Immune-checkpoint inhibitors (ICI) have transformed oncological therapy. Up to 20% of all non-small cell lung cancers (NSCLCs) show durable responses upon treatment with ICI, however, robust markers to predict therapy response are missing. Here we show that blood platelets interact with lung cancer cells and that PD-L1 protein is transferred from tumor cells to platelets in a fibronectin 1, integrin α5β1 and GPIbα-dependent manner. Platelets from NSCLC patients are found to express PD-L1 and platelet PD-L1 possess the ability to inhibit CD4 and CD8 T-cells. An algorithm is developed to calculate the activation independent adjusted PD-L1 payload of platelets (pPD-L1Adj.), which is found to be superior in predicting the response towards ICI as compared to standard histological PD-L1 quantification on tumor biopsies. Our data suggest that platelet PD-L1 reflects the collective tumor PD-L1 expression, plays important roles in tumor immune evasion and overcomes limitations of histological quantification of often heterogeneous intratumoral PD-L1 expression.
Immune-checkpoint receptors like CTLA4 and PD-1 are crucial for preventing excessive immune responses and autoimmunity.\textsuperscript{1,2} Seminal discoveries made by Allison and Honjo provided preclinical proof of concept data that blockage of CTLA4 and PD1 signaling unleashes marked anti-tumor immune responses.\textsuperscript{3,4} Clinical evaluation revealed remarkable therapeutic potential of immune-checkpoint inhibition in human cancer patients and for the first time allowed for long term survival of patients with advanced metastasized solid tumors.\textsuperscript{3–5} Besides melanoma patients, especially patients suffering from non-small cell lung cancer (NSCLC) benefit from treatment with antibodies inhibiting the PD1 and CTLA4 immune checkpoints.\textsuperscript{6–7} Nevertheless, simple and robust biomarkers to predict therapy responses towards ICI are still missing.

With 1.8 million deaths per year, lung cancer represents one of the most frequent and lethal cancers worldwide.\textsuperscript{10} In the US 254,170 new lung cancer cases are expected to be diagnosed in 2021.\textsuperscript{11} Given the high frequency of lung cancer and the cost of checkpoint inhibitory therapies, the lack of robust biomarkers to select patients who best possibly benefit from ICI represents a major burden for our health systems. Histological quantification of intratumoral PD-L1 expression is routinely performed in an attempt to predict therapy responses towards ICI, however, only an insufficient correlation between detection of PD-L1 expression in tumor biopsies and the overall response rate (ORR) was found.\textsuperscript{12} In lung cancer, evaluation of smoking history, tumor mutational burden (TMB), microsatellite instability (MSI), high expression of CTLA4, low expression of CX3CL1 and infiltration of CD8+ T cells within the tumor microenvironment (TME) seems to be superior in predicting therapy responses towards anti-PD-1/PD-1 directed ICI\textsuperscript{13–15} when compared to histopathological PD-L1 quantification, however these markers so far could not be translated into a robust and clinically easy to use biomarker signature.

Here, we show that platelets, during their frequent interaction with tumor cells, ingest PD-L1 and present it on their surface, a function of platelet PD-L1 (pPD-L1) correlates with tumor stage/grade and the occurrence of blood of NSCLC patients. The functionality of platelet PD-L1 with tumor cells, ingest PD-L1 and present it on their surface, a expression of CTLA4, low expression of CX3CL1 and infiltration of CD8+ T cells within the tumor microenvironment (TME) seems to be superior in predicting therapy responses towards anti-PD-1/PD-1 directed ICI\textsuperscript{13–15} when compared to histopathological PD-L1 quantification, however these markers so far could not be translated into a robust and clinically easy to use biomarker signature.

Here, we show that platelets, during their frequent interaction with tumor cells, ingest PD-L1 and present it on their surface, a process which is dependent on fibronectin, α5β1 and GPIIbPA. PD-L1 expressing platelets are detected in the TME and peripheral blood of NSCLC patients. The functionality of platelet PD-L1 (pPD-L1) is confirmed by inhibition of CD4+ and CD8+ activity. pPD-L1 correlates with tumor stage and the occurrence of metastases. We develop an algorithm allowing to calculate the total PD-L1 payload of platelets (pPD-L1\textsuperscript{Ad}.) without the need of artifact prone in vitro stimulation procedures. Strikingly, in our study pPD-L1\textsuperscript{Ad} is shown to be superior in predicting response to ICI when compared to immunohistochemistry-based quantification of PD-L1 on tumor biopsies.

Results

Tumor cells transfer PD-L1 to platelets. To address whether the immune regulatory protein PD-L1 can be transferred from tumor cells to platelets, we co-incubated platelets obtained from healthy donors with four different NSCLC tumor cell lines harboring varying expression levels of PD-L1 (NCI-H23, A549: PD-L1 low/ negative, NCI-H226, NCI-H460: PD-L1 positive) (Fig. 1a, b). PD-L1 positivity was determined by flow cytometry and defined as PD-L1 expression in ≥ 5% of all tumor cells. PD-L1 expression on platelets (pPD-L1) was observed after co-incubation with the PD-L1 expressing NCI-H226 and NCI-H460 cells but not after co-incubation with the PD-L1 low/negative cell lines NCI-H23 and A549 (Fig. 1c–e). Results were validated using a flow cytometry-based approach (Fig. 1f). Co-incubation of platelets with all tumor cell lines resulted in pplatelet activation, as indicated by P-selectin (CD62P) induction (Fig. 1g), however only co-incubation with PD-L1 positive NCI-H226 and NCI-H460 cells resulted in an increased PD-L1 expression on the platelet surface (Fig. 1h). To ensure that platelets from healthy donors, used in this assay, do not harbor relevant amounts of endogenous PD-L1 we conducted western blot analyses on platelet whole-cell lysates. Indeed, Western Blot data confirmed that platelets from healthy donors do not express relevant PD-L1 levels (Supplementary Fig. 5b).

Of note, conditioned medium from tumor cells induced platelet activation but did not result in increased levels of PD-L1 protein on the platelet surface (Supplementary Fig. 3c, d), suggesting that PD-L1 transfer from tumor cells to platelets is dependent on a direct cell-cell contact between both cell types. Of note, frequent interaction with platelets was not restricted to adherent tumor cells but could for example also be observed for non-adherent A549 lung cancer cells (Supplementary Fig. 3e, f).

To gain deeper insights into the interaction of platelets and lung cancer cells, we took advantage of a live-cell imaging platform, where platelets are added to the medium and circulate through an imaging chamber that contains human NSCLC cells. Real time video microscopy revealed distinct interactions of tumor cells and platelets (Fig. 1i, j and Supplementary Movie 1). Strikingly, platelets remained fully agile and re-entered the circulation after contacting the tumor cell membrane (Fig. 1k–j and Supplementary Movies 1, 2). These data suggest that platelets can re-circulate after tumor cell attachment and activation and are in line with studies by Cloutier and Michaelson et al.\textsuperscript{16,17}

While platelets are anuclear, protein translation from RNA can nevertheless occur within platelets.\textsuperscript{18–19} We therefore set out to investigate whether PD-L1 expression in platelets depends on a transfer of PD-L1 protein from tumor cells to platelets or whether a transfer of PD-L1 mRNA with subsequent protein synthesis within the platelet is involved. Transfection of vectors encoding for PD-L1-GFP and FLAG-GFP fusion proteins into PD-L1 negative A549 cells (Fig. 1m–o) resulted in high numbers of GFP positive platelets upon co-incubation (Fig. 1p–s). As inhibition of protein translation in platelets by cycloheximide did not result in a reduction of PD-L1-GFP expression in platelets (Fig. 1t), our data suggest that PD-L1 protein transfer and not mRNA transfer is underlying the observed pPD-L1 expression after interaction of platelets and tumor cells.

