PD-L1 marks a subset of melanomas with a shorter overall survival and distinct genetic and morphological characteristics.

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PD-L1 marks a subset of melanomas with a shorter overall survival and distinct genetic and morphological characteristics

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Background: Programmed cell death ligand 1 (PD-L1) is a cell surface molecule that plays a critical role in suppressing immune responses, mainly through binding of the PD-1 receptor on T lymphocytes. PD-L1 may be expressed by metastatic melanoma (MM). However, its clinical and biological significance remains unclear. Here, we investigated whether expression of PD-L1 in MM identifies a biologically more aggressive form of the disease, carrying prognostic relevance.

Patients and methods: PD-L1 expression was analyzed by immunohistochemistry using two different antibodies in primary tumors and paired metastases from 81 melanoma patients treated at a single institution. Protein expression levels were correlated with PD-L1 mRNA, BRAF mutational status and clinical outcome. PD-L1^{+} and PD-L1^{-} subsets of the A375 cell line were stabilized *in vitro* and compared using gene expression profiling and functional assays. Results were confirmed using xenograft models.

Results: PD-L1 membrane positivity was detected in 30/81 (37%) of patients. By multivariate analysis, Breslow thickness and PD-L1 membrane positivity were independent risk factors for melanoma-specific death {PD-L1 5% cutoff [hazard ratio (HR) 3.92, confidence interval (CI) 95% 1.61–9.55 $P < 0.003$], PD-L1 as continuous variable (HR 1.03, 95% CI 1.02–1.04 $P < 0.002$)}. PD-L1 expression defined a subset of the BRAF-mutated A375 cell line characterized by a highly invasive phenotype and by enhanced ability to grow in xenograft models.

Conclusions: PD-L1 is an independent prognostic marker in melanoma. If confirmed, our clinical and experimental data suggest that PD-L1^{+} melanomas should be considered a disease subset with distinct genetic and morpho-phenotypic features, leading to enhanced aggressiveness and invasiveness.

Key words: metastatic melanoma, PD-L1, prognostic markers

introduction

In its early-stages melanoma can be cured by surgical resection, but once it progresses to the metastatic stage it remains an incurable disease [1]. The finding of somatic mutations in the *BRAF* oncogene in ~40%–50% of melanoma patients [2, 3] paved the way to the introduction of BRAF inhibitors (BRAFi) as a standard treatment in locally advanced or metastatic melanoma (MM) patients (MMP) with BRAF^{V600} mutation [4]. While clinical responses to BRAFi may be dramatic and some patients treated with BRAF and MEK inhibitors (MEKi) may stay in remission for years, the median duration of response is 7 months for patients treated with BRAFi alone and 11 months for patients treated with BRAFi and MEKi. For this reason, there is intense investigation into alternative or complementary therapeutic strategies, including novel immunomodulatory agents. Among these drugs, anti-PD-1 and anti-programmed

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cell death ligand 1 (PD-L1)-directed therapies show significant clinical promises [5–7]. PD-L1/CD274 is one of the two ligands for the T-cell inhibitory receptor PD-1 [8]. It may be expressed on different cell types, including hematopoietic and epithelial cells, and is upregulated in response to proinflammatory cytokines, such as IFN-γ and IL-4 [9]. It may also be expressed by tumor cells, as well as by non-neoplastic elements in the tumor microenvironment [10–12]. In agreement with the proposed function of the PD-1/PD-L1 axis in the induction and maintenance of peripheral tolerance [11], surface expression of PD-L1 in some tumors has been reported to be an independent predictor of adverse clinical outcome [13]. Furthermore, expression of the molecule appears to correlate with response to treatment, at least in some tumor models [12].

In MM, PD-L1$^+$ cells co-localize with tumor-infiltrating lymphocytes and IFN-γ production, suggesting that expression of this molecule is part of the resistance strategy orchestrated by the tumor against the host immune response [12]. However, the prognostic significance of PD-L1 expression in melanoma remains incompletely explored [14, 15].

