrangement of TCR β-chain, then underwent proliferation and expression of complete TCRβ [53]. In the next step, double-positive (DP) thymocytes are educated further by positive and negative selection. In the positive selection, 'death by neglect' happened, and a small number of thymocytes with TCR affinity to self-peptide loaded MHC (self-pMHC) survived [56]. Among the survivors of positive selection, thymocytes with too strong TCR affinity to self-pMHC, chosen once more by negative selection, are removed [57]. During the positive and negative selection, the $CD8^+$ T cells are continuously receive self-TCR signals by contact with thymic epithelial cells (TECs), which is known to assign the expression level of CD5 [58]. In general, CD5 acts as a negative regulator of self-TCR signals received during the thymic selection. Although the level of CD5 on $CD8^+$ T cells remains stable in periphery, the physiological function in periphery is not clear [59].

Thus, $CD8^+$ T cells with fixed CD5 levels through thymic selection act as a defense against external antigens in periphery. Indeed, research is needed to determine how the levels of CD5 in thymus can affect their defensive actions.

2.1.2. Phenotypic categorization of $CD8^+$ T cell

About 1 million mature $CD8^+$ T cells, egressed from thymus to periphery every day in mice, are maintained the pool to ~25 million cells that contains under 100 microbial peptide specific cells per every clone [60]. Mature $CD8^+$ T cell

pool maintains quiescence and stays in homeostasis. It was noting that $CD8^+$ T cells at the quiescent stage expressed CD44 and CD62L that are known to be the marker for the activation status of $CD8^+$ T cells. However, they have also been used widely to categorize the population of $CD8^+$ T cells into naïve ($CD44^{lo}CD62L^{hi}$) and memory phenotype (MP; $CD44^{hi}CD62L^{-/+}$) [61]. Recently, the expression level of CD5 has been used as a surface marker to categorize sub-population of naïve $CD8^+$ T cells as well. While surface expression of CD44 and CD62L can be rapidly changed, CD5 maintains at relatively stable level in thymus even though T cells are proliferating. During the thymic selection, CD8 single-positive (SP) cells express broad spectrum of CD5 molecules gained by entire avidity of self-peptide loaded MHC (self-MHC) and TCR [58]. It has been well reported that, in thymus, the expression of CD5 acted as a negative regulator of TCR signaling [58, 62], while it is unclear in periphery [59]. So, the expression level of CD5 in periphery is broadly accepted as a surrogate marker of TCR reactivity, not functional molecule [63].

However, because its expression on naïve $CD8^+$ T cells is actively maintained by continuous and obligatory contact with self-pMHC, researchers are still wondering why $CD8^+$ T cells maintain their CD5 levels consistently, and suggesting the study need to be continued.

2.2. Features of naïve $CD8^+$ T cells

2.2.1. Homeostatic maintenance

It is quite important to maintain number of naïve $CD8^+$ T cells to construct immune defense system against foreign antigen. Thus, the study of survival factors to maintain adequate number of naïve $CD8^+$ T cells has been ongoing [64]. The best known essential factors, required to survival of naïve $CD8^+$ T cells, are TCR signals from self-pMHC (shortly, self-TCR signals) and IL-7 receptor (IL-7R) signals [65]. It has been reported that depriving TCR contact with MHC class I molecules [66, 67] or ablating TCR expression [68, 69] causes naïve $CD8^+$ T cells to die within several weeks. Also naïve $CD8^+$ T cells die within 1–2 weeks of transfer into IL-$7^{-/-}$ hosts [70] or after conditional deletion of IL-7R [71]. Both self-TCR and IL-7R signals have been well reported to upregulate pro-survival molecules (e.g. Bim, Bcl-2, and Bcl-x_L etc. [72]) and inhibit pro-apoptotic molecules (e.g. Bax, Bak, and caspase 9 etc. [73]). In addition to survive longer, another mechanism for maintaining the pool of naïve $CD8^+$ T cells is to maintain numbers through division in periphery. Every peripheral naïve $CD8^+$ T cells intermittently do the slow turnover, called homeostatic proliferation, around 20 times in their lifetime after emigrant from the thymus [65]. Interestingly, the previous studies showed that naïve $CD8^+$ T cells which have deficient or reduced self-TCR and IL-7R signals showed a reduced homeostatic proliferation in lymphopenic condition in mouse model [74-77]. Therefore, self-TCR and IL-7 are crucial factors to not only survival but also homeostatic proliferation of naïve $CD8^+$ T cells.

Another suggested role of homeostatic proliferation in some naïve T cells is to conversion into memory phenotype cells. When transferring naïve $CD8^+$ T cells into RAG recombinase deficient ($RAG^{-/-}$) mice or severe combined immunodeficiency (SCID) mice, a few cells showed a very rapid division rate in a IL-7 dependent manner [78]. In addition, such fast proliferation of naïve $CD8^+$ T cells seems to be similar to the natural lymphopenia-induced proliferation seen in neonatal mice that is a gut-microbiota dependent [79]. Then, the naïve $CD8^+$ T cells acquire the activation markers as if they were activated by foreign antigen stimulation to form a memory phenotype pool [80].

Even though local high concentrations of common γ-chain cytokines (e.g. IL-2, IL-7, and IL-15) [81], transient absence of negative signals (e.g. CD24, TAM receptors, PD-1 and CTLA-4) [82, 83], TCR revision by continuous stimulation of self-antigens [84] have been known to induce the conversion from naïve to memory phenotype, it is still unclear why a very little proportion of naive T cells continue to proliferate slowly and form memory phenotype cells in normal unimmunized mice.

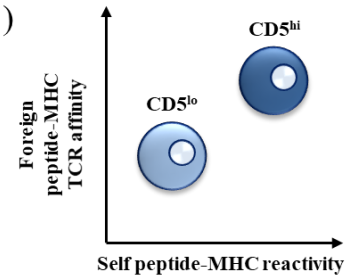
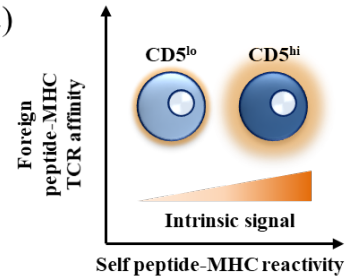
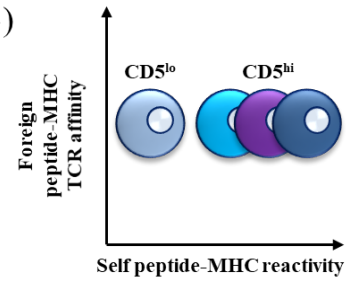
2.2.2. Differential expression of CD5 and consequential immune response

As aforementioned, crucial role of CD5 in thymocytes has been well reported, however it remained still unclear its role in peripheral naïve T cells. Moreover, functional ligand of CD5 is unknown although a few past studies suggested

CD72 and CD5L as possible ligands [85, 86]. So it has been widely accepted that CD5 is thought to reflect only the strength of self-reactivity, and persisting on peripheral naïve T cells as a footprint of thymic selection [58]. Actually, the naïve T cells express broad spectrum of CD5 level, and it is reported that the responsiveness of the cells are changed according to the high and low expression level of CD5 in external stimulation, such as homeostatic proliferation and foreign antigens [52]. It has been reported that $CD5^{hi}$ cells (naïve T cells expressing high level of CD5) have a stronger reactivity to γc cytokines (*e.g.*, IL-2, IL-7, and IL-15) than $CD5^{lo}$ cells (naïve T cells expressing low level of CD5) [87, 88]. So $CD5^{hi}$ cells have been known to have a faster homeostatic proliferation than $CD5^{lo}$ cells because reactivity to γc cytokines is the most important factor to determine the speed of homeostatic proliferation [87, 88]. Consistent with these studies, it has been also proven that $CD5^{hi}$ cells have superior responsiveness to viral or bacterial infection than $CD5^{lo}$ cells, thus consequently much more vigorous generation of long-term memory cells [89-91]. The studies have suggested different interpretations of why $CD5^{hi}$ cells are more reactive than $CD5^{lo}$ cells. It was suggested that $CD5^{hi}$ $CD4^{+}$ T cells have higher TCR binding affinity for foreign antigen epitopes than $CD5^{lo}$ $CD4^{+}$ T cells in *Listeria monocytogenes* (LM), LCMV, and influenza virus infection (Table 2.1) [89]. Another study using $CD4^{+}$ T cells in *L. monocytogenes* infection has suggested that $CD5^{hi}$ $CD4^{+}$ T cells have higher signaling intensity for the same stimulus than $CD5^{lo}$ $CD4^{+}$ T cells, and thus have high responsiveness, even though they have had a similar TCR binding affinity

(Table 2.2) [90]. A study using $CD8^+$ T cells in LCMV and *L. monocytogenes* infection has suggested that $CD5^{hi}$ cells have a more heterogeneous population which contains cells ready to be activated than $CD5^{lo}$ cells, and thus have high responsiveness although they have had a similar TCR binding affinity (Table 2.3) [91].

Table 2. Responsiveness of $CD5^{lo}$ and $CD5^{hi}$ T cells in infection studies.

Used cell	Infection model	Interpretation
$CD4^+$ T cell	*L. monocytogenes* LCMV Influenza	1) 
$CD4^+$ T cell	*L. monocytogenes*	2) 
$CD8^+$ T cell	*L. monocytogenes* LCMV	3) 

As mentioned above, although several researchers have struggled to interpret the different characteristics between $CD5^{lo}$ and $CD5^{hi}$ cells, it still remains to be questioned what makes $CD5^{hi}$ cells different from $CD5^{lo}$ cells.

2.2.3. Classical linear differentiation pathway

Previous studies have suggested that the differentiation process of effector and memory $CD8^+$ T cells from naïve $CD8^+$ T cells are accompanied through the series of linear differentiation like Naïve → Effector → Memory [92]. In this model, most of T cell pool is thought to have relatively homologous features during effector phase of infection, then the potential to memory differentiation is also expected to quite equivalent within effector T cell pool. Therefore, it was thought that the competition for, or withdrawal from environmental resources (e.g. antigen, cytokines, nutrients, and growth factor etc.) can limit the magnitude of T cell response, survival, and memory maintenance (Figure 1) [93].

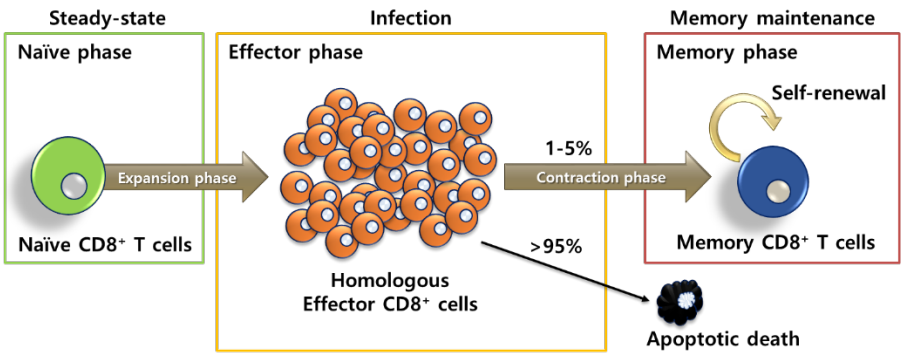


Figure 1. Cartoon of linear memory differentiation model.

After the linear differentiation model, another model was subsequently suggested that effector T cells progressively lose their potential to be memory cells by receiving prolonged TCR stimulation during the infection [94]. Although the linear differentiation model had been perfectly fitted to interpret fate of T cell differentiation during chronic infection [95], it had a fatal limitation that only antigenic stimulation was considered to determine the fate of T cells.

2.2.4. Further perspective: new beginning at the effector precursor

To complement the linear differentiation model, it has been suggested that T cell fates are committed early after the activation by strength of signals, such as TCR, cytokines, and co-stimulatory molecules [96]. By these strength of signals at the early phase of infection, effector $CD8^+$ T cell fate is committed by each effector precursor with entirely different feature. Before the proposal for effector precursors, effector $CD8^+$ T cells were known to have homogenous functions and phenotypes (e.g. $CD44^{hi}$ and $CD11a^{hi}$) [97]. Later, effector $CD8^+$ T cells were categorized by expression of several differential molecules, such as KLRG1 and CD127 (IL-7R), since then effector $CD8^+$ T cells need to be classified into two major subsets as memory precursor effector cells (MPEC; $KLRG1^{lo}CD127^{hi}$) and short-lived effector cells (SLEC; $KLRG1^{hi}CD127^{lo}$) [98, 99]. It has been demonstrated that, at the peak of $CD8^+$ T cell response during the acute infection, 5~10% of effector $CD8^+$ T cells highly express IL-7R (IL-

7R^{hi}) with better memory potential than cells expressing low IL-7R (IL-7R^{lo}) [99-101]. Therefore, based on the expression level of IL-7R, effector CD8^{+} T cells can be categorized into MPEC and SLEC. The MPECs are relatively smaller subsets within the effector CD8^{+} T cells and not fully committed to long-term memory cells, even they possess certain effector functions [96]. MPECs have properties to produce more IL-2 and have better multi-potency, especially be differentiated into long-term memory cells, than SLECs [102-104]. On the other hand, SLECs are the most abundant subsets among effector CD8^{+} T cells, and most of them are going to die after effector period of the infection [54]. Because SLECs are terminally differentiated status within effector CD8^{+} T cells, it is conceivable that SLECs are more sensitive to cell death than MPECs and no longer be able to differentiate into long-term memory cells (Figure 2) [98, 104].

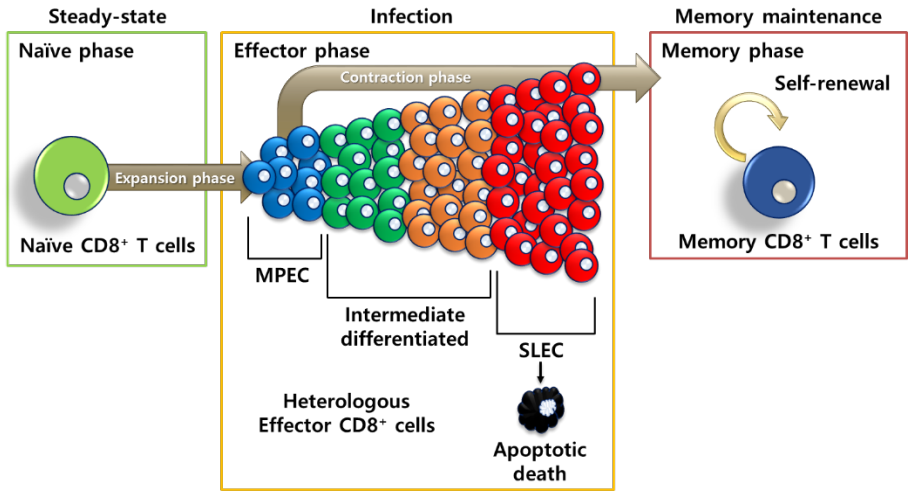


Figure 2. Cartoon for effector precursor differentiation model.

Then it had been wondered which transcription factors could determine the fate of the effector $CD8^+$ T cells. It was reported that mTOR is one of the most crucial factor to decide fate of effector $CD8^+$ T cells through the experiment that to administrate rapamycin into mice by drinking water has enhanced generation of memory $CD8^+$ T cells in LCMV infection model [105]. Since then, it has been broadly accepted that reduction of mTOR signals could enhance the generation of memory $CD8^+$ T cells. Since mTOR signals were provoked by TCR signals, several studies suggested the strength of TCR signals at the early period of infection is the key factor for the commitment of memory cells during the T cell fate decision [106-108]. Together with issue of TCR engagement, there are key transcription factors to regulate differentiation of effector precursor for MPEC and SLEC [109, 110]. MPECs appeared to require IL-7, IL-15 to maintain their specific transcription factors, such as Eomes, Bcl-6, ID3, and STAT3 etc. [111-116]. Whereas, it has been reported that pro-inflammatory cytokine cues (e.g. IL-2, IL-12, and type I IFN) are required to SLEC commitment and induce the transcription factors, such as T-bet, Blimp-1, ID-2, and STAT4 [46, 47, 98, 117, 118].

One of the most important defense mechanisms of $CD8^+$ T cells against foreign antigens is to generate memory cells that can respond quickly to the same antigens they have experienced. Therefore, numerous studies are designed to find factors related to the mechanism of memory differentiation and to answer how to generate many memory cells within immunized host. At the end of this effort, they could define an effector precursor model and identify several

external factors that could affect MPEC and SLEC differentiation. However, it is still unknown which factors in naïve $CD8^+$ T cells can predict their differentiation into MPEC or SLEC.

In conclusion, future studies should demonstrate the following: 1) A classification scheme for naïve $CD8^+$ T cell subsets should be established. 2) Factors influencing naïve $CD8^+$ T cell subset formation and characterization should be identified. 3) The classification scheme should be used to demonstrate which naïve $CD8^+$ T cells can differentiate into effector precursors specialized for long-term memory cell formation.

3. Research rationale

The fate of $CD8^+$ T cell at the naïve stage is passing through effector phase and a memory $CD8^+$ T cell, at which each phase undergoes a significant change. Peripheral naïve $CD8^+$ T cells developed in the thymus are transformed into high-heteorogeneity populations through the process of survival, self-renewal and post-thymic maturation by continuous self-TCR and homeostatic cytokine signals. The naïve $CD8^+$ T cells, which have a high-heterogeneous pool, participate in various immune responses provoked in hosts with certain condition such as infection, autoimmune disease and cancer.

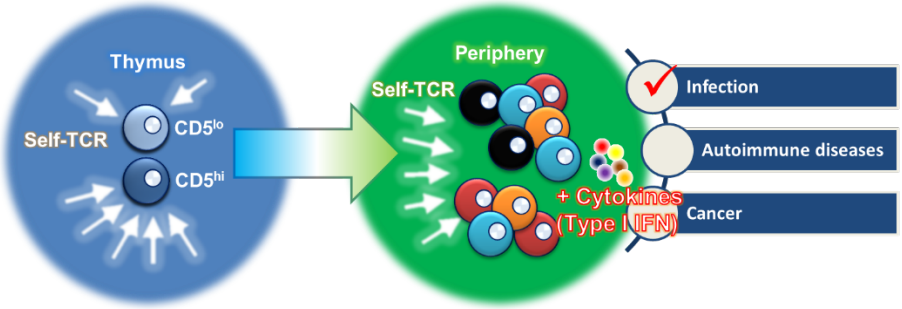


Figure 3. Cartoon for summary of research rationale.

Based on this, the hypothesis of this study was set to "Self-TCR reactivity and exposure to constitutive type I IFN can highly impact to naïve $CD8^+$ T cell pool and its functionality in LCMV infection". By verifying the hypothesis, I finally proposed the effects of self-reactivity and constitutive type I IFN on the differentiation of naïve $CD8^+$ T cells into long-term memory $CD8^+$ T cells in the LCMV infection model

II. Introduction

The contents herewith will be published elsewhere

as a partial fulfillment of Young-Jun Ju's Ph.D. program

Naïve $CD8^+$ T cells, regard as a guardian against foreign antigen after becoming active cytotoxic $CD8^+$ T cells, go through clonal expansion, differentiation, and then finally maintained to long-term memory CD8 T cells [109]. It has been well known that ~1 million of naïve $CD8^+$ T cells egress from thymus to periphery until before mice arrived to thymic atrophy, and they are maintained to ~25 million cells of naïve $CD8^+$ T cell pool which contains to ~100 microbial peptide specific cells per every clone in mice [60].

Those $CD8^+$ T cells in the pool commonly express CD44 and CD62L molecules. The high and low levels of CD44 molecules can define the $CD8^+$ T cells as "naïve ($CD44^{lo}$)" and "memory phenotype ($CD44^{hi}$; MP)", and the MP is also divided into central MP (T_{CM}; $CD44^{hi}CD62L^{hi}$) and effector MP (T_{EM}; $CD44^{hi}CD62L^{lo}$) based on the expression level of CD62L [61]. Together with CD44 and CD62L, expression level of CD5 has been studied to divide naïve $CD8^+$ T cells into more subsets.