While the transfer of PD-L1-GFP or FLAG-GFP was robustly observed across various NSCLC cell lines, we nevertheless noted differences in protein transfer efficacies. For example, platelets showed low levels of FLAG-GFP and PD-L1-GFP after co-incubation with NCI-H322, NCI-H522 and NCI-H23 cells, while HOP-62 and HOP-92 cells displayed significantly higher protein transfer rates (Fig. 2a–d). Given that our data indicated that a platelet-tumor-cell contact is necessary for a sufficient transfer of PD-L1 from tumor cells to platelets, we hypothesized that expression levels of adhesion molecules might determine the efficacy of protein transfer from tumor cells to platelets. Along these lines we found that PD-L1 transfer rates positively correlated with fibronectin (FNI) message expression levels, while no significant correlation was found for fibronectin alpha chain (FGA) or tissue factor (F3) message expression (Fig. 2e–g).

Of note, fibronectin expression also correlated with platelet-tumor cell interaction in vitro (Fig. 2h–j). Immunofluorescence staining as well as analysis of protein–protein interaction via proximity ligation assay (PLA) revealed close proximity of fibronectin and PD-L1 (Fig. 2k–n) at the cell surface. In line with these observations, we found that siRNA mediated knockdown of fibronectin resulted in a significant reduction of PD-L1 transfer, thus functionally validating fibronectin as a key regulator of protein transfer from tumor cells to platelets (Fig. 2o–q).
Platelet adhesion to fibronectin is known to be mediated via several molecules including GPIbα, integrin α5β1 or GPIIbIIIa21,22. We therefore set out to address whether inhibition of these adhesion molecules on platelets reduces adhesion to fibronectin and PD-L1 uptake from tumor cells. Strikingly, while monoclonal antibodies against GPIbα and integrin α5β1 prevented platelet adhesion to fibronectin (Fig. 2r–t) and PD-L1 protein transfer (Fig. 2u, v), inhibition of GPIIbIIIa by Tirofiban only marginally reduced platelet adhesion (Fig. 2r–t).
Detection of functional PD-L1 on platelets of NSCLC patients.

To address the significance of our findings for human cancers, we next quantified PD-L1 expression on platelets in healthy lung tissue or NSCLC tumor tissue. While platelets were detected in high abundance in healthy lung tissue and PD-L1 negative NSCLC, we could not observe any relevant PD-L1 expression on these platelets (Fig. 3a, b, d, e). In contrast PD-L1 positive platelets were observed in high abundance in tissue sections from patients suffering from PD-L1 positive NSCLC (Fig. 3c–e). To quantify the number of PD-L1 positive platelets outside the tumor, we next isolated platelets from the peripheral blood of a cohort of 64 healthy donors and 128 NSCLC patients. Fluorescence-Activated Cell Sorting (FACS) revealed threefold higher numbers of PD-L1 positive platelets in NSCLC patients as compared to healthy donors (median pPD-L1 expression in healthy donors 0.29 (95%CI: 0.21 – 0.44), median pPD-L1 expression in NSCLC patients 0.89 (95%CI: 0.61–1.21) (Fig. 3f). The detected differences were even higher, when total pPD-L1 levels were determined using a quantitative enzyme-linked immunosorbent assay (ELISA). While platelet rich plasma (PRP) from NSCLC patients in average contained 108.3 pg/mL PD-L1, PRP from healthy volunteers only contained 1.8 pg/mL (Fig. 3g, h). Differences in pPD-L1 expression in NSCLC patients versus healthy volunteers were also confirmed using western blot analysis (Supplementary Fig. 5b–d). Interestingly, PD-L1 expression was highest in patients with advanced (UICC stage IV) tumors (Supplementary Fig. 5e). Of note, immunofluorescence (Fig. 3i) and immunoelectron microscopy (Fig. 3j) revealed frequent PD-L1 clusters in platelets obtained from peripheral blood of a PD-L1 positive NSCLC patient, further underlining functionality of pPD-L1, as immune ligand clustering has been described to be a prerequisite for proper binding to its receptor.

Prompted by these results, we next explored whether pPD-L1 exerts immune-inhibitory functions. We stimulated human T cells from healthy donors with EBV/CMV-derived peptides in the presence or absence of PD-L1 positive platelets obtained from NSCLC patients. T cell activation was evaluated using an enzyme-linked-immuno-Spot (ELISpot) assays determining the effector cytokines IFNγ and TNFα. In line with published data we observed that platelets dampen T cell activity independent of their PD-L1 expression status (Fig. 4a–c and Supplementary Fig. 6a, b). However, when PD-L1 expressing platelets were pre-treated with the anti-PD-L1 mAb Atezolizumab their T cell inhibitory effect was abolished (Fig. 4a–c). Next, we expanded our work towards tumor-associated antigens. New York esophageal squamous cell carcinoma 1 antigen (NY-ESO-1) belongs to the family of cancer-testis antigens, but is also aberrantly expressed in many tumor entities including NSCLC. Stimulation of T cells from healthy donors with NY-ESO-1 peptides predominantly resulted in a clonal expansion of NY-ESO-1 specific CD4+ T cells (CD62L−/CD45RO+ and CD27−/CD28+) (Fig. 4d), which were further specified as CD4+ effector memory T cells (T EM, CD62L−/CD45RO+ and CD27−/CD28+) (Fig. 4d, e). Remarkably, T EM activity, as determined by IFNγ and TNFα release, decreased significantly upon co-incubation with PD-L1 positive platelets. However, T cell activity could be restored when pPD-L1 positive platelets were pre-treated with anti-PD-L1 (Fig. 4f–k).

To investigate a potential impact of PD-L1 positive platelets on other immune cells, we also characterize changes in the overall immune cell composition (peripheral blood) in 10 NSCLC patients and five healthy controls (Supplementary Fig. 7a). In NSCLC patients pPD-L1 tended to be correlated negatively with the total number of NK (p = 0.1), CD4+ T cells (p = 0.09) and CD8+ T cells (p = 0.02) (Supplementary Fig. 7b). Moreover, in NSCLC patients more PD-L1 and PD-L1 was expressed on dendritic cells (DCs), natural killer (NK) cells and CD4+ and CD8+ T cells (Supplementary Fig. 7c, d). In our analyses we did not observe a correlation of PD-1 or PD-L1 expression and pPD-L1 in DCs, NK cells or CD4+ T cells (Supplementary Fig. 7e, f). However, we detected a positive correlation of pPD-L1 and PD-1 on CD8+ T cells (p = 0.02). We also quantified T cells and T cell infiltration in the TME in eleven NSCLC patients with different levels of pPD-L1 using the MACSimma ultradepth tissue profiling platform. Noteworthy, in patients with high pPD-L1 we observed lower numbers of T cells in the TME and less infiltrating T cells (Fig. 5a–d). In contrast to our findings in the peripheral blood, we observed an inverse correlation of PD-1 on T cells and pPD-L1 (Fig. 5e, f).

