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Programmed cell death 1-expressing CD56-negative natural killer (NK) cell expansion is a hallmark of chronic NK cell activation during dasatinib treatment

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Abstract

Dasatinib treatment markedly increases the number of large granular lymphocytes including natural killer (NK) cells in a proportion of Ph^+ leukemia patients, which associates with a better prognosis. In-depth immune profiling of NK cells can predict therapeutic response in these patients. In the present study, we showed that CD56-negative ($CD56^{neg}$) NK cells increased exclusively in cytomegalovirus-seropositive (CMV^+) patients treated with dasatinib. The increase longitudinally paralleled with progressive differentiation of $CD56^{dim}$ NK cells during dasatinib therapy driven by CMV reactivation as shown by principal component analysis on 19 NK cell markers. The $CD56^{neg}$ NK cells showed downregulation of NK-activating receptors, upregulation of PD-1, and lower cytotoxicity and cytokine production, indicating that these cells are anergic and dysfunctional as seen in chronic infections with HIV-1 or hepatitis C virus. Moreover, cytolytic activity of $CD56^{dim}$ and $CD56^{neg}$ NK cells against leukemia cells was partially restored by nivolumab in proportion to the frequency of $PD\text{-}1^+$ NK cells. The proportion of patients who achieved deep molecular responses at 2 years was significantly higher in dasatinib-treated patients with ≥3% $CD56^{neg}$ NK cells than in those with fewer $CD56^{neg}$ NK cells (54.5% vs 15.8%, $P = .0419$). These findings suggest that $CD56^{neg}$ NK cells may be an exhausted population induced by chronic activation through CMV reactivation during dasatinib therapy. Expansion of $CD56^{neg}$ NK cells is a hallmark of chronic NK cell activation in patients treated with dasatinib and may predict a better clinical outcome. Furthermore, PD-1 blockade may enhance anti-leukemia responses of such NK cells.

KEYWORDS

CD56, chronic myeloid leukemia, dasatinib, NK cell, PD-1

Abbreviations: ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; CMV, cytomegalovirus; DA, dasatinib-treated; DMR, deep molecular response; HCV, hepatitis C virus; HD, healthy donor; HIV, human immunodeficiency virus; IFN, interferon; IL-2, interleukin 2; KIR, killer-cell immunoglobulin-like receptor; NK cell, natural killer cell; OTKI, other tyrosine kinase inhibitor-treated; PCA, principal component analysis; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand; Ph, Philadelphia chromosome; TKI, tyrosine kinase inhibitor; TNF, tumor necrosis factor.

TABLE 1 Study population

Group	No.	Gender	Age[a] (y)	Treatment duration[a] (mo)
CMV^+ dasatinib (CMV^+ DA)	30	18 M/12 F	55 (24-84)	17 (5-49)
CMV^- dasatinib (CMV^- DA)	6	4 M/2 F	41 (31-52)	22 (8-58)
CMV^+ imatinib or nilotinib (CMV^+ OTKI)	26	14 M/12 F	57 (32-84)	38 (7-119)
CMV^+ healthy donor (CMV^+ HD)	10	5 M/5 F	42 (34-82)	N/A
CMV^- healthy donor (CMV^- HD)	5	3 M/2 F	34 (32-43)	N/A

Abbreviations: CMV^+, CMV-seropositive; CMV^-, CMV-seronegative; DA, dasatinib; N/A, not applicable; OKTI, other tyrosine kinase inhibitor-treated.

[a]Shown are the median (range) values.

1 | INTRODUCTION

Tyrosine kinase inhibitors targeting BCR-ABL kinase have revolutionized the treatment for Ph^+ leukemia, including CML and a subset of ALL. Among TKI for Ph^+ leukemia, dasatinib distinctively causes a unique immunological phenomenon, that is, an increase in the number of cytotoxic lymphocytes, $CD8^+$ T cells and NK cells, in a substantial proportion of Ph^+ leukemia patients, which associates with a better prognosis.[1,2] We and others have shown that dasatinib-induced expansion of $CD8^+$ T cells and NK cells most likely involves CMV reactivation.[3,4] In our previous study, we applied PCA to a dataset of a wide range of NK cell markers to quantify the degree of CMV reactivation-driven NK cell differentiation. By this method, we showed that progressive NK cell differentiation induced by chronic NK cell activation through CMV reactivation occurred in CMV-seropositive (CMV^+) patients exclusively during dasatinib treatment.[4]

Levels of NK cell activation that lead to NK cell differentiation during TKI therapy are different among individuals, possibly affecting immunological responses to leukemia cells. Therefore, biological markers to trace NK cell differentiation might be relevant to predict not only therapeutic response but also successful discontinuation of TKI in CML therapy. In our previous study, CMV^+ patients with NK cells with more differentiated phenotype (as determined by PCA) at diagnosis showed a significantly greater NK cell expansion during dasatinib treatment and achieved a major molecular response more frequently than those without such NK cells.[4] Furthermore, several clinical studies have shown that an increased proportion of differentiated NK cells is associated with successful discontinuation of imatinib or dasatinib in CML patients.[5,6] Although our PCA, based on detailed profiling of NK cell markers, was useful to evaluate the NK cell activation status and predict therapeutic responses to dasatinib therapy, such detailed profiling is cumbersome and challenging in clinical settings.[4]

The NK cell population consists of immature $CD56^{bright}$ NK cells, mature $CD56^{dim}$ NK cells, and rare CD56-negative ($CD56^{neg}$) NK cells.[7] The latter has been reported to increase in patients with chronic viral infection such as HIV-1 and HCV and has defective capacity in cytotoxicity and cytokine production.[7-9] In this study, we examined whether $CD56^{neg}$ NK cells also increased in CMV^+ patients treated with dasatinib, but not in those with other TKI or in CMV^- patients, hypothesizing that $CD56^{neg}$ NK cells may represent one of the hallmarks of NK cell activation during dasatinib treatment in CMV^+ patients.

Current findings that PD-1 or PD-L1 blockade can restore cytotoxic T-lymphocyte activity against tumor cells encouraged us to explore the potential of blockade therapy on NK cells in leukemia. Several recent studies have reported the presence of $PD\text{-}1^+$ NK cells in tumor patients, as well as in a small proportion of healthy individuals.[10-14] So far, findings of PD-1 and PD-L1 interaction on human NK cells have been limited and confined to $CD56^{dim}$ NK cells.[10,13,15] The role of PD-1 on $CD56^{neg}$ NK cells remains unexplored, although PD-1 expression was frequently detected on $CD56^{neg}$ NK cells.

In the present study, we examined the phenotypic and functional properties of $CD56^{neg}$ NK cells in dasatinib-treated patients. Our findings suggest that an accumulation of PD-1-expressing $CD56^{neg}$ NK cells is a hallmark of chronic NK cell activation in CMV^+ CML patients treated with dasatinib and demonstrate the potential effects of PD-1 or PD-L1 blockade targeting NK cells in combination therapy with dasatinib.

2 | MATERIALS AND METHODS

2.1 | Patients and healthy donors

We assessed NK cell subsets in the same cohort as described previously.[4] The study population was dasatinib-treated (DA) CML or Ph^+ ALL patients (n = 36), other TKI (imatinib or nilotinib)-treated (OTKI) patients (n = 26), and healthy donors (HD) (n = 15). The patients and donors were divided into 5 groups according to the TKI used and CMV serology (Table 1). Blood sampling was carried out every 1-3 months after the start of dasatinib therapy. Written informed consents were obtained from all the patients and healthy donors. The study was conducted in accordance with the principles of the Helsinki Declaration and was approved by the Kyoto University Hospital Ethics Committee.

2.2 | Cell line

K562 cells (an MHC class I-negative CML-derived cell line, ATCC_CCL-243) were cultured in RPMI-1640 with 10% FCS and 100 U/mL penicillin and streptomycin (Sigma-Aldrich). Enhanced green fluorescent protein-transfected K562 (EGFP-K562) cells were generated by electroporation with plasmid EGFP-N3 (Clontech Laboratories) using a Neon transfection system (Invitrogen) according to the manufacturer's protocol and were selected with 1 mg/mL G418 (Nacalai Tesque). Human PD-L1 cDNA (GenBank: NC_000009.12) established from a healthy donor was amplified by PCR and subcloned into the pEGFP-N3 vector (Clontech Laboratories). PD-L1-transfected K562 (PD-L1-K562) cells were established using the same protocol as for EGFP-K562 cells. The expression of PD-L1 was confirmed by flow cytometry.