By using qRT-PCR and immunohistochemistry, PD-L1 expression was evaluated in a longitudinal cohort of MMP. The finding of a negative prognostic role for PD-L1 is backed by *in vitro* data showing that the PD-L1$^+$ fraction of the A375 melanoma cell line may be considered distinctly a more aggressive disease genetically, morphologically and phenotypically.

materials and methods

patient characteristics

Cohort characteristics are reported in supplementary Materials and Methods, available at *Annals of Oncology* online (Immunohistochemistry (IHC), DNA extraction from formalin-fixed paraffin-embedded tissues (FFPE), B-RAF mutation detection, mRNA extraction and detection by quantitative and real time PCR). Detailed protocols are reported in supplementary Materials and Methods, available at *Annals of Oncology* online.

cells and reagents

The cell lines and the antibodies used for this work are detailed in supplementary Materials and Methods, available at *Annals of Oncology* online.

Induction of PD-L1/CD274 expression by lentiviral technology, flow cytometry, western blot, confocal microscopy and *in vitro* assays.

Detailed protocols are reported in supplementary Materials and Methods, available at *Annals of Oncology* online .

gene expression profiling and analysis

RNA extraction, labeling and analysis were carried out as detailed in supplementary Materials and Methods, available at *Annals of Oncology* online.

xenograft models

Xenograft models are described in supplementary Materials and Methods, available at *Annals of Oncology* online.

statistical analysis

Disease-free survival (DFS) was calculated as the time from first melanoma diagnosis to first recurrence or death. Overall survival (OS) was calculated as the time from first melanoma diagnosis to death. Cox proportional-hazards models were used for univariate and multivariate analyses. Results are expressed as hazard ratios (HRs) with 95% confidence intervals (95% CIs).

Additional statistical methods are delineated in supplementary Materials and Methods, available at *Annals of Oncology* online.

results

PD-L1 expression in melanoma tissues

PD-L1 expression was studied by IHC in 81 consecutive, well characterized MMP. PD-L1 expression was considered either as a continuous or discontinuous variable using the 5% published [16] or the 17.5% cutoff, determined here by recursive partitioning analysis (RPA), yielding comparable results.

In 73/81 MMP (90%), both ab58810 polyclonal (Abcam) and 5H1 monoclonal anti-PD-L1 antibodies were tested, showing a high concordance rate (Cohen's κ 72%, $P < 0.0001$). Representative examples of PD-L1 immunostaining in melanoma tissues are illustrated in Figure 1 and supplementary Figures S1–S4, available at *Annals of Oncology* online.

Complete clinico-pathological information of the antecedent primary melanoma was available for 81 patients (supplementary Table S1, available at *Annals of Oncology* online). No meaningful associations were highlighted between PD-L1 expression and the degree of tumor-infiltrating lymphocytes, scored according to the current classification [brisk, non-brisk and absent (supplementary Figures S2 and S3, available at *Annals of Oncology* online)]. These results were consistent when PD-L1 was considered as continuous variable or with the 17.5% cutoff determined by RPA. The frequencies of PD-L1 membrane immunohistochemical positivity in paired primary and metastatic tumor samples are reported in supplementary Table S4, available at *Annals of Oncology* online. Overall, 40.3% of MM samples were PD-L1$^+$, when compared with 14% of primary melanomas ($P = 0.001$, supplementary Table S5a and b, available at *Annals of Oncology* online).

The PD-L1 positivity barely correlated with BRAF mutation, although did not reach statistical significance ($P = 0.051$, supplementary Table S6, available at *Annals of Oncology* online).

Lastly, PD-L1 mRNA levels did not correlate with membrane protein expression in 34 samples analyzed (Cohen's $\kappa < 3\%$, $P = 0.4273$, supplementary Table S7 and Figure S5, available at *Annals of Oncology* online), suggesting that protein expression must be used for diagnostic and prognostic purposes.

correlation between PD-L1 expression, DFS and OS

DFS was not significantly different between PD-L1$^+$ and PD-L1$^-$ lesions (Figure 2A and B), independently of the cutoff used. As expected, well-established prognostic factors of the primary melanoma, such as Breslow thickness [Breslow thickness 4.01–6.0 versus ≤2: HR 2.91, 95% CI 1.18–7.21, $P = 0.021$], and ulceration [HR 2.26, 95% CI 1.19–4.31, $P = 0.013$], correlated with DFS at multivariate analysis (supplementary Table S8, available at *Annals of Oncology* online).