Regulation of pPD-L1 during platelet activation. As it is well established that expression levels of platelet surface proteins...
correlate with the platelet activation status, we reasoned that different degrees of platelet activation might underlie varying levels of pPD-L1 expression on the platelet surface. Indeed, when we analyzed the platelet activation marker CD62P, we observed varying CD62P expression levels which showed a strong positive correlation with pPD-L1 expression (Fig. 6a). Of note, while PD-L1 expression in general was lower in unstimulated platelets, we were able to robustly detect pPD-L1 on the platelet surface of resting (CD62P negative) platelets (Supplementary Fig. 5a). In line with this, we also detected pPD-L1 in α-granules (Fig. 6b).

As even highly standardized blood collection procedures can result in varying levels of shear-stress mediated platelet activation and therefore complicates standardization, we hypothesized that different levels of platelet pre-activation might complicate the interpretability and comparability of pPD-L1 levels on freshly collected platelets from different patients. We therefore reasoned that a controlled in vitro activation of platelets with subsequent maximization of pPD-L1 expression might most adequately uncover the total payload of platelet PD-L1 and best possibly allow a comparison between different patients. Indeed, we found that pPD-L1 expression was maximized upon controlled platelet

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**Figure 6**

(a) Graph showing the correlation between CD62P expression and pPD-L1 expression on platelets. (b) Graph showing the correlation between PD-L1-GFP expression and pPD-L1 expression on platelets. (c) Graph showing the correlation between Fibronectin 1 expression and pPD-L1 expression on platelets. (d) Graph showing the correlation between Integrin α5β1 expression and pPD-L1 expression on platelets. (e) Graph showing the correlation between FN1 expression and pPD-L1 expression on platelets. (f) Graph showing the correlation between untreated PD-L1-GFP and pPD-L1 expression on platelets. (g) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (h) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (i) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (j) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (k) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (l) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (m) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (n) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (o) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (p) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (q) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (r) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (s) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (t) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (u) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets. (v) Graph showing the correlation between α5β1 blocking and pPD-L1 expression on platelets.
stimulation with the PAR1 agonist TRAP-6 (Fig. 6c–f and Supplementary Fig. 8 a-d) or other platelet activation agents such as ADP or collagen (Fig. 6g, h) and thus might allow for a better comparability of pPD-L1 levels between different patients. However, controlled platelet activation and subsequent measurement of CD62P and pPD-L1 is technically demanding and might prevent the use of pPD-L1 as a biomarker in clinical routine. We therefore set out to explore whether a normalized pPD-L1 level on the platelet surface could be calculated without in vitro manipulation of platelets. To do so we developed an adjustment model based on the calculation of ∆PD-L1 (ratio of PD-L1 before and after stimulation) as a function of pPD-L1 expression in unstimulated platelets and the degree of pre-activation (CD62P expression) (Fig. 6i, j). Specifically, we devised a matrix which allows us to calculate PD-L1Adj. for subgroups of patients harboring different levels of platelet pre-activation (CD62P expression) and pPD-L1 expression (Fig. 6) and Supplementary Fig. 9). Taking advantage of our matrix, corrected PD-L1 levels, designated pPD-L1Adj., were determined for all patients (Fig. 6k).

Adjusted platelet-derived PD-L1 serves as a prognostic and predictive marker in NSCLC. We first used the calculated pPD-L1Adj. levels and performed a receiver-operating characteristic (ROC) analysis for overall survival (OS). We found that pPD-L1Adj. levels in the subgroup of maximal platelet activation (CD62P 80–100%) showed highest accuracy in predicting OS and were superior in predicting OS compared to pPD-L1 (Fig. 7a). Since no cut-off value for pPD-L1Adj. had been established so far, we analyzed OS in pPD-L1Adj. quartile groups (Q1–4) using the Kaplan-Meier method (Fig. 7b, c). Details regarding characteristics of our patient population are provided in Supplementary Table 1. The median observation time for monitoring OS in our study was 23.5 months (95% CI: 3.4–67.55 months). At data cutoff for overall survival, 42 of 128 patients (32.8%) were still alive. Strikingly, patients with high pPD-L1Adj. levels showed a significantly shortened OS (Fig. 7b). The median survival in Q1 (low pPD-L1Adj. levels) was 43 months compared to only 24 months in Q3 (high pPD-L1Adj. levels) (hazard ratio (HR) for death Q1 vs. Q3: 2 (95% CI: 1.3–3.9) and 14 months in Q4 (very high pPD-L1Adj. levels) (hazard ratio (HR) for death Q1 vs. Q4: 3.64 (95% CI: 1.97–6.72)). Importantly, the observed differences in OS were not restricted to the time since initial diagnosis but were still significant when analyzing the time period since platelet analysis (Fig. 7c).

It has been reported that mutations in key oncogenic drivers do not only fuel proliferation via cell intrinsic cues but also impact tumor biology via modulation of the tumor microenvironment29–31. Along these lines, we found increased pPD-L1Adj. levels in patients suffering from KRAS mutated NSCLC as compared to those with KRAS wildtype status (Fig. 7d). In contrast, mutations in EGFR, ALK fusions and ROSI fusions or mutations showed no association with pPD-L1Adj. levels, respectively (Fig. 7e, f).

We also explored a potential correlation of pPD-L1Adj. with other clinical parameters. For example, we found that patients with higher tumor stages (T, p = 0.03), higher degrees of lymph node invasion (N, p = 0.04) and a higher tumor grading (G, p = 0.002) expressed more PD-L1 on the platelet surface (Fig. 7f–h). No association was found between pPD-L1Adj. and the region of tumor origin (central vs. peripheral, Fig. 7i). However, pPD-L1Adj. strongly correlated with the occurrence of metastases (p < 0.001), especially liver (p = 0.005) and bone metastasis (p = 0.001) (Fig. 7j–l). In line with previous studies32, we also found pPD-L1Adj. to be positively correlated with smoking history and the amount of pack years (Supplementary Fig. 10c, d). Moreover, pPD-L1Adj. was positively correlated with platelet count, LDH and CRP (Supplementary Fig. 10j–l).

