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Original Article

CD5 Expression Dynamically Changes During the Differentiation of Human CD8⁺ T Cells Predicting Clinical Response to Immunotherapy

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ABSTRACT

Defining the molecular dynamics associated with T cell differentiation enhances our understanding of T cell biology and opens up new possibilities for clinical implications. In this study, we investigated the dynamics of CD5 expression in CD8⁺ T cell differentiation and explored its potential clinical uses. Using PBMCs from 29 healthy donors, we observed a stepwise decrease in CD5 expression as CD8⁺ T cells progressed through the differentiation stages. Interestingly, we found that CD5 expression was initially upregulated in response to T cell receptor stimulation, but diminished as the cells underwent proliferation, potentially explaining the differentiation-associated CD5 downregulation. Based on the proliferation-dependent downregulation of CD5, we hypothesized that relative CD5 expression could serve as a marker to distinguish the heterogeneous CD8⁺ T cell population based on their proliferation history. In support of this, we demonstrated that effector memory CD8⁺ T cells with higher CD5 expression exhibited phenotypic and functional characteristics resembling less differentiated cells compared to those with lower CD5 expression. Furthermore, in the retrospective analysis of PBMCs from 30 non-small cell lung cancer patients, we found that patients with higher CD5 expression in effector memory T cells displayed CD8⁺ T cells with a phenotype closer to the less differentiated cells, leading to favorable clinical outcomes in response to immune checkpoint inhibitor (ICI) therapy. These findings highlight the dynamics of CD5 expression as an indicator of CD8⁺ T cell differentiation status, and have implications for the development of predictive biomarker for ICI therapy.

Keywords: CD5 antigen; CD8-positive T-lymphocyte; T-lymphocyte differentiation; Peripheral blood mononuclear cell; Non-small cell lung cancer; Immune checkpoint inhibitor

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
CCR7, C-C chemokine receptor type 7; CI, confidence interval; CTV, CellTrace Violet; DCB, durable clinical benefit; ICI, immune checkpoint inhibitor; KLRG1, killer cell lectin-like receptor subfamily G member 1; MFI, mean fluorescence intensity; mPFS, median progression-free survival; NDB, no durable benefit; ns, not significant; NSCLC, non-small cell lung cancer; Tcm, central memory T cells; TCR, T cell receptor; Tem, effector memory T cells; Temra, CD45RA re-expressing effector memory T cells; TIL, tumor-infiltrating lymphocyte; Tn, naïve T cells; Tpex, precursor exhausted T cells; Tscm, stem cell-like memory CD8+ T cells.

Author Contributions
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INTRODUCTION

T cells are a highly heterogeneous population and can be classified into distinct subpopulations based on their functional and phenotypic characteristics. By utilizing well-established surface markers, human T cells can be categorized into naïve T cells (Tn; $CCR7^{hi}$ $CD45RA^{hi}$), central memory T cells (Tcm; $CCR7^{hi}$ $CD45RA^{lo}$), and effector memory T cells (Tem; $CCR7^{lo}$) (1). While most of the Tem express low levels of CD45RA ($CCR7^{lo}$ $CD45RA^{lo}$), some of them re-express CD45RA (Temra; $CCR7^{lo}$ $CD45RA^{hi}$) (1). The prevailing consensus suggests that T cells follow a differentiation sequence of Tn, Tcm, Tem, and Temra, based on their self-renewal capacity (2), pluripotent potential (3), and cytotoxicity (4). However, the complex immunological history generates heterogeneity even within these subpopulations, allowing further stratification based on various phenotypic markers such as CD5, CD27, CD28, CD57, and CD95 (1,2,5-8).

CD5 has been extensively studied as a marker to define the heterogeneity within Tn (5,8-11). Its unique feature lies in its T cell receptor (TCR)-dependent upregulation mechanism, which allows the dissection of Ag-inexperienced Tn based on their affinity to self-Ags (5,8,11-14). Tn with high expression level of CD5 ($CD5^{hi}$), which exhibit a relatively high affinity to self-Ags, have been shown to possess superior proliferation ability in response to TCR or cytokine stimulation (15-17), as well as a more robust response to viral infection (15,16). While most studies on CD5 have been conducted in murine models, certain characteristics have also been demonstrated in humans. Both human thymocytes and peripheral T cells have been shown to upregulate CD5 expression in a TCR strength-dependent manner (10). Furthermore, human $CD4^+$ Tn were shown to have different functionalities depending on their relative CD5 expression (10).

As CD5 expression is markedly increased upon TCR stimulation, its level and utility as a surrogate marker of affinity for self-Ags are less applicable to Ag-experienced cells. Consequently, the regulation of CD5 expression in Ag-experienced memory T cells remains relatively unexplored. Nevertheless, a recent study reported that CD5 in human memory $CD8^+$ T cells inhibits the mTOR pathway, thereby suppressing IL-15-induced proliferation (18). Similarly, another study suggested that IL-15 induces the selective expansion of memory $CD8^+$ T cells (19). Despite these studies, the precise regulatory mechanisms underlying CD5 expression and the biological significance of heterogeneous CD5 expression in memory T cells remain largely elusive. In this study, using PBMCs from healthy donors and patients with non-small cell lung cancer (NSCLC), we demonstrated previously unrecognized regulatory dynamics of CD5 expression during $CD8^+$ T cells differentiation with potential clinical implications for developing a predictive biomarker for cancer immunotherapy.

MATERIALS AND METHODS

Human samples

Human blood samples for this study were provided by the Korean Red Cross and the Biobank of Chonnam National University Hwasun Hospital, a member of the Korea Biobank Network, in accordance with a protocol approved by the Institutional Review Boards of Chonnam National University Hwasun Hospital (CNUHH-2018-036 and CNUHH-2021-045). All patients provided written informed consent. PBMCs were obtained from whole blood by density gradient centrifugation using Lymphoprep (Alere Technologies GmbH, Koln, Germany).