2.3 | Immunophenotyping of NK cells

For identifying and characterizing NK cell subsets, cell staining was carried out with the antibodies seen in Table 2. The strategy to gate NK cell subsets is shown in Figure S1. NK cells were defined as $CD3^-$, $CD4^-$, $CD14^-$, $CD19^-$, and $CD16^+$ or $CD56^+$ cells in peripheral blood. NK cell marker expressions were compared between $CD56^{dim}$ and $CD56^{neg}$ NK cells in CMV^+ DA patients with ≥5% $CD56^{neg}$ NK cells (n = 17). Samples were acquired using a 7-color flow cytometer (LSRFortessa; BD Biosciences), and the data were analyzed with FlowJo software ver. 7 (Tree Star).

2.4 | Natural killer cell profiling by PCA

Principal component analysis was carried out using princomp function in R software 3.1.3 (The R Foundation for Statistical Computing). We exploited the PCA dataset of 19 NK cell markers expressed on $CD56^{dim}$ NK cells in the previous study[4] and used the eigenvectors (principal component 1: PC1) to measure the overall activation status of $CD56^{dim}$ NK cells. Longitudinal changes in the PC1 value were calculated as differences between PC1 values during dasatinib treatment and the baseline PC1 value at the start of dasatinib treatment. To visualize the relationship between NK cell subsets, we conducted PCA with a covariance matrix of expression percentages for 19 phenotypic markers on $CD56^{bright}$ NK cells, $CD56^{dim}$ NK cells, and $CD56^{neg}$ NK cells.

2.5 | Analysis of single-cell RNA sequencing data in the public database

To characterize transcriptional profiles of NK cell subsets, we used a single-cell RNA sequencing (scRNA-seq) dataset deposited in NCBI GEO with the accession number GSE144191.[16] To analyze the dataset, we used Seurat (v 3.0) in R software 3.1.3. We confirmed that the rds file contained the dataset of 7964 peripheral blood NK cells from 2 healthy donors with and without IL-2 stimulation, 16 367 genes, assay, and dimension-reduced matrix data. We validated the retrieved data by visualizing the dimension-reduced dataset that had been generated by the Seurat alignment strategy,[16] with t-distributed stochastic neighbor embedding (t-SNE), which recapitulated the t-SNE plot identical to the one published (Figure S4).[16]

TABLE 2 Monoclonal antibodies for phenotyping natural killer (NK) cells

Antibody	Fluorescence	Clone	Company
CD3	APC.Cy7	UCHT1	BioLegend
CD4	FITC or APC	PRA-T4	BioLegend
CD14	PerCP.Cy5.5	M5E2	BioLegend
CD16	PE.Cy7	B73.1	BioLegend
CD19	PE.Cy5	HIB19	BioLegend
CD56	BV421	HCD56	BioLegend
CD57	APC	HCD57	BioLegend
CD94	PE	DX22	BioLegend
NKG2D	APC	1D11	BioLegend
NKp30	APC	P30-15	BioLegend
NKp46	APC	9E2	BioLegend
DNAM-1	APC	11A8	BioLegend
Tim-3	APC	F38-2E2	BioLegend
LIR-1	APC	GHI/75	BioLegend
KIR2DL2/L3/S2	FITC	DX27	BioLegend
KIR2DL1/S1	FITC	HP-MA4	BioLegend
KIR3DL1	FITC	DX9	BioLegend
PD-1	PE	eBioJ105	eBioscience
NKG2A	APC	131411	R&D
NKG2C	PE or APC	134591	R&D
KIR2DL1	APC	143211	R&D
KIR2DL3	APC	180701	R&D
KIR3DL2	PE	539304	R&D
Granzyme B	FITC	GB11	BD Pharmingen
Perforin	FITC	dG9	BD Pharmingen

2.6 | Degranulation assay

Two hundred thousand freshly isolated PBMC were incubated with 4×10^4 target K562 cells at 37°C and 5% CO_2 in the presence of FITC-conjugated anti-CD107a mAb (H4A3; BioLegend) to monitor degranulation. After 2 hours of incubation, monensin (GolgiStop; BD Biosciences) and Brefeldin A (GolgiPlug; BD Biosciences) were added, and the incubation was continued for an additional 4 hours. The cells were stained for cell surface markers, and then fixed (BD Cell Fix; BD Biosciences), permeabilized (PBS with 0.5% BSA and 0.1% saponin), and stained with Brilliant Violet 605-conjugated anti-IFN-γ (X40; BioLegend) and phycoerythrin (PE)-conjugated

anti-TNF-α (Mab11; BioLegend). FlowJo software and its Boolean gate function were used to determine NK cell functionality. Degranulation was compared between $CD56^{dim}$ and $CD56^{neg}$ NK cells in CMV^+ DA patients with ≥5% $CD56^{neg}$ NK cells. To determine the effects of PD-1 blockade, PBMC were incubated in the presence of the anti-PD-1 antibody, nivolumab (Ono Pharmaceutical) for 1 hour prior to coculture with PD-L1-K562 cells.

2.7 | Cytotoxicity assay

Sorted $CD56^{dim}$ and $CD56^{neg}$ NK cells (Figure S1) were resuspended in RPMI-1640 medium containing 10% FCS with 100 IU/mL IL-2 (Peprotech) and added to the targets at effector/target ratios from 10:1 to 0.6:1, each in duplicate. Cells were incubated for 4 hours at 37°C in a 5% CO_2 incubator. Cells were stained with APC-conjugated Annexin V (BioLegend). Percentages of Annexin V^+ EGFP-positive K562 cells were measured. Spontaneous lysis was determined by incubating target cells without effectors. Specific lysis was calculated as [(lysis in the well containing target cells and effector cells − spontaneous lysis)/ (100 − spontaneous lysis)] × 100. NK cells were incubated for 1 hour in the presence of 1 μg/mL nivolumab prior to coculture with PD-L1-K562 cells. Specific lysis was calculated as described above. Samples were acquired by flow cytometry (FACSCalibur; BD Biosciences), and the data were analyzed with FlowJo software ver. 7 (Tree Star).

2.8 | Assessment of deep molecular response

The therapeutic effects of dasatinib were evaluated based on peripheral blood *BCR-ABL* mRNA expression. *BCR-ABL* mRNA expression was measured by real-time quantitative-PCR adjusted to the international reporting scale (IS) using an ODK-1201 kit (Otsuka Pharmaceutical Co.) or an Amp-CML test (Mitsubishi Chemical Medicine Corp.). Major molecular responses (MMR) were defined when the Amp-CML test detected <50 copies/μg RNA or *BCR-ABL* transcript levels in peripheral blood were IS ≤0.1%. DMR were defined as *BCR-ABL* transcript levels in peripheral blood of IS ≤0.01%.

2.9 | Statistical analysis

Counts of NK cell subsets were compared between the CMV^+ DA group and the other groups using the Wilcoxon rank sum test and adjusted by Holm's method. The Wilcoxon signed-rank test was used to compare NK cell marker expression and functional analysis between $CD56^{dim}$ NK cells and $CD56^{neg}$ NK cells. Comparison of therapeutic responses was examined by the Fisher's exact test. Statistical significance was defined as $P < .05$ with a two-tailed test. All statistical analyses were carried out using R software 3.1.3 (The R Foundation for Statistical Computing, Vienna, Austria).

3 | RESULTS

3.1 | $CD56^{neg}$ NK cells increase exclusively in CMV^+ DA patients

As previously reported by our group and others, dasatinib treatment induces lymphocytosis in a proportion of Ph^+ leukemia patients. Increased lymphocytes are morphologically large granular lymphocytes, and phenotypically $CD8^+$ T cells or NK cells. In our previous study, we carried out a detailed analysis of peripheral blood lymphocytes in TKI-treated patients and found that NK cell expansions were more dominant than those of $CD8^+$ T cells and observed exclusively in CMV^+ DA patients. While we focused on the phenotype of the $CD56^{dim}$ NK cell subset in that study, we noted that the $CD56^{neg}$ NK cell subset also expanded in a proportion of patients treated with TKI.

Using the same cohort as in our previous study (Table 1),[4] we compared the cell number of each NK cell subset in peripheral blood among the 5 groups, focusing on the $CD56^{neg}$ NK cells. $CD56^{neg}$ NK cells were defined as $CD3^-CD4^-CD14^-CD19^-CD16^+CD56^-$ lymphocytes. The number of $CD56^{neg}$ NK cells, as well as $CD56^{dim}$ NK cells, was significantly increased in CMV^+ DA patients (Figure 1A). $CD56^{bright}$ NK cells were also increased in a small proportion of CMV^+ DA patients, but there were no statistically significant differences compared to the patients in other groups. $CD56^{neg}$ NK cell counts were strongly correlated with total NK cell counts and $CD56^{dim}$ NK cell counts in CMV^+ DA patients, but not in CMV^+ OTKI patients (Figure 1B), suggesting that $CD56^{neg}$ NK cell subset expansion may reflect the activation status of the whole NK cell population in Ph^+ leukemia patients treated with dasatinib.