To further elaborate on the potential of pPD-L1Adj. as a predictive biomarker in NSCLC, we conducted sequential measurements of pPD-L1Adj. in 12 patients undergoing conventional chemotherapy or ICI (Supplementary Fig. 11a, b). Details on therapeutic regimens are provided in Supplementary Fig. 4. In these patients baseline pPD-L1Adj. levels were determined prior to the first cycle of the respective 1st line treatment. The second measurement was conducted in parallel to the first CT scan. In patients treated with a platinum-based chemotherapy the tumor responses were quantified according to the Response Evaluation Criteria in Solid Tumors (RECIST) guidelines33. For patients receiving ICI IRECIST guidelines34 were used. In both groups...
response evaluation was performed 6–8 weeks after initial treatment. Remarkably, a significant drop in pPD-L1<sub>Adj</sub> levels was detected upon initiation of therapy in those patients whose tumors were later identified to have undergone at least partial remission (PR) (<i>p</i> = 0.02, Supplementary Fig. 11a). In contrast, patients who were later identified to have progressed despite therapy displayed a significant increase of pPD-L1<sub>Adj</sub> already in early measurements after therapy initiation (<i>p</i> = 0.04, Supplementary Fig. 11b). Of note, the predictive value of pPD-L1<sub>Adj</sub> was robust regardless of the used therapeutic regime. In two patients receiving ICI we determined pPD-L1<sub>Adj</sub> at multiple time points. Remarkably, pPD-L1<sub>Adj</sub> expression changes correlated with...
Fig. 3 Platelets from NSCLC patients show increased PD-L1 levels. **a** Left panel, left, Immunohistochemistry for CD61 in healthy human lung tissue (black arrow highlights CD61+ platelets). Scale bar 200 µm. Left panel, right, Representative micrograph of healthy lung tissue (H&E). Scale bar 500 µm. Right panel, Immunofluorescence microscopy for CD41+ (green), PD-L1− (red) platelets in healthy lung tissue (n = 3). Scale bar left 100 µm, right 10 µm. **b** Immunofluorescence staining for CD41 (green) and PD-L1 (red) on platelets in a PD-L1− NSCLC patient tumor sample (n = 3). Scale bar left 500 µm, center left 50 µm, center right and right 10 µm. **c** Upper, Representative micrographs of NSCLC adenocarcinoma (H&E) (n = 3). Scale bar left 250 µm, center 50 µm, right 50 µm. Lower, Immunofluorescence staining for CD41 (green) and PD-L1 (red) (n = 3). Scale bar left 500 µm, center left 50 µm, center right and right 10 µm. **d** Immunofluorescence staining for CD41 (green) and PD-L1 (red) on a platelet derived from a NSCLC patient counter stained with phalloidin (upper) or CD41 (lower). Scale bar left 10 µm, right 2 µm (n = 1). **e** Platelets of a NSCLC patient, assessed by transmission electron microscopy. PD-L1 stained with post-embedding immunogold-labelling. PD-L1 gold particles densely accumulating on the platelet membrane (black dots). Upper scale bar 2 µm, lower 100 nm (n = 1). **f–h** Statistical significance was calculated by one-way ANOVA and Tukey’s multiple comparisons test. **f, g** Statistical significance was calculated by Mann–Whitney test. Source data are provided as a Source Data file.

disease activity routinely determined via CT-scan (Supplementary Fig. 11c–f). As genomic alterations in EGFR and ALK represent independent factors influencing OS and progression-free survival (PFS), especially in patients receiving ICI, we additionally analyzed the role of pPD-L1Adj in the respective subgroups with or without such alterations. Whereas we were not able to detect a significant difference regarding OS (Supplementary Fig. 12a–c), in pPD-L1Adj high patients harboring an EGFR or ALK alteration who received a platinum-based chemotherapy, PFS tended to be worse compared to patients without EGFR and ALK alteration (Supplementary Fig. 12d, e). This might be explained by the fact that these patients had already shown a tumor progression upon first-line treatment with a tyrosine kinase inhibitor (TKI). In patients with EGFR and ALK alteration who received TKI, pPD-L1Adj was not predictive for PFS (Supplementary Fig. 12f). Since in our cohort none of the patients receiving ICI showed EGFR or ALK aberrations, the role of pPD-L1Adj could not be investigated in this cohort.

Finally, we set out to probe whether the pre-therapeutically determined pPD-L1Adj level can predict the therapy response of NSCLC patients to immune-checkpoint blocking antibodies. To do so we analyzed the PFS of patients either treated with only conventional chemotherapy or immunotherapy blockade. pPD-L1 positive and negative subgroups were defined according to the median pPD-L1Adj level. In patients receiving conventional chemotherapy we observed a significantly higher PFS when pPD-L1 levels were low (Fig. 7m). Interestingly, in patients treated with ICI (Pembrolizumab or Nivolumab), high pPD-L1Adj predicted a PFS benefit (HR 4.74, p = 0.003) (Fig. 7n and Supplementary Fig. 12j). Strikingly, when the predictive power of pPD-L1Adj was compared to conventional histological PD-L1 quantification (TPS > 50% and ≥1% in tumor biopsies), pPD-L1Adj was found to much better predict therapy response towards ICI (Fig. 7o, Supplementary Fig. 12k, l). In summary, our data suggest that pre-therapeutically measured pPD-L1Adj levels accurately predict the therapeutic response towards immune-checkpoint blocking antibodies. Prospective clinical trials are warranted to validate our findings and to justify the implementation of pPD-L1Adj as a biomarker in clinical routine.

Discussion

Human cancers are heterogenous and biomarkers based on histopathological analyses of single tumor biopsies are often lacking robustness. Histological quantification of intratumoral PD-L1 expression is routinely performed on NSCLC biopsy material as an attempt to predict responses towards immune-checkpoint inhibition, however, the correlation between expression levels and the overall response rate (ORR) is limited. In our present study we show that blood platelets are in frequent contact with lung cancer cells in vitro and in vivo and take up PD-L1 from the cancer cells in a fibronectin, integrin α5β1 and GPIba dependent manner. Our data provides mechanistic explanation for recent reports describing PD-L1 on platelets from patients suffering from different types of cancers. Interestingly, while there is comprehensive literature describing tumor cell induced platelet aggregation (TCPA) and tumor cell-associated thrombus formation, our herein presented data suggest that platelet-tumor cell contact can occur without substantial platelet activation and degranulation. Since pPD-L1 has not only been detected on the surface of activated platelets but also in resting platelets, it is tempting to speculate on an equilibrium between intracellularly stored pPD-L1 in α-granules and cell surface pPD-L1. Indeed, a similar mechanism has been described for the uptake and redistribution of fibrinogen and immunoglobulins.

Importantly, as pPD-L1 is found to inhibit T cell function, it is likely that pPD-L1 plays a distinct role in systemic immunomodulation. Of note, pPD-L1 has recently been described in patients suffering from tumors which were classified as PD-L1 negative in biopsies. Our herein presented data as well as other published studies on tumor heterogeneity suggest that immunohistochemistry-based quantification of protein expression on tissue sections from single biopsies should be interpreted with caution, as protein expression might differ spatially and temporally. Obviously, while our herein presented data suggest a highly efficient uptake of PD-L1 from lung cancer cells into platelets, it does not exclude that some pPD-L1 might be derived from other sources such as endothelial or other non-malignant cell types.

As the total blood volume is circulated up to 1000 times through the body each day, we reasoned that platelets might mirror the collective PD-L1 payload of a tumor and thus might open up venues for novel biomarker strategies. In this regard it is striking that pPD-L1 not only correlates with tumor stage/grade and the occurrence of metastases but is found to be superior in predicting response towards immune-checkpoint inhibition when compared to standard histological PD-L1 quantification on tumor biopsies. Since in particular lung cancer represents one of the most frequent and lethal cancers worldwide, further clinical investigation of pPD-L1 as a biomarker in NSCLC does not only hold the promise to unburden our health systems by avoiding costly and unnecessary therapies with ICI but, even more
important, will avoid side effects of ICI in patients who would not benefit from this kind of therapy.

It should be mentioned that our exploratory study suffers from some limitations. Owing to the fact that we used an exploratory cohort of NSCLC patients with unequal representation of tumor stages and different cycles of various treatment regimens for the development of the calculation algorithm, pPD-L1^adj^ may not have yielded its maximum performance. Expectedly, while pPD-L1 can robustly detected in patients treated with TKI or platinum-based chemotherapy, pPD-L1 could not be detected in patients treated with anti-PD-L1 mAbs. Of note, this observation is in line with previous data and can be explained by binding of
Fig. 4 PD-L1 on platelets shows functional relevance via decreasing T-cell activity. 