Flow cytometry

PBMCs were stained for flow cytometric analysis with the following Abs (purchased from BioLegend [San Diego, CA, USA], eBioscience [San Diego, CA, USA], and BD Biosciences [Franklin Lakes, NJ, USA]): CD11b (M1/70), CD56 (5.1H11), CD3 (OKT3), CD19 (HIB19), $CD8^+$ (SK1), CD4 (RPA-T4), CD5 (UCHT2), killer cell lectin-like receptor subfamily G member 1 (KLRG1; 14C2A07), PD1 (29E2A3), CD95 (DX2), CD44 (IM7), CD183 (G025H7), CD39 (A1), T cell factor 1 (TCF1; 7F11A10), CD62L (DREG-56), CD27 (LG.7F9), perforin (B-D48), and granzyme B (GB11). Flow cytometry samples were run using CytoFLEX LX (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

In vitro $CD8^+$ T cell activation

Total $CD8^+$ Tn were purified using MagniSort™ Human $CD8^+$ Naïve T cell Enrichment Kit (Invitrogen, Waltham, MA, USA). $CCR7^+CD45RA^+CD95^-CD8^+$ Tn were sorted into $CD5^{lo}$ and $CD5^{hi}$ subsets (based on the lower or upper 20% of CD5 expression) using CytoFLEX SRT (Beckman Coulter). For some experiments, purified cells were labelled with 2.5 μM of Cell Trace™ Violet (ThermoFisher, Waltham, MA, USA) or Carboxyfluorescein succinimidyl ester (ThermoFisher). Purified cells were cultured in anti-CD3 (5 μg/ml) (clone OKT3; eBioscience) and anti-CD28 (2 μg/ml) (clone CD28.2; Invitrogen) coated Clear Flat-Bottom Immuno Nonsterile 96-Well Plate (ThermoFisher) for indicated time points. In some experiments, 10 ng/ml of cytokines (human IL-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12, IL-15, IL-18, IL-21, IL-23, TGF-β, IFN-β, IFN-γ, or GM-CSF; PeproTech, Cranbury, NJ, USA) were supplemented in the culture.

Intracellular staining

PBMCs were plated in 96-well Clear Round Bottom TC-treated Microplate (Corning, Corning, NY, USA) and stimulated with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors) (Invitrogen) for 4 h in a 37°C CO_2 incubator. Cells were stained for surface markers, then fixed and permeabilized with BD Cytofix/Cytoperm buffer (BD Biosciences). Cells were then stained for the indicated intracellular molecules and analyzed by flow cytometry. For analysis of transcription factors, *ex vivo* PBMCs were fixed and permeabilized with eBioscience Foxp3/Transcription Factor Staining Buffer Set (eBioscience), then stained for the indicated molecules.

Statistics

Samples were tested for a normal distribution using normality tests. For normally distributed samples, paired 2-tailed Student's *t*-test were performed. For samples that did not pass the normality tests, Wilcoxon matched-pairs signed rank test were performed. The statistics used for each figure are indicated in the legends of each figure. All statistics were performed using Prism (GraphPad Software). Values of $p<0.05$, $p<0.01$, $p<0.001$, $p<0.0001$ were considered significant. 95% confidence interval (CI) was calculated using R package (survival) for progression-free survival, and normal approximation for durable clinical benefit.

RESULTS

Human $CD8^+$ T cells exhibit dynamic CD5 expression during their differentiation

The regulatory mechanisms underlying CD5 expression in human T cell populations remain largely unexplored, although they have been well-documented in murine models (20,21). Therefore, we initially examined CD5 expression levels in various lymphoid lineage cells derived from PBMCs of healthy donors using flow cytometry (**Supplementary Fig. 1A and B**).