During the thymic selection, CD8 single-positive (SP) cells express broad spectrum of CD5 molecules gained by entire avidity of self-peptide loaded

MHC (self-MHC) and TCR [58]. While CD44 can be easily upregulated and CD62L be downregulated upon proliferation of $CD8^+$ T cells, CD5 designated during thymic selection is known to have stable expression. CD5 expression stably maintained via continuous contact with peripheral self-MHC, suggesting that the level of its expression represents to TCR sensitivity of naïve $CD8^+$ T cells [58, 119]. For the last decade, it has been broadly accepted that CD5 high expressing T cells ($CD5^{hi}$) have better response than CD5 low expressing T cells ($CD5^{lo}$) to lymphopenia-induced homeostatic proliferation [88]. When compared with responsiveness to foreign antigens, such as *Listeria monocytogenes* and lymphocytic choriomeningitis virus (LCMV), $CD5^{hi}$ cells are more proliferative than $CD5^{lo}$ cells [89-91].

The studies have postulated different reasons why $CD5^{hi}$ cells are more reactive than $CD5^{lo}$ cells. 1) $CD5^{hi}$ cells have a more heterogeneous population which contains cells ready to be activated than those of $CD5^{lo}$ cells [91]. 2) $CD5^{hi}$ cells have higher T cell receptor (TCR) binding affinity for foreign antigen epitopes than $CD5^{lo}$ cells [89]. 3) $CD5^{hi}$ cells have higher signaling intensity for the same stimulus than $CD5^{lo}$ cells [90]. However, it remains to be answered what makes $CD5^{hi}$ cells different from $CD5^{lo}$ cells, and whether CD5 can fully classify heterogeneous naive $CD8^+$ T cells.

Type I IFN produced constitutively in mice after the exposure to pathogen, tissue remodeling and damage. It has been well-reported that constitutively produced type I IFN affects to biological function of immune cells (e.g., proliferation of hematopoietic stem cells [31, 32], developments of NK and B

cells [33, 34], function and homeostasis of macrophages [35, 36]). Most studies on T cell have addressed the role of type I IFN in activating or activated T cells. But, recently, it was reported that absence of type I IFN signaling strongly affects the phenotype, function and age-dependent expansion of $CD44^{hi}CD49d^{lo}$ virtual memory (VM) $CD8^+$ T cells via eomesodermin (eomes)-dependent fashion [37]. And CD4 single-positive (CD4SP) and CD8 single-positive (CD8SP) thymocytes in the type I IFN signal deficient mice showed their phenotypic abnormality, including reduced absolute cell number, and low expression of STAT1 during final maturation of thymocytes [10]. Even though aforementioned studies have partially revealed on importance of type I IFN in the development of $CD8^+$ T cells and thymocytes, any phenotypic or functional changes of peripheral T cells due to type I IFN deficiency are not fully understood. Thus, it needs to be unveiled whether the constitutive type I IFN in the steady-state, uninfected, condition affects functional modification of peripheral naïve $CD8^+$ T cells.

Recent studies dealing with functionality of $CD8^+$ T cells have suggested the model defines two types of effector precursor named as SLEC (short-lived effector cell) expressing low KLRG1 (killer cell lectin like receptor G1) and high CD127 (IL-7Ra) and MPEC (memory precursor effector cell) expressing high KLRG1 and low CD127 [109]. It has been well-reported that SLEC is very sensitive to cell death, has low pluripotency and low IL-2 production capacity, whereas MPEC has strong resistance to cell death, high pluripotency and high IL-2 production capacity [120]. As mentioned above, because these two

effector precursors have quite distinct feature, fate decision of effector $CD8^+$ T cells between SLEC and MPEC has been one of the most important issues to understand the functionality of $CD8^+$ T cells. Several studies have found out the transcription factors regulate the differentiation of SLEC or MPEC. It has been suggested that IL-2, IL-12, and type I IFN stimulation provoked in microbial infection actively induce SLEC with upregulation of T-bet, Blimp-1, and Zeb2 [117, 121-123]. In addition, the inflammatory cytokines suggested above downregulates transcription factors important for MPEC differentiation such as Il7r, Sell, Tcf7, Lef1, and Bach2 by inhibiting FOXO1 signal, and consequently leads to SLEC differentiation [124-127].

In this study, the cells were distinguished using CD5 level which has been known to represent self-reactivity within naïve $CD8^+$ T cells, and the analysis were performed to check differences in signaling intensity and phenotype for type I IFN signals depending on the level of CD5 expression. It was confirmed that these difference in signaling intensity and phenotype confers genetic features that are more responsive to external stimuli of naive $CD8^+$ T cells, especially in response to pro-inflammatory cytokine stimulation. Also the LCMV infection model was used to determine whether the genetic features and responsiveness to cytokines could affect the immune response to foreign antigens. In the LCMV infection model, it was analyzed whether the differentiation of effector precursor could vary depending on the expression level of CD5 and the effect of constitutive type I IFN exposed during the steady-state period.

Consequently, the objective of this study is to investigate the role of constitutive type I IFN based on difference of self-reactivity in naïve $CD8^+$ T cells.

III. Materials and Method

Mice

C57BL/6 (B6) mice were provided from animal facility of POSTECH purchased from Jackson laboratory. P14, OT-1, IFNAR1$^{-/-}$, STAT1$^{-/-}$, IFNGR$^{-/-}$, and RAG1$^{-/-}$ mice were provided from Garvan Institute of Medical Research (Australia). Then, IFNAR1-/- x IFNGR-/-, P14 IFANR1-/-, P14 Rag1-/- mice were bred using each pure strain. All animal experiments were performed following regulation by IACUC guideline of POSTECH and IBS (institute for basic science).

Reagents and antibodies

Recombinant mouse IL-2, IL-4, IL-6, IL-7, IL-12, IL-15, IL-21, IL-23, IFN-γ and TGF-β were purchased from PeproTech. Mouse IFN-β was from PBL Biomedical Laboratories. Peptides (KAVYNFATM (GP33) was purchased from Bioneer. All of the following monoclonal antibodies (mAbs) was purchased from eBioscience and BD Biosciences. Anti-CD3ε (145-2C11), anti-CD4 (RM4-5), anti-CD8α (53-6.7), anti-CD5 (53-7.3), anti-CD24 (1M/69), anti-CD25 (PC61.5), anti-CD27 (O323), anti-CD28 (37.51), anti-CD38 (90), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD45RA (HI100), anti-CD45RB (C363.16A), anti-CD62L (MEL-14), anti-CD90 (5E10),

anti-CD90.1 (HIS51), anti-CD90.2 (53-2.1), anti-CD98 (RL388), anti-CD103 (2E7), anti-CD122 (5H4 and TM-β1), anti-CD123 (6H6), anti-CD124 (X2/45-12), anti-CD126 (D7715A7), anti-CD127 (A7R34), anti-CD130 (KGP130), anti-CD132 (TUGm2 and 4G3), anti-CD183 (CXCR3-173), anti-Ly6C (HK1.4), anti-TCRβ (H57-597), anti-β2 (TS1/18), anti-β7 (FIB504), anti-GITR (DTA-1), anti-S1P (JM16-66), anti-IFNAR1 (13222-MM08-P; SinoBiological), anti-KLRG1 (2F1), anti-CX3CR1 (2A9-1), and anti-Ki67 (SolA15). Flow cytometry samples were run using a LSRII (customized to use 5 laser), LSRII (4 laser) or FACSCanto II (BD Biosciences) and analyzed by FlowJo software (Tree Star).

T cell preparation, *in vitro* Ly6C induction, and *in vivo* proliferation assay.

Cells were purified from pooled lymph nodes (LN) or spleen (SP) following sorting procedures referred in below. For *in vitro* culture, sorted cells were plated in 96-well plates in complete RPMI 1640 medium. The cultures were supplemented with the cytokines, such as IFN-β (as indicated), IL-2 (10 ng/ml), IL-4 (10 ng/ml), IL-6 (10 ng/ml), IL-7 (10 ng/ml), IL-12 (10 ng/ml), IL-15 (10ng/ml), IL-21 (10 ng/ml), TGF-β (10 ng/ml), and IFN-γ (10 ng/ml), or soluble anti-CD3 mAb (1μg/ml). For proliferation analysis *in vivo* using CTV, purified naive $CD8^+$ T cells were labeled with CTV (CellTrace™ Violet) (ThermoFisher) as described [88]. Cell proliferation was analyzed by measuring CTV dilution using flow cytometry.

Intracellular staining.

For intracellular cytokine staining, cells were cultured in vitro with the indicated stimuli (PMA (100 ng/ml), Ionomycin (1000 ng/ml), IL-2 (500 ng/ml), IL-12 (10 ng/ml), IL-18 (10 ng/ml), gp33 peptide (as indicated)), then GolgiPlug (BD Biosciences) was added during the last 5 hr of culture. The cells were stained for cell surface markers, fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences) and then stained with anti-IFN-γ (XMG1.2), anti-IL-2 (JES6-5H4), anti-TNFα (MP6-XT22) using Perm/Wash buffer (BD Biosciences) followed by analysis by flow cytometry; intracellular CD107a and granzyme B staining was examined using anti-CD107a (1D4B) and anti-human granzyme B mAb (GB11) (ThermoFisher).

Immunoblotting.

FACS-purified $CD5^{lo}$, CD5hiLy6C^{-}, and CD5hiLy6C^{+} subsets were incubated with IFN-β (0.1 ng/ml) for indicated time, cell lysates (Tris-Cl 50 mM, pH 7.4, NP-40 0.5%, 0.15 M NaCl, 2 mM EDTA, 10 mM NaF, 10 mM P2O7, 0.5 μM Na3VO4, 100 μg/ml PMSF, 1 μg/ml aprotinin and leupeptin) were prepared and equal amounts of protein were analyzed by 10% SDS-PAGE, transferred onto nitrocellulose membrane (Amersham), blocked for 1 h at 23°C in blocking buffer (5% dry nonfat milk in Tris buffered saline, pH 7.4, containing 0.05%

Tween 20), and immunoblotted with the following monoclonal Abs: phospho-Stat1, phospoho-Stat2. After immunoblotting with these Abs, immunodetection was performed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) and developed with an ECL kit (Amersham).

$CD8^+$ T cell enrichment

Generally, $CD8^+$ T cell enrichment was done by negative selection method of MACS or BD IMag $CD8^+$ enrichment kit. In polyclonal LCMV infection experiment, we used $Thy1.1^+$ host mice and $CD8^+Ly5.1^+$ donor cells, so primarily we excluded $CD4^+$ and $B220^+$ cells from total splenocytes by MACS negative selection kit using anti-CD4, anti-B220, and then secondly excluded host cells by anti-Thy1.1 because only host cells were expressing Thy1.1.

Cell sorting and adoptive transfer

Cell sorting for all experiments was done by Astrios or XDP (Beckman Coulter). To sort, naïve $CD8^+$ T cells were purified from pooled lymph nodes (LN) or spleen (SP). Generally, we sorted cells to isolate pure $CD5^{lo}$, $CD5^{hi}Ly6C^-$, and $CD5^{hi}Ly6C^+$ subsets. In some experiment, we further sorted to isolate $CD5^{lo}$, $CD5^{hi}Ly6C^-$, $CD5^{hi}Ly6C^+CD183^-$, and $CD5^{hi}Ly6C^+CD183^+$ subsets. Purity of sorted cells was routinely tested after sorting and was >98%. Detailed

procedure of each experiment was done by information indicated in each Figure cartoon and Figure legend.

BrdU incorporation assay

Assay in expansion phase of LCMV infection, we injected to BrdU (1 mg/mice) by intraperitoneal route at 2 hours before sacrificing the mice [47]. For the memory phase of LCMV infection, we fed BrdU (1 mg/ml) by drinking water for 7-10 days before sacrificing the mice. Assay was done by using a BrdU assay kit (eBioscience) according to the instruction provided by company.

Infection

LCMV Armstrong and LM-gp33 was provided by Dr. SangJun Ha (Yonsei University, Korea). Propagation and titration of each pathogen was done by ourselves following general protocol [128, 129]. Mice were infected by i.p. route with $2x10^5$ pfu/mice LCMV Armstrong, and by i.v. with $1\text{-}2x10^4$ cfu LM-gp33. And anti-NK1.1 (300 μg/mice) was injected once by i.p. route to host mice at the day of LCMV infection when IFNAR1$^{-/-}$ CD8$^+$ T cells were used [48].

Administration of anti-IFNAR1 antibody *in vivo*

Anti-IFNAR1 blocking antibody (MAR1-5A3) was purchased from BioXCell. In blocking experiment, mice were injected with the antibody for 7-10 days (200-300 μg/once/mice, every 2-3 days) by i.p. route.

RNA preparation and sequencing

Cells from spleens and lymph nodes were sorted by flow cytometry. Over 1 × 10^6 sorted cells were used for RNA extraction with an NucleoZOL (Macherey-Nagel). Biotinylated cRNA were prepared from 0.55 μg total RNA using the Illumina TotalPrep RNA Amplification Kit (Ambion). Following fragmentation, 0.75 μg of cRNA were hybridized were to the Illumina Expression Beadchip according to the protocols provided by the manufacturer.

Real-time PCR

Cells from lymph nodes or thymus were sorted by flow cytometry. Over 2 x 10^5 sorted cells were used for RNA extraction with NucleoZOL (Macherey-Nagel) and stored at -80℃ before the further steps. Isolation of mRNA was done according to the manufacturer's' instruction. cDNA synthesized with the M-MLV reverse transcriptase and oligo dT (TAKARA). Real-time RT-PCR is done with the TaqMan Gene Expression Master Mix using StepOnePlus Real-Time PCR System (Applied Biosystems) with TaqMan probes. The following TaqMan probes (Applied Biosystems) were used: *Tbx21* (Mn00450960_m1),

Eomes (Mm01351984_m1), *IL-18Rap* (Mm00516053_m1), and *CCL5* (Mm01302427_m1).

Metabolic assay

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed using a 96-well XF Extracellular flux analyzer, according to the manufacturer's instruction (Seahorse Bioscience). SRC, OCR/ECAR ratios were defined as previously described [130, 131].

Statistical analysis

Unless indicated in the figure legend, a two-tailed, unpaired Student's t-test was performed on log-transformed data with Prism (GraphPad Software). For multiple comparisons, one-way ANOVA with Dunnett's multiple-comparison post-test was used.

IV. Results

Naïve CD8$^+$ T cells with high CD5 expression constitutively express Ly6C

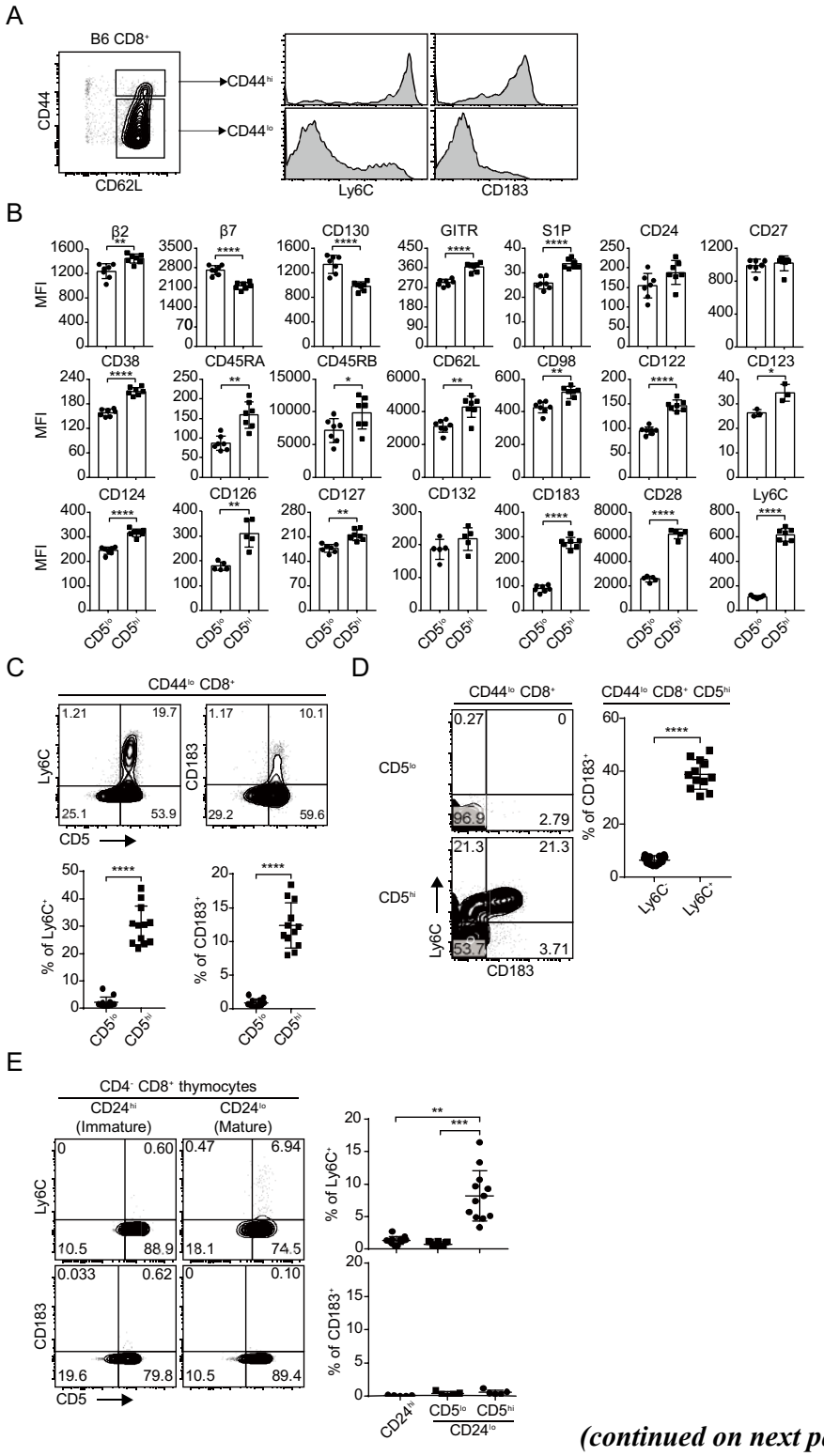
It has been noted that naïve CD8$^+$ T cells express broad spectrum of CD5 level, of which the higher CD5 expressing cells the stronger reactivity to both self and foreign antigens. Beside high CD5 expressing cells (CD5^{hi}) include more pre-activated heterogenic population than those of low CD5 expressing cells (CD5^{lo}). To find appropriate markers to further classify heterogenic CD5^{hi} cells, expression pattern of surface molecules on CD44^{lo}, CD44^{lo}CD5^{lo} and CD44^{lo}CD5^{hi} naïve CD8$^+$ T cells was analyzed. Naïve CD8$^+$ T cells, in spleen, expressed Ly6C and CD183, which are known as molecules expressed on activated or memory CD8$^+$ T cells (Figure 4A). When screening the surface molecule expression of CD44^{lo}CD5^{lo} and CD44^{lo}CD5^{hi} cells, β2, β7, CD130, GITR1, S1P, CD38, CD45RA, CD45RB, CD62L, CD98, CD122, CD123, CD124, CD126, CD127, CD183, CD28, and Ly6C were expressed significantly differently between CD44^{lo}CD5^{lo} and CD44^{lo}CD5^{hi} cells (Figure 4B). Especially, Ly6C and CD183 were highly expressed on CD5^{hi} cells only, not CD5^{lo} cells (Figure 4C). And most of the cells expressing CD183 appeared in the subset with CD5^{hi}Ly6C$^+$ phenotype (Figure 4D). These results suggested that peripheral naïve CD8$^+$ T cells can be classified to CD5^{lo}, CD5^{hi}Ly6C$^-$, and CD5^{hi}Ly6C$^+$(CD183$^-$ and CD183$^+$).