**a** IFNγ ELISPOT assay of peptide-specific T-cells co-incubated with PD-L1+ platelets with or without anti-PD-L1 mAb pre-treatment (n = 3). 

**b** Quantification of the IFNγ ELISPOT assays (n = 3). 

**c** Flow cytometry-based quantification of indicated cytokines and surface markers for peptide stimulated CD8+ T-cells co-incubated with PD-L1+ platelets with or without anti-PD-L1 mAb pre-treatment (n = 3). 

**d** Representative FACS plots showing the gating strategy and the T-cell subpopulations after pre-sensitization, enrichment, and expansion. 

**e** Quantitative sub-phenotyping of NY-ESO-1 specific T cells using flow cytometry (n = 2). 

**f** Representative FACS plots displaying CD4+ TEM activity levels measured by INFγ expression after co-incubation with PD-L1+ platelets with or without anti-PD-L1 mAb pre-treatment (n = 3). 

**g** Quantification of INFγ+ CD4+ TEM (n = 3). 

**h** INFγ fold change in CD4+ TEM (n = 3). 

**i** Representative FACS plots displaying CD4+ TEM activity levels measured by TNFα expression after co-incubation with PD-L1+ platelets with or without anti-PD-L1 mAb pre-treatment (n = 3). 

**j** Quantification of TNFα+ CD4+ TEM (n = 3). 

Data are mean ± SEM. Statistical significance was calculated by two-tailed Student’s t test. Source data are provided as a Source Data file.

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Fig. 5 pPD-L1 correlates with T cell infiltration in NSCLC. 

**a, b** Upper, Representative micrographs of NSCLC adenocarcinoma (H&E) (n = 11). Scale bar 500 µm. Lower, Immunofluorescence staining for T-cells (CD2: red, CD3: orange and PD-1: white) in the TME of a NSCLC patient presenting with low pPD-L1 (a) and high pPD-L1 (b). Each image is representative for at least two regions of interest (ROIs) in each tumor sample. ROI were selected based on manual pre-staining of DAPI. Scale bar 100 µm (n = 11). 

**c**–**f** Quantification of T cells per FoV (c), PD-1+ T cells per FoV (%) (d), infiltrating T cells per FoV (e) and PD-1+ infiltrating T cells per FoV (%) (f). pPD-L1 high vs. low was defined according to the median expression in this cohort. A total number of n = 22 ROIs (small symbols) were analyzed out of a total of n = 11 patients (large symbols). Data are mean ± SEM. Statistical significance was calculated by two-tailed Student’s t test (d–f) or two-tailed Mann–Whitney test (c). Source data are provided as a Source Data file.
Fig. 6 Platelets from NSCLC patients show increased PD-L1 protein levels upon activation. a Correlation between platelet-derived PD-L1 (pPD-L1) and platelet activation (CD62P expression) in 128 NSCLC patients. Each dot represents a single patient. b Platelets from a NSCLC patient assessed by transmission electron microscopy. PD-L1 stained with post-embedding immunogold-labeling. Upper and lower right, PD-L1 gold particles densely accumulate in α-granules. Scale bar left 1 μm, right 100 nm (n = 1). c, d Changes in CD62P (c) and the pPD-L1 (d) levels upon platelet stimulation with 10 μM TRAP-6 for 2 min in NSCLC patient samples (n = 128). Boxes represent median and 25th to 75th percentiles, whiskers are minimum to maximum. Statistical significance was calculated by two-tailed Mann-Whitney test. e CD62P expression change (ΔCD62P) in the different pPD-L1 quartile groups identified in unstimulated platelets of NSCLC patients (n = 128). a, P < 0.0001; b, P < 0.0001. Data are mean ± SEM. Statistical significance was calculated using Friedman and Dunn's multiple comparisons test. f pPD-L1 expression change (ΔpPD-L1) in the different pPD-L1 quartile groups identified in unstimulated platelets of NSCLC patients (n = 128). Data are mean ± SEM. Statistical significance was calculated by Friedman and Dunn’s multiple comparisons test. g CD62P expression change (ΔCD62P; CD62P in stimulated platelets in % - CD62P in unstimulated platelets in %) after platelet activation with 10 μM TRAP-6 (n = 24), 2.5 μM ADP (n = 24), or 5 μg/ml collagen (n = 24). Boxes represent median and 25th to 75th percentiles, whiskers are minimum to maximum. h pPD-L1 expression change (ΔpPD-L1) (pPD-L1 in stimulated platelets in % - pPD-L1 in unstimulated platelets in %) in NSCLC patients after platelet activation. Statistical significance was calculated by Kruskal-Wallis test. Boxes represent median and 25th to 75th percentiles, whiskers are minimum to maximum. i Correlation between pPD-L1 expression in unstimulated platelets of 128 NSCLC patients and the ΔpPD-L1 upon platelet stimulation with 10 μM TRAP-6. Each dot represents a single patient. Heatmap showing the calculated ΔpPD-L1 depending on the CD62P activation ranges (y-axis) and the quartiles of pPD-L1 level (x-axis) calculated in pooled data from 128 NSCLC patients. For details of subsampling and calculation used, see Methods and Supplementary Fig. 4. j Adjusted pPD-L1 levels in all 128 NSCLC patients upon calculated platelet pre-activation ranges (by CD62P expression level). a, i Correlation was determined by simple linear regression analysis. Source data are provided as a Source Data file.

anti-PD-L1 mAbs to pPD-L1 expressing platelets. However, this complicates an exact determination of pPD-L1 in these patients. Even if we did not observe a significant correlation of pPD-L1 and genetic alterations beyond KRAS, this exploratory study cohort of consecutively analyzed NSCLC patients might not be ideal to study the predictive role of pPD-L1 in NSCLC patients harboring genomic alterations including EGFR, ALK and ROS1.