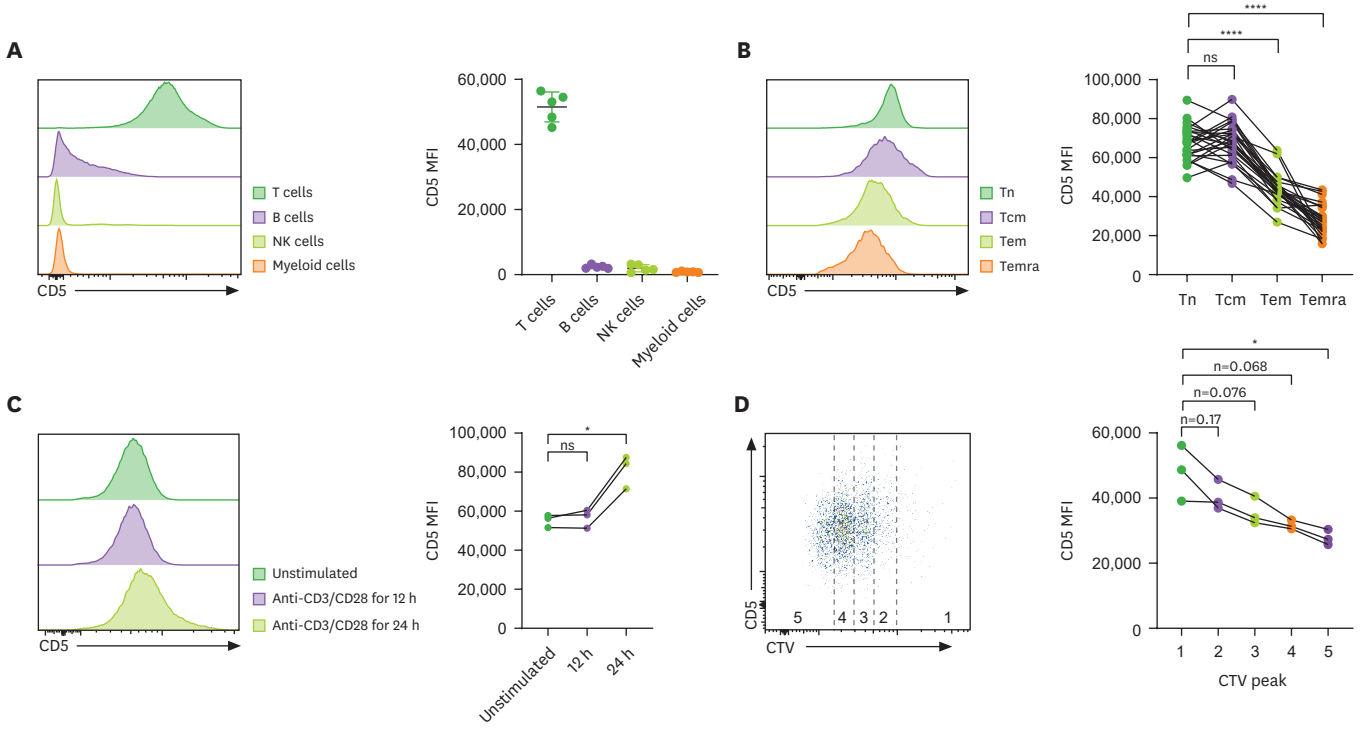

Figure 1. Characterization of CD5 expression in human PBMCs. (A) CD5 expression in T cells, B cells, NK cells and myeloid cells was assessed. Representative histogram data (left) and summary of CD5 MFI for each subset (right) are shown (n=5). (B) CD5 expression levels of CD8⁺ T cell subpopulations in PBMCs were evaluated. Representative histogram data (left) and summary of CD5 MFI of subpopulations (right) are shown (n=29). (C) CD8⁺ Tn were purified and stimulated with anti-CD3 (5 μg/ml), and anti-CD28 (2 μg/ml) for 0, 12, or 24 h. Changes in CD5 expression was assessed. Representative histogram data (left) and summary of CD5 MFI at each time point are shown (n=3). (D) CD8⁺ Tn were purified and labeled with CTV, then stimulated with anti-CD3 (5 μg/ml), and anti-CD28 (2 μg/ml) for 4 days. The changes in CD5 expression were assessed during the proliferation. Representative flow cytometric data (left) and summary of CD5 MFI of each CTV peak (right) are shown (n=3). (B-D) Statistical significance was performed with paired Student's *t*-test.
Values of *p<0.05, ****p<0.0001 were considered significant.

Notably, high CD5 expression was observed only in the T cell compartment (**Fig. 1A**), with the exception of certain B cell subsets that expressed CD5 at low levels (**Fig. 1A**). Within the T cell population, CD4⁺ T cells exhibited slightly higher CD5 expression compared to CD8⁺ T cells (**Supplementary Fig. 1C**). These findings indicate that the CD5 expression patterns of human PBMCs within broadly categorized lymphoid lineages are consistent with those observed in murine models (10,20,21).

Next, we investigated whether there were differences in CD5 expression based on T cell differentiation status (**Supplementary Fig. 1B**). CD4⁺ T cells exhibited nearly constant CD5 expression throughout differentiation (**Supplementary Fig. 1D**). Interestingly, however, human CD8⁺ T cells showed a stepwise decrease in CD5 expression throughout the differentiation process (**Fig. 1B**). To elucidate this phenomenon, we explored the regulatory mechanisms governing CD5 expression in human CD8⁺ T cells. First, we isolated CD8⁺ Tn from PBMCs of healthy donors and stimulated them with anti-CD3/CD28 Abs. CD8⁺ Tn increased CD5 expression after 24 h of TCR stimulation (**Fig. 1C**), confirming the presence of a TCR-mediated mechanism for upregulating CD5 expression. Next, we hypothesized the existence of an additional mechanism responsible for the downregulation of CD5 expression after its initial upregulation in CD8⁺ T cells. To investigate this, we assessed CD5 expression in CellTrace Violet (CTV)-labeled CD8⁺ Tn following 5 days of TCR stimulation. Notably, CD5 expression declined as CD8⁺ Tn underwent proliferation (**Fig. 1D**). This proliferation-associated downregulation of CD5 was observed even when various cytokines were treated

alongside TCR stimulation (**Supplementary Fig. 1E**). Collectively, these data suggest that proliferation-associated downregulation of CD5 may be a mechanism responsible for decreased CD5 expression as differentiation progresses.

CD5^{hi} effector memory CD8^{+} T cells display a less differentiated phenotype in comparison to their CD5^{lo} counterparts

Considering the observed decrease in CD5 expression as CD8^{+} T cells undergo proliferation, we hypothesized that relative CD5 expression might serve as an indicator of their proliferation history. In particular, effector memory T cells, which have participated in numerous immunological encounters, represent a highly heterogeneous population ranging from recently generated cells to senescent cells (22-24). We aimed to explore whether the relative CD5 expression within Tem could elucidate this diversity. To achieve this, we categorized Tem into 2 subsets based on their relative CD5 expression, namely CD5^{hi} and CD5^{lo} Tem (top 20% and bottom 20%, respectively) (**Supplementary Fig. 2A**), and subsequently conducted a comparative analysis of their phenotypic and functional characteristics.

First, to clarify that CD5^{hi} and CD5^{lo} Tem are not simply derived from CD5^{hi} and CD5^{lo} Tn, respectively, but rather diverge based on their distinctly different proliferation histories, we conducted *in vitro* stimulation of CD5^{hi} and CD5^{lo} Tn and evaluated their CD5 expression. Indeed, CD5 expression after TCR stimulation was primarily influenced by proliferation rather than the initial cellular origin prior to stimulation (**Supplementary Fig. 2B and C**). Next, we conducted a comparison of phenotypic markers between CD5^{hi} and CD5^{lo} Tem. Numerous molecules have been reported to exhibit differential expression throughout the differentiation process (**Fig. 2A**) (1,4). For instance, KLRG1, a marker associated with T cell senescence (1,4,25), displayed an increase as Tn differentiated into Temra (**Fig. 2A**). In addition, the expression of several other molecules (PD1, CD95, CD44, CD183, CD39, TCF1, CD62L, and CD27) generally diminished as differentiation progressed, although some of them (PD1, CD95, CD44, and CD183) experienced upregulation in the early stages of differentiation (**Fig. 2A**). Interestingly, CD5^{hi} Tem exhibited a molecular phenotype resembling less differentiated cells compared to CD5^{lo} Tem (**Fig. 2B-E**, **Supplementary Fig. 2D**). Likewise, CD5^{hi} and CD5^{lo} Temra also displayed a similar tendency, although some molecules such as KLRG1, CD95, or TCF1 was less apparent (**Fig. 2B, F-H**, **Supplementary Fig. 2E**). These findings strongly suggest an inverse relationship between the CD5 expression in Tem and the observed phenotypic changes associated with differentiation.

Cytotoxic function serves as a compelling indicator for discerning the differentiation status of human CD8^{+} T cells (4). We thus examined the expression of both perforin and granzyme B, within different subsets of human CD8^{+} T cells, and observed an increase of these cytotoxic molecules as differentiation progressed (**Fig. 3A**). Notably, within Tem, the CD5^{lo} subset exhibited significantly higher levels of perforin and granzyme B expression compared to the CD5^{hi} subset (**Fig. 3B and C**). Similar findings were also observed in Temra (**Fig. 3D and E**). Together, these findings support the notion that compared to CD5^{lo} cells, CD5^{hi} cells have phenotypic characteristics that indicate a less proliferative and less differentiated state within the heterogeneous pool of Tem.