3.2 | Expansion of $CD56^{neg}$ NK cells was associated with expansion of CMV-associated NK cells

We followed changes of the NK cell subset distribution before and after dasatinib treatment. Figure 2A shows representative data from a CML patient who received first-line dasatinib treatment for 12 months and then switched to nilotinib because of dasatinib intolerance. Before dasatinib treatment, there was only a very low percentage of $CD56^{neg}$ cells in the NK cell population. However, after the start of dasatinib, the percentage of $CD56^{neg}$ NK cells dramatically increased, reaching up to 16% at 6 months and was maintained thereafter during dasatinib treatment. Importantly, the $CD56^{neg}$ subset rapidly decreased after switching to nilotinib, highlighting that the $CD56^{neg}$ NK cell expansion was clearly associated with dasatinib treatment. Such transient expansions of $CD56^{neg}$ NK cells were observed exclusively in CMV^+ DA patients, not in CMV^- DA patients, although the degrees of expansion were variable (Figure 2B).

To verify the association between the increase in $CD56^{neg}$ NK cells and the underlying NK cell activation, we exploited the dataset in our previous study.[4] In that study, PCA of 19 NK-cell markers expressed on $CD56^{dim}$ NK cells identified the value of principal component 1 (PC1)

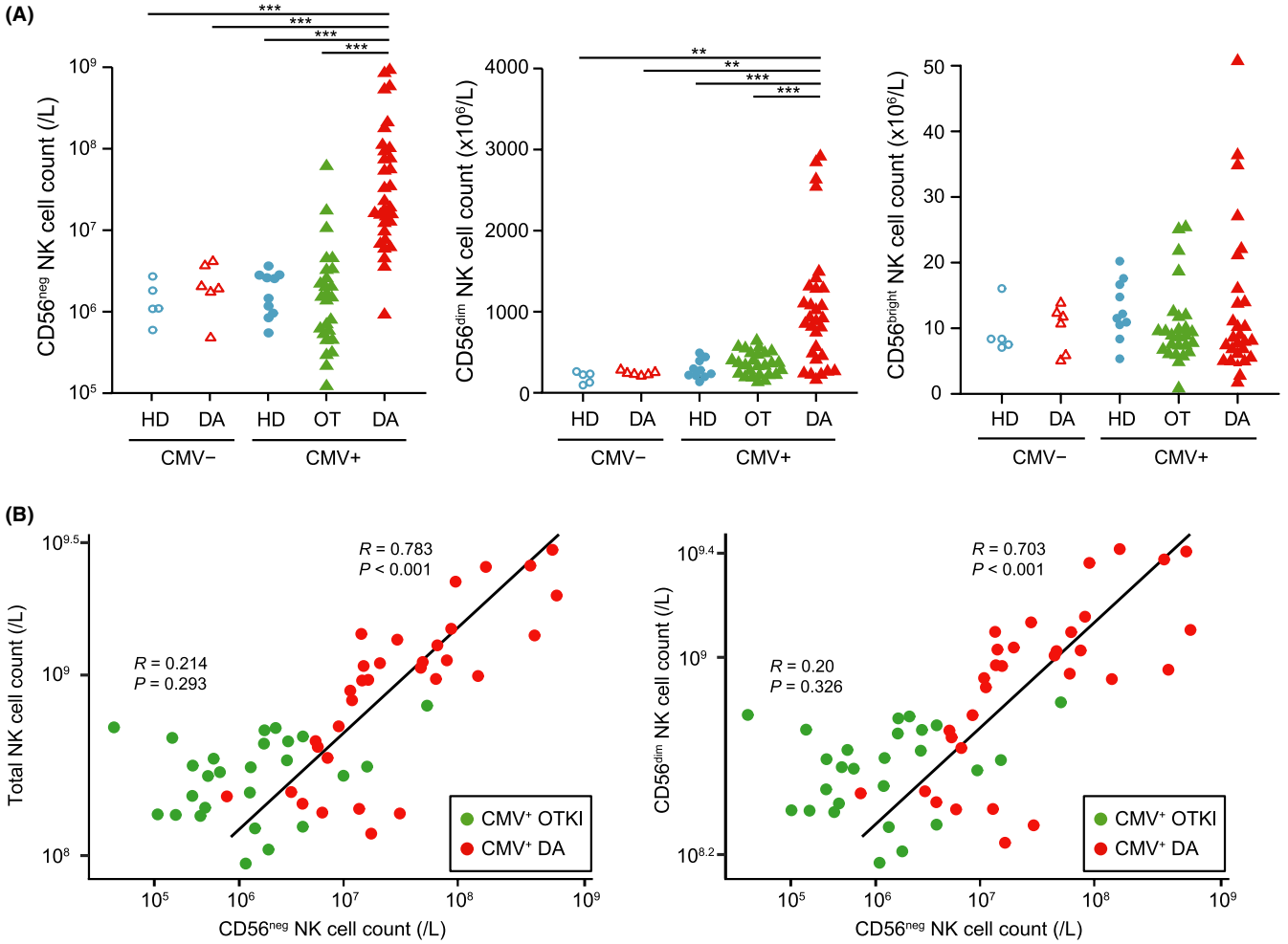

FIGURE 1 CD56^{neg} natural killer (NK) cells increase exclusively in cytomegalovirus-seropositive (CMV$^+$) dasatinib (DA) patients. A, Absolute counts of NK cell subsets. Red triangles: DA patients (filled, CMV$^+$; empty, CMV$^-$). Green triangles: other tyrosine kinase inhibitor-treated (OTKI) patients. Blue circles: healthy donors (HD). The Wilcoxon rank sum test was used to compare the CMV$^+$ DA group with the other groups. $^{**}P < .01$, $^{***}P < .001$. B, Correlation between CD56^{neg} NK cell counts and total NK cell counts, or CD56^{dim} NK cell counts in CMV$^+$ DA and OTKI patients. The scatter plot is displayed as a log-log plot. A solid line represents the correlation for CMV$^+$ DA patients only. Correlations for each group were analyzed with the non-parametric Spearman test

as an indicator of CD56^{dim} NK cell differentiation driven by CMV reactivation, as the PC1 value was positively correlated with the expression level of NKG2C, CD57 and LIR-1, and negatively correlated with that of NKp30 and NKp46: phenotypic changes observed in differentiating NK cells in association with CMV infection.[17] To extend those findings, we here evaluated the relationship between percentages of the CD56^{neg} NK subset, changes in the PC1 value, and the duration of dasatinib treatment in a 3D chart (Figure 2C). The chart shows that increases in CD56^{neg} NK cells synchronized with increases in the PC1 value, showing that the CD56^{neg} NK cell increases were accompanied by the CMV reactivation-driven CD56^{dim} NK cell differentiation during dasatinib therapy. This correlation was corroborated by the finding that some CMV$^+$ and all CMV$^-$ DA patients with limited changes in the PC1 value did not show a CD56^{neg} NK cell increase (dashed lines in Figure 2C). Notably, CD56^{neg} NK cells began to decrease when the therapy was switched from dasatinib to other TKI (representative examples with asterisk in Figure 2B,C). These CD56^{neg} NK cell decreases were accompanied by a reduction in the PC1 value (Figure 2C). Collectively, these data showed that dynamic changes of CD56^{neg} NK cells were closely associated with CMV reactivation-driven increases and phenotypic changes in the CD56^{dim} NK cell subset, strongly suggesting that CD56^{neg} NK cell increases were also induced and maintained by chronic CMV reactivation during dasatinib therapy.