Nevertheless, besides the tremendous potential of pPD-L1 (Adj) as a biomarker, we believe that platelet pPD-L1 might also represent a potential target for therapeutic intervention. This presumption is supported by our observation that pPD-L1 in NSCLC patients correlates with the number of T cells in TME and the number of infiltrating T cells. Similar observations in a mouse model support this finding. Along these lines it is tempting to speculate that pPD-L1 might be involved in formation of the premetastatic niche by generating an immunotolerant environment at sites distant from the primary tumor (Supplementary Figs. 1, 2). Inhibition of pPD-L1 could prevent the formation of metastasis and such a concept would warrant the investigation of immune-checkpoint blocking antibodies in order...
Fig. 7 Platelet-derived PD-L1 (pPD-L1) as prognostic and predictive marker in NSCLC. a Combined estimate of platelet CD62P (0–100%) and pPD-L1 levels predicts overall survival (OS) in 128 NSCLC patients; receiver-operating characteristics (ROC) analysis. b Kaplan–Meier analysis of overall survival (OS) dependent on the pPD-L1adj as defined by quartile groups (very low (Q1), low (Q2), high (Q3) and very high (Q4)). Survival data refer to the time point of primary diagnosis (n = 128). c Overall survival (OS) in different pPD-L1adj quartile groups according to the time point of platelet analysis (n = 128). b, c Statistical significance was calculated by log-rank test. d, e Association of pPD-L1adj levels and different genetic alterations (KRAS (mut = 38, wt = 90), EGFR (mut = 19, wt = 109), EML-4-ALK (alteration = 5, no alteration = 123) and ROS-1 (alteration = 6, no alteration 122)) (wt = wildtype, mut = mutation). Data are mean ± SEM. Statistical significance was calculated by two-tailed Mann–Whitney test. f-h pPD-L1adj levels in patients with different tumor stage (T1 = 8, T2 = 23, T3 = 33, T4 = 50), lymph node invasion (N1 = 9, N2 = 13, N1 = 11, N2 = 40, N3 = 55), and grade (G1 = 19, G2 = 48, G3 = 61). Each dot represents a single patient. Statistical significance was calculated by Kruskal–Wallis test and Dunn’s multiple comparisons test. i Association of pPD-L1adj levels with tumor origin (central = 42, peripheral = 79). j-l pPD-L1adj is associated with the occurrence of metastasis (in general (j), M0 = 25, M1 = 103) and at specific sites, including liver (confirmed = 31, no metastasis = 94) and brain (confirmed = 37, no metastasis = 91). m-o Each dot represents a single patient. Boxes represent median and 25th to 75th percentiles, whiskers are minimum to maximum. Statistical significance was calculated by two-tailed Mann–Whitney test. m Kaplan–Meier curves estimates of PFS in patients with a pPD-L1adj level > median (red) and pPD-L1adj level < median (blue) treated with conventional chemotherapy (n = 62). n Kaplan–Meier curves estimates of PFS in patients with a pPD-L1adj level > median (red) and pPD-L1adj level < median (blue) treated with anti-PD-1 therapy (n = 20). o Kaplan–Meier curves estimates of PFS in patients with a TPS score > 1% (red) and TPS < 1% (blue) treated with anti-PD-1 therapy (n = 20). m–o Statistical significance was calculated by log-rank test. Source data are provided as a Source Data file.
to prevent metastasis when tumors with high metastatic risk are treated in a curative intention. Of note, clinical trials investigating the perioperative administration of ICI in NSCLC have reported reduced relapse and metastasis and our herein presented data might offer a mechanistic explanation for the observed results.\(^{4,45}\) Last but not least, as pPD-L1\(^{13}\) is shown to be prognostic and predictive in NSCLC, pPD-L1 might additionally serve as a liquid biomarker for early tumor detection or recurrence, an approach which warrants future clinical testing.

**Methods**

**Study design and selection of patients.** During 2016–2019, 173 consecutive patients with non-small lung cancer (NSCLC) treated in the Department of Medical Oncology and Hematology and Department of Internal Medicine VIII, University Hospital Tuebingen, Germany were prospectively included in the study (screening cohort = 5C). In order to preclude the influence of antiangiogenics like aspirin (ASA), low molecular weight heparin (LMWH) or other heparinoids and non-vitamin K antagonistic oral anticoagulants (NOACs), long-term medication of each patient was considered. In our cohort 12 patients with LMWH and 28 patients taking ASS and/or clopidogrel were excluded. In Supplementary Fig. 4, a detailed flowchart of patient selection is given. In all cases, sample collection was performed prior to the next application of the respective therapy. Tumor characteristics are based on baseline clinical staging. In order to take disease progression better into account the occurrence of metastasis was double checked at the time point of study inclusion. Our cohort comprised 71 male and 57 female patients with a mean age of 65.7 years (range 19–87). The diagnosis of a NSCLC was histologically confirmed in all cases. NSCLC adenocarcinoma was identified in 95 patients (72.7%), in 35 patients (27.3%) another histologic tumor type was found. The distribution of the tumor characteristics is summarized in Supplementary Table 1. Written informed consent was given in all cases. Sample collection of healthy participants were in accordance with the ethical standards of the institutional review committee (Ethic committee of the Faculty of Medicine of the Eberhard Karls University Tuebingen and of the University Hospital Tuebingen vote 13/2007V). The observational study in NSCLC patients was approved by the ethics committee of the Faculty of Medicine of the Eberhard Karls University Tuebingen and of the University Hospital Tuebingen and was conducted in accordance with the Declaration of Helsinki (vote 456/BO2).

**Preparation of platelets.** Platelets were obtained from healthy donors (not taking any medication for at least 10 days) and NSCLC patients after informed writing consent. Citrated blood was briefly centrifuged for 20 min at 120 × g, the upper fraction was harvested as platelet-rich plasma (PRP). Platelets were washed twice with citrate wash buffer (128 mmol/L NaCl, 11 mmol/L glucose, 7.5 mmol/L Na\(_2\)HPO\(_4\), 4.8 mmol/L sodium citrate, 4.3 mmol/L NaH\(_2\)PO\(_4\), 2.4 citric acid, 0.35% lipase) to remove all traces of calcium, and resuspended in platelet wash buffer (154 mmol/L NaCl, 5.4 mmol/L KCl, 0.4 mmol/L NaHCO\(_3\), 1.25 mmol/L MgSO\(_4\), 2.5 mmol/L CaCl\(_2\), 0.2 mmol/L EDTA, 0.2% FCS, 10 mmol/L HEPES, 0.5% glucose). In order to influence pPD-L1 we stimulated platelets with 100 U/ml IL-1β for 18 h before use. For other measurements the platelets were used as purchased without any further incubation.

**Flow cytometry.** Flow cytometry was performed using fluorescence-conjugates or specific mAb and their controls followed by species-specific conjugate (Supplementary Table 2) using a FACS Cantor II flow cytometer (Beckton Coulter) or a LSRII Fortessa (Becton Dickinson) from the flow cytometer facility Tuebingen.

**Histopathology, immunohistochemistry and immunofluorescence staining of paraffin-embedded tissue samples.** Tissue samples were fixed in 4% formalin and paraffin-embedded (FFPE) at the Department of Pathology (University Hospital Tuebingen). The sections were cut in 3 μm sections and stained with Hematoxylin/Eosin and CD16 (clone: 2C9.G3) following standard protocols. For immunofluorescence microscopy, sections were deparaffinized and hydrated in a first step. The heat-induced antigen retrieval method was performed using sodium citrate buffer (pH 6.0) for 30 min. Antigen blocking was performed with Blocking Solution (Zymed) for 60 min. Primary antibodies that were included anti-CD41, mouse, 1:250 (clone: HIP8) and anti-PD-L1, rabbit, 1:200 (clone:28-8), Secondary antibodies include Alexa-Fluor 594 labeled anti-rabbit (1:1000, Invitrogen) and Fluor 488 labeled anti-mouse (1:1000, Invitrogen). DAPI (1:1000, Biologend) was used for nuclear staining prior mounting the slides with H-1500 Vectashield Hardset. Microscopic analysis was done with an Olympus BX63 microscope and a DP80 camera (Olympus).

**Immunofluorescence staining of platelets and tumor cells.** For immunostaining tumor cells and/or platelets were fixed in 2% PFA in PBS (pH 7.4) for 10 min at –20 °C. After three washing steps in PBS cells were incubated with a BSA blocking solution (2% BSA, 0.2% Triton X-100, 0.1% Tween) for 1 h. Primary antibodies were anti-PD-L1, rabbit (1:250, clone: 28-8), anti-CD41, mouse (1:1000 clone: HIP8), anti-CD61, rabbit (1:250, clone: S1-F-9), anti-GFP, rabbit (1:200, clone: EPR141D4), anti-fibronectin, mouse (1:200, clone: P1H11); as secondary antibodies Alexa-Fluor 488/594 labeled anti-rabbit (1:1000, Invitrogen) and Fluor 488/594 labeled anti-mouse (1:1000, Invitrogen) were used. Afterwards slides were mounted in fluorescent mounting medium containing DAPI (1:1000, Biologend) counter-stain. For the plasma membrane staining CellMask™ (ThermoFisher) and Dil (ThermoFisher) were used according to manufacturers’ instructions. For nuclear staining NucBlue™ (ThermoFisher) was used. Image acquisition was performed using an Olympus BX63 microscope and a DP80 camera (Olympus) and CellSens Dimension software. Quantification of platelets, fibronectin, and tumor cells were performed via counting fluorescence positive signals using an ImageJ script (v.1.51n and v.1.52).