CD5 expression on effector memory CD8^{+} T cells is a predictor of clinical response to ICI therapy

The advent of ICIs has revolutionized the treatment of cancer patients (26-29). However, the relatively low response rate to ICI treatment remains a major challenge (26,27), and

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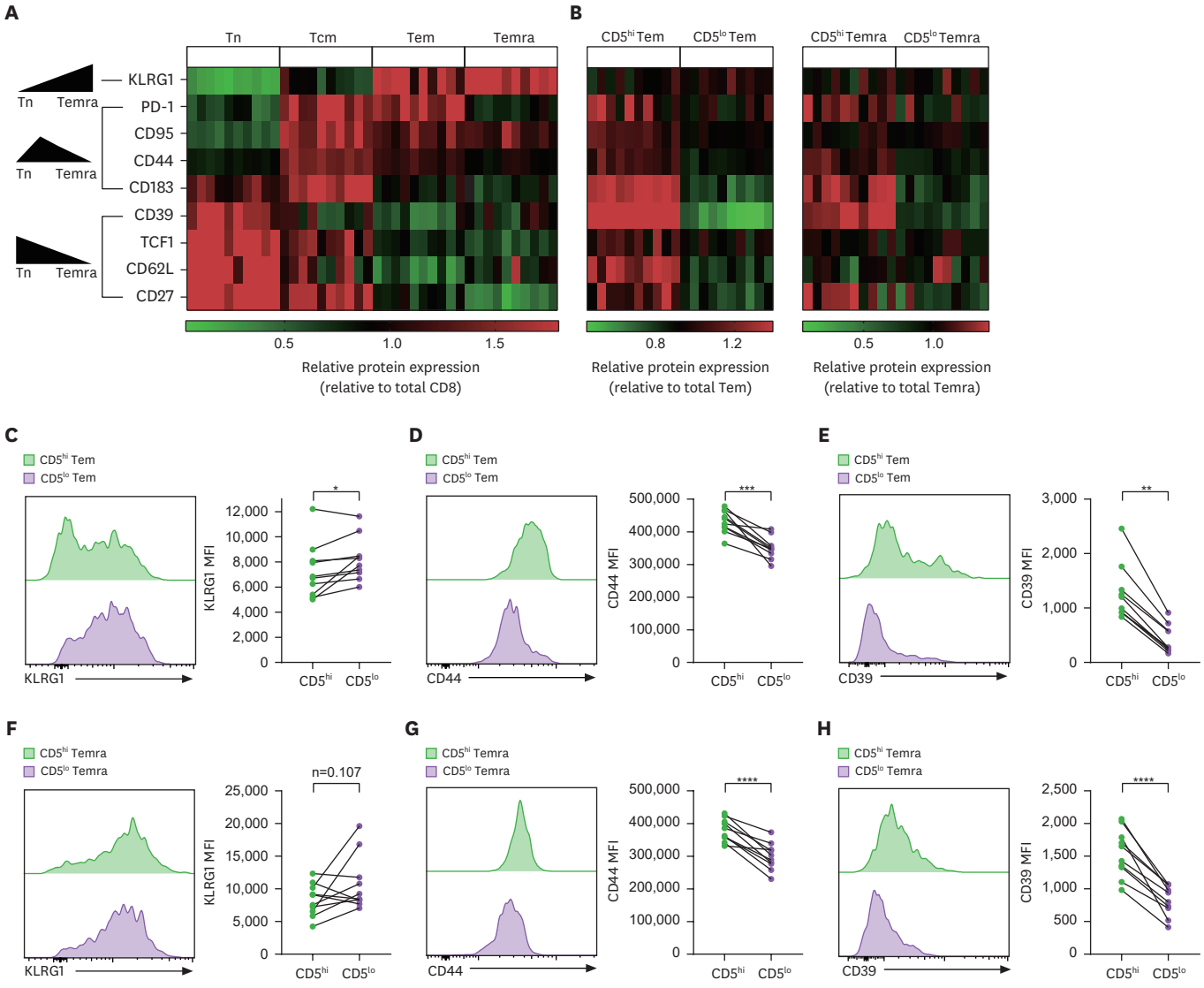

Figure 2. Differential molecular expression in CD5^{hi} and CD5^{lo} effector memory CD8^{+} T cells. (A, B) Heatmaps representing relative molecular expressions in (A) Tn, Tcm, Tem and Temra or in (B) CD5^{hi} and CD5^{lo} Tem (left) and Temra (right) are shown. The relative expressions are color-coded from green (low) to red (high). To determine relative expression, the MFI within each subset was divided by the MFI of total CD8^{+} T cells, Tem, or Temra (n=10). (C-H) The expressions of (C, F) KLRG1, (D, G) CD44, and (E, H) CD39 in CD5^{hi} and CD5^{lo} (C-E) Tem and (F-H) Temra are shown. Representative histogram data (left) and summary of expressions of the molecules (right) are shown (n=10). (C-H) Statistical significances were performed with (C, D, F-H) paired Student's *t*-test or (E) Wilcoxon matched-pairs signed rank tests.
Values of *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 were considered significant.

there is therefore growing interest in identifying the characteristics of individuals who are likely to respond to ICI treatment. Accumulating evidence highlights the importance of less differentiated CD8^{+} T cells in the context of ICI treatment (30-35). Given that CD5 expression has been shown to effectively reflect the differentiation status of effector memory CD8^{+} T cells, we sought to investigate whether CD5 expression correlates with ICI responsiveness. To accomplish this, we performed a retrospective analysis of CD8^{+} T cells from PBMCs in a cohort of patients with NSCLC (n=30) (**Fig. 4A**, **Supplementary Table 1**). Consistent with findings in healthy donors, we observed a similar tendency for CD5 expression on peripheral blood CD8^{+} T cells to decrease as their differentiation progressed from naïve to effector

