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Enhanced thrombopoiesis supplies PD-L1 to circulating immune cells via the generation of PD-L1-expressing platelets in patients with lung cancer

Sung-Woo Lee,[1,2,3] Saei Jeong,[1,2,3,4] Young Ju Kim,[1,2,3,4] Jeong Eun Noh,[1,2,3,4] Kyung Na Rho,[1,2,3,4] Hee-Ok Kim,[5] Hyun-Ju Cho,[6,7] Deok Hwan Yang,[6,7] Eu Chang Hwang,[7,8] Woo Kyun Bae,[6,7] Sook Jung Yun,[7,9] Ju Sik Yun,[7,10] Cheol-Kyu Park,[6,7] In-Jae Oh,[6,7] Jae-Ho Cho [1,2,3,4]

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S-WL and SJ are joint first authors.

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For numbered affiliations see end of article.

Correspondence to
Dr Sung-Woo Lee;
swl526@jnu.ac.kr

Professor In-Jae Oh;
droij@jnu.ac.kr

Professor Jae-Ho Cho;
jh_cho@chonnam.ac.kr

ABSTRACT

Background The increased expression of programmed cell death ligand 1 (PD-L1) on a subset of immune cells in the peripheral blood has been frequently observed in patients with cancer, suggesting a relationship with PD-L1 expression in tumor tissues. In this study, we investigated the mechanisms underlying PD-L1 expression on various types of immune cells in the peripheral blood of patients with cancer.

Methods PD-L1 expression on various immune cell populations was analyzed in peripheral blood mononuclear cells of 112 patients with non-small cell lung cancer (NSCLC) using flow cytometry. A mouse model of X-ray-induced acute thrombocytopenia was used to investigate the relationship between thrombopoiesis and PD-L1-expressing platelet generation. The clinical significance of PD-L1-expressing platelets was analyzed in a cohort of patients with stage IV NSCLC who received a combination of anti-programmed cell death 1 (PD-1) therapy and chemotherapy.

Results All immune cell populations, including monocytes, T cells, B cells, and NK cells, showed higher PD-L1 expression in patients with cancer than in healthy controls. However, this increased frequency of PD-L1-expressing cells was not attributed to the expression of the cells themselves. Instead, it was entirely dependent on the direct interaction of the cells with PD-L1-expressing platelets. Notably, the platelet-dependent acquisition of PD-L1 on circulating immune cells of patients with lung cancer was observed in various other cancer types and was mechanistically associated with a surge in thrombopoiesis, resulting in the increased production of PD-L1-expressing reticulated platelets. Clinically, patients with enhanced thrombopoiesis and concurrently high PD-L1-expressing platelets exhibited a better response to anti-PD-1 therapy.

Conclusions These findings highlight the role of tumor-associated thrombopoiesis in generating PD-L1-expressing platelets that may serve as a resource for PD-L1-positive cells in the circulation and act as a predictive biomarker for anti-PD-1/PD-L1 therapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Programmed cell death ligand 1 (PD-L1) expression on various cellular components in the blood, including monocytes, T cells, B cells, and platelets, has been reported to be elevated in patients with cancer. However, how all these different cell types gain PD-L1 expression is unknown.

WHAT THIS STUDY ADDS

⇒ The binding of PD-L1-expressing platelets was identified as the key mechanism underlying PD-L1 acquisition in various immune cell types in the blood. The acquisition of PD-L1 expression on platelets relied on enhanced thrombopoiesis. Consequently, patients with enhanced thrombopoiesis and concurrently high PD-L1-expressing platelets were more responsive to anti-programmed cell death 1 (PD-1) therapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides insight into the regulatory mechanism of PD-L1 expression in the blood that is regulated distinctly from that of the tumor microenvironment. This mechanism adds another layer of tumor evasion systemically in the circulation, suggesting that considering and modulating this evasion could be critical for anticancer immunotherapy.

BACKGROUND

Immune checkpoints play a crucial role in regulating immune responses to foreign antigens and even self-antigens, thereby preserving effective immune clearance of invading pathogens while maintaining tolerance to self-antigens.[1] The interaction of programmed cell death 1 (PD-1) and its ligand (PD-L1) is one of the best-known and most effective immune checkpoints through which tumors adapt to evade the immune system. It has therefore been a major target

of various solid cancer therapies.[2–4] Anti-PD-1/PD-L1 therapy has been highly successful in cancer treatment; however, low response rates and acquired resistance remain a challenge.[5–7] Moreover, although the level of PD-L1 expression on tumor tissues (known as tumor proportion score, TPS) has been used as a key biomarker for anti-PD-1/PD-L1 therapy,[8 9] a clear link between PD-L1 TPS and therapeutic efficacy remains to be established. In several cases, patients with cancer whose tumor tissue is negative for PD-L1 have been responsive to anti-PD-1/PD-L1 therapy,[8 9] suggesting a role of PD-L1 in tissues or cells other than the tumor itself.

PD-L1 expression on various components of peripheral blood, including monocytes, T cells, B cells, platelets, extracellular vesicles, and even cell-free plasma, has been examined in previous studies.[10–15] These studies have revealed that increased PD-L1 expression on these circulating components in patients with cancer correlates with responses to anti-PD-1/PD-L1 therapy. Whether PD-L1 expression levels across these components circulating in the blood are mechanistically related to one another or simply reflect a mere correlation remains unknown.

A possible mechanism underlying PD-L1 expression on various circulating immune cells is that inflammatory cytokines, such as interferon (IFN)-γ, IFN-α/β, and interleukin (IL)-6, induce PD-L1 expression on tumor cells as well as several tumor-infiltrating or—resident immune cells, including monocytes/macrophages, dendritic cells (DCs), natural killer (NK) cells, and T cells.[4 16–23] These cytokines may be enriched in the tumor microenvironment (TME) and thus be relevant for PD-L1 expression on cells within the TME; however, their role in circulating immune cells in the blood remains elusive.

In this study, we investigated PD-L1 expression on various circulating immune cell populations of patients with non-small cell lung cancer (NSCLC) and found a mutually shared mechanism for increasing PD-L1 expression. All immune cell populations showed higher PD-L1 expression in cancer patients than in healthy donors; however, this upregulation was almost exclusively dependent on the physical attachment and aggregation of PD-L1-expressing platelets on the surface of these cells. Mechanistically, PD-L1 expression on platelets was irrelevant to PD-L1 expression on tumor cells but was associated with a surge in thrombopoiesis that may occur in the context of tissue damage or trauma during tumor growth. Further retrospective analysis of a cohort of patients with lung cancer revealed a close relationship between platelet PD-L1 and clinical response to anti-PD-1 therapy. Thus, platelet PD-L1 is an effective biomarker for predicting anti-PD-1 therapy response independently of tumor PD-L1. These findings highlight a previously unappreciated role of thrombopoiesis in generating PD-L1-expressing platelets in lung cancer patients. These PD-L1-expressing platelets may serve as a resource of PD-L1-positive cells in the blood and act as a predictive biomarker for anti-PD-1/PD-L1 therapy.

MATERIALS AND METHODS

Patients

Peripheral blood mononuclear cells (PBMCs) of three cohorts of patients with cancer were retrospectively analyzed in this study: first, patients with stage IV NSCLC who received anti-PD-1 therapy in combination with chemotherapy as the first-line treatment; second, patients with NSCLC who underwent surgery; third, a mixture of patients with stage IV cancer with cancers originating from different organs (lung, skin, kidney, liver, bladder, and bile duct). In addition, peripheral blood of patients diagnosed with thrombocytosis without any malignancy was used. All patient samples for this study were provided by the Biobank of Chonnam National University Hwasun Hospital, a member of the Korea Biobank Network. No criteria such as sex, age, or weight were considered for exclusion. Healthy blood samples were provided by the Korean Red Cross. Information about these healthy donors was inaccessible due to local regulations.

Clinical analysis

For patients with NSCLC who received anti-PD-1 therapy, follow-up CT scans were performed every two to three cycles. The clinical response to anti-PD-1 therapy was evaluated by Response Evaluation Criteria in Solid Tumors V.1.1. The best response was defined 3 months after treatment.

Sample preparation

Peripheral whole blood was collected from the cephalic vein using BD Vacutainer (BD) and immediately processed with Lymphoprep (Alere Technologies) to obtain PBMCs. PBMCs were fast frozen at −80°C using 10% dimethyl sulfoxide (Merck) in fetal bovine serum (Gibco), then thawed in a 37°C water bath on experiment.

Flow cytometry

Thawed PBMCs were used for flow cytometry. Samples were stained with fluorochrome-conjugated antibodies and a viability dye (Ghost Dye Violet 510 Fixable Viability Dye (Cell Signaling, #59863S)) for 30 min in ice. For analysis of mitochondria contents, samples were stained with MitoTracker Green FM Dye (Invitrogen, #M46750) for 30 min. Following antibodies for flow cytometry were used: anti-human PD-L1 (29E2A3, BioLegend, #329708), anti-human CD45 (HI30, BioLegend, #304048), anti-human CD14 (63D3, BioLegend, #367112), anti-human CD3 (OKT3, BioLegend, #317322), anti-human CD19 (HIB19, BioLegend, #302232), anti-human CD56 (5.1H11, BioLegend, #362550), anti-human PSGL-1 (KPL-1, BioLegend, #328818), anti-human CD206 (15–2, BioLegend, #321152), anti-human HLA-DR (L243, BioLegend, #307640), anti-human CD11b (M1/70, BioLegend, #101292), anti-human CD8 (RPA-T8, eBioscience, #45-0088-42), anti-human CD4 (A161A1, BioLegend, #357416), anti-human CD45RA (HI100, BioLegend, #304123), anti-human CD28 (CD28.2, BioLegend, #302968), anti-human CD27 (M-T271, BD,

#741145), anti-human IgD (IA6-2, BioLegend, #348206), anti-human CD41 (HIP8, BioLegend, #303724), anti-human CD42b (HIP1, BioLegend, #303906), anti-human CD62P (AK4, BioLegend, #304920), anti-mouse CD41 (MWReg30, BioLegend, #133932), anti-mouse CD274 (B7-H1, BioLegend, #124312), anti-mouse CD45 (30-F11, BioLegend, #103149), anti-mouse CD11b (M1/70, eBioscience, #11-0112-82). Samples were run using CytoFLEX S and CytoFLEX LX (Beckman Coulter) and analyzed using FlowJo software (Tree Star).