**Cyclical immunofluorescence staining of NSCLC patient samples.** Paraffin-embedded patient samples were cut in 2–5 μm slices and collected on object slides. Subsequently, sections were subjected to deparaffinization and rehydration. Slides were treated with xylene for 10 min, followed by rehydration using an ethanol concentration series of 100%, 50%, 50% for 5 min each. One last change was performed using deionized water. Heat-induced antigen retrieval was performed using a Sodium-Citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0) and boiling the samples for 20 min. Samples were cooled down and stored in MACSima™ Running Buffer (Miltenyi Biotech, 130-121-565) until initial DAPI staining (Miltenyi Biotech, 130-121-554). The MACSima™ buffer is a Tris-HCl based high content cyclic IF device which allows for fully automated IF imaging. Iteratively, the device performs fluorescent staining with multiple labeled antibodies, image acquisition, and bleaching per cycle. Images were generated according to the manufacturer’s instructions and analyzed with the Qi Tissue Image Analysis Software. For quantification at least two ROI were selected based on manual pre-staining of DAPI.

**Tracking platelet-tumor cell interaction using live-cell imaging.** For live-cell imaging analysis A549 cells (cultured as stated above) were used. Tumor cells were co-incubated with platelets at a platelet-tumor cell ratio of 1:1000. Platelets were added to the tumor cells directly prior image acquisition. Platelet-tumor cell interactions were analyzed using phase-contrast live-cell microscopy with frame intervals of 30 s for up to 40 min (Leica Microsystems, Thumed Image 3D Assay; HC PL APO 40 ×/0.95) using adaptive focus control. Cell positions were assigned by their center-of-mass coordinates.

**Electron microscopy and immunoelectron microscopy.** For transmission electron microscopy, platelets from one representative pPD-L1 high expressing NSCLC patient were used. Platelets were centrifuged and the resulting pellets were fixed for 24 h in Karnovsky’s fixative. As previously described, Ultrathin sections were examined with a LIBRA 120 (Zeiss) operating at 120 kV.\(^{46}\) For immunoelectron microscopy, platelets were fixed and embedded in Lowicryl K4M (Polysciences)\(^{37}\). Samples were stained with anti-PD-L1 antibody (Abcam) and examined using a LIBRA 120 transmission electron microscopy (Zeiss) at 120 kV.

**ELISA.** Protein levels of PD-L1 were measured using a human PD-L1 ELISA kit (Abcam, clone: 28-8) according to the recommendations of the manufacturer. All concentrations are expressed as means ± SEM of triplicates.

**Western blot.** Whole-cell extracts were prepared using RIPA buffer and protein concentration was analyzed using the BioRad DC assay. 25-50 μg of protein were transferred onto a 0.15% SDS-PAGE gel and visualized on a PVDF membrane) with a wet blot system. The membrane was blocked for 1 h at room temperature with Roti-Block, followed by overnight incubation with the following antibodies: anti-PD-L1, rabbit (1:2000, clone: 28-8), anti-fibronectin, mouse (1:250, clone: P1H11), anti-Vinculin, mouse (1:10,000, clone: hVIN-1), anti-α tubulin (1:10,000, clone: AM01, clone: 1:2000, clone: AC) - blots were visualized using ECL reagents (GE Healthcare) or the Super Signal West Kit (Thermo Scientific) and the ChemiDocTM MT Imaging System using the ImageLab v5.2.1 software.
Real-time PCR. To determine mRNA abundance in several tumor cell lines we extracted mRNA in Trizol (Peprob) according to the manufacturer’s instructions. After 60 s of initial denaturation reverse transcription of the mRNA was performed using random hexamers (Roche Diagnostics) and SuperScriptII reverse transcriptase (Invitrogen). Amplification of the respective genes by real-time polymerase chain reaction (RT-PCR) was performed in a total volume of 20 μl using 40 ng of cDNA, 500 nM forward and reverse primer and 2X GoTaq qPCR Master Mix (Promega) according to the manufacturer’s instructions. Negative controls were always included. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Biorad). All experiments were performed in duplicates.

Cytokine and cell surface marker staining. Peptide-specific T cells were further analyzed by intracellular cytokine and cell surface marker staining. PBMCs were incubated with 10 μg/mL of peptide, 10 μg/mL of brefeldin A (Sigma-Aldrich) and 1 μg/mL of Golgistop (BD Biosciences) for 12-16 h. Intracellular Cytometry-Perm™-perm solution (BD), anti-CD4, mouse (1:100, clone: RPA-T4), anti-CD8, mouse (1:400, clone: B9.11), anti-TNF, mouse (1:120, clone: Mab11) and anti-IFN-γ, mouse (1:200 dilution, clone: 4S3B). PMA (5 μg/mL) and ionomycin (1 μM, Sigma-Aldrich) served as positive control. Viable cells were determined using Aqua live/dead (1:400 dilution, Invitrogen). Samples were analyzed using a FACS Canto II cytometer (BD) and evaluated using FlowJo software v10.08 (BD).

Generation of NY-ESO-1-specific CD4+ T cells. The generation of NY-ESO-1-specific CD4+ T cells was performed using a protocol described previously. Briefly, PBMCs from a healthy donor (1 × 10^{6}/ml) were stimulated using pools of NY-ESO-1-overlapping peptides (1 μg/mL). The NY-ESO-1-overlapping peptide pool of 15 amino acid length (11 amino acid overlap) was purchased via Miltenyi Biotec. The cells were cultured in RPMI 1640 containing 10% human AB serum and 1% l-glutamine in the presence of 10 μ/mL recombinant IL-2 and 10 ng/mL IL-7. Culture medium was replaced every third day. After a pre-sensitization period of 7-14 days, NY-ESO-1-specific, IFNγ+ T cells were enriched after re-stimulation with NY-ESO-1 peptide pool for 6 h using CliniMACS (Miltenyi Biotec) technique as reported previously. After enrichment, NY-ESO-1-specific T cells were expanded for 14 days in the presence of IL-7 (10 ng/mL), IL-15 (10 ng/mL) and IL-2 (50 μU/mL). T cell specificity was analyzed via intracellular IFNγ staining as stated above. For further characterization of the T cells the differentiation markers markers CD45RA/RO, mouse (1:200, clone: HI100), anti-CD69, mouse (1:400, clone: DREG-56), anti-CD28, mouse (1:200, clone: CD28.2) and anti-CD27, mouse (1:200, clone: M-T271) were co-analyzed by flow cytometry. The NY-ESO-1-specific T cells (5 × 10^{5}/ml) were cultured in TexMACS GMP Medium (Miltenyi Biotec). Six hours prior analysis T cells were co-incubated with platelets of NSCLC patients or healthy donors (ratio 1:200) and re-stimulated with NY-ESO-1 peptides (1 μg/mL) in order to investigate the functional role of PD-L1 on platelets surfaces. PD-L1 positive platelets from NSCLC patients were pre-treated with T cells and platelet T-cell co-incubation was performed using a 0.4% trypan blue solution (Fluka). The tumor cell lines A549 (CRM-CCL-18), NCI-H460 (HTB-177), NCI-H23 (CRL-5800), NCI-H226 (CRL-826), NCI-H322 (CRL-8060), NCI-H522 (CRL-8810), HOP-62 and HOP-92 were obtained from the American Type Culture Collection (ATCC).