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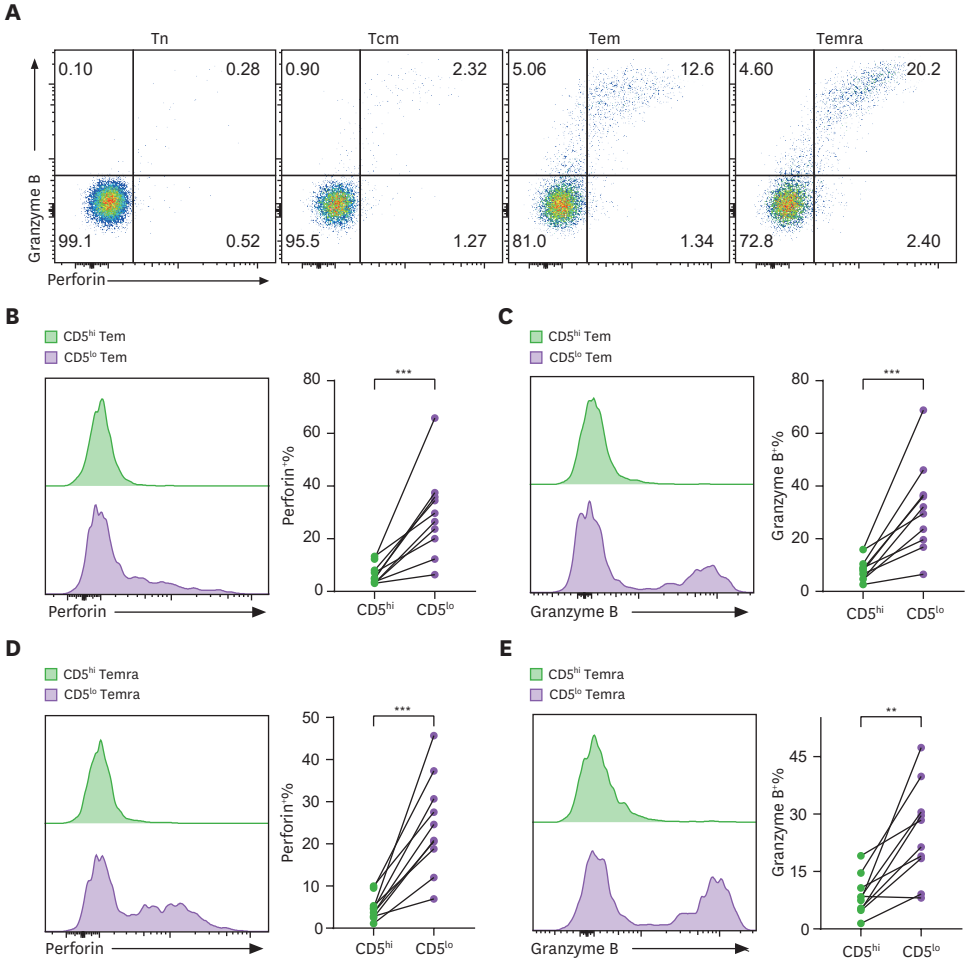

Figure 3. Perforin and granzyme B expression in CD5^{hi} and CD5^{lo} effector memory CD8^{+} T cells. (A) Representative flow cytometric data for the expression of perforin and granzyme B in CD8^{+} T cell subpopulations (Tn, Tcm, Tem, and Temra) are shown. (B-E) The expressions of (B, D) perforin and (C, E) granzyme B in CD5^{hi} and CD5^{lo} (B-C) Tem and (D-E) Temra were assessed. Representative histogram data (left) and frequencies of perforin- and granzyme B-expressing cells (right) are shown (n=10). (B-E) Statistical significance was performed with paired Student's *t*-test.
Values of **p<0.01, ***p<0.001 were considered significant

memory states (**Fig. 4B**). Moreover, the superior cytotoxic function of CD5^{lo} cells compared to their CD5^{hi} counterparts was also evident (**Fig. 4C-F**).

Next, we determined whether the observed differences in CD5 expression levels between patients could be used as a marker of distinct differentiation states, similar to the differences observed between CD5^{hi} and CD5^{lo} cells within the same individual. Notably, strong inverse correlations were observed between CD5 expression in Tem (and Temra) and the expression levels of perforin and granzyme B across different individuals (**Fig. 4G and H**). These data indicate that CD5 expression on effector memory CD8^{+} T cells can be a surrogate marker to predict the degree of differentiation between individual patients. Therefore, we further investigated the relationship between the CD5 expression level and the clinical response to ICI therapy. The patient cohort was divided into 2 groups based on the level of CD5 expression on Tem: Tem-CD5$^{hi\text{-}individual}$ and Tem-CD5$^{lo\text{-}individual}$ (higher and lower, respectively, than the average mean fluorescence intensity [MFI] of CD5 expression on CD8^{+} Tem from patients with NSCLC), and clinical outcomes were compared after ICI treatment (**Fig. 5A**). Notably, compared to Tem-CD5$^{lo\text{-}individual}$, Tem-CD5$^{hi\text{-}individual}$ exhibited significantly longer