Next, to know whether $CD5^{hi}Ly6C^{+}$ subsets were generated from thymus, Ly6C and CD183 expressing cells were analyzed from thymus of adult mice. In consistent with peripheral naïve $CD8^{+}$ T cells, $Ly6C^{+}$ cells were found in only $CD5^{hi}$ fraction of $CD24^{lo}$ mature thymocytes. In contrast, there was no $CD183^{+}$ cells in both $CD5^{lo}$ and $CD5^{hi}$ cells of mature thymocytes (Figure 4E). To confirm such discrepancy was induced by age-dependent thymic atrophy, spleen and thymus were obtained from neonatal and over 1-year-old mice. The results showed that around 5-10% of $CD5^{hi}Ly6C^{+}$ cells were constantly generated from the thymus during neonatal period while there was more $CD5^{hi}Ly6C^{+}$ cells in spleen than those being generated from thymus (Figure 4F). In consistent with results from adult thymus, a few $CD5^{hi}CD183^{+}$ cells were detected from the thymus during neonatal period (Figure 4G).

Next, the question was if $CD5^{hi}Ly6C^{+}$ cells were induced from thymus and egressed out to the periphery, the $CD5^{hi}Ly6C^{+}$ cells might be accumulated in periphery with age. Interestingly, inconsistent with the expectation, there was a comparable proportion of $CD5^{hi}Ly6C^{+}$ cells in secondary lymphoid organs of young and old mice (Figure 4H). Thus I asked whether biased generation of $Ly6C^{+}$ cells within $CD5^{hi}$ fraction was forced by clonal differences of T cell. At this end, the expression of TCR alpha and beta within three naïve $CD8^{+}$ T cells subsets, CD5lo, $CD5^{hi}Ly6C^{-}$, and $CD5^{hi}Ly6C^{+}$, were examined. The result showed that the expression of TCR alpha and beta chain was comparable among three naïve $CD8^{+}$ T cell subsets (Figure 4I).

Collectively, the results suggested that naive $CD8^{+}$ T cells can be classified

based on the expression of CD5 as $CD5^{lo}$ and $CD5^{hi}$ cells, and the $CD5^{hi}$ cells can be further classified by the expression of Ly6C and CD183. Therefore, naïve $CD8^{+}$ T cells could have been classified to three subsets as $CD5^{lo}$, $CD5^{hi}Ly6C^{-}$, and $CD5^{hi}Ly6C^{+}$(including $CD183^{-}$ and $CD183^{+}$) cells. And $CD5^{lo}$, $CD5^{hi}Ly6C^{-}$, and $CD5^{hi}Ly6C^{+}$ cells are stably and continuously produced in the thymus and migrated to periphery.



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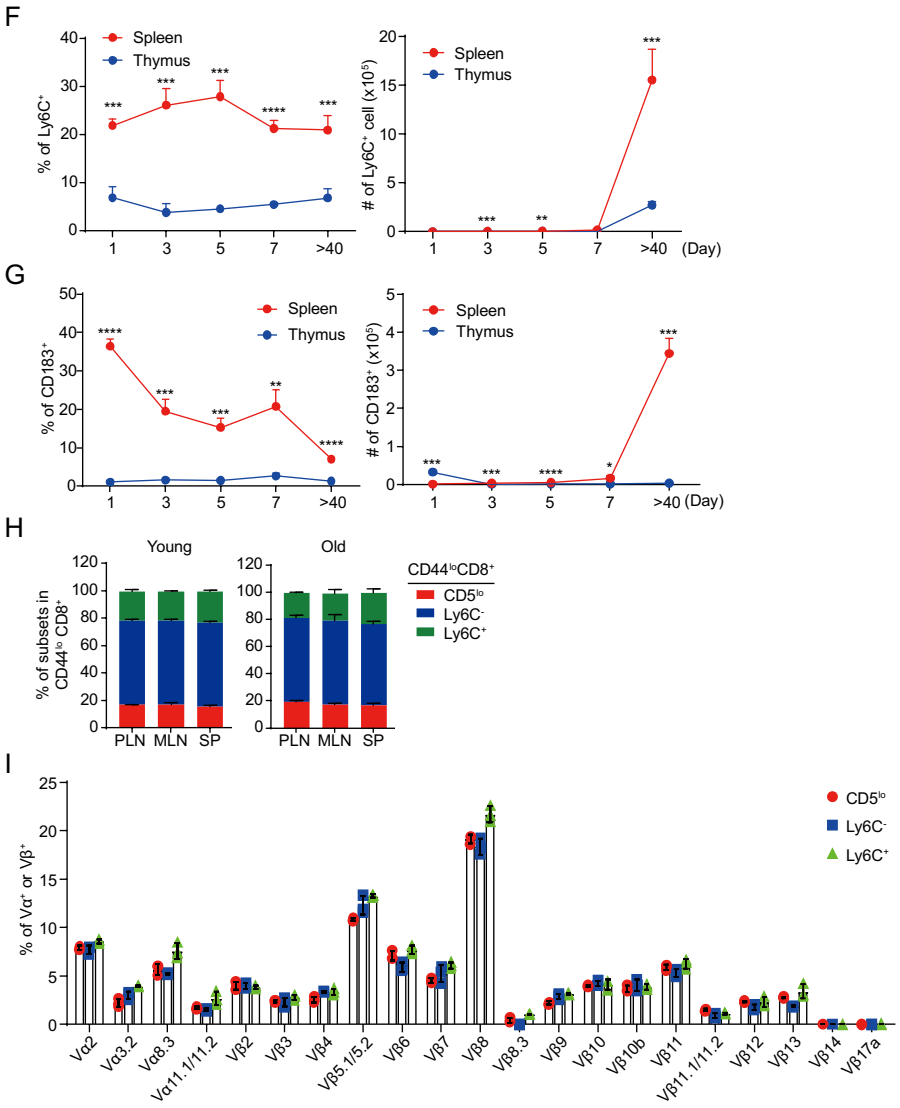


Figure 4. Naïve CD8$^+$ T cells maintain heterogeneous phenotypes developed following their CD5 expression.

Cells were analyzed by flow cytometry isolated from indicated organs. (A) Differential expression of surface molecules in CD44^{lo} and CD44^{hi} CD8$^+$ T cells. (B) Expression of surface molecules differently expressed in CD44^{lo}CD5^{lo} and CD44^{lo}CD5^{hi} naïve CD8$^+$ T cells. (C) Sub-population of naïve CD8$^+$ T cells

classified by differential expression of several surface molecules on highly CD5 expressing ($CD5^{hi}$) naïve $CD8^+$ T cells. (D) Proportion of $CD183^+$ cells in $Ly6C^-$ and $Ly6C^+$ cells. (E) Generation of $CD5^{hi}Ly6C^+$ and $CD5^{hi}CD183^+$ cells in thymus. (F-G) Proportional changes of (F) $CD5^{hi}Ly6C^+$ and (G) $CD5^{hi}CD183^+$ cells in spleen and thymus during neonatal period. (H) Existence of naïve $CD8^+$ T cell subsets in young and aged mice. (I) Expression of several TCR variable chain repertoire in naïve $CD8^+$ T cell subsets. Data are representative of at least two independent experiments. Unpaired Student's t-test was used for the statistical analysis. *P<0.05; **P<0.01; ***P<0.005 ****P<0.001.

Generation of Ly6C^{+} subsets is regulated by constitutive type I IFN and self-TCR engagement

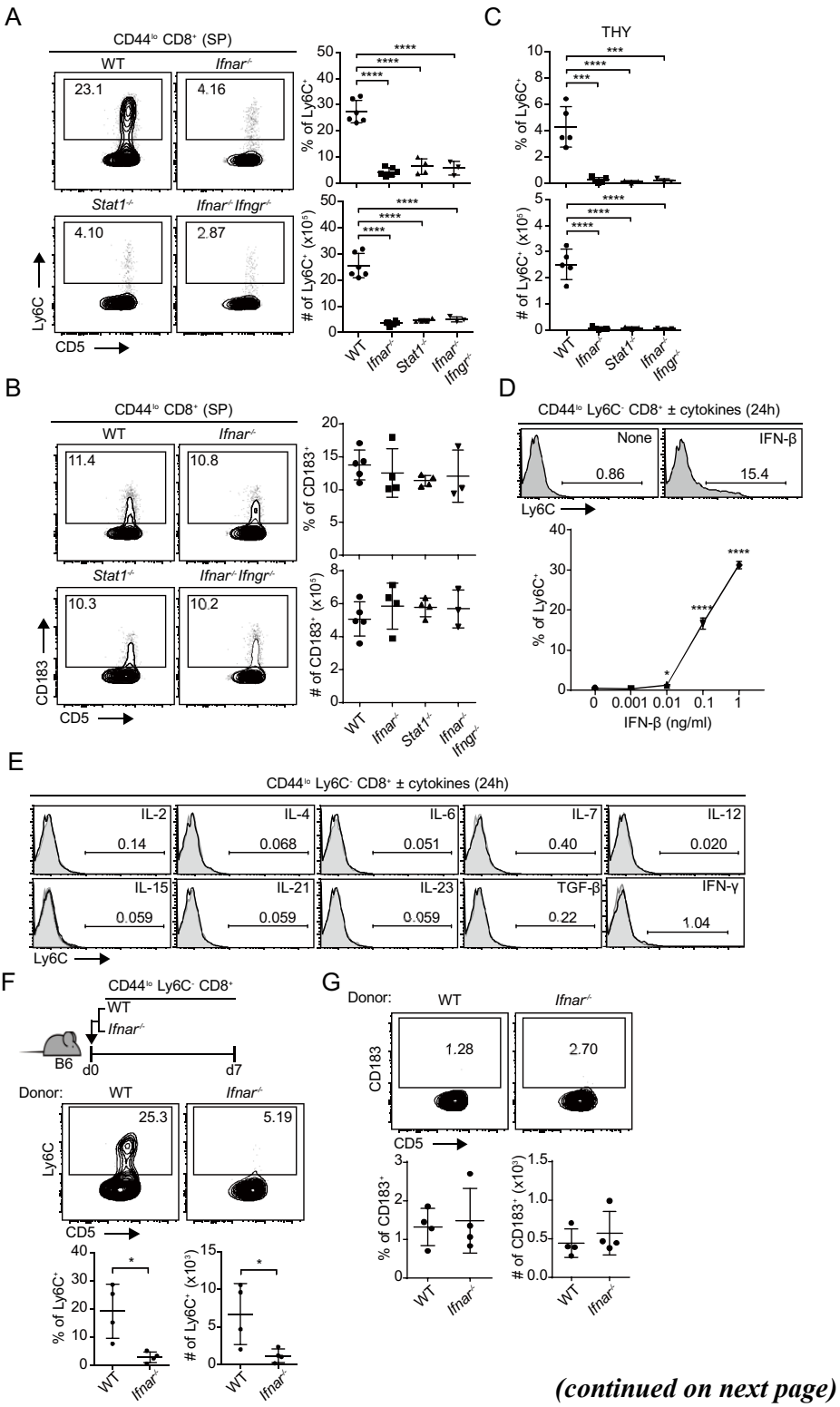
It has been suggested that the Ly6C can be upregulated on T cells stimulated with IFN-β [132-134]. Therefore, to investigate whether type I IFN is essential for the generation of CD5^{hi}Ly6C^{+} cells, their expression was analyzed in several kinds of type I IFN signal-deficient mice. CD5^{hi}Ly6C^{+} cells were almost disappeared in Ifnar1$^{-/-}$, Stat1$^{-/-}$, and Ifnar1$^{-/-}$Ifngr$^{-/-}$ mice compared to WT (B6) mice (Figure 5A), whereas there were comparable CD5^{hi}CD183^{+} cells in the type I IFN signal-deficient mice (Figure 5B). Furthermore, it was evident that, in thymus, type I IFN is an essential factor for the generation of CD5^{hi}Ly6C^{+} cells (Figure 5C). To confirm whether type I IFN can directly induce Ly6C molecules to naïve CD8^{+} T cells, induction of Ly6C was examined from purified Ly6C^{-} naïve CD8^{+} T cells cultured with IFN-β or other cytokines. The results clearly showed that IFN-β can induce Ly6C in a dose-dependent manner (Figure 5D), but not by other cytokines, including IFN-γ (Figure 5E). Because it was shown that proportion and absolute number of CD5^{hi}Ly6C^{+} cells are higher in spleen compared to thymus after the birth (Figure 4F), it could be hypothesized that type I IFN-dependent Ly6C induction happened not only in thymus but also in periphery. To prove the hypothesis, purified Ly6C^{-} naïve CD8^{+} T cells from WT and Ifnar1$^{-/-}$ mice were co-transferred to B6 host, then the donor cells were analyzed at 7 days after the transfer. The results showed Ifnar1$^{-/-}$ Ly6C^{-} donor cells could not express Ly6C, while WT Ly6C^{-} donor expressed Ly6C in periphery (Figure 5F). Meanwhile, there was no difference

in CD183 expression between WT and Ifnar1$^{-/-}$ Ly6C^{-} donor cells (Figure 5G). Also, when the cells were sorted into CD5^{lo} Ly6C^{-} and CD5^{hi}Ly6C^{-} were cultured with IFN-β, CD5^{hi}Ly6C^{-} cells showed much better Ly6C expression than those of CD5^{lo} cells in both protein (Figure 5H) and mRNA (Figure 5I). In addition, CD5^{hi}Ly6C^{-} cells showed higher phosphorylation of STAT1 and STAT2 upon IFN-β stimulation than that of CD5^{lo} cells. Based on these results, it showed that CD5^{hi}Ly6C^{-} cells have higher IFN-β sensitivity than CD5^{lo} cells (Figure 5J).

While it was proven that CD5^{hi}Ly6C^{+} cells are generated by constitutive type I IFN in periphery, it was still remaining to question what is the role of self-TCR contact to Ly6C induction. So it was hypothesized if the self-TCR engagement is correlated to Ly6C induction, CD5^{hi}Ly6C^{-} cells might be influenced by self-TCR signal better than CD5^{lo} cells because it had been broadly accepted that the CD5 level in periphery represents to self-TCR reactivity [58, 88]. To prove the hypothesis Ly6C^{-} naïve CD8^{+} T cells were transferred to WT and TAP1$^{-/-}$ host mice respectively, then Ly6C induction was analyzed on the donor cells at 5 days after the transfer. The result showed that there was significantly less induction of Ly6C^{+} subset in the donor cells in TAP1$^{-/-}$ mice than those in WT (Figure 5K). Also, when the Ly6C^{-} naïve CD8^{+} T cells were cultured with IFN-β and soluble anti-CD3 antibody, type I IFN-dependent Ly6C induction was enhanced (Figure 5L). In addition, positive role of self-TCR engagement was confirmed by competitive Ly6C induction. The Ly6C^{-} cells purified from B6, P14 and OT-1 mice were mixed, then adoptively transferred to another B6 and

P14 host mice. The highest Ly6C induction was observed in OT-1 donor cells for 7 days in B6 host mice, followed by P14 and B6 donor cells. It suggested that because, in the naïve $CD8^+$ T cell pool of B6 host mice, the frequency of clones competing with OT-1 and P14 donor cells for self-TCR contact is very small, the OT-1 and P14 donor cells may be received a relatively high intensity of self-TCR contact, and generate more $Ly6C^+$ cells than that of B6 donor cells. On the other hand, the result from P14 host mice showed the P14 donor cells gain $Ly6C^+$ just as much as that of B6 donor cells (Figure 5M). It suggested that because, in the naïve $CD8^+$ T cell pool of P14 host mice, the frequency of clones competing with P14 donor cells for self-TCR contact is quite abundant, the P14 donor cells may be taken low intensity of self-TCR contact, and generate less $Ly6C^+$ cells than that of OT-1 donor cells.

Taken together, the generation of $CD5^{hi}Ly6C^+$ cells are tightly regulated by constitutively produced type I IFN in both thymus and periphery, and self-TCR engagement acts positively to type I IFN-dependent Ly6C induction.



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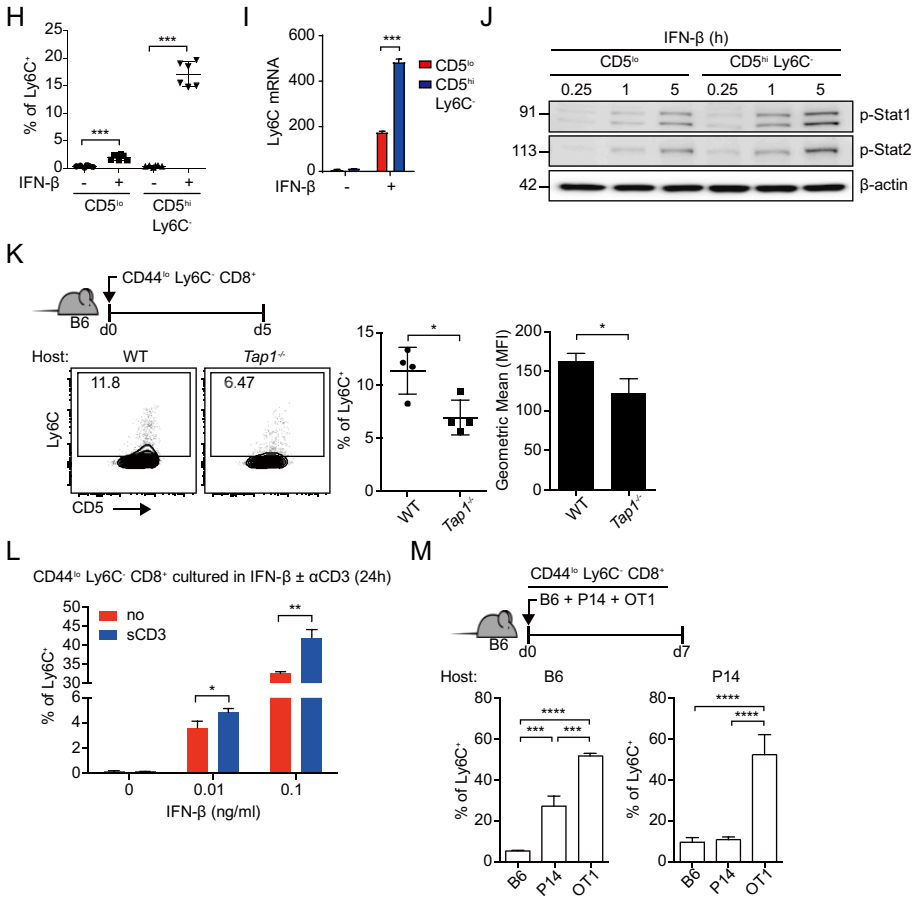


Figure 5. Generation of Ly6C$^+$ naïve CD8$^+$ T cells is tightly regulated by Type I IFN and self-TCR engagement.