Generation of peripheral blood mononuclear cells (PBMC) and tumor cell lines. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated using Ficoll/Paque (Biochrom) density gradient centrifugation after informed consent. All tumor cell lines were cultured with 10% FCS in Roswell Park Memorial Institute (RPMI) 1640 Medium at 37°C and 5% CO2. Cell proliferation was quantified using a Neubauer chamber; for viability testing Trypan blue was used (1:400 dilution, Invitrogen). Samples were analyzed on a FACS Canto II cytometer and evaluated using FlowJo software v10.08 (BD).

In vitro platelet-tumor cell-co-incubation and platelet adhesion. Tumor cells were coated with platelets as described previously with slight modifications. Briefly, tumor cells were grown in 2% PFA in PBS (pH 7.4) for 10 min at 20°C. Platelet adhesion to bronectin matrices and platelet blocking of GPIb-IX-V complex, AATACGACCAAATCCGT. Real-time PCR amplification and analysis were performed as described above. For the quantification of non-adherent platelets cells were fixed in 2% PFA in PBS (pH 7.4) for 10 min at 20°C prior staining.

Preparation of fibronectin matrices and platelet blocking. To prepare fibronectin matrices, plates were coated with a human plasma fibronectin purified protein (R&D, Minneapolis, MN, USA) concentration 50 μg/cm² for 120 min. For blocking of GpIIb-IIIa complex, a5b1 or GPIbIIa, washed platelets (8 × 10^{8}/ml) were pre-treated with 5 μg/ml anti-CD42b (clone: AK2), 20 μg/ml anti-integrin a5 (clone: PAC10) and anti-Integrin a5 (clone: IB5) or corresponding control IgG2 (20 μg/ml) for 30 min at 37°C and 7% CO2. Tumor cells were incubated in platelets at a platelet-tumor-cell ratio of 1:1000 for 30 min at 37°C and 7% CO2. For immunoﬂuorescence microscopy and FACS analysis cells were fixed in 2% PFA in PBS (pH 7.4) for 10 min at −20°C prior staining.

Plasmid construction, transfection and knockdown of NSCLC cells. For over-expression of PD-L1 (CD274) a True-ORF-GFP-tagged expression vector was used (Origene, RG213071, Rockville, MD, USA). Control cells were transfected using a FLAG tag. The FLAG cDNA was generated by PCR and cloned into the PD-L1-GFP vector (Origene). Peptide stimulation ex vivo. Patients were categorized into pPD-L1 quartile groups. For siRNA knockdown of Fibronectin, Lipofectamine TM 3000 and mincine (Mabtech) was used (1 μg/mL). SEB (Toxin Technology, Sarasota, FL, USA) at 10 μg/mL was used as positive control. NY-ESO-1 specific T cell activity was determined by intracellular TNFα and IFNγ quantified via flow cytometry as described above.

In situ proximity ligation assay (PLA). PLA. HOP-62 and NCI-H323 cells were grown on glass bottom plates. After two washing steps cells were fixed in 1% PFA in PBS for 10 min at room temperature. Cells were incubated with a BSA blocking solution (5% BSA, 0.2% Triton X-100, 0.1% Tween) for 30 min. In situ PLA was performed using the Duolink PLA kit (Sigma-Aldrich) according to the manufacturer’s instructions. In brief, after blocking cells were incubated with anti-FD-L1, rabbit (1:250, clone: 28-15) and anti-fibronectin, mouse (1:200, clone: PH111) for 2 h at room temperature. After three washing steps with PBST (phosphate buffered saline, 0.1% Tween), anti-mouse PLUS and anti-rabbit MINUS PLA probes were linked to the primary antibodies for 1 h at 37°C. After three times washing steps with buffer A (0.01 M Tris, 0.15 M NaCl, and 0.05% Tween-20), PLA probes were ligated for 60 min at 37°C. After two washing steps with buffer A, amplification using Duolink In Situ Detection Reagents (Sigma) was performed. The Duolink signal was visualized at 37°C for 120 min. The slides were washed three times for 5 min with wash buffer B (0.2 M Tris 0.1% NaCl). Cells were then co-cultured with Duolink Mounting Medium containing DAPI. Image acquisition was performed using an Olympus BX63 microscope and a DP80 camera (Olympus).

Establishment of an activation-independent calculation matrix for platelet PD-L1. Since platelet pre-activation levels differ due to sample collection/preparation and protein surface expression depends on the platelet activation state, accurate determination of total protein expression on platelet surfaces is challenging. As a result, the platelet pre-activation level acts as a confounding factor and thus impairs the suitability of pPD-L1 as a promising biomarker in NSCLC. To circumvent this dilemma, we established an activation-independent calculation matrix of platelet PD-L1 expression (Fig. 5). IFNγ was used to stimulate platelets of NSCLC patients. Cytokine and platelet blocking of GPIb-IX-V complex, AATACGACCAAATCCGT. Real-time PCR amplification and analysis were performed as described above. For the quantification of non-adherent platelets cells were fixed in 2% PFA in PBS (pH 7.4) for 10 min at −20°C prior staining. For the quantification of non-adherent platelets cells were fixed in 2% PFA in PBS (pH 7.4) for 10 min at −20°C prior staining.

In vitro platelet-tumor cell-co-incubation and platelet adhesion. Tumor cells were coated with platelets as described previously with slight modifications. Briefly, tumor cells were grown in 2% PFA in PBS (pH 7.4) for 10 min at −20°C prior staining. For the quantification of non-adherent platelets cells were fixed in 2% PFA in PBS (pH 7.4) for 10 min at −20°C prior staining. For the quantification of non-adherent platelets cells were fixed in 2% PFA in PBS (pH 7.4) for 10 min at −20°C prior staining.
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**Author contributions**
C.H., H.-G.K., O.B., and L.Z. conceived and designed the study. C.H., E.M., M.S., St.Ma., M.L., Me.He., So.Sc., C.S. conducted in vitro experiments. B.W. performed live-cell imaging. T.B., M. L., and J.W. conducted in vitro T cell experiments. C.H., J.S., Ma.Hi., J.H. Ma.Hso., Sv.Ma., St.Si., and F.F. conducted patient data and sample collection as well as medical evaluation and analysis. C.H., M.L., J.H., and So.Sc. analyzed data and performed statistical analyses. C.H., B.W., So.Sc., Ma.Hi., Me.He, and T.B. prepared figures and tables; C.H. wrote the first draft of the manuscript. R.H., H.S., H.-G.K., O.B., and L.Z. contributed to data interpretation and manuscript edit. L.Z., R.H., J.W., and O.B. gave technical support and conceptual advice. All authors critically reviewed, read, and approved the final manuscript.

**Competing interests**
The authors declare no competing interests.

**Additional information**

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