Confocal microscopy

CD41$^+$ PBMCs were purified using CytoFlex SRT (Beckman Coulter). The purified cells were seeded on a slide glass and fixed with intracellular (IC) fixation buffer (eBioscience, #00-8222-49) for 20 min at room temperature. After blocking non-specific binding with phosphate-buffered saline supplemented with 1% bovine serum albumin (Bioshop, #ALB001.100), samples were stained with anti-PD-L1 antibody (MIH1, Invitrogen, #14-5983-82) overnight in a 4°C humid chamber. The next day, samples were stained with anti-mouse Alexa Fluor 594 secondary antibody (Invitrogen, #A11032) and DAPI (Merck, #MBD0015). Then, ProLong Gold antifade reagent (Invitrogen, #P36934) was applied followed by a cover slide. Samples were left overnight at room temperature and analyzed using an LSM900 with Airyscan2 (ZEISS). The z-stacked images were processed with ZEN 2.3 SP1 (black) program for maximum intensity projection.

Platelet detachment

PBMCs were resuspended in 5 mL of complete media and stirred in a glass vial at 37°C in an incubator with a magnetic stirrer (MTOPS, #MS20312) for 30 min. After stirring, the samples were stained and analyzed using flow cytometry.

Mice

C57BL/6J mice were maintained under specific pathogen-free conditions in a 12-hour light/dark cycle at 25°C, with 40% humidity and free access to food and water. All mice used in the experiments were sex-matched and aged between 8 and 15 weeks.

Irradiation-induced thrombocytopenia

Mice were exposed to X-irradiation using an X-Rad320 (Precision) (320 kV, 12.5 mA, 600 rad). Blood samples were harvested using heparinized capillary tubes (Kimble, #41B2501) into 1.5 mL microcentrifuge tubes (Neptune, #4445.X) containing Alserver's solution. For some experiments, bone marrow cells were harvested from the right femur. Samples were treated with ACK lysing buffer before antibody staining. All samples were analyzed using flow cytometry.

T-cell activation in the presence of PD-L1 expressing platelets

Platelets from the plasma of patients with stage IV NSCLC were assessed for PD-L1 expression. Platelets with high PD-L1 expression (≥30%) were pooled and used as PD-L1^{hi} platelets. T cells were stimulated with plate-coated anti-CD3 (HIT3a, BioLegend, #300302, 5 µg/mL) and anti-CD28 (CD28.2, eBioscience, #16-0289-81, 2 µg/mL) in the presence of PD-L1^{hi} platelet and blocking antibodies (either anti-IgG (MRG1-58, BioLegend, #407402, 2.5 µg/mL) or anti-PD-L1 (MIH1, eBioscience, #14-5983-82, 2.5 µg/mL)). For short-term activation, T cells were assessed 1 hour after stimulation. For long-term activation, T cells were assessed 3 days after stimulation. Pan-T cells were used for short-term activation, while naïve CD8 T cells were used for long-term activation. In some experiments, total PBMCs instead of T cells were used for activation.

Statistics

All statistics were performed using Prism (GraphPad Software). The statistical significances were tested with Student's t-test (parametric) or Mann-Whitney (non-parametric) test for unpaired samples, Wilcoxon matched-pairs signed rank test for paired samples, and two-way analysis of variance for comparisons involving more than three samples. Values of ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 were considered significant.

RESULTS

Circulating immune cells in patients with lung cancer exhibit increased PD-L1 expression in a coordinated manner

To assess PD-L1 expression profiles on circulating immune cells in patients with cancer, we conducted an extensive retrospective analysis of PBMCs from 112 patients with NSCLC (online supplemental table 1) and 50 healthy donors. While PBMCs from healthy donors rarely expressed PD-L1, a significant proportion of PBMCs from patients with NSCLC expressed PD-L1 (figure 1A). PD-L1$^+$ PBMCs in these patients consisted of various cell types, particularly monocytes and to a lesser extent T cells, B cells, and NK cells (figure 1B and online supplemental figure 1A). Notably, among these cell types, monocytes exhibited the highest frequency of PD-L1 expression (figure 1C). In contrast, a small fraction of PD-L1$^+$ PBMCs was detected in healthy donors; however, these PD-L1$^+$ cells were not predominantly restricted to monocytes, but the frequency of PD-L1$^+$ T cells was also similar in healthy donors (figure 1B).

Given the different cellular compositions of PD-L1$^+$ cells between healthy donors and patients with cancer, we determined whether monocytes are the primary source of increased PD-L1 expression in patients with cancer. PD-L1$^+$ monocytes were significantly more prevalent in patients with cancer than in healthy donors (figure 1D). However, we noted a smaller but consistent increase in PD-L1$^+$ cells among other immune cell populations, including T cells, B cells, and NK cells (figure 1E–G). Interestingly, the frequency of PD-L1$^+$ monocytes correlated with the frequency of other PD-L1$^+$ immune cells (figure 1H). Patients with high PD-L1 expression

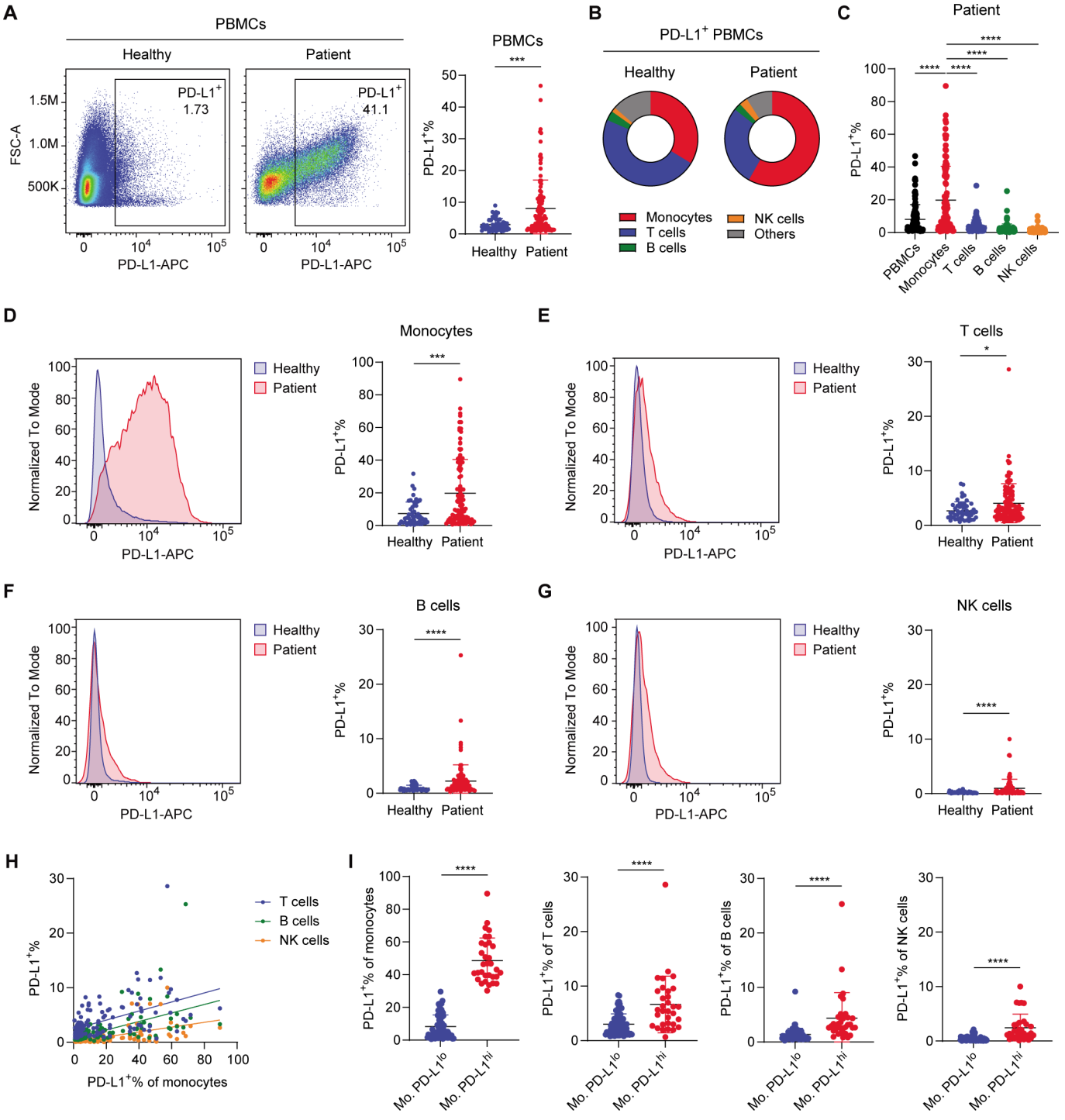

Figure 1 Immune cell populations in the peripheral blood of patients with NSCLC show increased PD-L1 expression in a coordinated manner (A) Frequency of PD-L1$^+$ cells in PBMCs from patients with NSCLC (n=112) and healthy donors (n=50). (B) Pie charts showing the distribution of immune cell populations among the PD-L1$^+$ PBMCs from patients with NSCLC and healthy donors. (C) Frequency of PD-L1$^+$ cells in various immune cell populations of patients with NSCLC. (D-G) Frequency of PD-L1$^+$ cells on (D) monocytes, (E) T cells, (F) B cells, and (G) NK cells from patients with NSCLC and healthy donors. (H) Correlation between PD-L1$^+$ frequencies on monocytes and other immune cells (T cells, B cells, and NK cells). Lines represent linear regressions. (I) Frequency of PD-L1$^+$ cells on various immune cell populations in two patient groups divided by PD-L1 expression on monocytes (Mo.PD-L1^{lo} and Mo.PD-L1^{hi}; n=80, 32, respectively). All bar graphs represent mean±SD. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. APC, Allophycocyanin; Mo.PD-L1^{lo}, low monocyte PD-L1; Mo.PD-L1^{hi}, high monocyte PD-L1; NK, natural killer; NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear cells; PD-L1, programmed cell death ligand 1.

on monocytes (Mo.PD-L1^{hi}; higher than that of healthy donors) also exhibited significantly higher PD-L1 expression on all other immune cell populations compared with patients with lower monocyte PD-L1 expression (Mo.PD-L1^{lo}) (figure 1I). These findings imply that the increased PD-L1 expression in patients with lung cancer broadly involves various circulating immune cell populations in a coordinated manner, with monocytes being the predominant cell type.