(A-B) Cells were analyzed by flow cytometry isolated from indicated organs. (A) Ly6C$^+$ and (B) CD183$^+$ cells in SP from different Type I IFN signal deficient mice. (C) Ly6C$^+$ cells in THY from different Type I IFN signal deficient mice. (D-E) Induction of Ly6C$^+$ cells from CD44loLy6C- cells. CD44^{lo}Ly6C$^-$ cells were purified from pooled lymph node (LN) or SP and cultured with (D) IFN-β and (E) various homeostatic cytokines. (F-G) Induction of (F) Ly6C$^+$ and (G) CD183$^+$ cells from WT and Ifnar$^{-/-}$ CD44^{lo}Ly6C$^-$

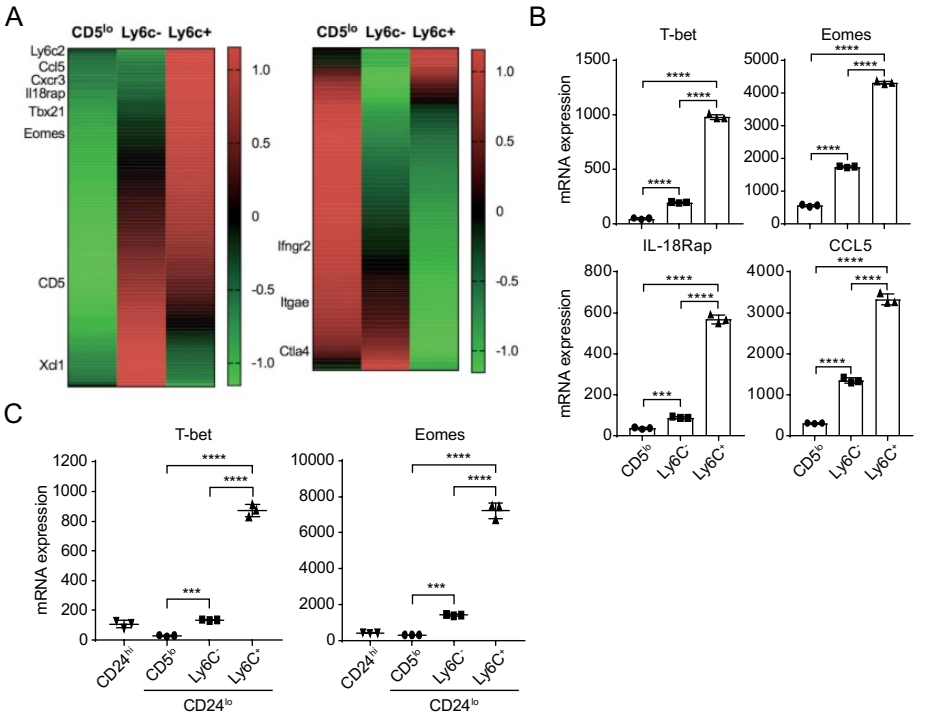
cells. $CD44^{lo}Ly6C^{-}$ cells were purified from LN or SP of WT (Thy1.1/1.1, Ly5.2/5.2) and $Ifnar^{-/-}$ (Thy1.2/1.2, Ly5.2/5.2) mice. Purified cells were mixed ($5x10^5$ cells/each, 1:1 ratio), and adoptively transfer to B6 host (Thy1.2/1.2, Ly5.1/5.1) for 7 days. (H-I) Comparison of Ly6C expression in $CD5^{lo}$ and $CD5^{hi}$ fraction of naïve $CD8^{+}$ T cells. $CD5^{lo}(Ly6C^{-})$ and $CD5^{hi}Ly6C^{-}$ cells were purified from pooled LN or SP of at least three C57BL/6 mice, then cultured with IFN-β. Induction of $Ly6C^{+}$ cells were analyzed by (H) flow cytometry and (I) quantitative real-time PCR. (J) Phosphorylation of Stat1 and Stat2 in $CD5^{lo}$ and $CD5^{hi}Ly6C^{-}$ cells treated with/without IFN-β were examined using Immunoblot assay. (K) Induction of $Ly6C^{+}$ cells in WT and $Tap1^{-/-}$ host mice. $CD44^{lo}Ly6C^{-}$ cells were purified from LN or SP of B6 mice ($Thy1.1^{+}$) and adoptively transferred to WT and $Tap1^{-/-}$ host for 5 days ($5x10^5$ cells/mice). Then, induction of $Ly6C^{+}$ cells were analyzed by flow cytometry. (L) Effect of weak TCR signals to induction of $Ly6C^{+}$ cells. $Ly6C^{-}$ cells were purified from LN, and cultured with IFN-β with/without soluble anti-CD3. (M) Induction of $Ly6C^{+}$ cells in B6 and P14 host mice. $CD44^{lo}Ly6C^{-}$ cells were purified from B6 (Ly5.1/5.1), P14 (Ly5.1/5.2), and OT1 (Thy1.1/1.1) mice and mixed at 1:1:1 ratio ($5x10^5$ cells each), and adoptively transfer to B6 (Ly5.2/5.2, Thy1.2/1.2) and P14 (Ly5.2/5.2, Thy1.2/1.2) host mice for 7 days. And then, induction of $Ly6C^{+}$ cells were analyzed by flow cytometry. Data are representative of at least two independent experiments. Unpaired Student's t-test was used for the statistical analysis. *P<0.05; **P<0.01; ***P<0.005 ****P<0.001.

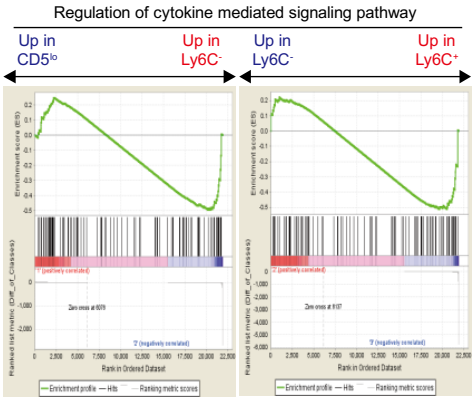
Type I IFN affects to unique genetic features of naïve $CD8^+$ T cell subsets

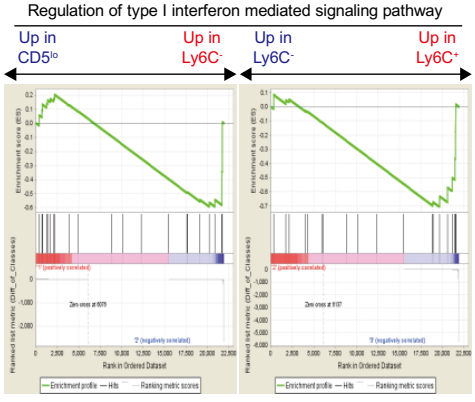
To understand further for genetic landscape, peripheral naïve $CD8^+$ T cells were analyzed by RNA-sequencing for $CD5^{lo}$, $CD5^{hi}Ly6C^-$ (in short, $Ly6C^-$), and $CD5^{hi}Ly6C^+$ (in short, $Ly6C^+$) subsets. The genetic landscape of each subset showed that $Ly6C^+$ cells express the highest level of genes related to T cell activation including *T-bet, Eomes, IL-18Rap,* and *Ccl5* followed by the order of $Ly6C^-$ and $CD5^{lo}$ cells, whereas the highest level of T cell inhibitory molecules, *Ctla4*, is expressed in $CD5^{lo}$ cells followed by $Ly6C^-$ and $Ly6C^+$ cells (Figure 6A). It was further confirmed by using quantitative PCR that $Ly6C^+$ cells express the highest level of *T-bet, Eomes, IL-18Rap,* and *Ccl5* followed by $Ly6C^-$ and $CD5^{lo}$ cells (Figure 6B). Especially, $Ly6C^+$ cells expressed the highest level of *T-bet* and *Eomes* followed by $Ly6C^-$ and $CD5^{lo}$ within $CD24^{lo}$ mature thymocytes (Figure 6C). It has been well defined that $CD5^{hi}$ naïve $CD8^+$ T cells have better responsiveness to γc cytokine, such as IL-2 and IL-7, than $CD5^{lo}$ naïve $CD8^+$ T cells [88, 91]. In similar line with the previous reports, by using gene set enrichment assay (GSEA), $Ly6C^-$ cells had better enrichment to regulation of cytokine-mediated signaling pathway than $CD5^{lo}$ cell, and, interestingly, $Ly6C^+$ cells showed even higher enrichment than $Ly6C^-$ cells (Figure 6D). In GSEA using gene sets of regulation of type I IFN-mediated signaling pathway and response to type I IFN, $Ly6C^+$ cells also showed the highest enrichment score to these gene sets followed by $Ly6C^-$ and $CD5^{lo}$ cells (Figure 6E and F).

To investigate how type I IFN affected to $CD5^{hi}$ subsets including $Ly6C^{-}$ and $Ly6C^{+}$ cells, up/down-regulated gene sets within $CD5^{hi}$ subsets were compared to well-established IFN-β responding gene set [135]. The results showed that up-regulated genes in $CD5^{hi}$ subset over $CD5^{lo}$ cells shared 99 genes with IFN-β responding gene set, and shared 73 down-regulated genes (Figure 6G).

Collectively, the results suggested that each naïve $CD8^{+}$ T cell subsets has a unique genetic landscape which have been obtained by sensitivity to constitutive type I IFN.







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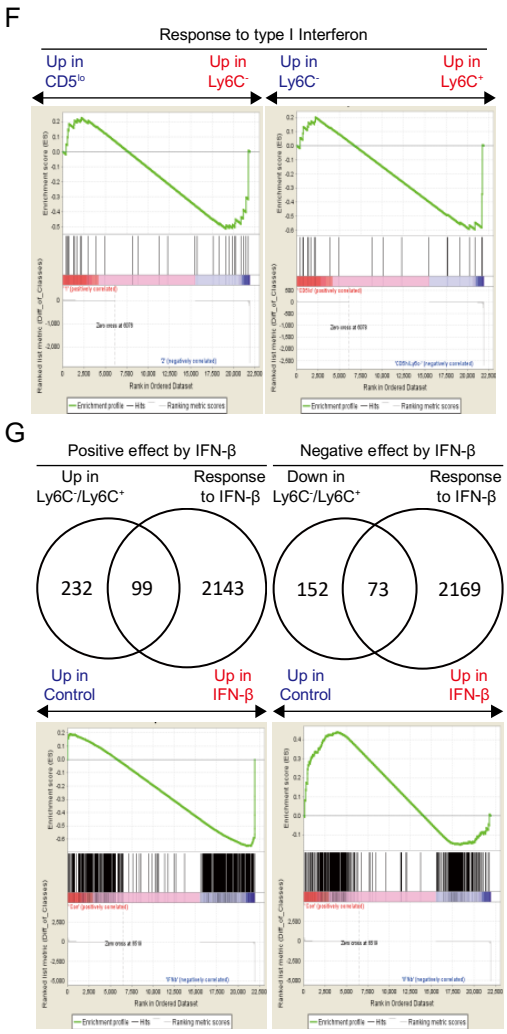


Figure 6. Naïve CD8 T cell subsets have distinguishable genetic landscape.

Expression levels of mRNA were measured by RNA-sequencing (A, D-G) and quantitative real-time PCR (B-C) of indicated sorted cells from B6 mice. (A) Total number of transcripts differentially expressed (up- or down-regulated; twofold cutoff) between the indicated comparison groups is shown. (B) Expression of highlighted genes in Ly6C^{+} cells (*T-bet, Eomes, IL-18Rap,* and *CCL5*) were measured in CD5^{lo}, Ly6C^{-}, and Ly6C^{+} cells isolated form pooled

LN. (C) Expression of highlighted genes in $Ly6C^+$ cells (*T-bet* and *Eomes*) were measured in $CD24^{hi}$, $CD24^{lo}CD5^{lo}$, $CD24^{lo}Ly6C^-$, and $CD24^{lo}Ly6C^+$ cells isolated form of THY. (D-F) Gene Set Enrichment Assay (GSEA) profile of indicated signature genes in $CD5^{lo}$ versus $Ly6C^-$ cells and $Ly6C^-$ and $Ly6C^+$ cells. (G) GSEA profile of indicated signature genes in IFN- β non-responding genes in $Ly6C^-$ and $Ly6C^+$ cells versus IFN- β responding genes in $Ly6C^-$ and $Ly6C^+$ cells. Data (B-C) are representative of at least two independent experiments. Unpaired Student's t-test was used for the statistical analysis. ***P<0.005 ****P<0.001.

Type I IFN enhances naïve CD8$^+$ T cell subsets to produce more IFN-γ

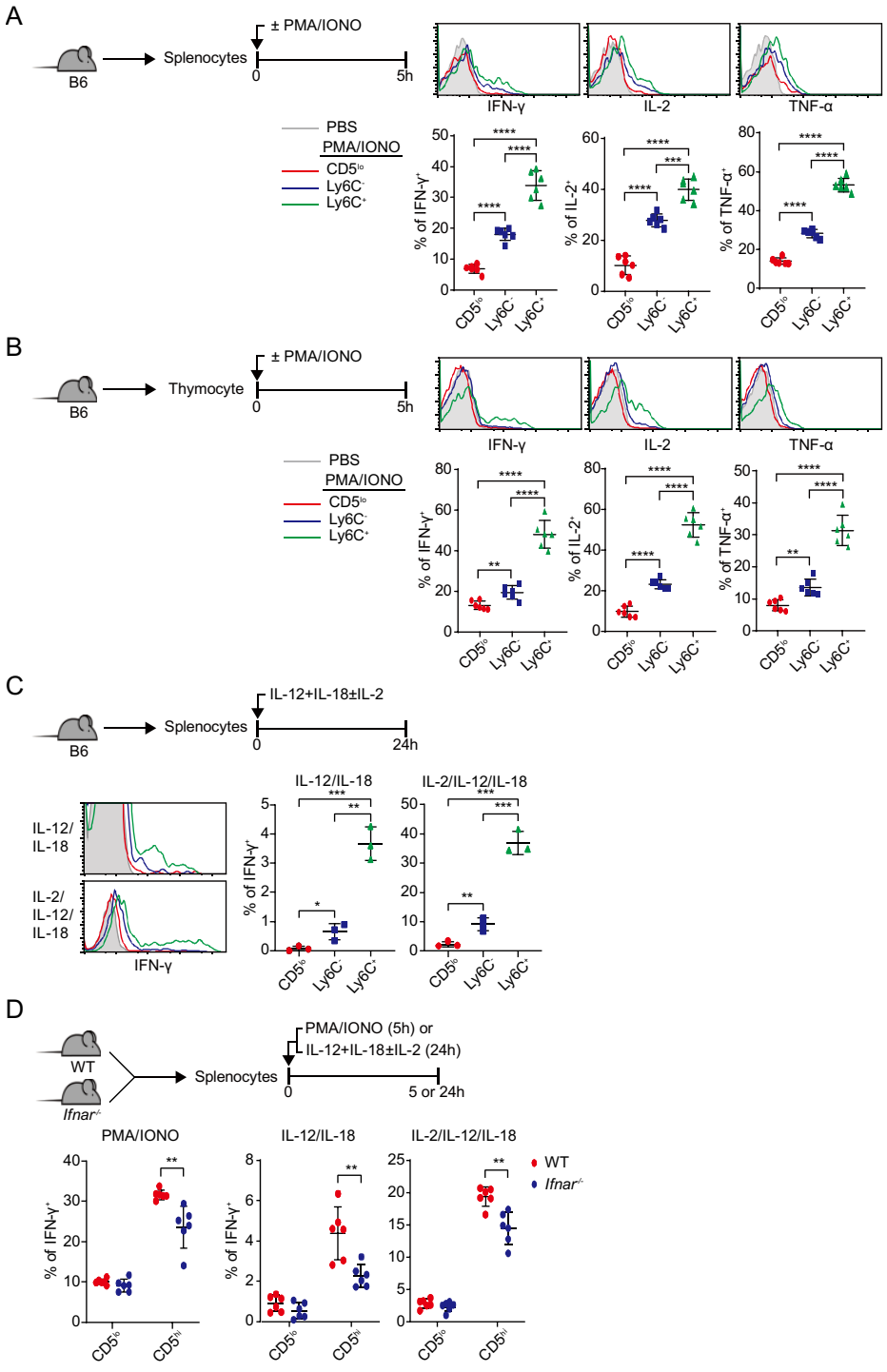
Based on the different genetic landscapes among naïve CD8$^+$ T cell subsets (Figure 6), it was wondered to differences in cytokine production among three naïve CD8$^+$ T cell subsets. It was reported that CD4$^+$ and CD8$^+$ T cells expressing high CD5 produce more IL-2 than CD5 low expressing cells via TCR signal bypass stimulation (i.e., PMA and Ionomycin) [90]. So, production of IFN-γ, IL-2, and TNF-α by PMA and Ionomycin stimulation was examined from peripheral naïve CD8$^+$ T cell subsets to investigate whether Ly6C$^+$ cells, expressing highlighted genes (*T-bet, Eomes,* and *IL-18Rap*) related to T cell activation, would have higher potential to produce cytokines than those of CD5^{lo} and Ly6C$^-$ cells. The results revealed that peripheral Ly6C$^+$ cells are the best producer of IFN-γ, IL-2, and TNF-α followed by Ly6C$^-$ and CD5^{lo} (Figure 7A). In consistent with this result, thymic mature CD8$^+$ T cell subsets showed a similar tendency of cytokine production (Figure 7B). Next, to verify whether Ly6C$^+$ cells can produce IFN-γ more than CD5^{lo} and Ly6C$^-$ cells after the stimulation with pro-inflammatory cytokines, splenocytes were cultured with combination of IL-2, IL-12, and IL-18 [136]. Similar to the results of PMA/Ionomycin stimulation, the highest production of IFN-γ was detected in Ly6C$^+$ cells, followed by Ly6C$^-$ and CD5^{lo} cells (Figure 7C).

Type I IFN seemingly affect both genetic features and phenotypic changes of naïve CD8$^+$ T cells (Figure 6). Next, I asked a question whether type I IFN can

also influence cytokine production in naïve $CD8^+$ T cell subsets. Splenocytes from WT and $Ifnar1^{-/-}$ mouse were cultured with PMA/Ionomycin or combination of pro-inflammatory cytokines. Since there are no $Ly6C^+$ cells in $Ifnar1^{-/-}$ mice (Figure 5), this was done in $CD5^{lo}$ and total $CD5^{hi}$ cells naturally excluding Ly6C expression. Interestingly, IFN-γ production was decreased only in $Ifnar1^{-/-}$ $CD5^{hi}$ cells compared to WT $CD5^{hi}$ cells in both stimulation conditions (Figure 7D). It was further confirmed that temporal treatment of type I IFN enhanced production of IFN-γ in naïve $CD8^+$ T cell subsets by both stimulations (Figure 7E). To validate the effect of constitutive type I IFN in peripheral naïve $CD8^+$ T cell subsets, IFN-γ production was compared in splenocytes purified from IFNAR1 blocked or control mice. The results showed that IFN-γ production was significantly decreased only in IFNAR1 blocked $Ly6C^+$ cells upon stimulation of pro-inflammatory cytokines (Figure 7F). Next, it was examined whether the acquisition of Ly6C phenotype could be achieved by obtaining the function of $Ly6C^+$ cells. The newly induced $Ly6C^+$ cells ($InLy6C^+$) *in vivo* can obtain the ability to produce IFN-γ compared with existing host $Ly6C^+$ (Host $Ly6C^+$) cells. In PMA/Ionomycin treatment, $inLy6C^+$ cells produced IFN-γ at the same level as $Ly6C^-$ subsets, which was lower than the IFN-γ production capacity of Host $Ly6C^+$ cells. The $InLy6C^+$ cells showed the ability to produce IFN-γ similar to the Host $Ly6C^+$ cells by pro-inflammatory cytokine stimulation with IL-2. However, stimulation of pro-inflammatory cytokine without IL-2 showed a higher IFN-γ production capacity than $Ly6C^-$ subsets, but did not catch up with IFN-γ production

capacity of Host Ly6C^{+} cells (Figure 7G).

Collectively, Ly6C^{+} cells had a better cytokine production capacity than CD5^{lo} and Ly6C^{-} cells. In particular, it was confirmed that exposure to constitutive type I IFN can enhance IFN-γ production capacity of InLy6C^{+} cells or Host Ly6C^{+} cells. Therefore, it could be suggested that type I IFN has a positive effect on the phenotypical changes of naive CD8^{+} T cells as well as the functional aspects of the cells exposed to it.