3.3 | Phenotypic features of CD56^{neg} NK cells in CMV$^+$ DA patients

To characterize phenotypic features of CD56^{neg} NK cells, we compared the expression of NK cell markers between CD56^{dim} and CD56^{neg} NK cells in 17 CMV$^+$ DA patients with ≥5% CD56^{neg} NK cells. There were no patients meeting this criterion in other groups. CD56^{neg} NK cells showed decreased expression of activation and differentiation of NK cell markers including CD57, NKG2C, NKG2D, NKp30, and NKp46, and cytotoxic granules including granzyme B and perforin (Figure 3A and Figure S2). These phenotypes of

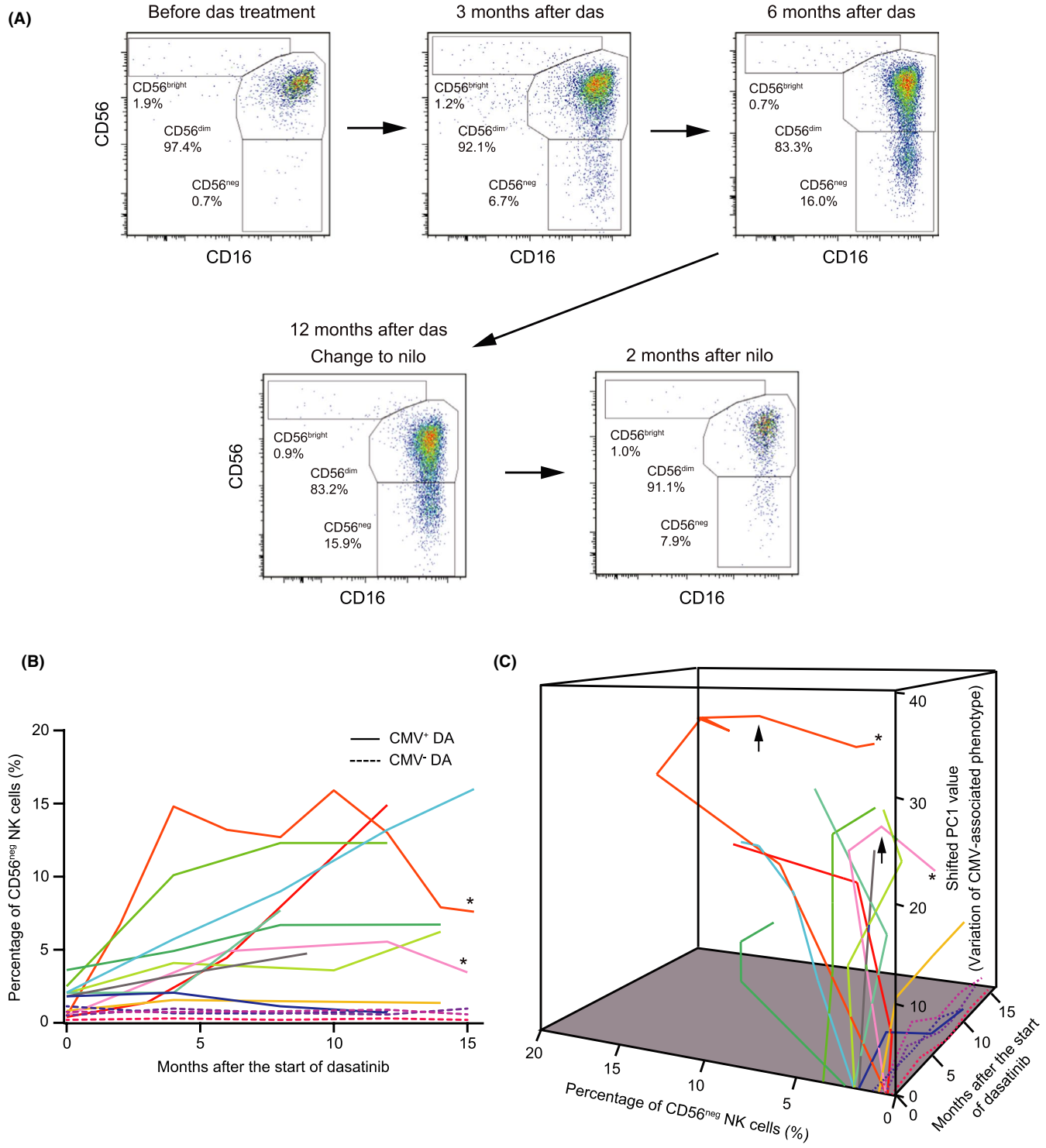

FIGURE 2 Longitudinal changes in the percentage of $CD56^{neg}$ natural killer (NK) cells. A, Dynamic changes of NK cell subsets in a representative patient that started dasatinib therapy. Percentages of $CD56^{bright}$, $CD56^{dim}$, and $CD56^{neg}$ NK cells are shown. The proportion of $CD56^{neg}$ NK cells decreased after the switch to nilotinib. Das, dasatinib; Nilo, nilotinib. B, Percentage of $CD56^{neg}$ NK cells after the start of dasatinib (DA). Solid lines: cytomegalovirus seropositive (CMV^+) DA patients (n = 11), dotted lines: cytomegalovirus seronegative (CMV^-) DA patients (n = 4). Asterisks indicate the patients that changed from dasatinib to other tyrosine kinase inhibitors (TKI). C, Three-dimensional line chart shows correlations in the percentages of $CD56^{neg}$ NK cells vs the CMV-associated NK cell activation (changes in the principal component 1 [PC1] value from the baseline) vs dasatinib therapy duration. Solid lines: CMV^+ DA patients (n = 11), dotted lines: CMV^- DA patients (n = 4). Asterisks indicate the patients that changed from dasatinib to other TKI. Arrows indicate the time point when switching from DA to other TKI. The colors are identical to those in Figure 2B