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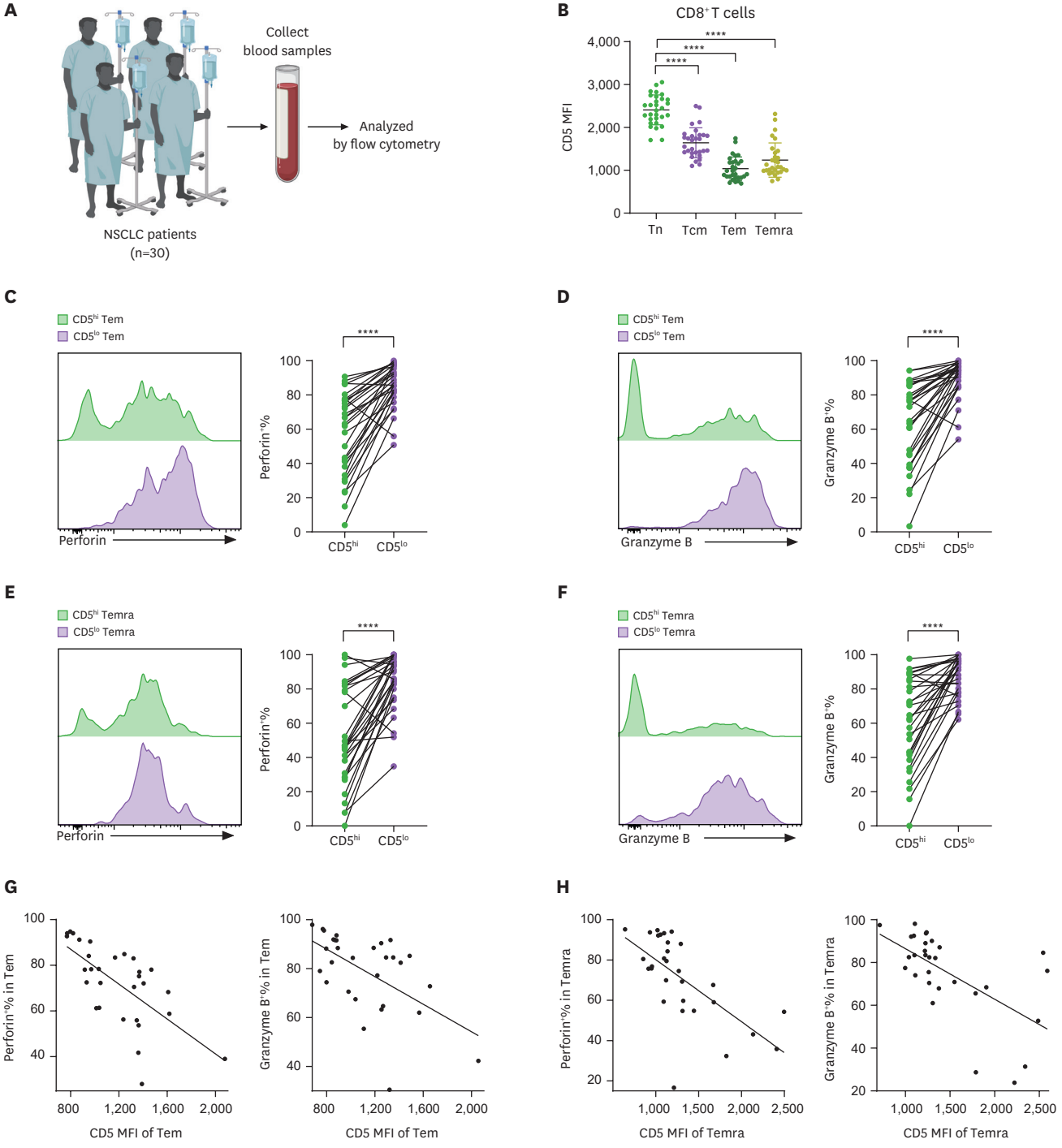

Figure 4. Correlation between CD5 expression in effector memory CD8⁺ T cells and cytotoxicity in NSCLC patients. (A) A schematic model illustrating the analysis process for the blood samples obtained from NSCLC patients is shown. (B) CD5 expression in CD8⁺ T cell subpopulations is evaluated in NSCLC patients. (C-F) Expression of (C, E) perforin and (D, F) granzyme B in $CD5^{hi}$ and $CD5^{lo}$ (C, D) Tem and (E, F) Temra is assessed. Representative histogram data (left) and frequencies of perforin- and granzyme B-expressing cells (right) are shown. (G, H) The correlation between CD5 MFI and the expression of perforin (left) and granzyme B (right) in (G) Tem and (H) Temra is examined. (C-F) Statistical significance was performed with (C, F) paired Student's *t*-test or (D, E) Wilcoxon matched-pairs signed rank test. Values of ****p<0.0001 were considered significant.

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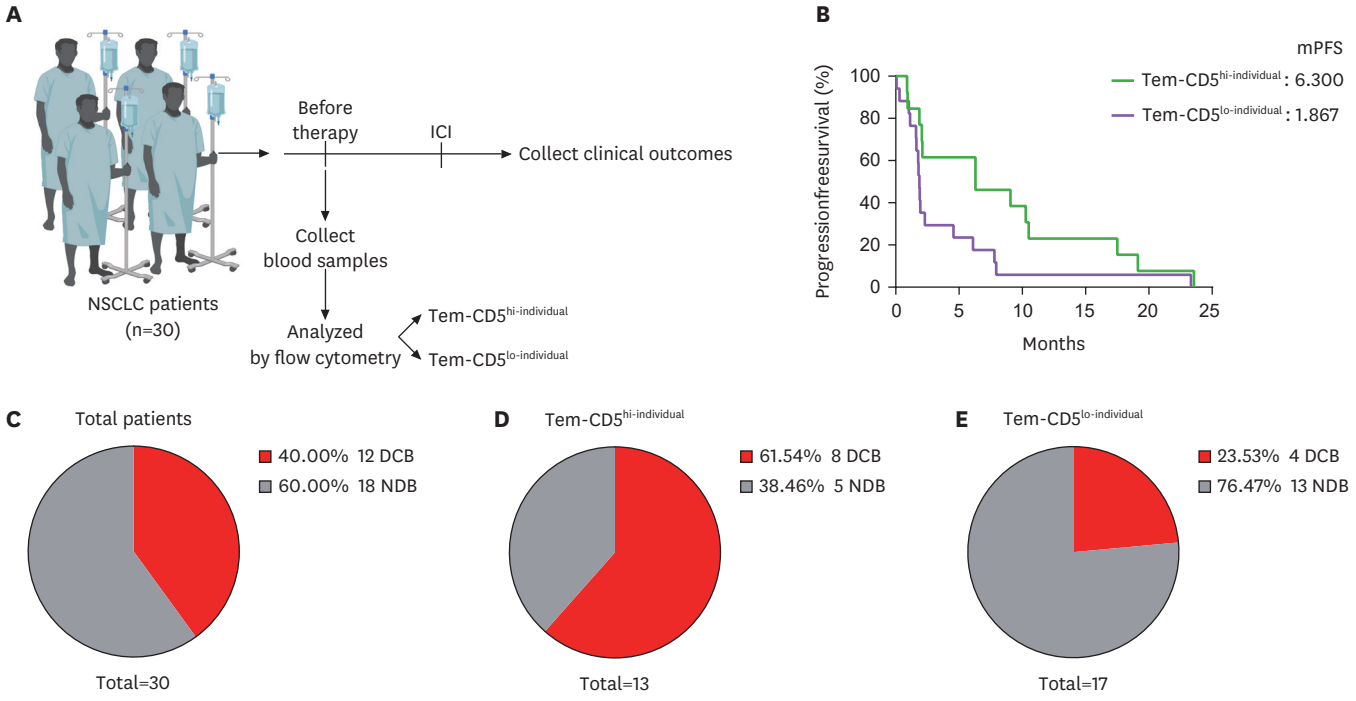