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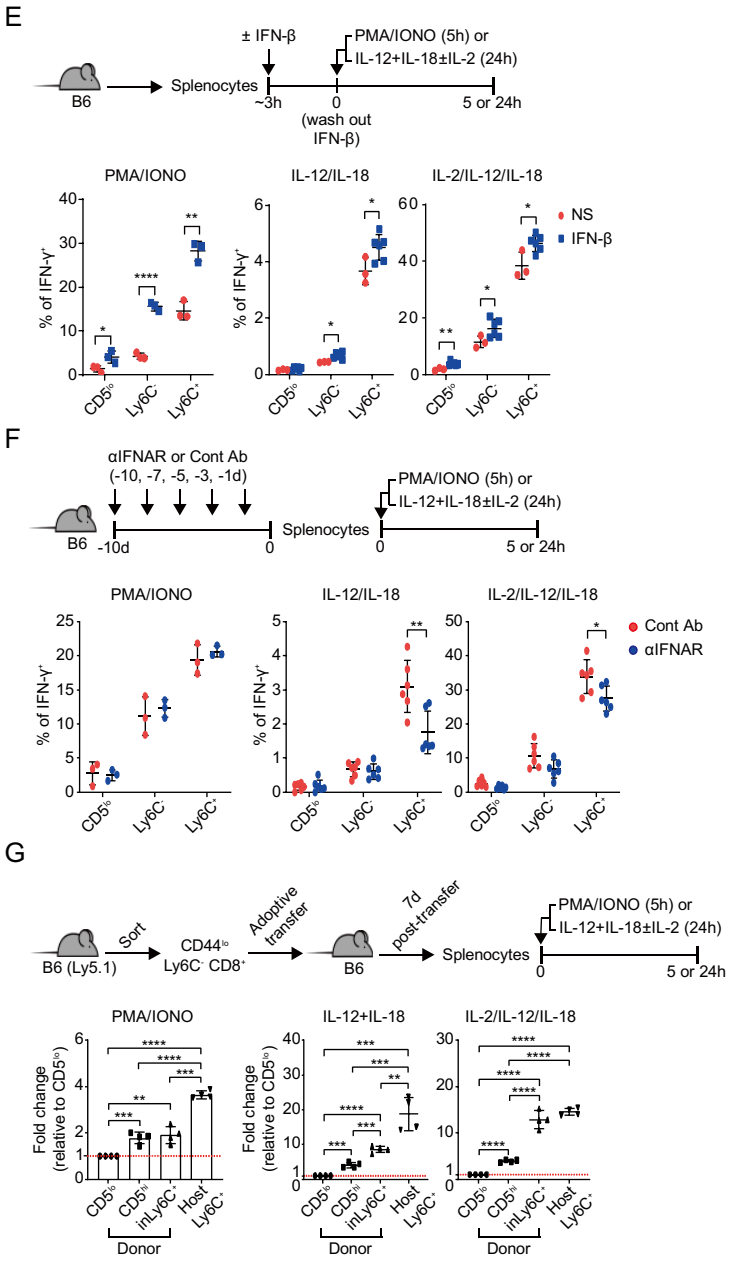


Figure 7. Type I IFN enhances to cytokine production ability of naïve CD8$^+$ T cell subsets.

(A-B) Intrinsic cytokine production in naïve CD8$^+$ T cell subsets from (A) SP and (B) THY. CD5^{lo}, Ly6C$^-$ and Ly6C$^+$ cells were cultured with PMA (100

ng/ml) and Ionomycin (IONO, 1000 ng/ml) for 5 hours. (C) IFN-γ production in naïve $CD8^+$ T cell subsets from SP. $CD5^{lo}$, $Ly6C^-$ and $Ly6C^+$ cells were cultured with combination of cytokines, IL-2 (500 ng/ml), IL-12 (10 ng/ml), and IL-18 (10 ng/ml). (D) IFN-γ production in naïve $CD8^+$ T cell subsets from WT and $Ifnar^{-/-}$ mice. Splenocytes from B6 and $Ifnar^{-/-}$ mice were cultured with PMA/IONO or indicated combination of cytokines. (E) IFN-γ production in naïve $CD8^+$ T cell subsets from IFN-β pre-treated splenocytes. Isolated splenocytes were pre-cultured with IFN-β for 3 hours, and washed by fresh complete media. And then, the cells were cultured with PMA/IONO or indicated combination of cytokines. (F) IFN-γ production in naïve $CD8^+$ T cell subsets from type I IFN signaling pre-blocked splenocytes. Splenocytes were purified from B6 mice administrated control antibody or IFNAR1 blocking antibody for 10 days (200-300 ug/once/mice, 5 times). And then, the cells were cultured with PMA/IONO or indicated combination of cytokines. (G) IFN-γ production potential of newly induced $Ly6C^+$ cells. $CD44^{lo}Ly6C^-$ cells were purified from B6 mice ($Ly5.1^+$), and adoptively transfer to B6 host ($Ly5.1^-$) for 7 days. Host mice were sacrificed after 7 days of transferring, then isolated splenocytes were cultured with PMA/IONO or indicated combination of cytokines. IFN-γ^+ cells of $CD5^{lo}$ cells were defined as standard and the fold change of IFN-γ^+ cells of $CD5^{hi}$ and newly induced $Ly6C^+$ of donor cells and $Ly6C^+$ host cells were shown. Data are representative of at least two independent experiments. Unpaired Student's t-test was used for the statistical analysis. *P<0.05; **P<0.01; ***P<0.005 ****P<0.001.

Ly6C^{+} naïve CD8^{+} T cells have the strongest primary response to acute LCMV infection

Next, responsiveness of naïve CD8^{+} T cell subsets to acute LCMV infection was examined. CD5^{hi} cells are more responsive to acute LCMV or LM-gp33 infection than CD5^{lo} cells because of their pre-activated phenotypes [91]. Since the Ly6C^{+} naïve CD8^{+} T cells showed higher expression of *T-bet* and *Eomes* and better cytokine production than CD5^{lo} and Ly6C^{-} cells, it was hypothesized that Ly6C^{+} cells may have superior responsiveness to acute LCMV infection over other subsets. To estimate the physiological number comprising less than 100 cells of LCMV-specific naïve CD8^{+} T cells per clone, 0.8-1x10^{6} cells of two subsets with different congenic marker were mixed. And the mixed donor subsets were adoptively transferred to B6 host, then infected with acute LCMV to check primary response of each naïve CD8^{+} T cell subsets. In mixed subsets Group 1 (CD5^{lo}+Ly6C^{-}) and Group 2 (CD5^{lo}+Ly6C^{+}), CD5^{lo} cells showed lower proportion of total CD8^{+} and tetramer^{+} donor recovery than Ly6C^{-} and Ly6C^{+} cells at 7 days post-infection (dpi). Interestingly, it was confirmed in Group 3 (Ly6C^{-}+Ly6C^{+}) that Ly6C^{+} cells were more proliferative than Ly6C^{-} cells at 7 dpi in total CD8^{+} and tetramer^{+} donor recovery even though Ly6C^{-} and Ly6C^{+} cells have expressed similar level of CD5 (Figure 8A). Even though the expansion capacity of subsets was evaluated by transferring polyclonal T cells, it could be a misinterpretation if the number of LCMV-specific clones

between the transferred subsets was initially different. Therefore, P14 TCR transgenic was used to ensure the identical TCR clone and number of cells transferred between the subsets. P14 $CD5^{lo}$ cells were mixed with $Ly6C^-$ (Group 1) or $Ly6C^+CD183^-$ (Group 2), or $Ly6C^+CD183^+$ (Group 3) and adoptively transferred to B6 host, then infected with acute LCMV. At 7 dpi, the results showed $Ly6C^+$ subsets, both $CD183^-$ (Group 2) and $CD183^+$ cells (Group 3), were more responsive than $CD5^{lo}$ cells whereas $Ly6C^-$ cells showed a comparable responsiveness with $CD5^{lo}$ cells (Figure 8B). Reactivity of P14 $Ly6C^+$ cells superior to P14 $CD5^{lo}$ and P14 $Ly6C^-$ cells was also confirmed in experiments in which each subset was transferred separately (Figure 8C).

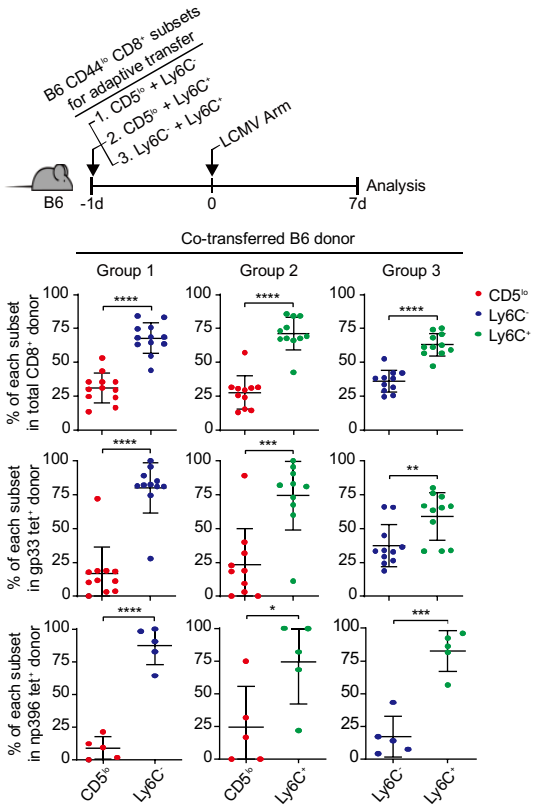
To determine why P14 $Ly6C^+$ cells showed better reactivity at 7dpi than other subsets, the division capacity of each subset was measured by BrdU incorporation assay [47]. At 6 dpi, P14 $Ly6C^+$ cells maintained the highest BrdU uptake capacity compared to P14 $CD5^{lo}$ and P14 $Ly6C^-$ cells (Figure 8D). Also Ki67, a marker expressed in dividing cells, was highly expressed in P14 $Ly6C^+$ cells compared to P14 $CD5^{lo}$ and P14 $Ly6C^-$ cells at 7 dpi (Figure 8E). To see whether $Ly6C^+$ cells can sustain division for longer than other subsets, CTV-labeled P14 subsets were separately transferred to LCMV infected host. At the 1.5 days after the transfer of P14 subset, the cells that divided more than three times upon LCMV infection were significantly higher in P14 $Ly6C^+$ cells than in the other subsets (Figure 8F).

Although the results in Figure 5F showed that $Ly6C^+$ cells can be generated from $Ly6C^-$ cells by constitutive type I IFN *in vivo*, it remains to be investigate

whether functional changes are also occurred during the phenotypic changing from Ly6C$^-$ to Ly6C$^+$ cells. To prove this, it was named to “Induced P14 Ly6C$^+$ cells (InLy6C$^+$)” which are newly generated Ly6C$^+$ cells from transferred P14 Ly6C$^-$ cells in B6 host mice for 7 or 21 days, then proliferative capacity between InLy6C$^+$ and freshly-sorted P14 Ly6C+ cells (FreshLy6C$^+$) in mice infected with LCMV was compared. Interestingly, InLy6C$^+$ cells (7d) did not show as high response as the freshly-sorted P14 Ly6C$^+$, but InLy6C$^+$ cells (21d) showed the same reactivity as the freshly-sorted P14 Ly6C$^+$ cells (Figure 8G).

Collectively the results suggested that Ly6C$^+$ cells have a better potential for clonal expansion during the expansion phase of LCMV infection compared to CD5^{lo} and Ly6C$^-$ cells. It is suggested that Ly6C$^+$ cells show the highest cell number during the expansion phase because Ly6C$^+$ cells can maintain longer proliferation than CD5^{lo} and Ly6C$^-$ cells. And it was suggested that better proliferative capacity of Ly6C$^+$ cells than other cells in LCMV infection were obtained during phenotypic change from Ly6C$^-$ cells to Ly6C$^+$ cells in steady-state condition mice.

A



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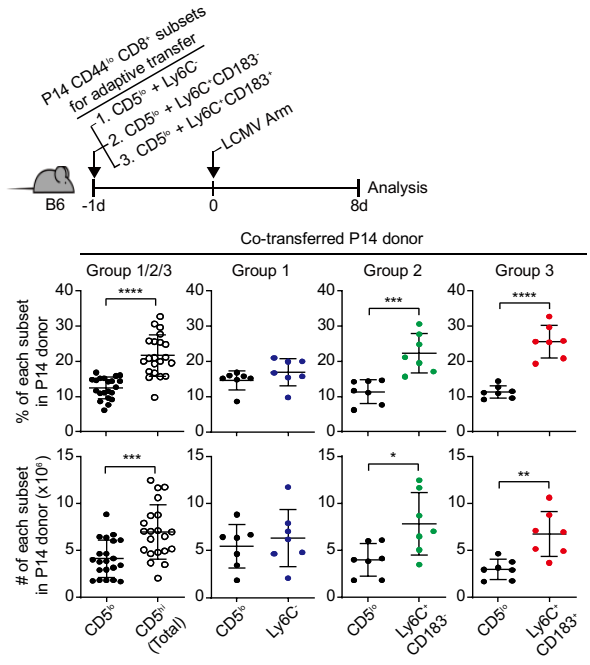

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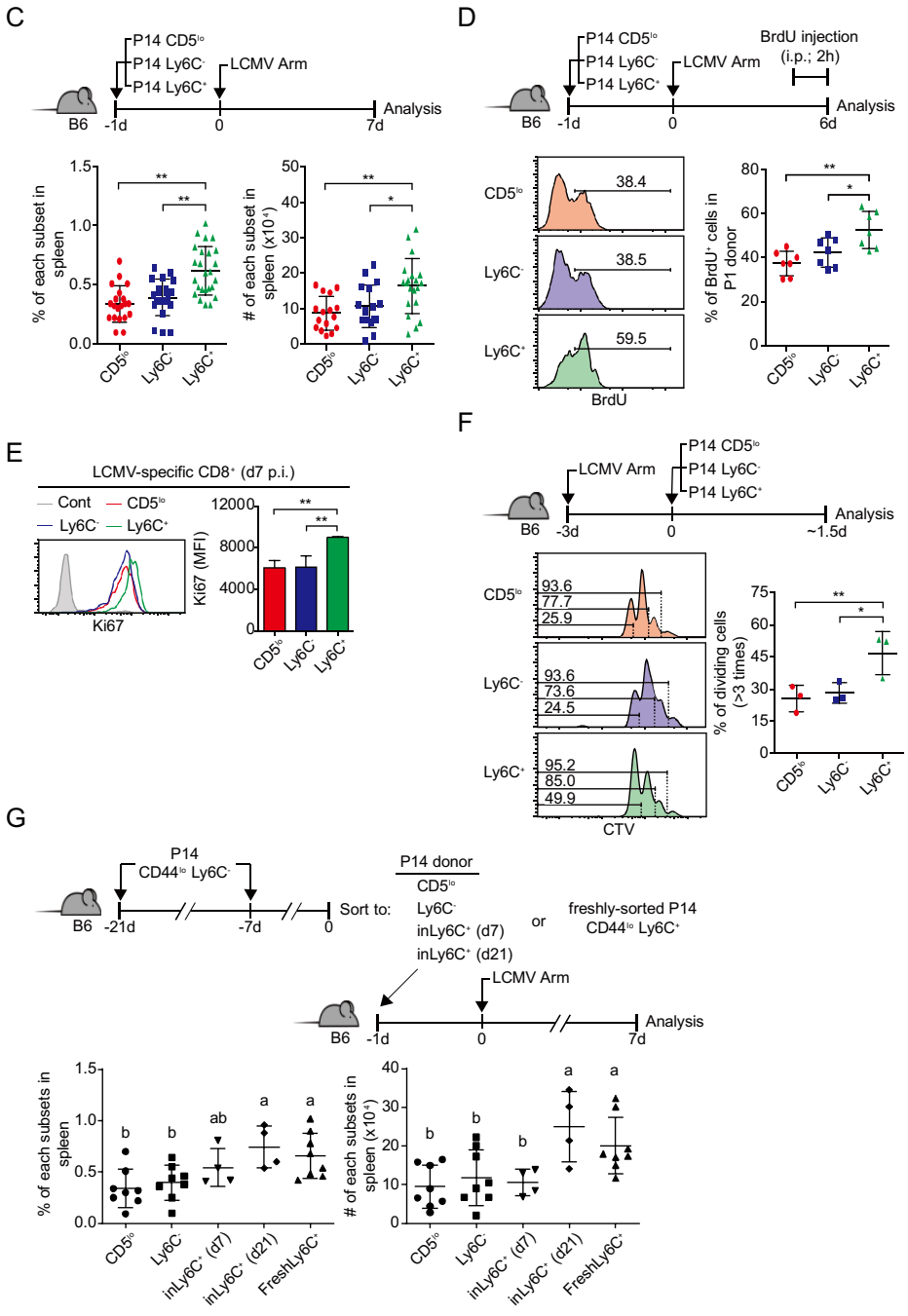


Figure 8. The expansion of Ly6C^{+} subsets is superior to Ly6C^{-} subsets during LCMV infection.

(A) Response of polyclonal mixed naïve $CD8^+$ T cell subsets in LCMV infection. Donor cells were purified from B6 mice (Ly5.1/5.1 or Ly5.1/5.2), and mixed each of two subsets at 1:1 ratio. The mixed cells were adoptively transferred to B6 host mice ($Thy1.1^+$), then the mice were infected with LCMV Armstrong (Arm) strain ($2x10^5$ pfu/mice, i.p) at a day after the cell transfer. The mice were sacrificed at 7 days-post infection (dpi), then splenocytes were taken and examined for total $CD8^+$ donor response (upper), gp33- (middle), and np396-$tetramer^+$ donor cells (lower). (B) Response of monoclonal mixed naïve $CD8^+$ T cell subsets in LCMV infection. Donor cells were purified from P14 TCR transgenic mice (P14 Thy1.1/1.1 or P14 Thy1.1/1.2), and mixed with each of two subsets at 1:1 ratio ($5x10^3$ cells). The mixed cells were adoptively transferred to B6 host mice (Thy1.2/1.2), then mice were infected with LCMV Armstrong strain at a day after the cell transfer. Mice were sacrificed at 7 dpi, then the proportion and absolute number of donor cells in spleen were analyzed. (C) Response of monoclonal single naïve $CD8^+$ T cell subsets in LCMV infection. Donor cells were purified from P14 $RAG1^{-/-}$ mice (P14 $Ly5.1^+$) and adoptively transferred to B6 host mice ($Ly5.1^-$), then mice were infected with LCMV Armstrong strain at a day after the cell transfer. Mice were sacrificed at 7 dpi, then proportion and absolute number of donor cells in spleen were analyzed. (D) BrdU incorporation of naïve $CD8^+$ T cell subsets in LCMV infection. Donor cells were purified from P14 $RAG1^{-/-}$ mice (P14 $Ly5.1^+$) and adoptively transferred to B6 host mice ($Ly5.1^-$), then mice were infected with LCMV Armstrong strain at a day after the cell transfer. At 6 dpi, BrdU (1 mg/ml)

was administrated by i.p route for 2 hours. Mice were sacrificed at 2 hours after BrdU administration, then the $BrdU^+$ donor cells were analyzed. (E) Expression of Ki67. Scheme was identical with Figure 5C. At 7 dpi of LCMV infection, geometric mean (MFI) of Ki67 were analyzed from donor cells by intracellular staining. (F) Prolonged proliferation of naïve $CD8^+$ T cells subsets in LCMV infection. Donor cells were purified from P14 mice ($Ly5.1^+$), and labeled with CTV. CTV-labeled P14 subsets ($1\text{-}2x10^5$ cells/mice) were adoptively transferred to host mice ($Thy1.1^+$) have been infected with LCMV 3 days before. Mice were sacrificed at 1.5 days after adoptive transfer, then the dividing donor cells were analyzed. (G) Response of induced $Ly6C^+$ cells in LCMV infection. $Ly6C^-$ cells ($1x10^6$ cells/mice) were purified from P14 mice ($Ly5.1^+$), then transferred to B6 host ($Ly5.1^-$). After 7 and 21 days of cell transfer, re-sorted the CD5lo, Ly6C-, and newly induced $Ly6C^+$ cells from have been transferred donor cells, and adoptively transferred to new B6 host ($Ly5.1^-$), then mice were infected with LCMV. Mice were sacrificed at 7 dpi, then proportion and absolute number were analyzed. Data are representative of at least two independent experiments. Unpaired Student's t-test and one-way ANOVA with Dunnett's multiple-comparison post-test was used for the statistical analysis. *P<0.05; **P<0.01; ***P<0.005 ****P<0.001.