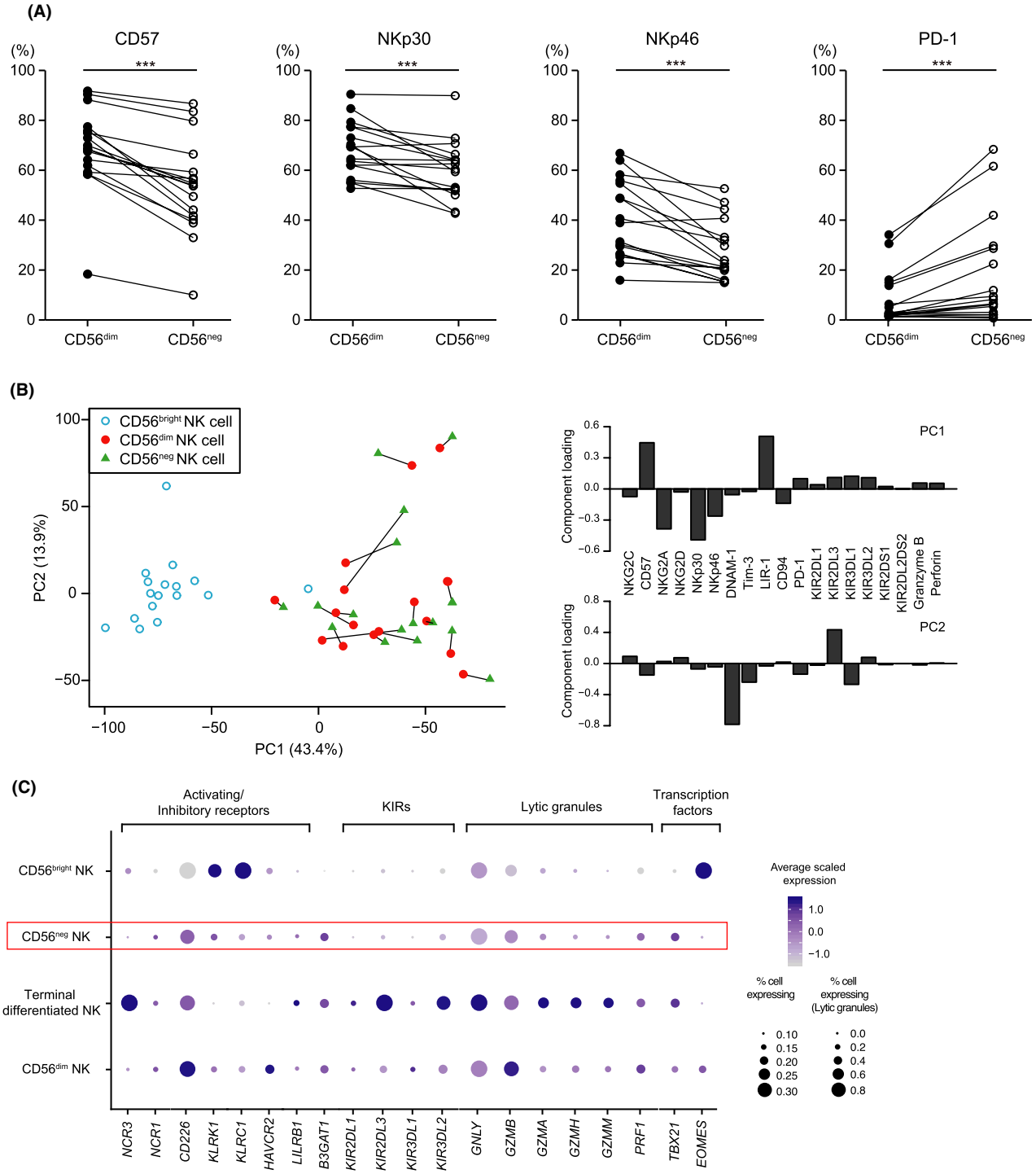

FIGURE 3 Phenotypic, transcriptional, and functional features of CD56^{neg}natural killer (NK) cells in cytomegalovirus seropositive (CMV^{+}) dasatinib (DA) patients. A, NK cell marker expression was compared between CD56^{dim}NK cells (filled circles) and CD56^{neg}NK cells (open circles) in CMV^{+}DA patients with ≥5% CD56^{neg}NK cells (n = 17). Statistical significance was analyzed with the Wilcoxon signed-rank test. B, principal component analysis (PCA) plot of CD56^{bright}, CD56^{dim}, and CD56^{neg}NK cells in 17 CMV^{+}DA patients. The dots representing CD56^{dim}and CD56^{neg}NK cells in the same patient are connected with a line. The component loading values are shown. C, Dot plot of selected markers of interest across the different NK cell clusters. Data are derived from a single-cell RNA sequencing data in the public database (GSE1144191). Size of the dot represents the percentage of cells expressing the markers and the color encodes the average scaled expression values. D, Functional assay of CD56^{dim}and CD56^{neg}NK cells from CMV^{+}DA patients (n = 14). Expression of surface CD107a and intracellular interferon (IFN)-γ and tumor necrosis factor (TNF)-α after coculture with K562 cells are shown. Statistical significance was analyzed with the Wilcoxon signed-rank test. E, Cytolytic activity of CD56^{dim}(black) vs CD56^{neg}NK cells (gray) against EGFP-transfected K562 at various effector-to-target (E/T) ratios. Data represent the average lytic activity ± SEM from CMV^{+}DA patients (n = 5). Statistical significance was analyzed with the Wilcoxon signed-rank test. $^{*}P < .05$, $^{***}P < .001$

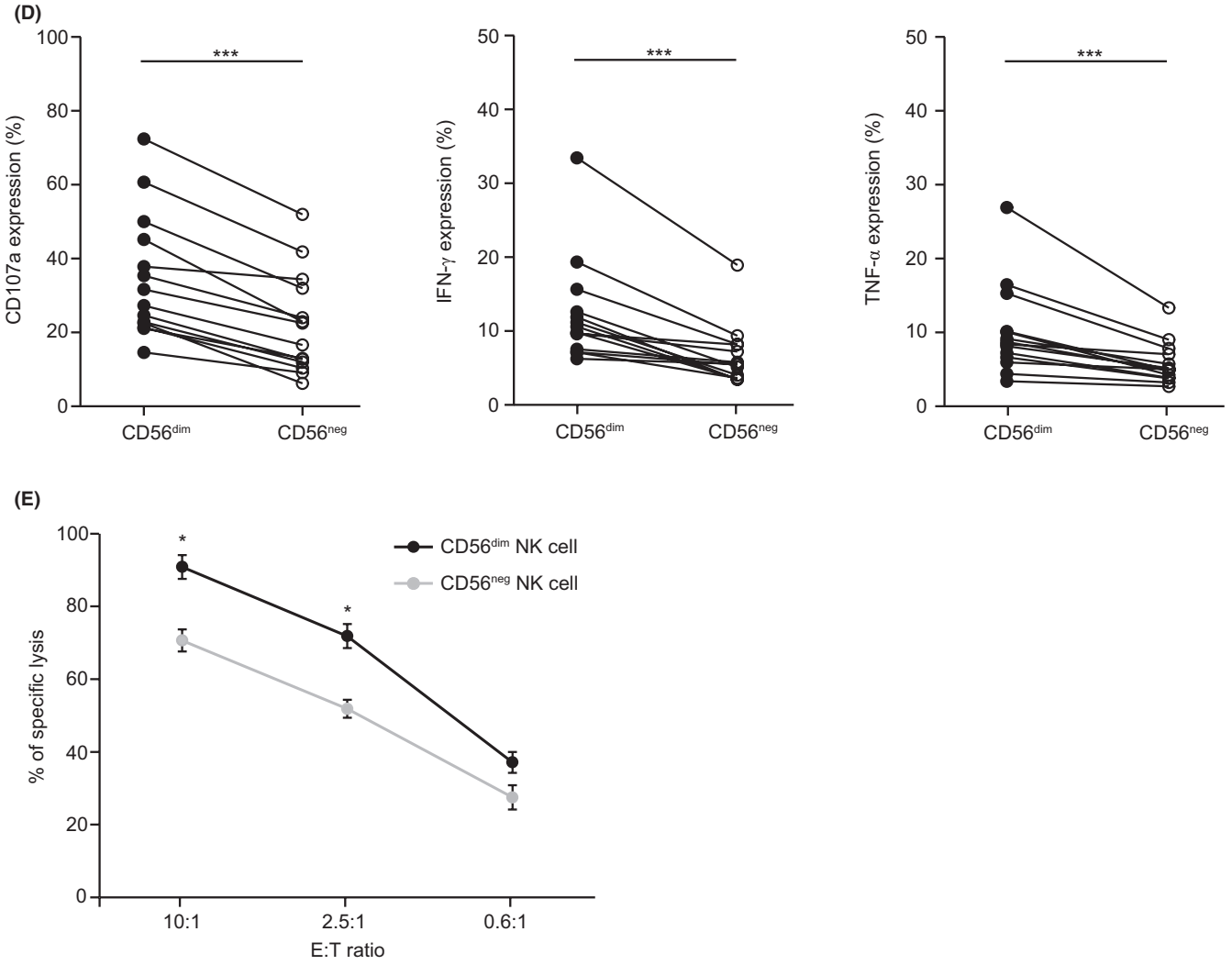

FIGURE 3. Continued

CD56^{neg} NK cells in CMV^{+} DA patients were largely similar to those of CD56^{neg} NK cells in HIV-infected viremic patients.[8]

Intriguingly, the expression of PD-1 was significantly upregulated in CD56^{neg} NK cells compared to that of CD56^{dim} NK cells (Figure 3A). Moreover, PD-1 expression of NK cells was exclusively observed in CMV^{+} DA patients (Figure S3).

3.4 | CD56^{neg} NK cells were closely related to CD56^{dim} NK cells and apparently distinct from CD56^{bright} NK cells

To visualize the relationship between NK cell subsets, we first conducted PCA of surface markers expressed on three NK cell subsets. The PCA showed a close relationship of CD56^{dim} and CD56^{neg} NK cells in each patient, definitively separating these two NK subsets from CD56^{bright} NK cells with large component loadings of CD57, NKG2A, NKp30, and LIR-1 (Figure 3B).

Next, we investigated gene expressions in these NK cell subsets, using a scRNA-seq dataset from GEO (accession number GSE144 4191).[16] To compare the expression of relevant genes among NK cell subsets, we extracted data of CD56^{dim} NK cells (clusters 0 and 1), terminally differentiated NK cells (cluster 2), CD56^{neg} NK cells (cluster 3, also described as type I IFN-responding NK cells), and CD56^{bright} NK cells (cluster 4) as defined in the previous study (Figure S4).[16] Consistent with our phenotypic analysis (Figure 3A and Figure S2), CD56^{neg} NK cells showed slightly lower gene expressions of activating NK receptors including *NCR3* (the gene encoding NKp30), *CD226* (DNAM-1), *HAVCR2* (Tim-3), *KIRs*, and lytic granules than CD56^{dim} NK cells (Figure 3C), validating that the dataset recapitulates the feature of NK cell subsets in our cohort. Importantly, despite these differences between CD56^{neg} and CD56^{dim} subsets, transcriptional profiles of these two subsets were mostly similar and apparently distinct from CD56^{bright} NK cells (cluster 4), which showed distinctive gene expressions of *KLRC1* (NKG2A), *KLRK1* (NKG2D), *LILRB1*, *B3GAT1*, *KIRs*, lytic granules, *TBX21* and *EOMES* (Figure 3C). Note that *SNCA* (PD-1) expression was barely detected in all NK cell subsets (data not shown), presumably because the scRNA-seq dataset was derived from CMV^{-} healthy donors. Collectively, phenotypic and transcriptional features of CD56^{neg} NK cells are more closely related to those of CD56^{dim} NK

cells rather than those of CD56^{bright} NK cells, implying that CD56^{neg} NK cells are derived from CD56^{dim} NK cells.