PD-L1 is primarily expressed on CD41-expressing PBMCs in patients with lung cancer

Considering the unique coordinated expression of PD-L1 among various cell types, we explored whether different PD-L1^{+} immune cells exhibit certain phenotypic similarities. First, we compared PD-L1^{+} and PD-L1^{-} subsets of monocytes and examined the phenotypic significance of PD-L1^{+} cells. While common monocyte markers (CD162, CD45, CD14, CD206, HLA-DR, and CD11b) exhibited no noticeable differences between PD-L1^{+} and PD-L1^{-} monocytes, the expression of CD41 was exceptionally high in PD-L1^{+} cells (figure 2A). Similarly, CD41 was the only differentially expressed marker in PD-L1^{+} and PD-L1^{-} subsets of other immune cell populations (online supplemental figure 2A). Thus, across all cell types, CD41^{+} cells exhibited significantly higher PD-L1 expression than CD41^{-} cells (figure 2B).

Next, we investigated whether the previously observed differences in PD-L1 expression among immune cell populations and the increased PD-L1 expression noted in patients with cancer are associated with CD41. Monocytes, which exhibited the highest PD-L1 expression in patients with cancer (figure 1C), also exhibited the highest frequency of CD41 expression among all immune cell populations (figure 2C). Moreover, CD41 expression was closely correlated with PD-L1 expression in the Mo.PD-L1^{hi} group, supporting the co-expression of CD41 and PD-L1 (figure 2D and online supplemental figure 2B).

However, we noticed intact CD41 expression despite the lack of PD-L1 expression in the Mo.PD-L1^{lo} group (figure 2D). Specifically, patients with high CD41^{+} monocytes within the Mo.PD-L1^{lo} group (Mo.PD-L1^{lo}CD41^{hi}; accounting for 81.3% of the patients in the Mo.PD-L1^{lo} group) exhibited similar CD41 expression levels to those in the Mo.PD-L1^{hi} group; however, PD-L1 expression levels significantly differed between the two groups (figure 2E). Moreover, healthy donors exhibited a high frequency of CD41^{+} monocytes, showing only a slight difference from patients with cancer (figure 2C). Thus, the higher frequency of PD-L1^{+} monocytes in the Mo.PD-L1^{hi} group than in the Mo.PD-L1^{lo} group or healthy donor group was primarily attributed to an increased proportion of PD-L1-expressing cells, particularly within the CD41^{+} populations (figure 2F and online supplemental figure 2C). These data suggest that while PD-L1 is primarily expressed on CD41-expressing cells, PD-L1 expression is not dependent on CD41 expression.

The increased frequency of PD-L1^{+} PBMCs in patients with lung cancer results from the direct attachment and aggregation of PD-L1-expressing platelets

To elucidate the close relationship between CD41 and PD-L1, we investigated the nature of CD41 expression. CD41 is a megakaryocytic lineage marker that is specifically expressed on platelets among the differentiated hematopoietic lineage cells in the peripheral blood.[24–26] Notably, several studies have demonstrated that platelets in patients with lung cancer express PD-L1.[12 27] Therefore, we examined PD-L1 expression on platelets and explored its relationship with PD-L1 expression on PBMCs. As platelets are present in the plasma, they are often included during the preparation of PBMCs because the buffy coat and plasma are adjacent in centrifuged blood samples[28] (figure 3A). We analyzed PD-L1 expression on these platelets and confirmed that the frequency of PD-L1^{+} platelets was significantly higher in patients with cancer than in healthy donors (figure 3B). Importantly, the frequency of PD-L1^{+} platelets showed an exceptionally high correlation with the frequency of PD-L1^{+} cells in CD41^{+} PBMCs but not in CD41^{-} PBMCs (figure 3C and online supplemental figure 3A). Consequently, compared with healthy donors, patients with high platelet PD-L1 (PLT.PD-L1^{hi}; higher than that of healthy donors) exhibited increased PD-L1 expression on circulating immune cell populations, while those with low platelet PD-L1 (PLT.PD-L1^{lo}) did not (figure 3D and online supplemental figure 3B). These data imply that PD-L1 expression on platelets is a crucial factor for determining PD-L1 expression on various immune cell populations of PBMCs.

Next, we investigated how platelets influence PD-L1 expression on PBMCs. In addition to their role in coagulation, platelets bind to various circulating immune cells—particularly monocytes—in the peripheral blood, thereby enabling these cells to acquire various platelet-derived surface molecules.[29 30] We therefore hypothesized that PD-L1 molecules on PBMCs are derived from those of platelets binding to these cells. To verify this, we assessed whether CD41^{+} PBMCs are platelet–leukocyte aggregates (PLAs). Considering the size of platelets (~3 µm in diameter),[31] we anticipated a noticeable increase in the size of PLAs. As expected, forward scatter and side scatter were significantly greater in CD41^{+} subsets than in CD41^{-} subsets across all circulating immune cell populations in the peripheral blood of patients with cancer (figure 3E and online supplemental figure 3C). Moreover, we observed high expression levels of other platelet-specific markers, namely CD42b and CD62P, on CD41^{+} PBMCs but not on CD41^{-} PBMCs (figure 3F). Notably, the expression levels of CD42b and CD62P were higher on CD41^{+} PBMCs than on platelets (figure 3F); this finding is consistent with the higher expression of PD-L1 on CD41^{+} PBMCs than on platelets (figure 3C). This relationship likely reflects the nature of platelets, which bind to and form aggregates on host cells, resulting in the attachment of multiple platelets to a single host cell.[30 32] Together, these data suggest that CD41^{+} PBMCs are PLAs that arise

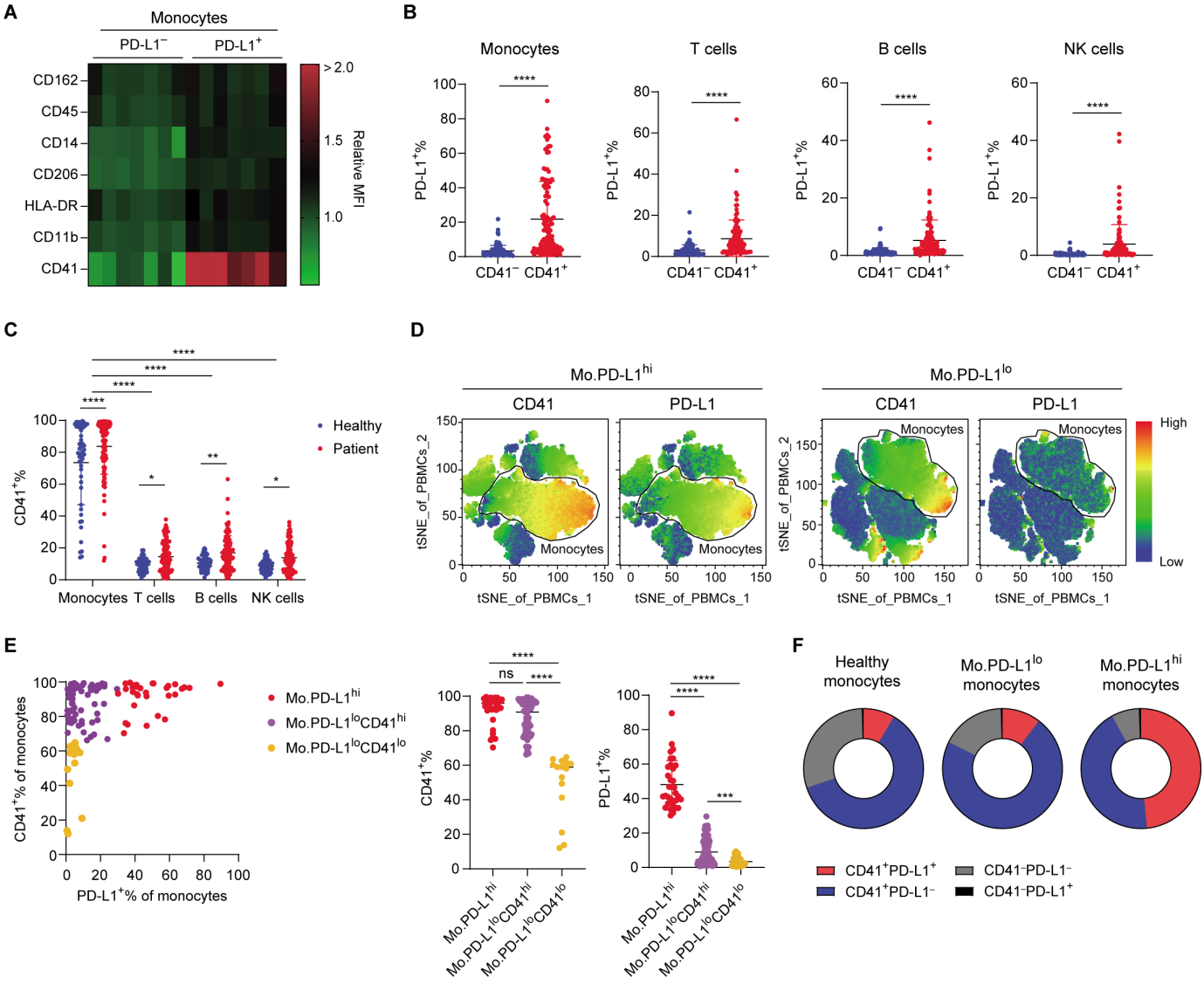

Figure 2 PD-L1 expression on circulating immune cells of patients with NSCLC is closely associated with CD41 (A) Heatmap representing various molecule expressions in PD-L1$^-$ and PD-L1$^+$ subsets of monocytes from patients with NSCLC (n=7). Relative MFI was calculated by dividing the MFI of monocyte subsets by the MFI of total monocytes. The expression was color-coded from green (low) to red (high). (B) Frequency of PD-L1$^+$ cells in CD41$^-$ and CD41$^+$ subsets of various immune cell populations (monocytes, T cells, B cells, and NK cells) of patients with NSCLC (n=112). (C) Frequency of CD41$^+$ cells in various immune cell populations from patients with NSCLC (n=112) and healthy donors (n=50). (D) t-SNE plot showing CD41 and PD-L1 expressions on PBMCs from Mo.PD-L1^{hi} and Mo.PD-L1^{lo} patients. The expression was color-coded from blue (low) to red (high). Monocytes were highlighted using solid lines. (E) Relationship between CD41 and PD-L1 in patients with NSCLC. Patients with NSCLC were divided into three groups (Mo.PD-L1^{hi}, Mo.PD-L1^{lo}CD41^{hi}, Mo.PD-L1^{lo}CD41^{lo}, n=32, 65, 15, respectively) and compared for CD41$^+$ frequency and PD-L1$^+$ frequency in monocytes. (F) Pie charts showing the distribution of monocyte subsets (CD41$^+$PD-L1$^+$, CD41$^+$PD-L1$^-$, CD41$^-$PD-L1$^-$, and CD41$^-$PD-L1$^+$) in healthy donors and patients with NSCLC (Mo.PD-L1^{lo} and Mo.PD-L1^{hi}). All bar graphs represent mean±SD. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. MFI, mean fluorescence intensity; Mo.PD-L1^{lo}, low monocyte PD-L1; Mo.PD-L1^{lo}CD41^{lo}, low monocyte PD-L1 and low monocyte CD41; Mo.PD-L1^{lo}CD41^{hi}, low monocyte PD-L1 and high monocyte CD41; Mo.PD-L1^{hi}, high monocyte PD-L1; NK, natural killer; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death ligand 1; t-SNE, t-distributed stochastic neighbor embedding.

from physical attachment between CD41$^+$ platelets and CD41$^-$ PBMCs.