Naïve $CD8^+$ T cell subsets differentiate into predetermined effector precursors upon LCMV infection

In the last decade, it has been focused on to evaluate the differentiation potential to long-term memory cell via effector precursor differentiation in infection model [96]. Among the various infection model, LCMV infection model has been the most widely established in the field of long-term memory cell and exhausted cell research of $CD8^+$ T cells [137]. Therefore, it was a suitable model to interpret the research results based on a lot of researches and apply it to the vaccine development[54] and chronic infectious disease[138].

Previously it has been suggested that $CD8^+$ T cells can differentiate into two types of effector precursors that are completely different in character under the acute infection situation, such as LCMV and *L. monocytogenes*. The two effector precursors can be distinguished by the differential expression of KLRG1 and CD127. In general, $KLRG1^{lo}CD127^{hi}$ cells are defined as MPEC (memory precursor effector cell) and $KLRG1^{hi}CD127^{lo}$ cells as SLEC (short lived effector cell) [96, 98, 99]. Also the studies have suggested that MPEC has higher long-term memory differentiation capacity and IL-2 production capacity than SLEC, while SLEC is more vulnerable to cell death than MPEC, so most of them disappear in the contraction phase of T cell response [104, 139].

To investigate the differentiation of naïve $CD8^+$ T cell subsets in LCMV infection, analysis was performed using well-established surface markers expression related to effector differentiation of $CD8^+$ T cell. In the present study,

P14 $CD5^{lo}$, P14 $Ly6C^{-}$, and P14 $Ly6C^{+}$ cells were transferred to B6 host mice, and then infected with acute LCMV. At 7 dpi, expression of KLRG1 and CD127 were measured. Interestingly, differentiation into SLEC was highest in P14 $Ly6C^{+}$ cells, followed by P14 $Ly6C^{-}$ and P14 $CD5^{lo}$ cells, whereas MPEC differentiation was highest in P14 $CD5^{lo}$ cells, followed by P14 $Ly6C^{-}$ and P14 $Ly6C^{+}$ cells (Figure 9A).

It has been suggested that effector $CD8^{+}$ T cells can be categorized by expression of CD27 and CX3CR1 as $CD27^{+}CX3CR1^{lo}$, $CD27^{+}CX3CR1^{int}$, and $CD27^{-}CX3CR1^{hi}$ cells where $CD27^{+}CX3CR1^{lo}$ cells have similar characteristics to MPEC, $CD27^{-}CX3CR1^{hi}$ cells to SLEC, and $CD27^{+}CX3CR1^{int}$ to intermediate characteristics [140].

In the present study, $CD27^{+}CX3CR1^{lo}$ cells appeared the highest in P14 $CD5^{lo}$ cells, followed by $Ly6C^{-}$ and $Ly6C^{+}$ cells, whereas $CD27^{-}CX3CR1^{hi}$ cells were detected higher in P14 $Ly6C^{+}$ cells than P14 $Ly6C^{-}$ and P14 $CD5^{lo}$ cells (Figure 9B). Also, similar results were obtained using donor cells from B6 mice instead of P14 mice (Figure 9C). Together with phenotypes, cell death was highest in P14 $Ly6C^{+}$ cells with high SLEC differentiation tendency, followed by P14 $Ly6C^{-}$ and P14 $CD5^{lo}$ cells (Figure 9D). Also, as in the studies mentioned above[140], higher IL-2 production, a characteristic of MPEC, was measured in P14 $CD5^{lo}$ cells (Figure 9E). So it was again confirmed that $CD5^{lo}$ cells prefer differentiation to MPEC and $Ly6C^{+}$ cells prefer to differentiate to SLEC.

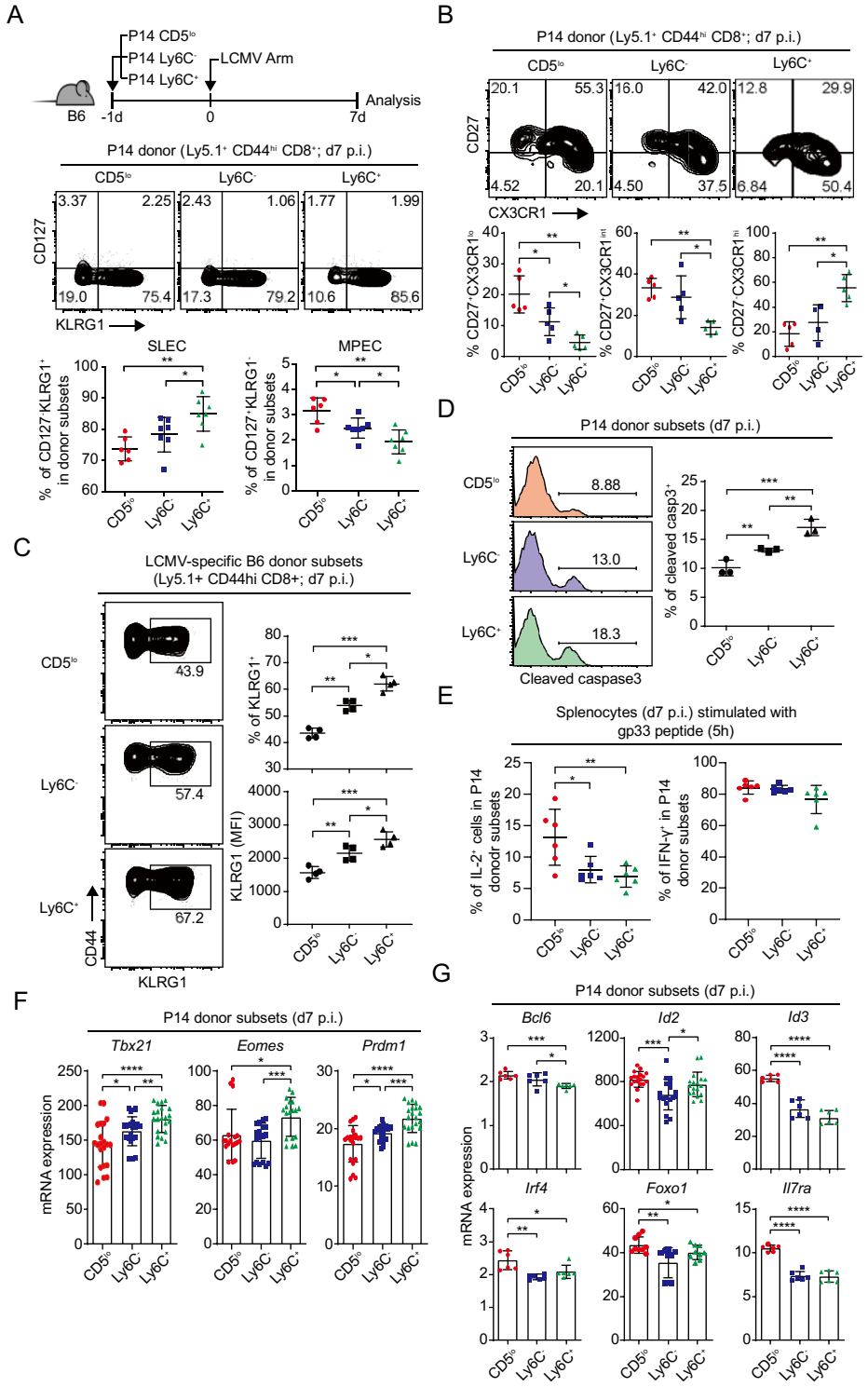
Next, to investigate whether each of the P14 subsets have SLEC and MPEC

features at the mRNA level, representative genes were measured by quantitative-PCR and RNA-seq from P14 subset donor at 7 dpi. The SLEC signature genes, *Tbx21*, *Eomes*, and *Prdm1*, were expressed higher in P14 Ly6C^{+} cells than P14 Ly6C^{-} and P14 CD5^{lo} cells (Figure 9F). On the other hand, MPEC signature genes, *Bcl6*, *Id2*, *Id3*, *Irf4*, *Foxo1*, and *Il7rα*, were expressed more in P14 CD5^{lo} cells than P14 Ly6C^{-} and P14 Ly6C^{+} cells (Figure 9G). Furthermore, it was validated by GSEA that SLEC signature gene set was more enriched in P14 Ly6C^{+} cells than P14 Ly6C^{-} and P14 CD5^{lo} cells (Figure 9H), whereas MPEC signature gene set in P14 CD5^{lo} and P14 Ly6C^{-} cells than P14 Ly6C^{+} cells (Figure 9I).

It has been reported that metabolic features differ according to the activation phase of CD8^{+} T cells such as naive, effector, and memory T cells. Such metabolic differences could be determined at the effector precursor stage, where SLEC showed higher extracellular acidification rate (ECAR) and MPEC with higher oxygen consumption rate (OCR) [141]. Consistent with previous reports, at 7 dpi, OCR of P14 CD5^{lo} was the highest, followed by P14 Ly6C^{-} and P14 Ly6C^{+}, whereas ECAR was lower than other subsets in P14 CD5^{lo} (Figure 9J). In addition to metabolic changes, the strength of mTOR signal was known to be closely related to memory cell differentiation [105]. The higher the intensity of mTOR signal, the more strongly induced differentiation into the terminal differentiated cells like SLEC [109]. As expected, it was confirmed that signature gene sets of mTOR signaling pathways are strongly enriched in P14 Ly6C^{+} cells with high differentiation rate to SLEC than P14 Ly6C^{-} and P14

$CD5^{lo}$ cells. (Figure 9K)

Collectively, the data suggested that differentiation of naïve $CD8^+$ T cells is a predictable event during acute LCMV infection, and that preference for differentiation may be predetermined. In addition, it is possible to predict the differentiation preference by measuring the expression level of CD5 and Ly6C in naive $CD8^+$ T cells.



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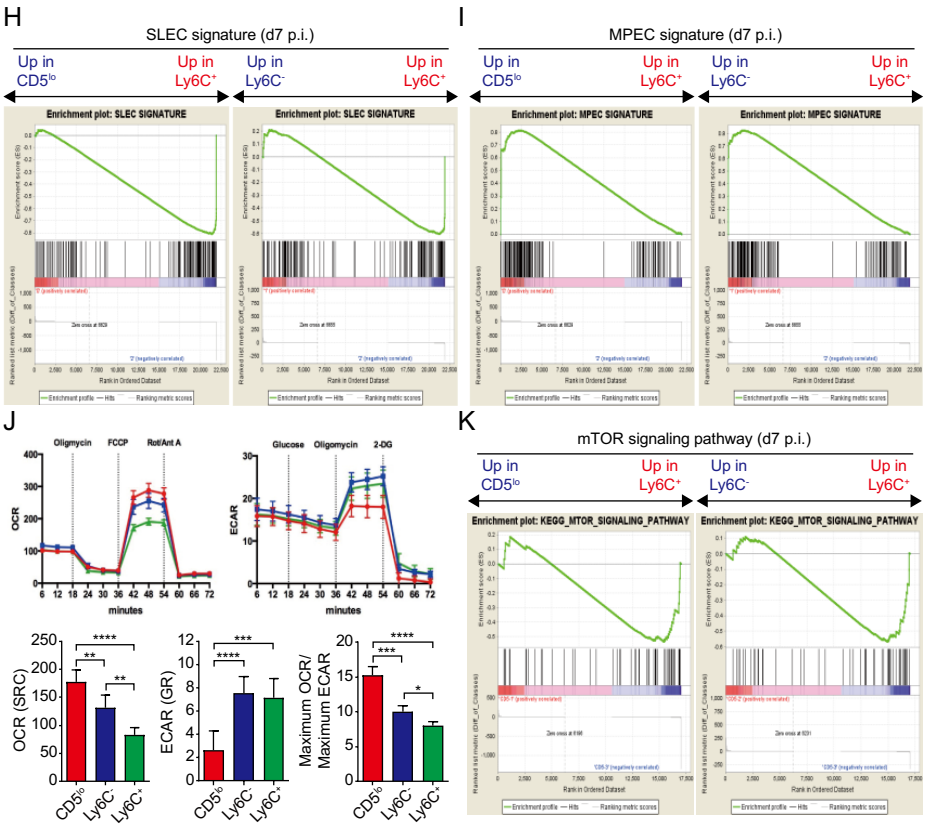


Figure 9. Naïve CD8$^+$ T cell subsets have distinct preference for effector precursor differentiation.

Preferred differentiation of naïve CD8$^+$ T cell subsets in LCMV infection. Donor cells were purified from P14 RAG1$^{-/-}$ mice (P14 Ly5.1$^+$) and adoptively transferred to B6 host mice (Ly5.1$^-$), then the mice were infected with LCMV at a day after the cell transfer. Mice were sacrificed at 7 dpi, then indicated analysis were done. Experimental schemes were identical to A-B and D-K. (A) Proportion of CD127$^-$KLRG1$^+$ (short lived effector cell; SLEC) and CD127$^+$KLRG1$^-$ (memory precursor effector cell; MPEC) were analyzed in donor cells. (B) Proportion of CD27$^+$CX3CR1^{lo}, CD27$^+$CX3CR1^{int}, and CD27$^-$

CX3CR1^{hi} cells were analyzed in donor cells. (C) Preferred differentiation of polyclonal naïve CD8^{+} T cell subsets in LCMV infection. Experimental schemes were identical with Figure 5A. CD44^{hi}KLRG1^{+} cells were analyzed in donor cells. (D) Expression of cleaved caspase 3 in LCMV infection. Cleaved caspase 3 were measured in LCMV 7 dpi donor cells. (E) Cytokine production in donor cells at LCMV 7 dpi. Splenocytes were isolated from LCMV 7 dpi mice and re-stimulated with gp33 peptide *in vitro* for 5 hours. IL-2 and IFN-γ production were analyzed in donor cells. (F-G) Expression of effector precursor signature genes in naïve CD8^{+} T cell subsets during LCMV infection. Donor cells were purified from splenocytes at LCMV 7 dpi, then expression of (F) SLEC signature genes (*Tbx21, Eomes,* and *Prdm1*) and (G) MPEC signature genes (*Bcl6, Id2, Id3, Irf4, Foxo1,* and *Il7ra*) were analyzed by quantitative real-time PCR. (H-I) GSEA profile of (H) SLEC and (I) MPEC signature genes in CD5^{lo} versus Ly6C^{+} cells and Ly6C^{-} and Ly6C^{+} cells at LCMV 7 dpi. (J) Metabolic differences in CD5^{lo}, Ly6C^{-}, and Ly6C^{+} cells at LCMV 7 dpi. Donor cells were sorted and examined their potential to oxidative phosphorylation (oxygen consumption rate, OCR) and glycolysis (extracellular acidification rate, ECAR). (K) GSEA profile of mTOR signaling pathway signature genes in CD5^{lo} versus Ly6C^{+} cells and Ly6C^{-} and Ly6C^{+} cells at LCMV 7 dpi. Data (A-G, J) are representative of at least two independent experiments. Unpaired Student's t-test was used for the statistical analysis. *P<0.05; **P<0.01; ***P<0.005 ****P<0.001.

CD5^{lo} cells have the best potential to become long-term memory cells

CD5^{lo} cells, which showed high MPEC differentiation rates compared with other subsets, are the most capable of differentiating into memory CD8^{+} T cells, independent of the proliferation capacity observed during the effector phase. Under the same setting as the previous experiments, at 120 dpi, the highest long-term memory CD8^{+} T cell formation was observed in the group that was transferred with P14 CD5^{lo} cells (Figure 10A). Several studies have reported that central memory CD8^{+} T cell (T_{CM}; CD44^{hi}CD62L^{hi}) can be finally more differentiated into long-term memory CD8^{+} T cells than the effector memory CD8^{+} T cell (T_{EM};CD44^{hi}CD62L^{lo}) [111]. When the ratio of T_{CM} and T_{EM} was investigated at 120 dpi, a high proportion of T_{CM} was observed in the order of P14 CD5^{lo}, P14 Ly6C^{-}, and P14 Ly6C^{+} cells, while T_{EM} showed the opposite order (Figure 10B). As with the results of 120 dpi, the highest memory CD8^{+} T cell and T_{CM} ratio was observed in P14 CD5^{lo} cells at 35 dpi (Figure 10C). These results suggest that CD5^{lo} cells have the best memory differentiation potential, although they have less proliferation capacity than Ly6C^{+} cells. The self-renewal capability, important factor leading to the formation of long-term memory CD8^{+} T cells [110], of T_{CM} is known to be superior to T_{EM}. When comparing the self-renewal ability through BrdU uptake of memory CD8^{+} T cells at 35 dpi, the highest BrdU uptake was observed in memory CD8^{+} T cells derived from P14 CD5^{lo} cells (Figure 10D). This result suggests that the reason of CD5lo cell form more long-term memory cells than other cell is because of not only prefer to differentiated into MPEC but also maintain higher ratio of

T_{CM} specialized for self-renewal.

Next, recall response of the memory $CD8^+$ T cells was investigating by using cognate pathogen infection. When the recall infection was given by LM-gp33 to the LCMV immunized mice containing with memory cells derived from P14 subset, memory $CD8^+$ T cells derived from P14 $CD5^{lo}$ cells showed the higher recall response than that derived from other cells (Figure 10E). Interestingly, however, when the fold change in the number of memory $CD8^+$ T cells was measured before and after the recall response, the highest fold change was observed in the memory $CD8^+$ cells derived from P14 $Ly6C^+$ cells, followed by from P14 $Ly6C^-$ and P14 $CD5^{lo}$ cells (Figure 10E).

Collectively, $CD5^{lo}$ cells, which had more MPECs in the effector phase, produced more long-term memory $CD8^+$ T cells with higher T_{CM} ratios than the other subsets. The results in recall infection showed that memory $CD8^+$ T cells derived from $CD5^{lo}$ cells showed the highest cell number at the peak response, but fold change was higher in memory $CD8^+$ T cells derived from $Ly6C^+$ cells than that of other cells. Thus, it is suggested that the number of cells that differentiate into memory cells during primary infection and the proliferation capacity during the secondary infection depend on which naïve $CD8^+$ T cells differentiated into the memory cells.