Figure 5. Positive correlation between CD5 expression in effector memory CD8⁺ T cells and clinical outcomes by immune checkpoint inhibitor therapy in NSCLC patients. (A) Schematic model describing how blood samples from NSCLC patients with ICI treatment are analyzed is shown. (B) Kaplan-Meier curve for progression free survival analyzed in Tem-CD5$^{hi\text{-}individuals}$ and Tem-CD5$^{lo\text{-}individuals}$ NSCLC patients. (C-E) Proportions of patients with durable clinical benefit are assessed in (C) total, (D) Tem-CD5$^{hi\text{-}individual}$, and (E) Tem-CD5$^{lo\text{-}individual}$ patients.

median progression-free survival (mPFS) (6.3 vs. 1.867 months; 95% CI, 2.07–10.5 months vs. 1.60–4.57 months) (**Fig. 5B**) and a higher proportion of patients with durable clinical benefit (DCB) (61.54% vs. 23.53%; 95% CI, 35.0–88.0% vs. 3.4–43.7%) (**Fig. 5C-E**). Similar results were also obtained when patients were divided based on the level of CD5 expression on Temra, although the difference was somewhat smaller than for those observed in Tem (**Supplementary Fig. 3A-D**). It is also important to note that this correlation was not associated with differences in the proportion of naive CD8⁺ (Tn) cells (**Supplementary Fig. 3E-G**) or differences in CD5 expression levels of Tn (**Supplementary Fig. 3H-J**). Collectively, these findings suggest that the CD5 expression level of effector memory CD8⁺ T cells in patients' blood is positively correlated with better clinical outcomes and can therefore be considered a predictive biomarker of ICI treatment.

DISCUSSION

The precise mechanisms of how CD5 expression levels are regulated in the human effector memory CD8⁺ T cell population remain largely unexplored. In this study, we investigated the dynamics of CD5 expression during the differentiation of human CD8⁺ T cells and observed a progressive decrease in CD5 expression as they differentiated. Importantly, CD5 expression was found to initially increase after TCR stimulation and then gradually decrease as cell division continued, which may explain the downregulation of CD5 expression during CD8⁺ T cell differentiation. Moreover, we showed that effector memory CD8⁺ T cells can be further stratified by CD5 expression levels, which are closely related to their proliferation/differentiation history. Indeed, CD5^{hi} Tem (and Temra) exhibited a less differentiated

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phenotype compared to their CD5lo counterparts, as evidenced by surface molecule expressions and cytotoxic functions. Furthermore, considering the association between less differentiated CD8+ T cells and favorable outcomes to ICI therapy, we also showed that CD5, as an indicator of less differentiated subsets within effector memory CD8+ T cell population, can be a useful biomarker for predicting clinical response to ICI therapy for patients with NSCLC.

CD5 has been extensively investigated due to its close relationship with TCR signaling (5,12,15,20,22). Its comprehensive investigation primarily focuses on Tn, and the mechanisms governing the regulation of CD5 expression on Ag-experienced effector/effector memory T cells are not fully understood. In murine models, it has been observed that Ag-inexperienced virtual memory T cells display higher levels of CD5 expression compared to Tn (36,37). However, in the case of Ag-experienced memory cells, CD5 expression has been shown to be lower compared to Tn (38). This pattern was also observed in human memory CD8+ T cells, which primarily consist of Ag-experienced memory cells and exhibit a gradual decline in CD5 expression (19). This phenomenon was intriguing, particularly considering the prevailing notion that CD5 expression is upregulated in response to TCR stimulation. However, we showed that following an initial upregulation of CD5 expression, its levels subsequently decline during the proliferation of CD8+ T cells. Notably, the observed decrease in CD5 expression was not attributed to the potential differences in the inherent proliferative capacity of distinct naive subsets, as both CD5hi and CD5lo Tn exhibited comparable proliferation. These findings therefore suggest that proliferation-dependent downregulation is a key mechanism by which CD5 expression levels on effector memory CD8+ T cells vary between different individuals and even within the same individual.

Given that human effector/effector memory CD8+ T cells can be generated in the context of various cytokine exposures, it is reasonable to speculate that these cytokines can influence CD5 expression levels. In fact, a study reported that human memory CD8+ T cells stimulated by IL-15 alone reduced CD5 expression in proliferation-dependent manner (19). However, it is important to note that although various cytokines tested in this study affected the overall dynamics of CD5 expression to some extent, proliferation-dependent downregulation of CD5 was still evident with all cytokines analyzed, indicating a strong inverse correlation between CD5 expression and proliferation.

The main implication from the above proliferation-dependent CD5 downregulation is that relative density of CD5 expression is a discriminative marker to define the cellular continuum of human effector memory CD8+ T cell population. Indeed, CD5hi Tem and Temra displayed a less differentiated phenotype in terms of expression of both activation-related and cytotoxic effector molecules compared to their CD5lo cells. We also confirmed similar phenomenon on effector memory CD8+ T cells from different individuals, extending the inverse association of CD5 density and differentiation status beyond inter-individual variation. It seems therefore clear that the absolute level of CD5 expression rather than its relative expression within individual can dictate a more differentiated subset of effector memory CD8+ T cells with enhanced cytotoxicity. Whether this phenomenon suggests a direct role of CD5 in modulating proliferation and cytotoxic function, possibly through its influence on TCR signaling (39) or cytokine responsiveness (15) needs to be further investigated.

The association between the differentiation status of CD8+ T cells and their responsiveness to ICI has been extensively demonstrated in various studies (30-35). For instance, adoptive transfer of stem cell-like memory CD8+ T cells (Tscm) was shown to induce potent anti-tumor

effects with ICI treatment (40,41). Similarly, several studies in mice have also highlighted the role of precursor exhausted T cells (Tpex), which possess self-renewing ability and sustain a long-term supply of exhausted T cells, as a potential cellular target responding to anti-PD-1/PD-L1 therapy and mediating potent anti-tumor effect (42-46). In addition, other studies have shown that CD28+ PD-1+ CD8+ tumor-infiltrating lymphocytes (TILs) retain proliferative capacity in response to anti-PD-1/PD-L1 therapy in contrast to their non-responsive CD28− PD-1+ CD8+ TIL counterpart (47,48). These findings highlight the enhanced responsiveness of less differentiated CD8+ T cells to ICI therapy.