Considering the aggregation of PD-L1-expressing platelets on PBMCs, we investigated whether PD-L1 expressed on PBMCs is in fact platelet-derived PD-L1. We confirmed that PD-L1 exhibits a co-expression pattern with all platelet-specific markers on PBMCs (figure 3G and online supplemental figure 3D). Next, using confocal microscopy, we observed the localization of PD-L1 on PBMCs. PD-L1 was mostly expressed on CD41-enriched, nucleus-free zones (figure 3H and online supplemental figure 3E). Finally, we assessed the frequency of PD-L1$^+$ circulating immune cell populations among CD41$^+$ PBMCs and CD41$^-$ PBMCs. CD41$^+$ PBMCs (including monocytes,

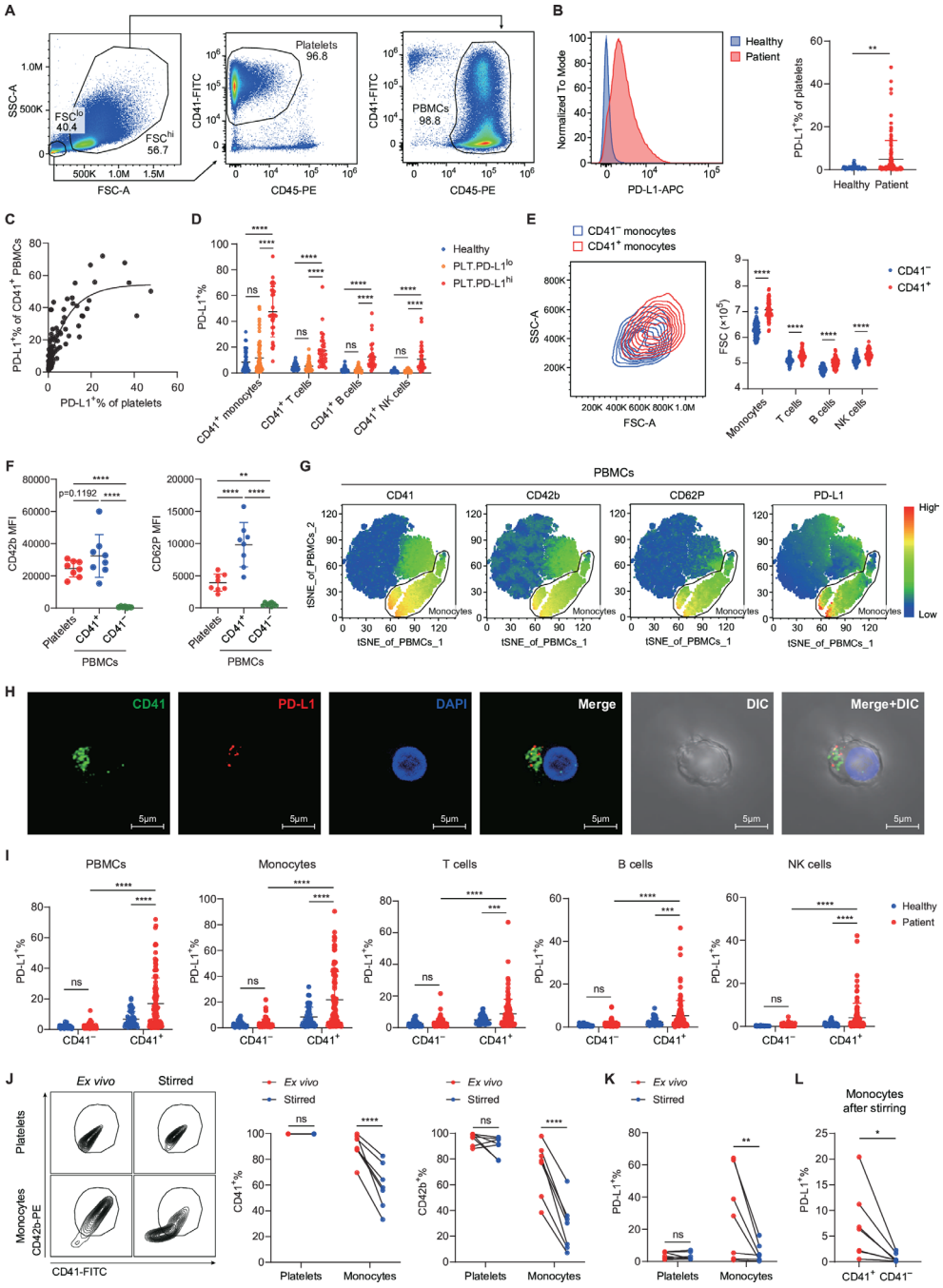

Figure 3 The increased PD-L1 expression on circulating immune cells of patients with NSCLC is not cell-intrinsic but relies on the binding of PD-L1-expressing platelets. (A) Gating strategy for identifying platelets among PBMCs. (B) Frequency of PD-L1$^+$ platelets in patients with NSCLC (n=112) and healthy donors (n=50). (C) Correlation between PD-L1$^+$ frequencies in platelets and CD41$^+$ PBMCs of patients with NSCLC (n=112). Line represents non-linear regression. (D) Frequency of PD-L1$^+$ cells in CD41$^+$ immune cell populations (T cells, B cells, and NK cells) of healthy donors (n=50) and patients (PLT.PD-L1^{lo} and PLT.PD-L1^{hi}; n=80, 32, respectively). (E) FSC of CD41$^-$ and CD41$^+$ immune cell populations from patients with NSCLC (n=112). Representative flow cytometric data shows CD41$^-$ and CD41$^+$ monocytes. (F) Expression of CD42b and CD62P in platelets, CD41$^-$ PBMCs, and CD41$^+$ PBMCs from patients with NSCLC (n=8). (G) t-SNE plot showing CD41, CD42b, CD62P, and PD-L1 expressions on PBMCs from a patient with NSCLC. The expression is color-coded from blue (low) to red (high). Monocytes are highlighted using solid lines. (H) Confocal microscopy image of a CD41$^+$ PBMC from a patient with NSCLC. Molecules are color-coded with green (CD41), red (PD-L1), and blue (DAPI). Scale bars are presented at the corner (5 μm). (I) Frequency of PD-L1$^+$ cells in CD41$^-$ and CD41$^+$ cells from patients with NSCLC (n=112) and healthy donors (n=50). (J-K) Frequency of (J) CD41$^+$, CD42b$^+$, and (K) PD-L1$^+$ cells in platelets and monocytes of patients with NSCLC before (ex vivo) and after mechanical stirring (stirred) (n=7). (L) Frequency of PD-L1$^+$ cells in CD41$^+$ and CD41$^-$ monocytes after stirring. All bar graphs represent mean±SD. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. FSC, forward scatter; MFI, mean fluorescence intensity; NSCLC, non-small cell lung cancer; NK, natural killer; PBMC, peripheral blood mononuclear cells; PD-L1, programmed cell death ligand 1; PLT.PD-L1^{lo}, low platelet PD-L1; PLT.PD-L1^{hi}, high platelet PD-L1; t-SNE, t-distributed stochastic neighbor embedding.

T cells, B cells, and NK cells) from cancer patients exhibited a significantly higher frequency of PD-L1$^+$ cells than those from healthy donors (figure 3I). In contrast, the frequency of PD-L1$^+$ cells in CD41$^-$ PBMCs was comparable between cancer patients and healthy donors (figure 3I). Collectively, these data suggest that the increased PD-L1 expression on PBMCs in patients with lung cancer is largely, if not entirely, caused by the aggregation of PD-L1-expressing platelets.

To determine the extent to which platelets are responsible for PD-L1 expression on PBMCs, we attempted to detach platelets from PBMCs and analyze PD-L1 expression on platelet-free PBMCs. Platelets bind to immune cells through interactions of receptors, such as the CD62P–PSGL1 axis.[29 30] Consistent with previous reports, monocytes exhibited the highest level of PSGL1 expression among all immune cell populations (online supplemental figure 3F), supporting the role of the CD62P–PSGL1 axis in different levels of platelet aggregation on various circulating immune cell types. These receptor-mediated platelet bindings are reversible, allowing platelets to re-enter the circulation after detachment.[33] To analyze PD-L1 expression on platelet-free PBMCs, we mechanically stirred PBMCs using magnetic beads for 30 min and assessed changes in PD-L1 expression. Monocytes readily decreased the levels of platelet markers on stirring, suggesting substantial levels of platelet detachment (figure 3J and online supplemental figure 3G). Importantly, PD-L1 expression on patient monocytes significantly decreased accordingly (figure 3K). Moreover, CD41$^-$ monocytes generated by stirring did not express PD-L1 at all (figure 3L). Collectively, these findings strongly suggest that the increased PD-L1 expression on PBMCs in patients with lung cancer heavily depends on PD-L1-expressing platelets that preferentially bind to and form aggregates on various circulating immune cells, particularly monocytes.

PD-L1$^+$ platelets are detected across various cancer types at variable degrees

Given the significant impact of platelets on PD-L1 acquisition on PBMCs, we investigated when and how PD-L1$^+$ platelets are detected during tumor development. We found that PD-L1 was expressed on platelets only in the peripheral blood of patients with stage III/IV metastatic NSCLC but not in that of patients with stage I/II NSCLC (figure 4A). Consequently, we exclusively detected increased PD-L1 expression on CD41$^+$ PBMCs but not on CD41$^-$ PBMCs of patients with stage III/IV metastatic NSCLC (figure 4B). Assessments based on other tumor-related criteria (tumor PD-L1 expression, epidermal growth factor receptor (EGFR) mutation, and histology) or patient-specific criteria (age and sex) did not reveal differences in PD-L1 expression on platelets or PBMCs among patients with stage IV NSCLC (figure 4C,D and online supplemental figure 4A–F). Moreover, we assessed PD-L1 expression in patients with extensive disease stage small cell lung cancer (SCLC) (online supplemental table 2). In contrast to patients with NSCLC, only a small fraction of patients with SCLC showed increased PD-L1 expression on platelets and CD41$^+$ PBMCs, resulting in no statistical significance compared with healthy donors (figure 4E,F). These results suggest the presence of cancer-type-dependent differences in PD-L1 expression on platelets.