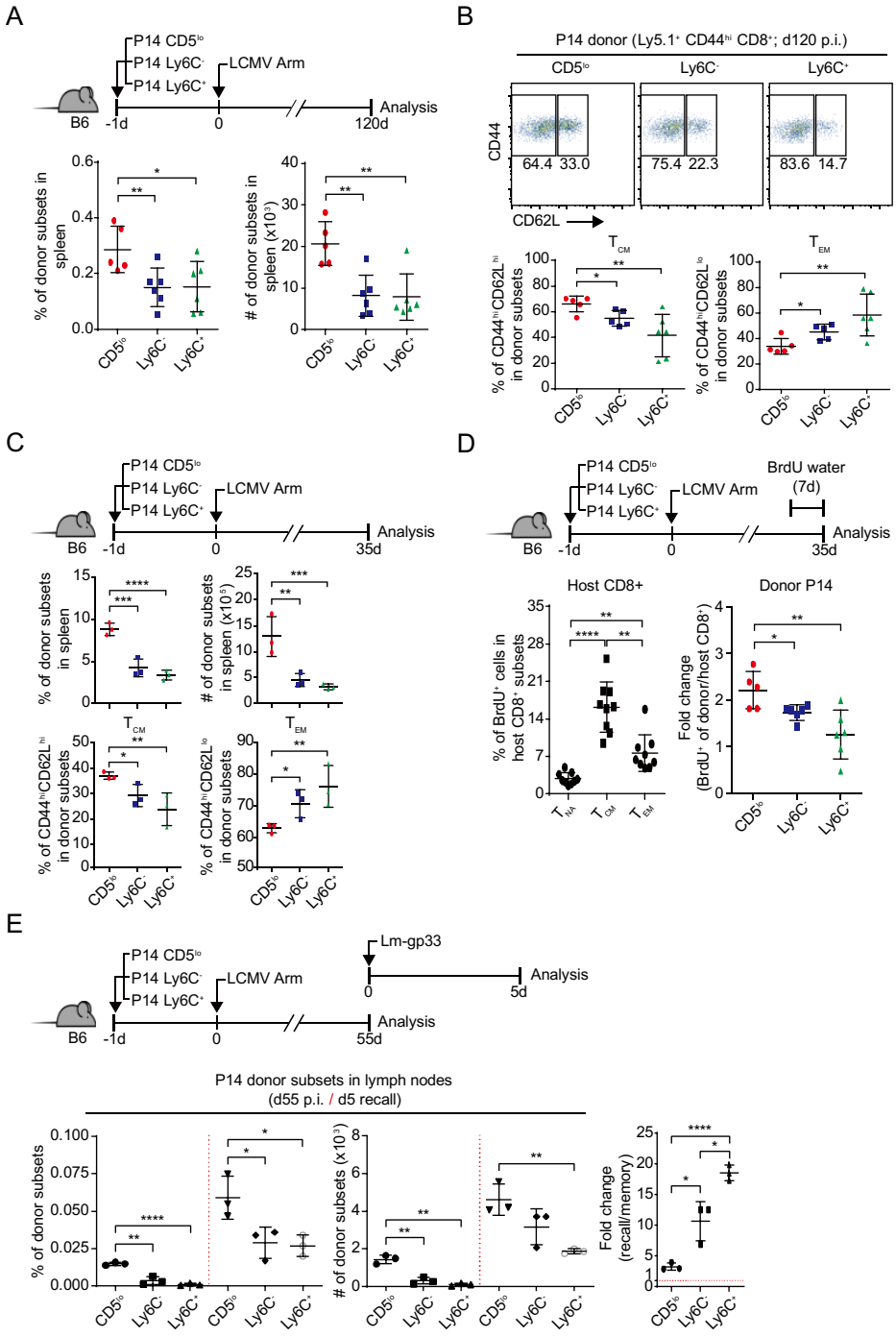


Figure 10. CD5^{lo} cells have the best potential in long-term memory generation among the three naive CD8^{+} T cell subsets.

(A-C) Long-term memory potential in naïve $CD8^+$ T cell subsets in LCMV infection. Donor cells were purified from P14 $RAG1^{-/-}$ mice (P14 $Ly5.1^+$) and adoptively transferred to B6 host mice ($Ly5.1^-$), and then the mice were infected with LCMV at a day after the cell transfer. (A) Mice were sacrificed at 120 dpi, then proportion, absolute number, and (B) phenotypes of memory cells were analyzed in donor cells. (C) Experimental schemes were identical with above. Mice were sacrificed at 35 dpi, then proportion, absolute number, and phenotypes of memory cells were analyzed in donor cells. (D) BrdU incorporation of memory $CD8^+$ T cells in LCMV infection. Donor cells were purified from P14 $RAG1^{-/-}$ mice (P14 $Ly5.1^+$) and adoptively transferred to B6 host mice ($Ly5.1^-$), and then the mice were infected with LCMV at a day after the cell transfer. BrdU was administrated by drinking water for 7 days, and the mice were sacrificed at 35 dpi. $BrdU^+$ cells were analyzed in host $CD8^+$ T cells and donor cells. (E) Recall response of memory $CD8^+$ T cells. Experimental schemes for memory generation were identical with above. At LCMV 55 dpi, host mice infected with LCMV were re-infected with listeria-monocytogenes expressing gp33 (LM-gp33) ($1\text{-}2x10^4$ cfu/mice) by i.v route. Donor cells were analyzed before and after the recall reaction. Data are representative of at least two independent experiments. Unpaired Student's t-test was used for the statistical analysis. *P<0.05; **P<0.01; ***P<0.005 ****P<0.001.

Constitutive type I IFN modulates pre-determined effector features of naïve CD8$^+$ T cell subsets, especial to Ly6C$^+$ cells in LCMV infection

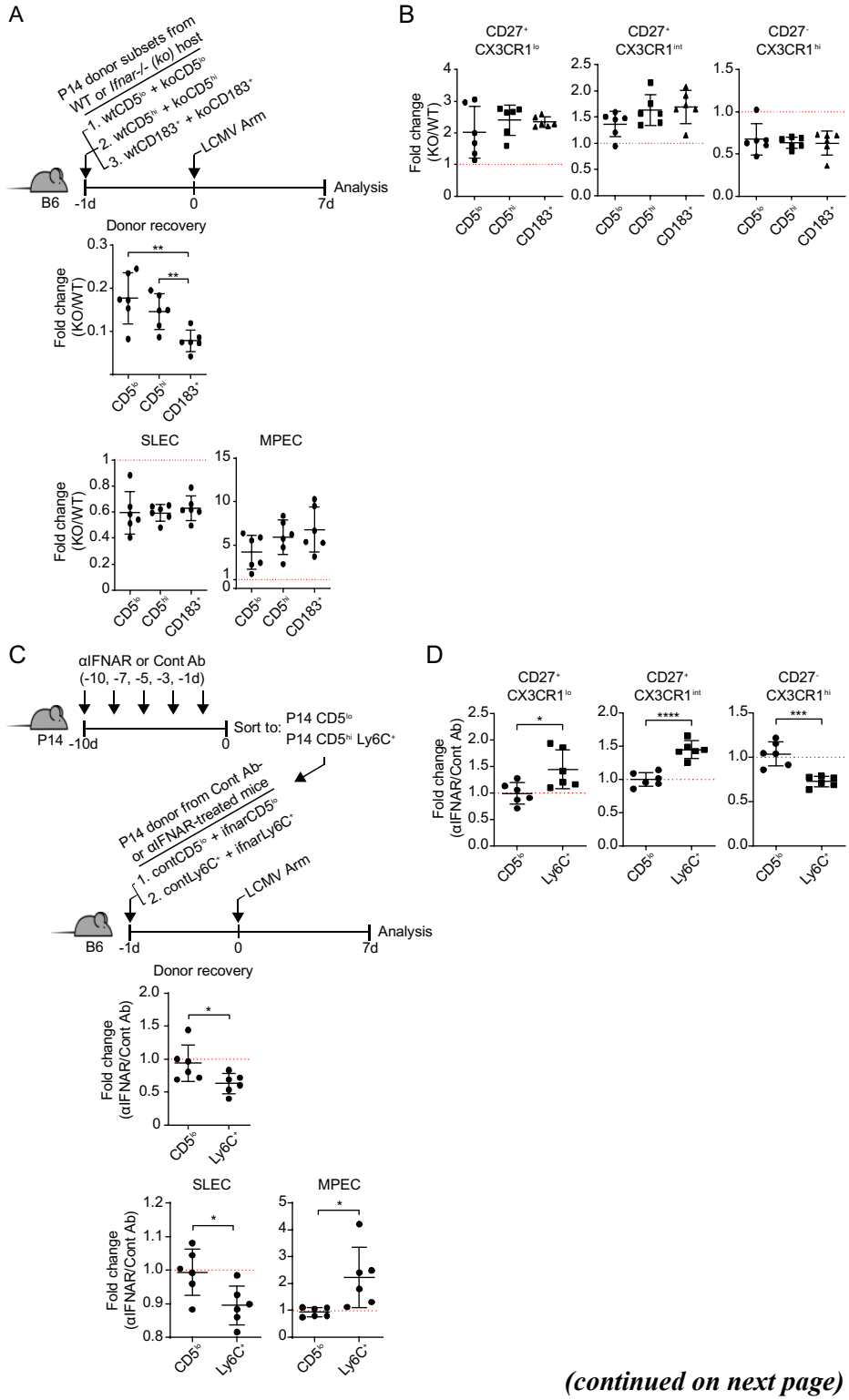
Momentary blocking of constitutive type I IFN reduced production of IFN-γ in Ly6C$^+$ cells upon stimulation of IL-2, IL-12, and IL-18 (Figure 7). Since these cytokines were well-reported to be secreted upon LCMV infection [142, 143], next question was asked whether exposure to type I IFN in periphery also had modulated pre-determined effector features such as clonal expansion and preference for effector precursor differentiation in acute LCMV infection. To verify the question, the same phenotype subsets isolated from congenically different WT and Ifnar1$^{-/-}$ P14 mice were mixed respectively and co-transferred to B6 host mice, then infected with acute LCMV. At 7 dpi, ratio of donor recovery tended to decrease significantly in Ifnar1$^{-/-}$ P14 cells compared to WT P14 cells, especially in mixture of CD183$^+$ cells. In addition, when measuring the effector precursor differentiation, SLEC was decreased in Ifnar1$^{-/-}$ P14 cells compared to that of WT P14 cells, whereas MPEC increased (Figure 11A). Based on the expression of CD27 and CX3CR1 showed that P14 CD27$^+$CX3CR1^{lo} and P14 CD27$^+$CX3CR1^{int} cells were increased while P14 CD27$^-$CX3CR1^{hi} cells were decreased in Ifnar1$^{-/-}$ P14 cells than WT P14 cells (Figure 11B). These results suggested that type I IFN can enhance SLEC differentiation and expansion of naïve CD8$^+$ T cell subsets. Yet, it was still concern that, if the experiments were carried out using Ifnar1$^{-/-}$ mice, the net effect of constitutive type I IFN on naive CD8$^+$ T cells could not be estimated,

because Ifnar1$^{-/-}$ cells did not receive constitutive type I IFN and, at the same time, type I IFN robustly secreted by LCMV infection. Thus, naive CD8^{+} T cell subsets were isolated from P14 mice that transiently blocked constitutive type I IFN with IFNAR1 blocking antibody. The antibody-blocked P14 CD5^{lo} and P14 Ly6C^{+} cells were mixed with the control antibody-treated cells, adoptively transferred into new B6 host mice, and infected with LCMV. Surprisingly, the ratio of donor recovery (αIFNAR1/Cont Ab) was lower than 1 in P14 Ly6C^{+} cell mixture group only, not in the P14 CD5^{lo} cell mixture group, indicating that IFNAR1-blocked P14 Ly6C^{+} cells showed a lower expansion than control P14 Ly6C^{+} cells. In addition, the ratio of effector precursor differentiation preference showed significant changes in P14 Ly6C^{+} cell mixture only, supporting the previous results that the SLEC differentiation of P14 Ly6C^{+} cells were reduced while MPEC differentiation were increased due to IFNAR1 blocking (Figure 11C). In the same context, it was confirmed that IFNAR1 blocking increased CD27^{+}CX3CR1^{lo} and CD27^{+}CX3CR1^{int} cells and decreased CD27^{-}CX3CR1^{hi} cells in P14 Ly6C^{+} cell mixture group (Figure 11D).

Next, experiment was performed to define whether the different expansion capacity and differentiation features of naïve CD8^{+} T cell subsets shown so far were stemmed from either at developmental stage in thymus or by effect of constitutive type I IFN within the periphery. When the donor cells were tested using thymus-derived subsets under the same condition as in Figure 8C, the results showed that there were no differences between subsets not only in donor expansion capacity but also in differentiation into effector precursors (Figure

11E).

In conclusion, the data suggested that constitutive type I IFN may act as an important factor in the post-thymic modulation of naïve $CD8^+$ T cells. Constitutive type I IFN altered the phenotype of naïve $CD8^+$ T cells as a major contributor to post-thymic modulation, and was shown to be closely related to the expansion and effector precursor differentiation of $Ly6C^+$ cells upon the LCMV infection.



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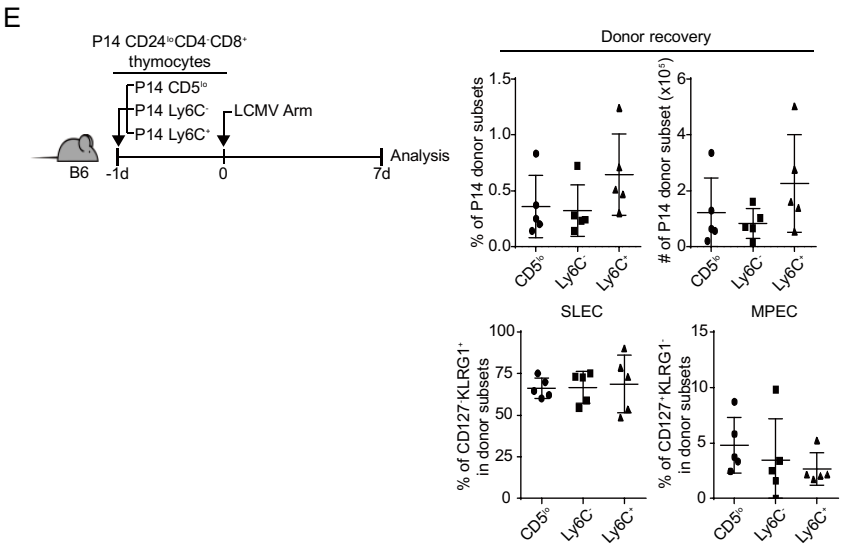

Figure 11. Type I IFN modulates to pre-determined unique traits of naïve CD8$^+$ T cell subsets in LCMV infection.

(A-B) Response of monoclonal mixed naïve CD8$^+$ T cell subsets from WT and Ifnar$^{-/-}$ mice in LCMV infection. Donor cells were purified from P14 WT and P14 Ifnar$^{-/-}$ mice (P14 Ly5.1/5.1 or P14 Ifnar$^{-/-}$ Ly5.1/5.2), and mixed with phenotypically same subset at 1:1 ratio (1x10^3). The mixed donor cells were adoptively transferred to B6 host mice (Ly5.2/5.2), then mice were infected with LCMV at a day after the cell transfer. The mice were sacrificed at 7 dpi, (A) then proportion of expansion and effector precursor differentiation (phenotypes of CD127$^-$KLRG1$^+$ and CD127$^+$KLRG1$^-$) between two types of donor cells were analyzed. (B) Proportion of CD27$^+$CX3CR1^{lo}, CD27$^+$CX3CR1^{int}, and CD27$^-$CX3CR1^{hi} cells were analyzed between two types of donor cells. (C-D) Response of monoclonal mixed naïve CD8$^+$ T cell subsets from control or IFNAR1 antibody administrated mice in LCMV infection.

Donor cells were purified from P14 mice pre-administrated with control antibody (Ly5.1/5.2) or IFNAR1 blocking antibody (Ly5.1/5.1) for 10 days (200-300 ug/once/mice, 5 times), then the purified cells were mixed with phenotypically same subsets ($1x10^3$ cells/subsets, 1:1 ratio). Mixed donor cells were adoptively transferred to B6 host mice (Ly5.2/5.2), then mice were infected with LCMV at a day after cell transfer. The mice were sacrificed at 7 dpi, (C) then proportion of expansion and effector precursor differentiation (phenotypes of $CD127^-KLRG1^+$ and $CD127^+KLRG1^-$) between two types of donor cells were analyzed. (D) Proportion of $CD27^+CX3CR1^{lo}$, $CD27^+CX3CR1^{int}$, and $CD27^-CX3CR1^{hi}$ cells were analyzed between two types of donor cells. (E) Response of monoclonal single matured CD8SP subsets in LCMV infection. Donor cells were purified from thymus of P14 $RAG1^{-/-}$ mice ($Ly5.1^+$). Purified cells ($1x10^3$ cells/mice) were adoptively transferred to B6 host mice ($Ly5.1^-$), then mice were infected with LCMV at a day after cell transfer. Mice were sacrificed at 7 dpi, then expansion and effector precursor differentiation were analyzed. Data are representative of at least two independent experiments. Unpaired Student's t-test was used for the statistical analysis. *P<0.05; **P<0.01; ***P<0.005 ****P<0.001.

V. Discussion

Role of type I IFN in T cell immunology has been incompletely understood both at their development in thymus and responsive against foreign antigen in periphery. Although it has been reported dysfunction of type I IFN in viral and bacterial infection studies by using IFNAR1 deficient mice, they are mainly deal with a strong and robust action of type I IFN, but not the constitutively produced level of type I IFN at steady-state. Therefore, it was questioned how the Ly6C$^+$ cells continuously maintain their proportion within the naïve CD8$^+$ T cells pool even though they do not have chance to exposure to type I IFN robustly produced by bacterial and virial infection. Indeed it is well-known that homeostatic factors required for survive, self-turnover in steady-state condition of naïve CD8$^+$ T cells are IL-7 and self-TCR contact [65, 144, 145]. On the other hand, it was suggested that type I IFN is also constitutively produced as one of homeostatic cytokines from thymus and, lymphoid and non-lymphoid organs [8], that plays a role for the development of thymic architecture and maturation of T cells [7, 10]. While aforementioned studies have suggested that type I IFN is essential to both development of thymus and thymocytes, it is still uncovered whether the constitutive type I IFN can directly modulate phenotypic and physiological features of naïve CD8$^+$ T cells.

In the present study, three steps of strategic approaches were established to investigate the hypothesis, constitutive type I IFN at steady-state in mouse

directly affects functionality of naïve $CD8^+$ T cells as following;

Part I. Generation and regulation of heterogenic naïve $CD8^+$ T cell pool by constitutive type I IFN.

Part II. Different functional features of three naïve $CD8^+$ T cell subsets.

Part III. Fate decision of the three naïve $CD8^+$ T cell subsets in LCMV infection.

In the parts I and II, the results suggested the constitutive type I IFN can regulate generation of the heterogenic naïve $CD8^+$ T cell pool and it also can affect functional features of naïve $CD8^+$ T cell subsets.

First, the naïve $CD8^+$ T cells were categorized based on the expression of CD5 and Ly6C, then subsets were defined as $CD5^{lo}$, $CD5^{hi}Ly6C^-$ ($Ly6C^-$), and $CD5^{hi}Ly6C^+$ ($Ly6C^+$) cells. While the Ly6C molecule is accepted only as a surface marker representing activated [65] or memory T cells [37], the results in Figure 4 clearly showed that the Ly6C molecules are also expressed on $CD44^{lo}CD62L^{hi}$ naïve $CD8^+$ T cells. Furthermore, it was interesting to note that $Ly6C^+$ cells appeared in both thymus and periphery at steady-state with constitutive type I IFN, especially only within $CD5^{hi}$ fraction of naïve $CD8^+$ T cells.

It has been reported that T cell clone pool size is crucial factor for intra-clonal competition in individual mice to self-peptide loading MHC class I within naïve $CD8^+$ T cells sharing similar TCR clone [146]. Because the naïve $CD8^+$ T cells having the same TCR compete for binding with self-peptide loaded MHC (self-

MHC) complex, the larger T cell clone pool size leads the less binding chance between T cell and self-MHC complex [147]. Based on the aforementioned self-MHC competition, congenically distinguishable B6, P14 and OT-1 Ly6C$^-$ cells were mixed to 1:1:1 ratio and adoptively transferred to another B6 or P14 host mice, then induction of Ly6C$^+$ cell was analyzed in each host mice as shown in Figure 5M. It is probable that if the adoptively transferred Ly6C$^-$ cells originated from one of the three donor mice (B6, P14, and OT-1) had been laid on the stronger self-MHC competition than Ly6C$^-$ originated from the others donor mice, the Ly6C$^-$ cells laid on stronger self-MHC competition than the other donor cells causing reduced self-TCR signals. The results of B6 host mice showed that the amount of Ly6C$^+$ cells were parallel with CD5 expression level of P14 and OT-1 mice donor cells (B6<P14<OT1) because there was a few self-MHC competition between each donor cell and host naïve CD8$^+$ T cells, [91]. On the other hand, in monoclonal P14 host data, Ly6C$^+$ cell generation of P14 donor cells was significantly suppressed similar to that of B6 donor cells. It suggested that the P14 donor cells experienced less chance to binding with self-MHC by self-competition because they have been facing into a huge pool size of clone expressing identical P14 TCR.