As CD56^{neg} NK cells have been described as "type I interferon-responding NK cells", which have a strong type I IFN signature,[16] we examined whether CD56^{neg} NK cells are induced by IFN-α stimulation. We cultured sorted CD56^{dim} NK cells in the presence of IFN-α, but neither CD56 downregulation or PD-1 upregulation was observed (Figure S5), suggesting that additional signals are involved in the generation of CD56^{neg} NK cells.

3.5 | CD56^{neg} NK cells showed lower functionality than CD56^{dim} NK cells

Next, to examine the functionality of CD56^{neg} NK cells in CMV^{+} DA patients, we carried out a functional assay using a CML-derived human leukocyte antigen (HLA)-deficient K562 cell line as a target. The degranulation marker CD107a was used to evaluate cytotoxic activity, and IFN-γ and TNF-α production was measured by intracellular cytokine staining. In CMV^{+} DA patients, CD56^{neg} NK cells showed lower frequencies of CD107a-positive and cytokine-producing cells than CD56^{dim} NK cells (Figure 3D). Consistent with this, cytotoxicity assay showed a significantly lower cytolytic activity in CD56^{neg} NK cells than in CD56^{dim} NK cells (Figure 3E). Taken together, these data indicate that CD56^{neg} NK cells that accumulate during dasatinib therapy are dysfunctional.

3.6 | PD-1 blockade enhances effector functions of NK cells in accordance with the expression level of PD-1, especially on CD56^{dim} NK cells

Dasatinib therapy upregulated PD-1 expression on CD56^{dim} NK cells as well as expanding dysfunctional CD56^{neg} NK cells that highly express PD-1 (Figure S3). Thus, the percentage of PD-1 expression on total NK cells was significantly correlated with the frequency of CD56^{neg} NK cells (Figure 4A).

We next investigated the role of the PD-1/PD-L1 axis on sorted CD56^{dim} and CD56^{neg} NK cells, because they have different expression levels of NK receptors and PD-1. As K562 cells express low levels of PD-L1, we transfected K562 cells with PD-L1 (Figure 4B) and tested their susceptibility to NK cells. Both sorted CD56^{dim} and CD56^{neg} NK cell populations containing PD-1^{+} NK cells showed lower cytolysis against PD-L1-K562 than control EGFP-K562 cells (Figure 4C,D), suggesting that the PD-1/PD-L1 axis can suppress NK cells cytolytic activity against leukemia cells.

To determine whether PD-1 blockade by nivolumab would enhance NK cell response, we first established the appropriate concentration of nivolumab to saturate the PD-1 molecules on the NK cells. Nivolumab competed with the anti-PD-1 antibody used for staining (clone: eBioJ105) in a dose-dependent method, showing that 1 μg/mL nivolumab efficiently binds to PD-1 on NK cells (Figure S6). Functional assay using bulk NK cells demonstrated enhancement of IFN-γ production by nivolumab in a dose-dependent way in both CD56^{dim} and CD56^{neg} subsets (Figure 5A). Furthermore, functional assay using sorted CD56^{dim} and CD56^{neg} NK cells showed enhancement of degranulation and cytokine-production by nivolumab in correlation with the percentages of PD-1 expression on CD56^{dim} and CD56^{neg} NK cells (Figure 5B). Importantly, the degrees of functional enhancement by nivolumab were significantly greater in CD56^{dim} NK cells than in CD56^{neg} NK cells, even at the similar level of PD-1 expression on each subset. In line with this, cytotoxicity assay showed enhanced cytolytic activity against PD-L1-K562 cells in the presence of nivolumab in both sorted CD56^{dim} and CD56^{neg} NK cells, with significantly greater enhancements in CD56^{dim} NK cells than in CD56^{neg} NK cells, although the percentages of PD-1-expressing cells were always higher in CD56^{neg} NK cells than in CD56^{dim} NK cells in each patient (Figure 5C). These data show that nivolumab can enhance the cytotoxic activity of both subsets, but more efficiently in the CD56^{dim} subset compared to the CD56^{neg} subset.

3.7 | Dasatinib-treated patients with CD56^{neg} NK cells experience better therapeutic responses

Finally, to evaluate the clinical significance of the CD56^{neg} NK cell increase in CML patients treated with dasatinib, we divided CML patients into two groups based on the percentage of CD56^{neg} NK cell subset (≥3%: n = 12, <3%: n = 19) at 6 months after the start of dasatinib. We compared therapeutic responses at 6 months, 12 months, and 2 years after starting first-line dasatinib treatment. There were no significant differences between the two groups in MMR rates at 6 and 12 months, respectively (75.0% vs 66.7%, $P = .694$; 83.3% vs 73.6%, $P = .675$). In contrast, however, there was a trend at 1 year and a statistically significant difference at 2 years that a higher proportion of patients with more CD56^{neg} NK cells achieved DMR than those with fewer CD56^{neg} NK cells (45.5% vs 15.8%, $P = .104$ at 1 year; 54.5% vs 15.8%, $P = .0419$ at 2 years). In subgroup analysis of CMV^{+} patients, the same trend and difference were observed only in the patient group treated with dasatinib (45.5% vs 7.1%, $P = .0561$ at 1 year; 54.5% vs 7.1%, $P = .0213$ at 2 years). In regard to the percentages of PD-1^{+} NK cells as a predictor of therapeutic response, there were no statistically significant differences at any time points (data not shown). Thus, the percentage of CD56^{neg} NK cell subsets can serve as a biomarker predicting therapeutic response in CML patients treated with dasatinib.

4 | DISCUSSION

CD56^{neg} NK cells are rare cells, representing at most a few percent of total NK cells in healthy individuals. In this study, we found that these unusual CD56^{neg} NK cells expanded exclusively in CMV^{+} Ph^{+} leukemia patients treated with dasatinib, but not in those treated with other TKI or in CMV^{-} patients treated with any TKI. Our data suggest that CD56^{neg} NK cells observed in CMV^{+} DA patients are an

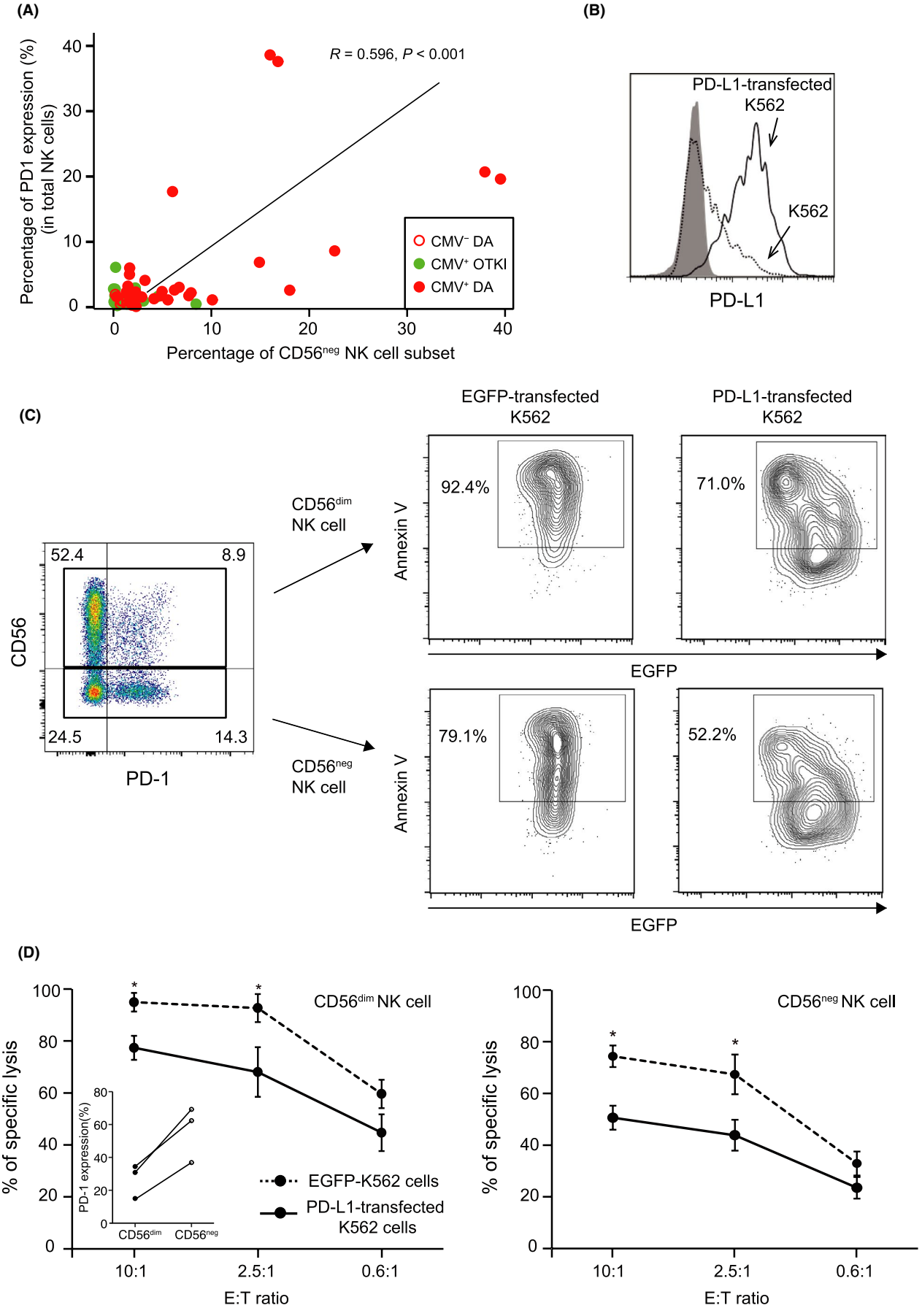
(A)
Percentage of PD1 expression (%) (in total NK cells)
R = 0.596, P < 0.001
CMV⁻ DA
CMV⁺ OTKI
CMV⁺ DA
Percentage of CD56neg NK cell subset
(B)
PD-L1-transfected K562
K562
PD-L1
(C)
EGFP-transfected K562
PD-L1-transfected K562
CD56dim NK cell
CD56neg NK cell
52.4
8.9
24.5
14.3
CD56
PD-1
92.4%
71.0%
79.1%
52.2%
Annexin V
EGFP
(D)
CD56dim NK cell
CD56neg NK cell
% of specific lysis
PD-1 expression(%)
EGFP-K562 cells
PD-L1-transfected K562 cells
10:1
2.5:1
0.6:1
E:T ratio