We further extended our investigation to different cancer types other than lung cancer (online supplemental table 3). Notably, we detected PD-L1-expressing platelets in some but not all cancer types. For example, patients with bladder and bile duct cancer contained PD-L1$^+$ platelets, whereas patients with skin, kidney, and liver cancer did not, even though all these patients were at stage IV (figure 4G). However, the frequency of PD-L1$^+$ cells in CD41$^+$ PBMCs, which tend to be more sensitive to PD-L1 detection than platelets themselves (figure 3C), was much more pronounced. Patients with liver and kidney cancer contained PD-L1$^+$ cells in CD41$^+$ PBMCs, unlike the result observed in platelets (figure 4H). On the other hand, patients with skin cancer did not contain noticeably higher PD-L1$^+$ cells than healthy controls even in CD41$^+$ PBMCs (figure 4H). Importantly, CD41$^-$ PBMCs never expressed PD-L1 in any case (figure 4H). Thus, while the platelet-dependent expression of PD-L1 on PBMCs is a pan-cancer phenomenon, the expression level of PD-L1 on platelets varies depending on the cancer type, even in metastatic conditions.

Reticulated platelets preferentially express PD-L1 in patients with cancer

Considering the variation of platelet PD-L1 in different cancer types, we investigated the mechanism underlying the upregulation of PD-L1 on platelets. We analyzed whether PD-L1$^+$ and PD-L1$^-$ platelets in patients with NSCLC exhibit any phenotypic differences. Interestingly, we found that PD-L1$^+$ platelets exhibited substantially greater CD45 expression than PD-L1$^-$ platelets, although at a lower level than PBMCs (online supplemental figure 5A). Therefore, we categorized platelets into CD45$^+$ and CD45$^-$ platelets and investigated their physiology (figure 5A and online supplemental figure 5B). CD45$^+$ platelets exhibited higher levels of the platelet markers CD41, CD42b, and CD62P than CD45$^-$ platelets (figure 5B and online supplemental figure 5C,D). Moreover, while CD45$^-$ platelets did not show any sign of CD14 expression, CD45$^+$ platelets unexpectedly expressed a high level of CD14 (figure 5C). These phenotypic differences suggest that CD45$^+$ platelets have high protein contents and express other lineage markers that are not normally expressed in typical platelets in the peripheral blood. Notably, these phenotypic characteristics match those of reticulated platelets, the youngest platelets in the circulation that are recently released from megakaryocytes (MKs) and therefore exhibit high protein contents and express a full spectrum of proteins found in MKs.[34–37] In contrast, CD45$^-$ platelets are similar to mature platelets, which exhibit reduced protein contents because

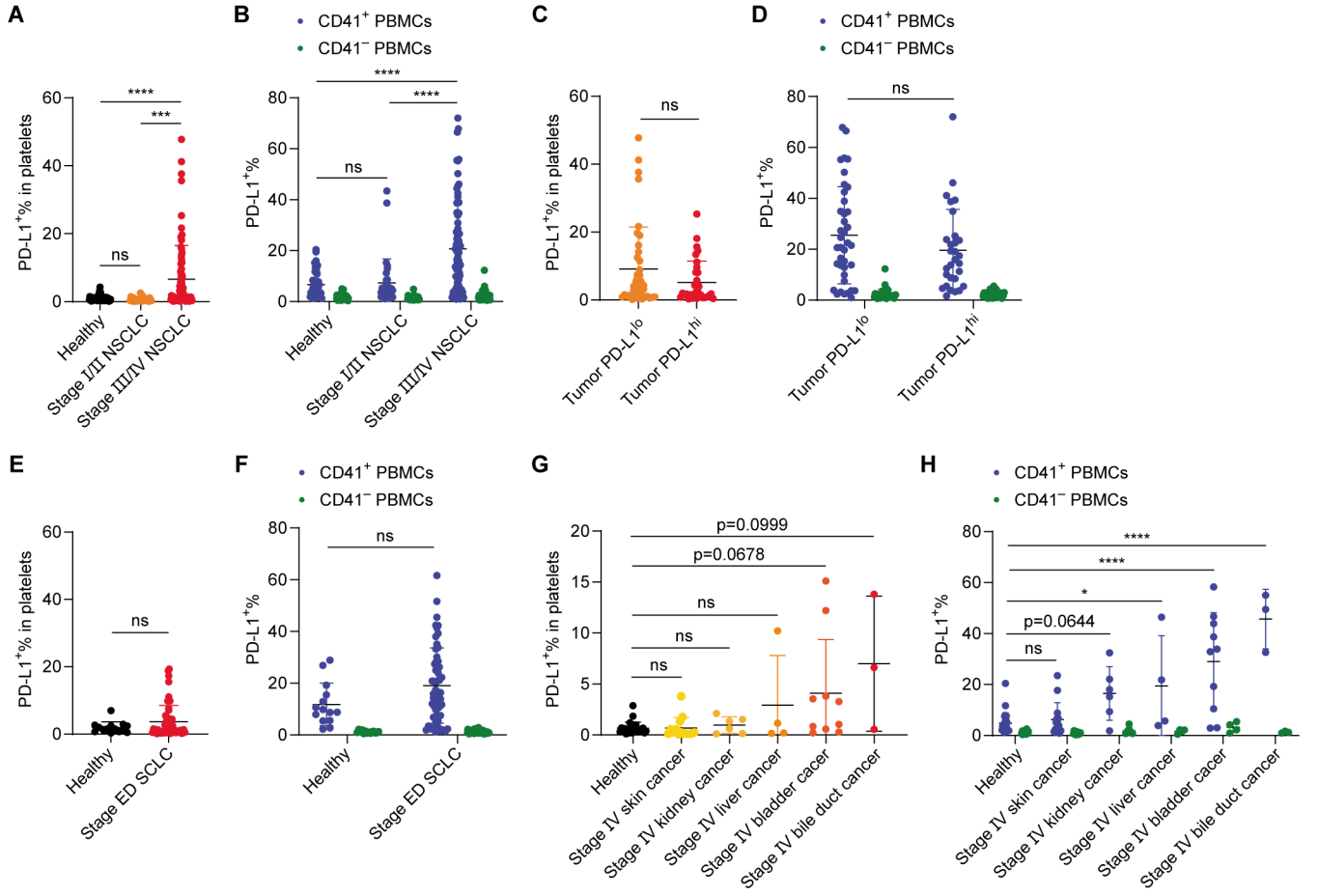

Figure 4 The platelet-dependent expression of PD-L1 on PBMCs is a pan-cancer phenomenon (A-B) Frequency of PD-L1$^+$ cells in (A) platelets, (B) CD41$^+$, and CD41$^-$ PBMCs in healthy donors (n=50), patients with stage I/II NSCLC (n=33), and patients with stage III/IV NSCLC (n=79). (C-D) Frequency of PD-L1$^+$ cells in (C) platelets, (D) CD41$^+$, and CD41$^-$ PBMCs in patients with stage IV NSCLC with high PD-L1 TPS (tumor PD-L1^{hi}) and low PD-L1 TPS (Tumor PD-L1^{lo}) (n=32, 39, respectively). (E-F) Frequency of PD-L1$^+$ cells in (E) platelets, (F) CD41$^+$, and CD41$^-$ PBMCs in healthy donors (n=14) and patients with stage ED SCLC (n=55). (G-H) Frequency of PD-L1$^+$ cells in (G) platelets, (H) CD41+, and CD41– PBMCs in healthy donors (n=25) and stage IV patients with cancers originated from skin, kidney, liver, bladder, and bile duct (n=14, 6, 4, 10, 3, respectively). All bar graphs represent mean±SD. ****p<0.0001, ***p<0.001, *p<0.05. ED, extensive disease; NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear cells; PD-L1, programmed cell death ligand 1; SCLC, small cell lung cancer; TPS, tumor proportion score.

of their anucleate state, resulting in the loss of lineage markers, such as CD45 and CD14, which are expressed in MKs.[24–26 37 38] The number of mitochondria decreases as platelets age.[37] Indeed, CD45$^+$ platelets showed higher levels of mitochondrial contents than CD45$^-$ platelets (figure 5D), further supporting that CD45$^+$ platelets are recently generated reticulated platelets.

Next, we analyzed these two platelet subsets in healthy donors and patients with NSCLC and melanoma (skin cancer). Surprisingly, PD-L1 expression was prominently increased on CD45$^+$ platelets but not on CD45$^-$ platelets from patients with NSCLC (figure 5E), a pattern resembling molecules derived from MKs. In contrast, PD-L1 was marginally expressed on CD45$^+$ platelets from patients with melanoma and healthy donors (figure 5E). Notably, specific PD-L1 expression on reticulated platelets was generally observed across various cancer types and conditions (figure 5F). Such preferential expression of PD-L1 on reticulated platelets suggests that thrombopoiesis is involved in the regulation of PD-L1 expression on platelets.