One of the key questions that still remains is why the Ly6C was expressed only in CD5^{hi}Ly6C$^-$ cells. It comes to two potential explanations, 1) differential expression of type I IFN receptor and 2) differential reactivity (*i.e.*, strength of the signal) for type I IFN. The possibility of difference in type I IFN receptor level was excluded since CD5^{lo} and CD5^{hi}Ly6C$^-$ cells expressed comparable

level of IFNAR1 (data not shown). On the other hand, the $CD5^{hi}Ly6C^{-}$ cells had more phosphorylation on STAT1 and STAT2 after type I IFN treatment than that of $CD5^{lo}$ cells. Therefore, it strongly suggested the difference in type I IFN reactivity can be answer to why Ly6C is induced only on $CD5^{hi}Ly6C^{-}$ cells. Then, obvious follow-up question would be how $CD5^{hi}Ly6C^{-}$ cells have stronger type I IFN reactivity than $CD5^{lo}$ cells even though they have similar level of type I IFN receptor expression. It has been reported that TCR signals increase the reactivity of cytokine stimuli, such as IL-2, by opening the closed chromatin of naïve $CD8^{+}$ T cells [148]. It has been also known that the expression level of CD5 represents strength of the self-TCR reactivity in steady-state mice [58]. Therefore, it is likely the mechanism that the $CD5^{hi}Ly6C^{-}$ cells have more open chromatin landscape than $CD5^{lo}$ cells on promotor regions of type I IFN-responding genes, such as Ly6C, via receiving stronger self-TCR stimulation in steady-state condition. So, further studies on histone modification of naïve $CD8^{+}$ T cell subsets will provide a new insight in understanding their role.

It was shown in this study that *in vitro* pre-treatment of type I IFN directly increases production of IFN-γ in naïve $CD8^{+}$ T cells. It is known that pSTAT1 homodimer complex binds to gamma-interferon activated site sequences (GASs) to provoke the expression of pro-inflammatory genes including IFN-γ [15, 16]. It would be interesting to examine that pre-treatment of type I IFN increases IFN-γ production in naïve $CD8^{+}$ T cells through the mechanism of pSTAT1 homodimer signaling. However, it remains to be revealed in further

study whether pre-treatment of type I IFN directly induces recruitment of pSTAT1 homodimer in naïve $CD8^+$ T cells.

Collectively, in the parts I and II, the results showed that constitutive type I IFN and self-TCR engagement regulate the generation of $Ly6C^+$ cells. Furthermore, constitutive type I IFN has enhanced IFN-γ production capacity of $Ly6C^+$ cells via upregulating the effector function-related gene profiles, especially *T-bet* and *Eomes* (Figure 12).

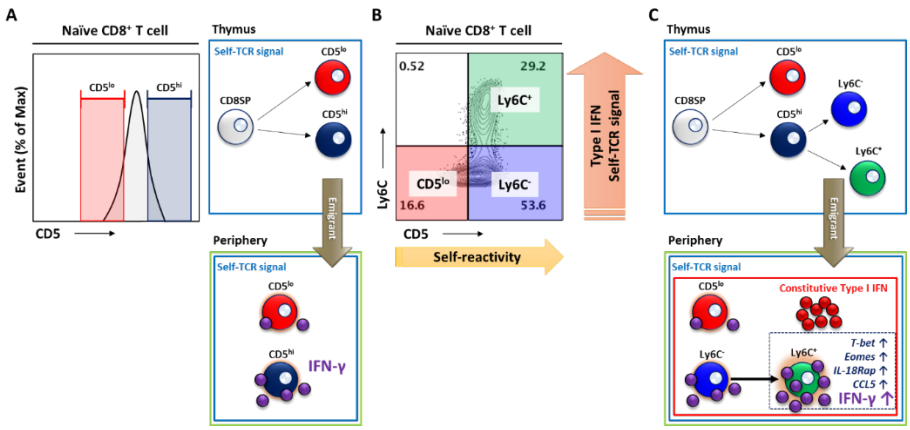

Figure 12. Novel findings from part I and II.

(A) Previous classification of naïve $CD8^+$ T cells and IFN-γ production potency of $CD5^{lo}$ and $CD5^{hi}$ naïve $CD8^+$ T cells. (B) New paradigm of heterogeneity in naïve $CD8^+$ T cells based on constitutive type I IFN and self-TCR signal dependent Ly6C expression. (C) Novel classification, suggested through the present study, of naïve $CD8^+$ T cells and IFN-γ production ability of $CD5^{lo}$, $Ly6C^-$ and $Ly6C^+$ naïve $CD8^+$ T cells.

In PART III, it has been suggested about the effect of constitutive type I IFN to the heterogenic naïve $CD8^+$ T cells through utilizing acute LCMV infection model.

Several studies have suggested that $CD5^{hi}$ cells show higher clonal expansion in mouse with LCMV infection than $CD5^{lo}$ cells [91], however no valid study has shown differentiation between $CD5^{lo}$ and $CD5^{hi}$ naïve $CD8^+$ T cells as a effector precursor. Up to date, it has been thought that naïve $CD8^+$ T cells can differentiate into every effector precursor similarly without keeping a stochastically preference to certain one [139, 149]. Bearing this in mind, the results in the present study showed that $CD5^{lo}$ prefers to be differentiated into MPEC ($KLRG1^{lo}CD127^+$), $Ly6C^+$ to SLEC ($KLRG1^{hi}CD127^-$), and $Ly6C^-$ showed intermediate level between $CD5^{lo}$ and $Ly6C^+$ cells. Each naïve $CD8^+$ T cell subsets appeared to have their own differentiation preference, keeping consistency with previous studies that showed better expansion of $CD5^{hi}$ cells than $CD5^{lo}$ cells in mouse with LCMV infection. In addition, the results showed that naïve $CD8^+$ T cell subsets have distinct differentiation preferences, even when perfectly identical TCR clones were used among the subsets to exclude possible misinterpretation due to differences in clones.

In general, the more effector $CD8^+$ T cells generate the more memory cells as a consequence [94]. However, recent studies on the differentiation of effector precursor cells during acute virus and bacterial infection have suggested that the naïve $CD8^+$ T cells which can be differentiated into MPEC ($KLRG1^{lo}CD127^{hi}$ or $CD27^+CX3CR1^{lo}$) remains more long-term memory cells

than SLEC (KLRG1^{hi}CD127^{lo} or CD27^{-}CX3CR1^{hi}) [99, 140]. At the best of my knowledge, the present study is the first to show that subsets of naive CD8^{+} T cells have different potentials for expansion and differentiation preference in mouse infected with LCMV. Thus, the differentiation preference of each subset in heterogenic naïve CD8^{+} T cell pool should be considered as a crucial factor for determining the potential of long-memory generation together with maximum magnitude of expansion during effector phase in LCMV infection. Collectively, results in the present study provide a new insight for importance of pre-determined differentiation preference in naive CD8^{+} T cell subsets.

Generally, strength of TCR signal is known to govern the differentiation of memory CD8^{+} T cells [108]. More specifically, the strength of mTOR signal after the TCR stimulation is known to be the most important factor for the differentiation of memory CD8^{+} T cells. Indeed, the highest memory CD8^{+} T cell differentiation occurred when an intermediate intensity mTOR signal is received during microbial infection [105]. The results in the present study also confirmed that mTOR signal-related gene set was enriched lower in CD5^{lo} cells than Ly6C^{-} and Ly6C^{+} cells, suggesting that this characteristic induced differentiation into MPEC and consequently form more long-term memory cells.

The IL-7 has been well-established to be a crucial survival factor of CD8^{+} T cells [65]. Especially in acute infection (e.g. LCMV or *Listeria monocytogenes*), IL-7 helps activated CD8^{+} T cells to survive during contraction and memory cell generation phase, not effector phase, because they lose IL-7Rα at effector

phase [99]. It has been reported IL-7Rα level can be re-acquired in MPEC within 10 dpi as high as on naïve T cell level, while SLEC only one-third [150]. Even though exact molecular mechanism still remains to be answered in further study, the present study suggested that $CD5^{lo}$ cells would have been re-acquired IL-7Rα faster than $Ly6C^{+}$ cells because $CD5^{lo}$ cells favored differentiation into MPEC rather than SLEC.

The present study revealed that the crucial factor to generate the $Ly6C^{+}$ cells is type I IFN, yet it was also questioned whether type I IFN can affect functional features to $Ly6C^{+}$ cells. Actually, previous study has shown that $Ifnar1^{-/-}$ effector $CD8^{+}$ T cells had an attenuated clonal expansion with low granzyme B production [45]. One of the reasons for essentiality of being received robust type I IFN signals in $CD8^{+}$ T cells during clonal expansion during the LCMV infection could be protection of the proliferating T cells against NK cells attack through upregulating MHC class I and natural cytotoxicity receptor (NCR) ligand [48, 49]. Despite those studies dealt with direct role of type I IFN in LCMV infection, the effect of constitutive type I IFN on naïve $CD8^{+}$ T cells at steady-state remained questionable. Since the previous experiments have been carried out using only $Ifnar1^{-/-}$ mice, no information is available to show the net effect of constitutive type I IFN on naive $CD8^{+}$ T cells. This is because experiments using $Ifnar1^{-/-}$ mice exclude not only the direct effects of type I IFN on naive $CD8^{+}$ T cells but also the indirect effects required for T cell activation in acute infection. In the present study, the experiment performed with IFNAR1 blocking antibody suggested that exposure to type I IFN I in

naïve $CD8^+$ T cells in steady-state is crucial to the expansion capacity and effector precursor differentiation of naïve $CD8^+$ T cell subset, especially $Ly6C^+$ cells. Collectively, this might be a direct evidence for demonstrating the effect of constitutive type I IFN induced functional modulation of naïve $CD8^+$ T cells.

It has been suggested that the post-thymic education of naïve $CD8^+$ T cells governs differentiation preference between MPEC and SLEC in LCMV infection [151]. Furthermore, adult naïve $CD8^+$ T cells express less effector function proteins, such as T-bet and Eomes, than neonatal naïve $CD8^+$ T cells, suggesting the possibility that changes could be educated during peripheral circulation [136]. Even though these previous studies have emphasized the importance of post-thymic education in gaining the functionality (i.e., clonal expansion and differentiation) of naïve $CD8^+$ T cells, the major factor of post-thymic education has been remained to be uncovered. The present study suggested that peripheral naïve $CD8^+$ T cell subsets gain the differential clonal expansion capacity and differentiation preference via exposure to constitutive type I IFN while there are no differences among thymic naïve $CD8^+$ T cell subsets. Therefore, the present study demonstrated that constitutive type I IFN would be the major factor to induce post-thymic education in naïve $CD8^+$ T cells.

Collectively, in part III, each naïve $CD8^+$ T cell subset has different clonal expansion capacity, differentiation into effector precursor, and memory generation potential via post-thymic education in mouse with LCMV infection model. Furthermore, it noting that the constitutive type I IFN as a major factor

for post-thymic education that regulates the fate decision of naïve $CD8^+$ T cells, especially $Ly6C^+$ cells, into MPEC or SLEC during the effector phase (Figure 13).

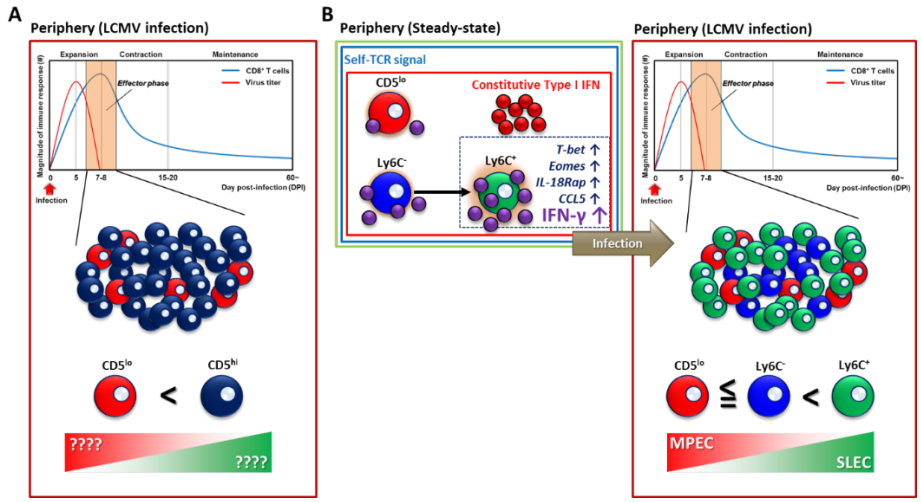

Figure 13. Novel findings from part III.

(A) Previously reported foreign-antigen reactivity of $CD5^{lo}$ and $CD5^{hi}$ naïve $CD8^+$ T cells in LCMV infection model. (B) Expansion and effector precursor differentiation in LCMV infection of naïve $CD8^+$ T cell subsets affected by constitutive type I IFN during steady-state.

The present study has been highlighting the two novel concepts as following; First, constitutive type I IFN can modulate the features of naïve $CD8^+$ T cells with diverse heterogeneity. Second, differentiation fate of naïve $CD8^+$ T cells in LCMV infection is stochastically pre-determined during post-thymic period.

It would be better if one could provide the underlying molecular mechanism on how the constitutive type I IFN changes the feature of naïve $CD8^+$ T cell subsets.

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VII. Summary in Korean

감염이나 질병이 없는 정상 상태(steady-state condition)에서 외부 항원을 한번도 경험한 적이 없는 naïve $CD8^+$ T 세포는 각각 CD44 및 CD62L의 발현 정도를 통해 $CD44^{lo}CD62^{hi}$의 표현형을 가지는 균일한 집단으로 정의될 수 있다. 그러나 최근의 연구들에서는 naïve $CD8^+$ T 세포들이 예전 연구들에서 보도된 것보다 더 다양한 세포막 단백질들을 발현하는 복잡한 집단으로 구성되어 있고 CD5의 발현 정도를 활용하여 naïve $CD8^+$ T 세포를 성격이 다른 $CD5^{lo}$ 세포 및 $CD5^{hi}$ 세포로 구분할 수 있다고 보고하였다. 또한 $CD5^{hi}$ 세포가 $CD5^{lo}$ 세포보다 높은 다양성(heterogeneity)을 지니는 동시에 박테리아나 바이러스 감염상황에서 더 높은 분열 능력을 보인다는 것도 보고되어 왔다.

본 연구에서 앞서 언급한 다양한 세포막 단백질 중 Ly6C 분자의 발현이 두드러짐을 확인할 수 있었고 특히, 생체 내(*in vivo*)에서는 높은 다양성을 보이는 $CD5^{hi}$ 세포에서만 Ly6C의 분자가 높게 발현되는 것을 확인하였다. 실제로 T 세포에서의 Ly6C 발현은 제 1 유형 인터페론(type I IFN)에 의해 증가한다고 알려져 왔고 정상상태의 생쥐에서 낮은 농도의 제 1 유형 인터페론이 항존적 사이토카인(constitutive cytokine)으로서 지속적으로 존재한다는 것이 알려져 왔

다. 그러나 이것이 흉선 내부 및 외부의 환경에서 어떻게 조절되는지 그리고 어떤 다른 항상성 요인(homeostatic factor)들과 관련이 있는지는 명확히 밝혀지지 않았었다.

본 연구에서는 제 1 유형 인터페론 알파 수용체 1 결핍 생쥐($Ifnar1^{-/-}$)의 흉선과 말초 면역 기관을 분석해본 결과 Ly6C를 발현하는 naïve $CD8^+$ 세포들이 사라진 것을 확인할 수 있었다. 또한 정상 생쥐와 제 1 유형 인터페론 알파 수용체 1 결핍 생쥐의 $Ly6C^-$ 세포들 분리하여 정상적인 숙주 생쥐에게 이식해주었을 때 정상 생쥐에서 유래한 $Ly6C^-$ 세포는 항존적 제 1 유형 인터페론에 의해 Ly6C를 획득한 반면에 제 1 유형 인터페론 알파 수용체 1 결핍 생쥐에서 유래한 $Ly6C^-$ 세포는 그렇지 못했다. 이를 통해 낮은 농도의 항존적 제 1 유형 인터페론이 흉선과 말초의 naïve $CD8^+$ T 세포 군집 내에서 $Ly6C^+$ 세포의 생성을 유도하는 중요한 요인임을 보여주었다. 또한 CD5의 발현이 T 세포의 자가 반응성(self-reactivity)을 대변할 수 있는 단백질임을 고려해보았을 때, $CD5^{hi}$ 세포에서 보여지는 높은 다양성은 아마도 자가 항원(self-ligands)과의 높은 결합력과 사이토카인에 대한 반응성, 특히 제 1 유형 인터페론에 대한 높은 반응성을 통해 획득되었을 가능성이 있음을 시사하였다.

또한 본 연구는 정상 상태의 항존적 제 1 유형 인터페론 신호는 $Ly6C^+$ 세포의 생성뿐만 아니라 *T-bet, eomes, IL-18Rap, CCL5*의 발현을

증가시켜서 독특한 유전적 특징을 형성시키며, Ly6C$^+$ 세포 특이적으로 인터페론-감마(IFN-γ) 생산 능력을 증가시킬 수 있음을 보여주었다. 더 나아가 림프구성 맥락수막염(Lymphocytic choriomeningitis; LCMV) 감염 모델에서는 항존적 제 1 유형 인터페론 신호가 Ly6C$^+$ 세포의 장기 기억 세포(long-term memory cell)로의 분화에도 영향을 줄 수 있음을 제안하였다. RNA 염기서열분석(RNA sequencing)을 활용한 유전자세트증폭분석(gene set enrichment assay; GSEA)을 통해 항존적 제 1 유형 인터페론에 가장 민감한 반응성 갖는다는 것이 확인된 Ly6C$^+$ 세포는 LCMV 감염 상황에서 Ly6C를 발현하지 않는 집단의 세포들보다 단기생존형 effector 세포(short-lived effector cell; SLEC)로 더 많이 분화하였으며, 더 적은 장기기억세포를 형성했다. 반면에 Ly6C$^+$ 세포와 유전적 및 기능적으로 가장 높은 이질성을 보였던 CD5^{lo} 세포는 기억세포 전구체 effector 세포(memory precursor effector cell; MPEC)로 더 많이 분화하였으며, 더 많은 장기기억세포를 형성함을 확인할 수 있었다. 그러나 흥미롭게도 정상상태에서 일시적으로 제 1 유형 인테페론 알파 수용체 1 차단 항체(blocking antibody)를 투여한 생쥐에서 분리한 Ly6C$^+$ 세포는 림프구성 맥락수막염 감염상황에서 기억세포 전구체 effector 세포로의 분화가 증가되고, 단기생존형 effector 세포로의 분화는 억제되는 것을 보였다. 이 실험을 통해 항존적 제 1 유형 인터페론이 naïve CD8$^+$ T 세포들의 장기기억세포로의 분화 잠재력을 조절할 수 있는 요인임을 시사할 수 있었다.

결론적으로, 본 연구는 항존적 제 1 유형 인터페론이 naïve $CD8^+$ T 세포의 자가 반응성과 밀접한 연관성을 가지고 있으며 그들의 표현형과 기능성에 영향을 줄 수 있다는 것을 보여주고 있다. 또한 더 나아가 naïve $CD8^+$ T 세포들의 장기기억세포로의 분화 가능성은 감염 상황에서 무작위로 부여되는 것이 아니라, naïve $CD8^+$ T 세포의 항존적 제 1 유형 인터페론에 대한 민감도에 따라 정상 상태에서 미리 결정되는 특성이라는 근거를 처음으로 제시한 연구일 것이다.