FIGURE 4 Natural killer (NK) cell killing of programmed cell death 1 ligand (PD-L1)-transfected K562 with programmed cell death 1 (PD-1) blockade. A, Correlation between the percentage of $CD56^{neg}$NK cell subset and the percentage of PD-1 expression in total NK cells from cytomegalovirus seropositive (CMV^+) dasatinib (DA) patients (n = 30), CMV^-DA patients (n = 6) and CMV^+other tyrosine kinase inhibitor-treated (OTKI) patients (n = 26). Solid line represents the correlation for CMV^+DA patients only. The correlation coefficient was analyzed with the Pearson test. B, Representative histograms showing PD-L1 expression on K562 cells (dotted line) and PD-L1-transfected K562 cells (solid line) compared to isotype-matched control Ig staining (shaded peak). C, Representative flow cytometry plots showing PD-1 expression and killing of EGFP (mock)- or PD-L1-transfected K562 cells (EGFP-K562, PD-L1-K562, respectively) by sorted $CD56^{dim}$and $CD56^{neg}$NK cells. Cytolytic activity was measured by Annexin V staining of target cells. D, Cytolytic activity of $CD56^{dim}$NK cells (left) and $CD56^{neg}$NK cells (right) against EGFP-K562 vs PD-L1-K562 at various effector-to-target (E/T) ratios. Data represent the average lytic activity ± SEM from CMV^+DA patients (n = 3). PD-1 expression on each NK cell subset is shown in the bottom left. Statistical significance was analyzed with the Wilcoxon signed-rank test. $^*P < .05$

exhausted and dysfunctional population that has accumulated most likely as a result of chronic CMV reactivation induced by dasatinib therapy.

Expansion of $CD56^{neg}$ NK cells has previously been reported in chronic viral infection, such as HIV-1 and HCV.[9,18] We speculate that $CD56^{neg}$ NK cell expansion in CMV^+ DA patients is also induced by chronic viral (CMV) reactivation, based on its similarities to these viral infections. First, phenotypic and functional features of $CD56^{neg}$ NK cells are quite similar between HIV-viremic and dasatinib-treated patients.[19] Second, kinetics and persistence of $CD56^{neg}$ NK cell expansion are also similar. In HIV-1 infection, $CD56^{neg}$ NK cell expansion is absent or modest in the acute phase, but develops and persists in the chronic phase, and rapidly disappears with the clearance of viremia by anti-retroviral therapy.[18] Similarly, in CMV^+ DA patients, expansion of $CD56^{neg}$ NK cells was gradually induced and persisted during dasatinib therapy, and rapidly returned to a normal level after dasatinib was switched to another TKI. These similarities suggest a common underlying mechanism for expansion of $CD56^{neg}$ NK cells in these clinical situations, that is, protracted exposure to viremia. This is corroborated by previous reports that dasatinib therapy actually induces chronic CMV reactivation at a subclinical level,[3,20] owing to its uniquely broad off-target inhibitory activities on tyrosine kinases which are key regulators of an immune response, such as src family kinases.[21-23] The spectrums of off-target inhibitions are different between TKI, explaining the exclusive occurrence of CMV reactivation that leads to $CD56^{neg}$ NK cell expansion in dasatinib therapy.

In contrast, there are differences between dasatinib therapy and chronic virus infection. It has been reported that in chronic HIV-1 and HCV infection, expansion of $CD56^{neg}$ NK cells is accompanied by decreases in numbers of $CD56^{dim}$ NK cells.[9,18] In contrast, in CMV^+ DA patients, $CD56^{dim}$ NK cells increased in proportion to the expansion of $CD56^{neg}$ NK cells. This discordance may reflect distinct immunostimulatory properties of different virus species (HIV-1 or HCV vs CMV) and the pharmacological effect of dasatinib on NK cell subsets. Whereas HIV-1 infection directly impairs $CD4^+$ T cells and concomitantly induces exhaustion of innate immune cells including NK cells,[24] CMV does not directly damage immune cells. In addition, some studies have shown that dasatinib has a potential effect to proliferate and mobilize NK cells, while inhibiting T cells and plasmacytoid dendritic cells.[22,23,25-27] Thus, a distinctive distribution of NK cell subsets in CMV^+ DA patients might be affected not only by CMV reactivation, but by the modification of immune cells by dasatinib.

Our PCA using flow cytometric data showed that $CD56^{neg}$ NK cells are phenotypically more closely related to $CD56^{dim}$ NK cells than $CD56^{bright}$ NK cells. Recently, two groups published reports that analyzed human NK cells by scRNA-seq.[16,28] In these transcriptomic analyses, $CD56^{neg}$ NK cells were distinctively recognized as a discreate cluster (named "inflamed NK"[28] or "type I IFN-responding NK"[16]), indicating a differentiation process involving epigenetic modifications. Using the dataset available in the public database, we confirmed that $CD56^{neg}$ cells display a transcriptional feature more similar to $CD56^{dim}$ than to $CD56^{bright}$ NK cells. These phenotypic and transcriptional profiles indicate that $CD56^{neg}$ cells are derived from $CD56^{dim}$ NK cells. Interestingly, both of the groups revealed that a strong IFN signature is found in $CD56^{neg}$ NK cells,[16,28] suggesting that chronic type I IFN stimulation induced by chronic viral infection might develop expansion of $CD56^{neg}$ NK cells. However, IFN-α stimulation could not induce CD56 downregulation or PD-1 upregulation in $CD56^{dim}$ NK cells. It seems likely that more complex in vivo processes are involved to induce $CD56^{neg}$ NK cells.