Patients with metastatic cancer often show enhanced thrombopoiesis to compensate for the constitutive consumption of platelets.[39 40] We investigated whether PD-L1 expression on reticulated platelets of patients with NSCLC is associated with such a phenomenon. Enhanced thrombopoiesis is characterized by the generation of larger platelets.[36 41 42] Indeed, CD45$^+$ platelets from patients with NSCLC were significantly larger than those from healthy donors and patients with melanoma (figure 5G). Importantly, PD-L1 expression on CD45$^+$ platelets exhibited a high correlation with their size; this was observed not only in patients with stage IV NSCLC but also universally across patients with other cancer types and conditions and even in healthy donors (figure 5H and online supplemental figure 5E–H). These results

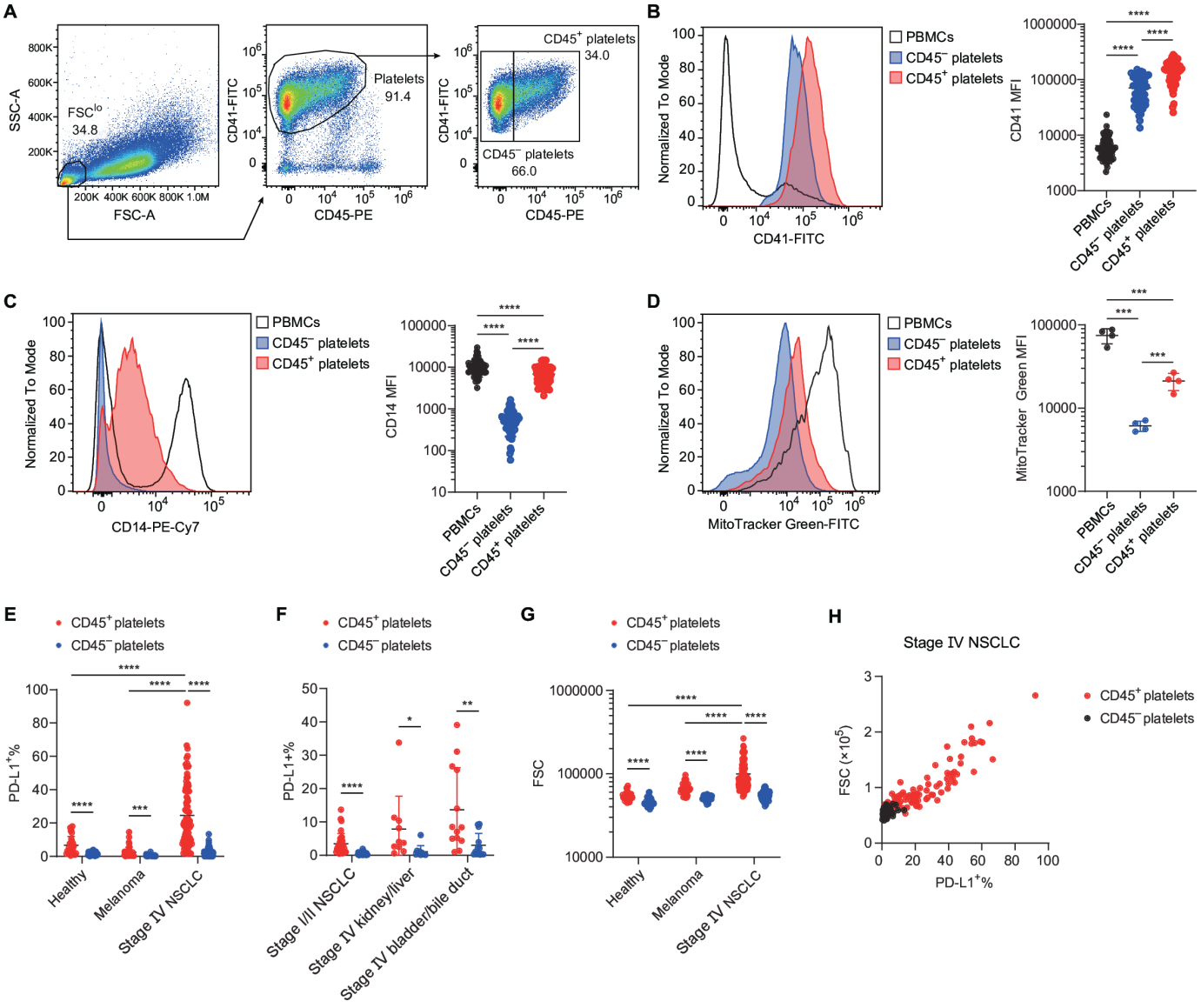

Figure 5 PD-L1 is preferentially expressed on reticulated platelets with a close correlation to their size. (A) Gating strategy of CD45⁻ and CD45⁺ platelets. (B-C) Expression of (B) CD41 and (C) CD14 in PBMCs, CD45⁻ platelets, and CD45⁺ platelets from patients with NSCLC (n=60). (D) MitoTracker Green expression in PBMCs, CD45⁻ platelets, and CD45⁺ platelets from patients with NSCLC (n=4). (E) Frequency of PD-L1⁺ cells in CD45⁻ platelets and CD45⁺ platelets from healthy donors (n=26), patients with melanoma (n=40), and patients with stage IV NSCLC (n=94). (F) Frequency of PD-L1⁺ cells in CD45⁻ platelets and CD45⁺ platelets from patients with stage I/II NSCLC (n=33), patients with stage IV kidney cancer and liver cancer (n=10), and patients with stage IV bladder cancer and bile duct cancer (n=13). (G) FSC of CD45⁻ platelets and CD45⁺ platelets from healthy donors, patients with melanoma, and patients with stage IV NSCLC. (H) Correlation between FSC and PD-L1⁺ frequency in CD45⁻ and CD45⁺ platelets from patients with stage IV NSCLC (n=94). All bar graphs represent mean±SD. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. FSC, forward scatter; NSCLC, non-small cell lung cancer; PBMC, peripheral blood mononuclear cells; PD-L1, programmed cell death ligand 1.

indicate that the preferential expression of PD-L1 on reticulated platelets is a common feature among patients with cancer, possibly driven by a shared mechanism of enhanced thrombopoiesis.

Enhanced thrombopoiesis generates PD-L1-expressing platelets as a general process

The acceleration of thrombopoiesis is a general phenomenon that is routinely observed in wound healing.[43] Therefore, we assessed whether the generation of PD-L1-expressing platelets by enhanced thrombopoiesis is a general phenomenon. For this purpose, we longitudinally analyzed platelets from patients with NSCLC who underwent surgical removal of their tumors (figure 6A and online supplemental table 4). Surgery would eliminate any tumor-originating factor while leaving deep wounds that trigger thrombopoiesis. Interestingly, CD45⁺ platelets exhibited increased PD-L1 expression levels at the first follow-up (2–8 months after the surgery; first FU). The levels returned to or even dropped below baseline levels at the second follow-up (9.5–15.5 months after

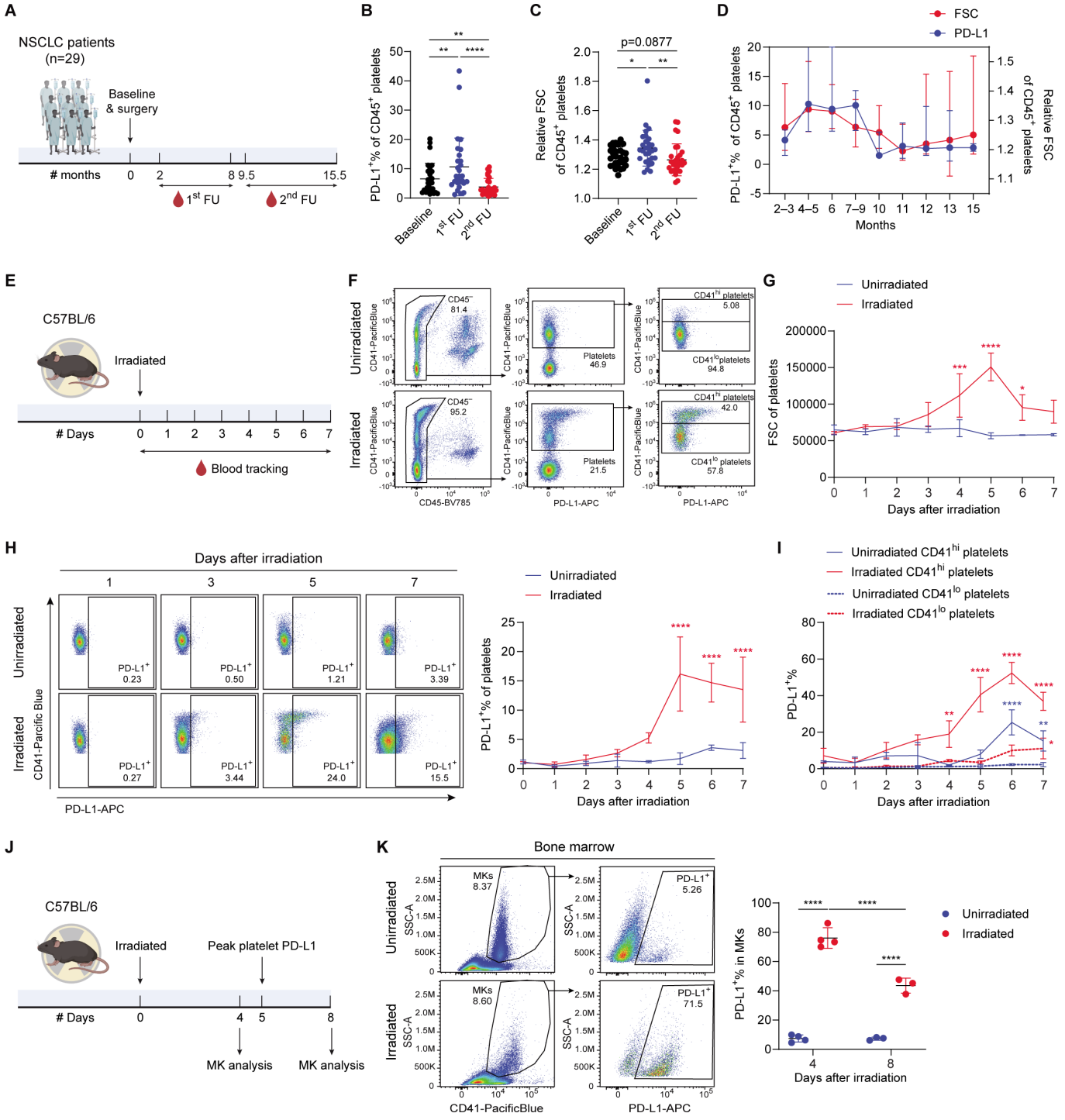

Figure 6 Enhanced thrombopoiesis generates PD-L1-expressing reticulated platelets and PD-L1-expressing megakaryocytes. (A) Blood collection scheme for patients with NSCLC who underwent surgery. Baseline blood was collected for all patients prior to surgery. Follow-up blood was collected twice for each patient. The first follow-up (first FU) was performed within 8 months after the surgery. The second follow-up (second FU) was performed at least 9 months after the surgery. (B-C) (B) PD-L1$^+$ frequency and (C) FSC of CD45$^+$ platelets at baseline, first FU, and second FU. Relative FSC was calculated by dividing FSC of CD45$^+$ platelets by FSC of CD45$^-$ platelets of the same sample. (D) Kinetics of PD-L1$^+$ frequency and FSC of CD45$^+$ platelets over time post-surgery. Error bars represent median and 95% CI. (E) Experimental scheme of mouse irradiation. (F) Gating strategy for platelets and platelet subsets in the mice. (G-H) (G) FSC and (H) PD-L1$^+$ frequency of platelets from irradiated (n=3) and unirradiated (n=3) mice at each time point. (I) PD-L1$^+$ frequency of CD41^{hi} and CD41^{lo} platelets from irradiated and unirradiated mice at each time point. (J) Experimental scheme of megakaryocyte analysis. (K) PD-L1$^+$ frequency of MKs analyzed 4 days (n=4) and 8 days (n=3) after irradiation. MKs from unirradiated mice were used as controls (n=4, 3, respectively). All bar graphs represent mean±SD unless addressed otherwise. ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. (G–I) Statistics for each time point were carried out against day 0 of the same group. FSC, forward scatter; FU, follow-up; MK, megakaryocytes; NSCLC, non-small cell lung cancer; PD-L1, programmed cell death ligand 1.

the surgery; second FU) (figure 6B). Moreover, these changes were closely associated with the changes in the size of CD45$^+$ platelets (figure 6C,D). These results suggest that the induction of PD-L1 expression on platelets is not limited to cancer or cancer-associated factors but is rather a general phenomenon driven by enhanced thrombopoiesis.