Building upon this perspective, we investigated whether the relative density of CD5 expression that is closely associated with differentiation status of effector memory CD8+ T cells could be a potential marker for predicting ICI responsiveness. Notably, in our retrospective analysis for patients with NSCLC who received anti-PD-1/PD-L1 therapy, individuals with higher CD5 expression on their Tem showed improved clinical outcomes to anti-PD-1/PD-L1 therapy compared to those with lower CD5 expression. However, it is important to note that our findings were not based on TILs but instead largely limited to PBMCs, where tumor-specific CD8+ T cells are likely to be much less enriched. While the relationship between peripheral blood CD8+ T cells and ICI responsiveness is still not fully understood, recent studies have demonstrated correlations between them (30,49-52). For instance, patients with NSCLC who exhibited increased proliferation of peripheral blood PD-1+ CD8+ T cells after ICI therapy demonstrated positive clinical outcomes (53). In addition, a recent study has shown the clonal replacement, suggesting that ICI therapy replenishes TILs with new clones derived from the periphery (51). Our findings, which highlight the correlation between CD5 expression in peripheral blood CD8+ Tem and positive clinical outcomes, further support this notion. It will also be interesting to see if similar relationships apply to other peripheral blood CD8+ T cell populations, particularly Tscm and Tpex, which will need to be addressed in the future.

There are also limitations in our study. The size of patient cohort is relatively small and thus will be important to further validate with large cohort studies in the future. In addition, it should be noted that it is difficult to determine the exact cut-off value of CD5 expression level, because CD5 expression on human T cell populations displays marked variability along a continuous spectrum, with a relatively wide range of expression levels across different individuals. Further improvement is thus needed for practical use as a predictive biomarker. Overall, this study highlights previously unappreciated aspects on the dynamics of CD5 expression associated with CD8+ T cell differentiation that have potential implications for the development of predictive biomarker for cancer immunotherapy.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Patient clinical characteristics.

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Supplementary Figure 1

CD5 expression in human PBMCs. (A, B) Gating strategies to distinguish (A) T cells, B cells, NK cells, and myeloid cells or (B) $CD4^+$ and $CD8^+$ T cell subpopulations (Tn, Tcm, Tem, and Temra) from PBMCs are shown. (C) CD5 expression in $CD4^+$ and $CD8^+$ T cells were assessed. Representative histogram data (left) and summary of CD5 MFI of each subset (right) are shown (n=29). (D) CD5 expressions of $CD4^+$ T cell subpopulations in PBMCs were assessed. Representative histogram data (left) and summary of CD5 MFI of subpopulations (right) are shown (n=29). (E) $CD8^+$ Tn were purified and labeled with CFSE, then stimulated with anti-CD3 (5 μg/ml), anti-CD28 (2 μg/ml), and various cytokines (10 ng/ml) for 5 days. The changes in CD5 expression were assessed during the proliferation. Flow cytometric data (left) and summary of CD5 MFI of each CFSE peak (right) are shown. (C-E) Statistical significance was performed with paired Student's *t*-test.

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Supplementary Figure 2

Differences in various molecules between $CD5^{lo}$ and $CD5^{hi}$ subset of peripheral blood effector memory $CD8^+$ T cells. (A) The expression of CD5 in $CD5^{hi}$ and $CD5^{lo}$ Tem (left) and Temra (right) are shown. CD5 expression of $CD5^{hi}$ (top 20%) and $CD5^{lo}$ (bottom 20%) of Tem or Temra. (B, C) $CD5^{hi}$ and $CD5^{lo}$ $CD8^+$ Tn were purified and labeled with CTV, then stimulated with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) for 5 days. (B) CD5 expression of $CD5^{hi}$ and $CD5^{lo}$ Tn before (left) or after (right) stimulation are shown. (C) The changes in CD5 expression were assessed during the proliferation of $CD5^{hi}$ and $CD5^{lo}$ Tn. (D, E) Summary of surface (PD-1, CD95, CD183, CD62L, and CD27) and intracellular (TCF1) molecule expression of $CD5^{hi}$ and $CD5^{lo}$ (D) Tem and (E) Temra are shown (n=10). (D, E) Statistical significance was performed with paired Student's *t*-test for data that passed normality tests and Wilcoxon matched-pairs signed rank test for data that did not passed normality tests.

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Supplementary Figure 3

Predictive ability of CD5 expression on peripheral blood effector memory $CD8^+$ T cells to ICI therapy. (A) Schematic model describing how blood samples from NSCLC patients with ICI treatment were analyzed is shown. (B-D) Thirty patients with NSCLC were categorized into Temra-$CD5^{hi\text{-}individual}$ and Temra-$CD5^{lo\text{-}individual}$ groups based on their CD5 expression in $CD8^+$ Temra. (B) Kaplan-Meier curve for progression-free survival is analyzed in Temra-$CD5^{hi\text{-}individual}$ and Temra-$CD5^{lo\text{-}individual}$ patients. (C, D) Proportions of patients with durable clinical

benefit are assessed in (C) Temra-CD5$^{hi\text{-}individual}$, and (D) Temra-CD5$^{lo\text{-}individual}$ patients. (E-G) 30 patients with NSCLC were categorized into Tn%-high and Tn%-low groups based on their proportion of Tn in total CD8⁺ T cells. (E) Kaplan-Meier curve for progression-free survival is analyzed in Tn%-high and Tn%-low patients. (F, G) Proportions of patients with durable clinical benefit are assessed in (F) Tn%-high, and (G) Tn%-low patients. (H-J) 30 patients with NSCLC were categorized into Tn-CD5$^{hi\text{-}individual}$ and Tn-CD5$^{lo\text{-}individual}$ groups based on their CD5 expression in CD8⁺ Tn. (H) Kaplan-Meier curve for progression-free survival is analyzed in Tn-CD5$^{hi\text{-}individual}$ and Tn-CD5$^{lo\text{-}individual}$ patients. (I-J) Proportions of patients with durable clinical benefit are assessed in (I) Tn-CD5$^{hi\text{-}individual}$, and (J) Tn-CD5$^{lo\text{-}individual}$ patients.

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