As PD-1 is a marker of exhausted T cells in chronic viral infection, PD-1-expressing $CD56^{neg}$ NK cells might also be an exhausted population generated under chronic CMV reactivation. Recently, Merino et al[29] demonstrated that chronic NKG2C signaling and IL-15 stimulation induces PD-1 expression by epigenetically reprogramming adaptive NK cells. This might be a mechanism of PD-1 upregulation on NK cells in CMV^+ DA patients. PD-1 blockade by nivolumab augmented function of NK cells more efficiently in $CD56^{dim}$ NK cells than in $CD56^{neg}$ NK cells. So far, several papers have reported enhancement of NK cell function by blocking PD-1 and PD-L1 interaction,[13,30,31] but this is the first paper that demonstrated that the degree of enhancement was dependent on the NK cell subset. Merino et al[29] also showed that in addition to PD-1, LAG-3 expression is induced on NK cells by chronic stimulation. It is possible that $CD56^{neg}$ NK cells are more profoundly suppressed by multiple immune checkpoints such as PD-1, LAG-3, TIGIT, and some inhibitory KIRs, compared with $CD56^{dim}$ NK cells, or that downregulation of activating receptors plays a critical role in diminishing the function of $CD56^{neg}$ NK cells. It will be of interest to determine the expression profile of immune checkpoint molecules on $CD56^{neg}$ NK cells and whether the function of these NK cells can be restored by a combination of immune checkpoint inhibitors. Finally, a recent paper reported that dasatinib has a synergistic effect with PD-1 blockade in tumor immunotherapy through DDR2 inhibition.[16] Our data suggest

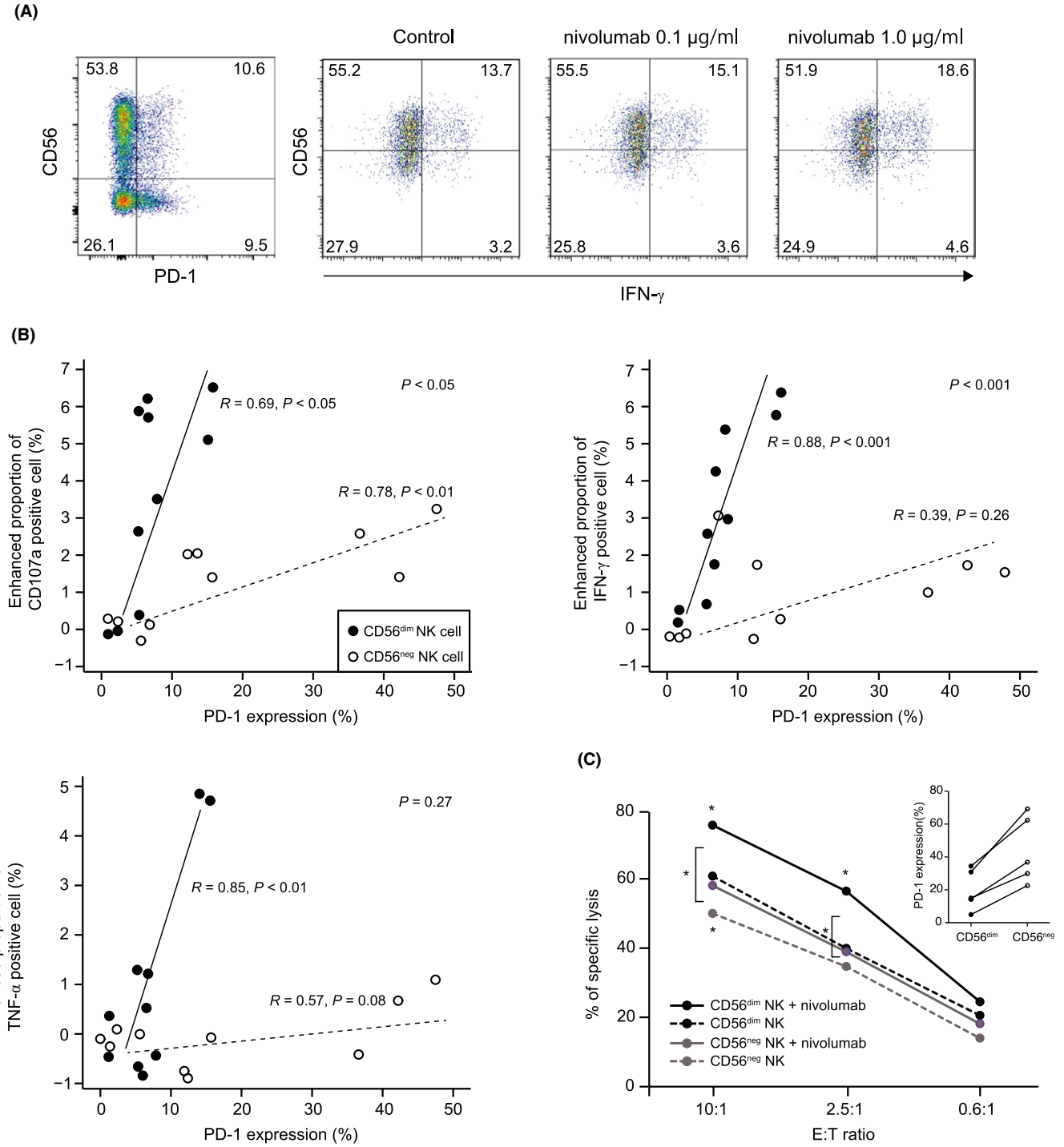

FIGURE 5 Enhancement of natural killer (NK) cell functionality by nivolumab. A, Representative flow cytometry plots showing enhancement of NK cell function by nivolumab. Interferon (IFN)-γ production by bulk NK cells against programmed cell death 1 ligand (PD-L1)-K562 was enhanced by pretreatment with 0.1 or 1 μg/mL nivolumab. The percentages in each quadrant are indicated on the plot. B, Correlation between programmed cell death 1 (PD-1) expression and functional enhancement by nivolumab in $CD56^{dim}$(filled circles) and $CD56^{neg}$NK cells (open circles) from cytomegalovirus seropositive (CMV^{+}) dasatinib (DA) patients (n = 10). The*x*-axis indicates the percentage of PD-1 expression on $CD56^{dim}$and $CD56^{neg}$NK cells. The*y*-axis indicates the difference between the percentages of the positive fraction with and without 1 μg/mL nivolumab pretreatment. Solid and dotted lines represent the correlation in $CD56^{dim}$and $CD56^{neg}$NK cells, respectively. Pearson correlation coefficients for each subset are indicated. The statistical significance of the mean slope difference between $CD56^{dim}$and $CD56^{neg}$NK cells was evaluated with Student's*t*test and is indicated in the top right. C, Cytolytic activity of $CD56^{dim}$(black) and $CD56^{neg}$(gray) NK cells against PD-L1-K562 with (solid line) or without (dotted line) 1 μg/mL nivolumab pretreatment. Data represent the average lytic activity from CMV^{+}DA patients (n = 5). Statistical significance of enhanced cytolytic activity by nivolumab is indicated by asterisks above or below the lines. Statistical significance of $CD56^{dim}$vs $CD56^{neg}$NK cells is indicated in the left of the dots. PD-1 expression of each NK cell subset is shown in the top right. Statistical significance was analyzed with Student's*t*test. *$P < .05$

another potential mechanism for the cooperative effect of dasatinib and PD-1 blockade by reversing the function of exhausted NK cells that has been induced during dasatinib therapy in CMV^+ patients.

We found that increases in $CD56^{neg}$ NK cells were associated with a higher rate of DMR, although they were an exhausted, dysfunctional population. We consider that this discrepancy can be explained by differential influences of dasatinib treatment on NK cell subsets. In contrast to dysfunctional $CD56^{neg}$ NK cells, functionality of $CD56^{dim}$ NK cells, which expanded in good correlation with $CD56^{neg}$ NK cells in CMV^+ DA patients, is enhanced during dasatinib treatment, as we have shown previously.[4] $CD56^{neg}$ NK cells constitute only a minor subset in the total population of NK cells, even in CMV^+ DA patients with an increased number of $CD56^{neg}$ NK cells. Thus, we assume that the major $CD56^{dim}$ NK cell subset plays an important role in contributing to anti-leukemia effects, bringing about a better clinical response in CMV^+ DA patients.

Despite the above mentioned, $CD56^{neg}$ NK cell expansion can be a predictive biomarker for therapeutic responses in DA patients. In our previous study, we showed that the PC1 value (CMV-associated phenotype of $CD56^{dim}$ NK cells) in PCA of 19 NK-cell markers could be a biomarker for predicting DMR in CML patients treated with dasatinib.[4] However, PCA requires flow cytometric analysis of a large number of NK-cell markers, which is complicated and cumbersome, especially in clinical laboratory settings. As shown in the present study, numbers of $CD56^{neg}$ NK cells had a good correlation with the PC1 value in the PCA, indicating that assessment of $CD56^{neg}$ NK cells is a simpler and more practical surrogate biomarker, which can be used as an equivalent of PCA for predicting therapeutic responses in CML patients.

In summary, a proportion of CMV^+ CML patients treated with dasatinib develops an accumulation of PD-1-expressing $CD56^{neg}$ NK cells that have a similar phenotype and functional impairment as seen in HIV-1- and HCV-infected patients, suggesting a common underlying mechanism involving chronic viral exposure. The accumulation of $CD56^{neg}$ NK cells may serve as a biomarker to predict a better clinical outcome in CML patients treated with dasatinib. It will be of interest to examine whether PD-1 blockade can further improve clinical outcome of these CML patients and whether a combination of dasatinib and PD-1 blockade will be effective in other types of cancers.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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