To further validate this relationship, we analyzed platelets in cancer-free conditions with enhanced thrombopoiesis. We initially examined patients with thrombocytosis that was not malignant. CD45$^+$ platelets from these patients exhibited increased PD-L1 expression and had an increased size (online supplemental figure 6A). Next, we used a mouse model to enhance thrombopoiesis and then assessed platelet PD-L1 expression. Whole-body irradiation induces acute thrombocytopenia, which in turn enhances thrombopoiesis.[44–46] We tracked the platelets of irradiated and unirradiated mice daily through blood sampling (figure 6E,F). The size of platelets increased and peaked at day 5 after irradiation, indicating the enhancement of thrombopoiesis by irradiation (figure 6G). Importantly, PD-L1 expression on platelets also increased accordingly in the irradiated mice (figure 6H). In contrast to human platelets, CD45 was not a good marker to discriminate PD-L1-expressing platelets (online supplemental figure 6B). Interestingly, however, increased PD-L1 expression was primarily observed on platelets with extremely high CD41 expression (CD41^{hi} platelets) (figure 6F,I). CD41^{hi} platelets also exhibited phenotypes of reticulated platelets, as evident by their higher expression levels of lineage markers (CD45 and CD11b) and larger size than platelets with relatively lower CD41 expression (CD41^{lo} platelets) (online supplemental figure 6C). Moreover, even the unirradiated mice showed increased PD-L1 expression on CD41^{hi} platelets after 6 days of blood tracking, presumably due to mild thrombocytopenia induced by consecutive blood harvests (figure 6I).

To prove that platelet PD-L1 is derived from MKs, we assessed PD-L1 expression on MKs after irradiation (figure 6J). MKs from irradiated mice showed significantly higher PD-L1 expression than those from unirradiated mice (figure 6K). Similar to platelet PD-L1 expression, which peaked at 5–6 days after irradiation and then declined (figure 6H), MKs exhibited higher PD-L1 expression at 4 days after irradiation than at 8 days after irradiation (figure 6K). Collectively, these findings indicate that enhanced thrombopoiesis generates PD-L1-expressing MKs, which in turn produce PD-L1-expressing reticulated platelets.

PD-L1 expression on platelets predicts treatment responses to anti-PD-1 therapy independently of PD-L1 TPS

The immune suppressive role of platelet PD-L1 is well established.[12 47] Consistent with this, the presence of PD-L1-expressing platelets significantly suppressed the expression of activation markers and cytotoxicity in in vitro T-cell activation (online supplemental figure 7). Additionally, previous studies have reported a correlation between platelet PD-L1 expression and response to anti-PD-1/PD-L1 therapies.[12 27 48] However, in light of new insights into the regulatory mechanism underlying PD-L1 expression on platelets and subordinate PD-L1 expression on circulating immune cells, this correlation clearly needs to be re-evaluated. Hence, we analyzed PD-L1 expression on platelets and PBMCs from pre-therapy blood samples of 62 patients with stage IV NSCLC who were treated with anti-PD-1 therapy in combination with chemotherapy as their first-line cancer treatment (online supplemental table 5). We initially analyzed the predictive power of PD-L1 on platelets. The threshold for high PD-L1 expression on CD45$^+$ platelets (CD45$^+$.PLT.PD-L1) was set to achieve a sensitivity of 0.33 in the CD45$^+$.PLT.PD-L1^{hi} group. Notably, 71.4% of the patients in the CD45$^+$.PLT.PD-L1^{hi} group exhibited a partial response (PR), which was significantly higher than the CD45$^+$.PLT.PD-L1^{lo} group (41.7%) (figure 7A). The area under the curve of the receiver operating characteristic curve for CD45$^+$.PLT.PD-L1 was 0.6167 (95% CI, 0.476 to 0.757) (figure 7B). PD-L1 expression on total platelets showed similar predictive power to CD45$^+$.PLT.PD-L1 as they are highly correlated (online supplemental figure 8A,B). Subsequently, we assessed the predictive power of PD-L1 expression on various circulating immune cell populations. As expected, PD-L1 expression on monocytes, T cells, B cells, and NK cells—both CD41$^+$ cells and total cells—showed similar predictive power to CD45$^+$.PLT.PD-L1 (figure 7A,B and online supplemental figure 8C). Moreover, most PR patients in the PD-L1^{hi} group of these immune cell populations overlapped with those in the CD45$^+$.PLT.PD-L1^{hi} group (online supplemental figure 8D). Thus, as PD-L1 expression on immune cell populations is mostly derived from platelets, their function as a biomarker practically mirrors the function of PD-L1 expression on platelets.

Next, we compared the predictive power of CD45$^+$.PLT.PD-L1 and PD-L1 TPS. Under the settings of low sensitivity (0.2), CD45$^+$.PLT.PD-L1 was a significantly better predictor than PD-L1 TPS (75% vs 54.5% in the PD-L1^{hi} group) (figure 7C,D). Increasing the sensitivity gradually decreased the percentage of PR patients in the CD45$^+$.PLT.PD-L1^{hi} group (75%, 71.4%, and 55% at 0.2, 0.33, and 0.5 sensitivity, respectively) (figure 7C). PD-L1 TPS remained a poor predictor across all settings (54.5%, 58.8%, and 53.8% at 0.2, 0.33, and 0.5 sensitivity, respectively) (figure 7D). These results suggest that CD45$^+$.PLT.PD-L1 is a better biomarker for predicting anti-PD-1/PD-L1 therapy responses than PD-L1 TPS.

As platelet PD-L1 expression results from enhanced thrombopoiesis and is thus regulated independently of tumor PD-L1 expression, we speculated that platelet PD-L1 and PD-L1 TPS could synergistically cooperate. PR patients in the CD45$^+$.PLT.PD-L1^{hi} group and those in the PD-L1 TPS^{hi} group barely overlapped (figure 7E). Therefore, we investigated whether patients with both

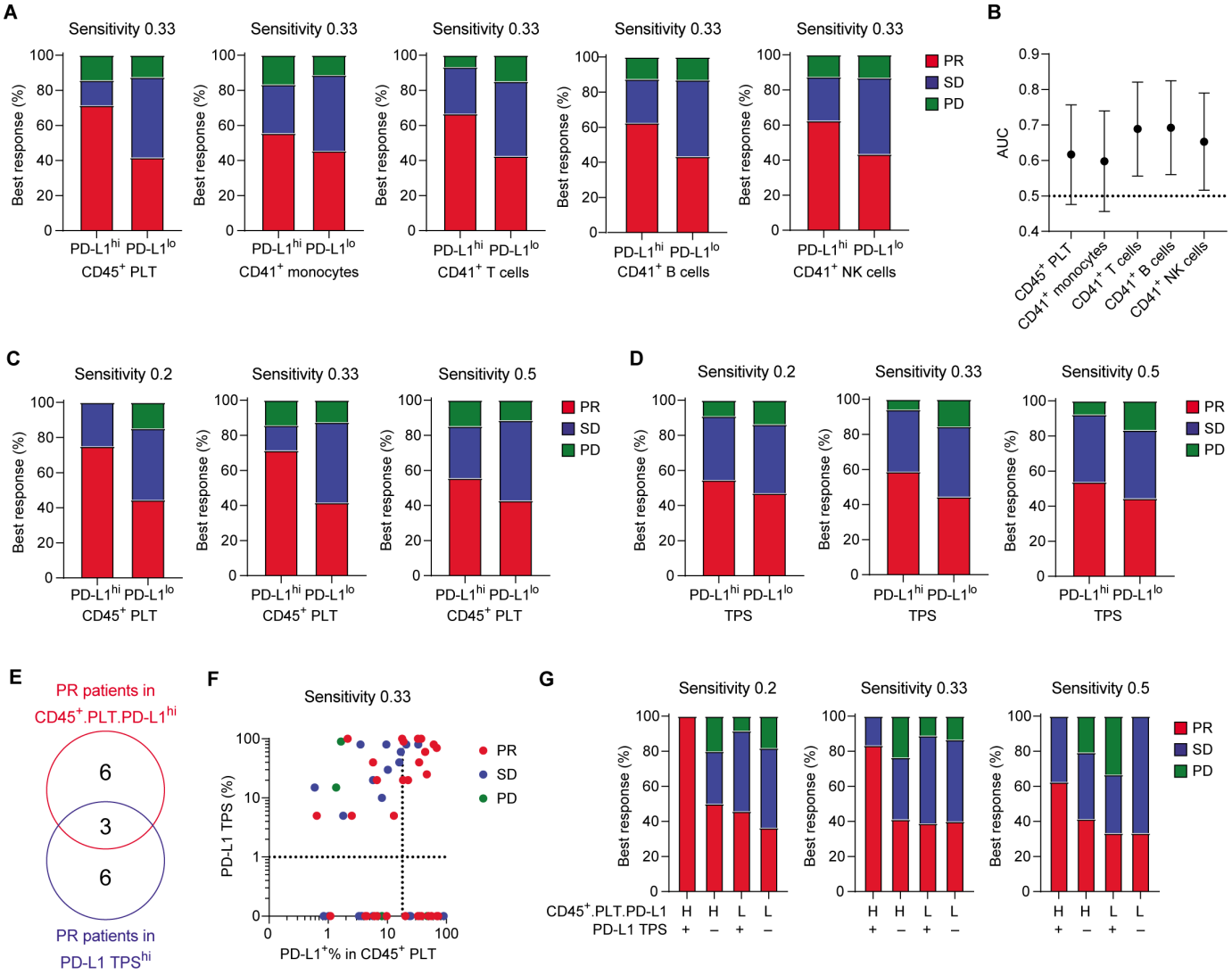

Figure 7 Platelet PD-L1 synergistically works with PD-L1 TPS as a biomarker for anti-PD-1 therapy. (A) Clinical response of patients with stage IV NSCLC who received pembrolizumab in combination with chemotherapy as first-line anticancer therapy (n=62). Patients were grouped by PD-L1 expression in CD45$^+$ platelets (PLT), CD41$^+$ monocytes, CD41$^+$ T cells, CD41$^+$ B cells, or CD41$^+$ NK cells. The threshold for PD-L1 was set to achieve a sensitivity of 0.33 for PD-L1^{hi} groups. (B) AUC of ROC curve of PD-L1 expression in various cell types in the blood. Error bars represent 95% CI. The dotted line represents 0.5 (no biomarker power). (C-D) Clinical response in patients grouped by (C) CD45$^+$.PLT.PD-L1 or (D) PD-L1 TPS. The threshold for PD-L1 was set to achieve a sensitivity of 0.2, 0.33, or 0.5 for PD-L1^{hi} groups. (E) Venn diagram representing the overlap between PR patients in the CD45$^+$.PLT.PD-L1^{hi} group (sensitivity 0.33) and PR patients in the PD-L1 TPS^{hi} group (sensitivity 0.33). (F) Patient grouping strategy using CD45$^+$.PLT.PD-L1 together with PD-L1 TPS. Dotted lines represent thresholds of PD-L1 expressions. The threshold of PD-L1 TPS was 1%. The threshold for CD45$^+$.PLT.PD-L1 was set to achieve a sensitivity of 0.33 in the Tumor PD-L1$^+$ CD45$^+$.PLT.PD-L1^{hi} group. (G) Clinical response in patients grouped by CD45$^+$ PLT and PD-L1 TPS. The threshold of PD-L1 TPS was 1%. The threshold for CD45$^+$.PLT.PD-L1 was set to achieve a sensitivity of 0.2, 0.33, or 0.5 in the tumor PD-L1$^+$ CD45$^+$.PLT.PD-L1^{hi} groups. AUC, area under the curve; CD45$^+$.PLT.PD-L1, PD-L1 expression in CD45$^+$ platelets; NK, natural killer; NSCLC, non-small cell lung cancer; PD, progressive disease; PD-L1, programmed cell death ligand 1; PLT, platelets; PR, partial response; SD, stable disease; TPS, tumor proportion score.

high CD45$^+$.PLT.PD-L1 and high PD-L1 TPS would exhibit the best clinical results (figure 7F). Considering PD-L1 expression in both cases resulted in significantly higher biomarker efficacy (100%, 83.3%, and 62.5% at 0.2, 0.33, and 0.5 sensitivity, respectively) than individual PD-L1 expression (figure 7G). Collectively, these findings suggest that platelets, being the independent source of PD-L1 in the peripheral blood, have high potential as an alternative to PD-L1 TPS or as a synergistic biomarker for predicting anti-PD-1/PD-L1 therapy responses.

DISCUSSION

While PD-L1 expression in the TME and its mechanisms have been extensively studied, the mechanisms underlying PD-L1 expression in the circulation remain largely

elusive. In this study, we found that various immune populations from the peripheral blood of patients with cancer, including monocytes, T cells, B cells, and NK cells, exhibited higher PD-L1 expression than those from healthy individuals. Notably, this increase was predominantly dependent on the physical attachment and aggregation of PD-L1-expressing platelets on the surface of these cells. In particular, we found that PD-L1 expression on platelets was directly related to thrombopoiesis, the process by which MKs produce platelets. Moreover, this process could be enhanced in patients with cancer. Consequently, PD-L1 expression on circulating immune cells bound to platelets closely mirrored PD-L1 expression on platelets themselves, which provided a better synergy in predicting response to anti-PD-1 therapy when combined with PD-L1 TPS. These findings strongly suggest that platelets serve as a major source of PD-L1 on various cell types circulating in the blood of patients with cancer and act as a predictive biomarker of response to anti-PD-1/PD-L1 therapy.

Platelets are very abundant in the blood. They exist at concentrations of up to 400×10^9/L in healthy humans; this value is approximately 40 times higher than that in white blood cells.[49] Consequently, platelet binding is a typical in vivo phenomenon that exhibits notable biological functions by providing platelet-derived molecules or cytokines to host cells.[29 30 32] In this regard, a notable finding of our study was that PD-L1-expressing platelets could directly supply their PD-L1 to various cell types, especially monocytes, within PBMCs through physical attachment and aggregation on the surface of these cells. Notably, although platelet-bound ($CD41^+$) PBMCs exhibited significantly higher PD-L1 expression than platelet-unbound ($CD41^-$) PBMCs, platelet-unbound PBMCs, particularly monocytes and T cells, still expressed PD-L1, suggesting that at least some PD-L1 was obtained by expression on the cells themselves. Importantly, however, PD-L1 expression on platelet-unbound PBMCs did not differ between patients with cancer and healthy donors. These data, together with data from $CD41^+$ PBMCs, indicate that nearly all PD-L1 expressed on the surface of circulating immune cells in the blood of patients was derived from PD-L1-expressing platelets.

Regarding the mechanism underlying PD-L1 expression on platelets, several studies have demonstrated that platelet PD-L1 is observed in patients with metastatic but not localized tumors, suggesting a direct relationship between the two.[32 50 51] Similarly, in this study, a significant increase in PD-L1 expression on platelets was noted in patients with metastatic NSCLC. In contrast, this pattern was not noted in several other metastatic malignancies, including SCLC and melanoma. Moreover, among patients with metastatic NSCLC, only 30%–40% exhibited higher platelet PD-L1 expression than healthy donors. Notably, some healthy donors showed small but significant PD-L1 expression on platelets, raising the possibility that metastatic tumors are not directly related to the generation of PD-L1-expressing platelets.

Our further analysis using irradiated mice revealed an unexpected role of thrombopoiesis and its regulatory mechanism for the generation of PD-L1-expressing platelets. We found that post-irradiation thrombopoiesis was accompanied by the generation of PD-L1-expressing platelets. Importantly, PD-L1 expression on platelets in this condition could not be detected on mature platelets. Instead, it was detectable on fresh reticulated platelets, the most recently generated platelets from MKs in the bone marrow. Furthermore, we noted a strong correlation between the size of platelets and PD-L1 expression on them, considering the fact that generation of larger platelets is a well-characterized indicator of enhanced thrombopoiesis.[36 41 42] We also confirmed that irradiation-induced thrombocytopenia increased PD-L1-expressing MKs and reticulated platelets. All these findings support a general role of enhanced thrombopoiesis in generating PD-L1-expressing platelets in the context of both thrombocytopenia and metastatic tumors.

Whether and how particular conditions of patients with cancer are responsible for promoting thrombopoiesis and the production of PD-L1-expressing platelets remains unclear. As metastatic tumors can cause significant damage and hemorrhage to various organs, it can be hypothesized that the process of repairing tissue damage results in increased platelet consumption, which in turn leads to increased thrombopoiesis and the production of PD-L1-expressing platelets.[43] It is thus conceivable that PD-L1-expressing platelets in the circulation play a role in dampening immune responses to self-antigens from damaged normal tissues and neoantigens from tumor tissues. Along with PD-L1 expression in the TME, malignant tumors may induce both systemic (platelet) and local (tumor) PD-L1-mediated immune suppression, which is well supported by our finding that patients with the highest PD-L1 TPS and platelet PD-L1 expression exhibited the best response to anti-PD-1 therapy. In this context, anticancer treatments, such as concurrent chemoradiation therapy, may inadvertently exacerbate systemic PD-L1-mediated immune suppression by inducing the release of $PD\text{-}L1^+$ platelets, potentially promoting tumor progression. Further investigation into the role of systemic PD-L1-mediated immune suppression across different anticancer therapies is crucial, as it could provide valuable insights and open important avenues for future research.

Because PD-L1 upregulation on platelets is a natural phenomenon under the conditions of enhanced thrombopoiesis and platelet PD-L1 may serve to regulate immune responses systemically in the circulation, thrombopoiesis-induced platelet PD-L1 expression is another example of a natural defense mechanism by which cancer cells evade immune responses. Hence, it will be interesting to explore why different cancer types induce varying levels of platelet PD-L1 (and thrombopoiesis) and whether such differences are related to the exceptional resistance to immunotherapies observed in some cancers, such as hematological malignancies. In

conclusion, our study demonstrates a previously unappreciated role of platelets as a primary source of PD-L1 expression in the peripheral blood that is regulated through thrombopoiesis, providing valuable insights for designing a good biomarker and developing better treatment strategies involving anti-PD-1/PD-L1 therapy.

Author affiliations
[1]Department of Microbiology and Immunology, Chonnam National University Medical School, Hwasun-eup, Hwasun-gun, Jeollanam-do, Korea (the Republic of)
[2]Medical Research Center for Combinatorial Tumor Immunotherapy, Chonnam National University Medical School, Hwasun-eup, Hwasun-gun, Jeollanam-do, Korea (the Republic of)
[3]National Immunotherapy Innovation Center, Chonnam National University Medical School, Hwasun-eup, Hwasun-gun, Jeollanam-do, Korea (the Republic of)
[4]BioMedical Sciences Graduate Program, Chonnam National University Medical School, Hwasun-eup, Hwasun-gun, Jeollanam-do, Korea (the Republic of)
[5]Selecxine Inc, Seoul, Korea (the Republic of)
[6]Department of Internal Medicine, Chonnam National University Medical School, Hwasun-eup, Hwasun-gun, Jeollanam-do, Korea (the Republic of)
[7]Chonnam National University Hwasun Hospital, Hwasun-eup, Hwasun-gun, Jeollanam-do, Korea (the Republic of)
[8]Department of Urology, Chonnam National University Medical School, Hwasun-eup, Hwasun-gun, Jeollanam-do, Korea (the Republic of)
[9]Department of Dermatology, Chonnam National University Medical School, Hwasun-eup, Hwasun-gun, Jeollanam-do, Korea (the Republic of)
[10]Thoracic and Cardiovascular Surgery, Chonnam National University Medical School, Hwasun-eup, Hwasun-gun, Jeollanam-do, Korea (the Republic of)

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ORCID iD
Jae-Ho Cho http://orcid.org/0000-0002-3081-7674

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