At a median follow-up of 147 months, the median OS for the whole group of patients was 42 months. At the time of the analysis, 20 patients were still alive and 61 dead. At multivariate analysis, PD-L1 membrane positivity was an independent risk factors for melanoma-specific death [PD-L1 5% cutoff (HR 3.92,

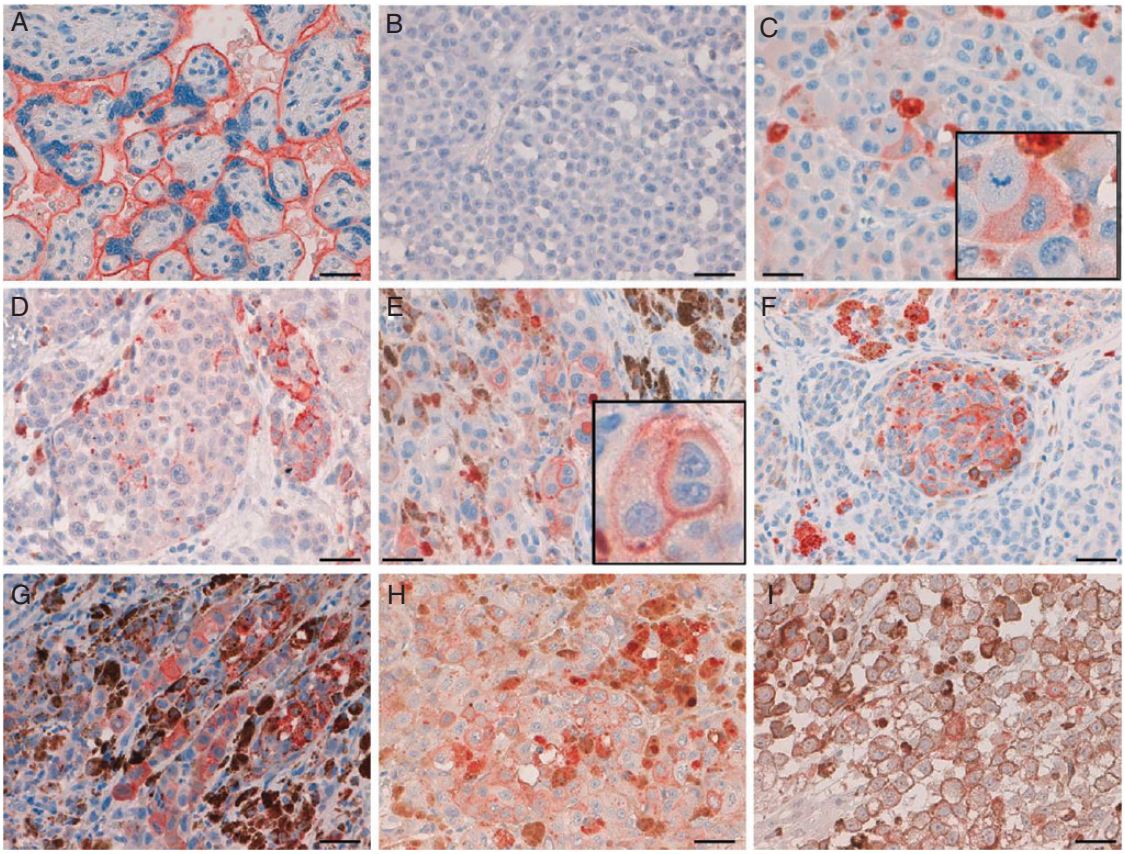

Figure 1. PD-L1 immunohistochemical expression using the mouse anti-human B7-H1 mAb (clone 5H1). (A) Staining of placental tissue, used as positive control. (B) PD-L1-negative melanoma. (C–I) Examples of PD-L1 staining in melanoma tissues. Scattered PD-L1$^+$ melanoma cells (C); focal and patchy PD-L1 staining (D); scattered melanoma cells showing PD-L1 expression and melanin granules (E); roundish aggregate of melanoma cells with moderate PD-L1 membrane expression (F); strong PD-L1 immunostain in melanoma cells easily recognizable from intra and extracellular granular melanin depositions (G); (H–I) staining of melanoma metastases. Note that cytoplasm of melanoma cells is pigmented due to abundant melanin deposition (original magnification ×40, scale bar 50 μm).

CI 95% 1.61–9.55, $P < 0.003$), PD-L1 as continuous variable (HR 1.03, CI 95% 1.01–1.05, $P < 0.002$), supplementary Tables S9 and S10, available at *Annals of Oncology* online, Figure 2C and D]. On the contrary, median OS did not differ in patients with cytoplasmic PD-L1$^-$ versus PD-L1$^+$ lesions (16 versus 12 months, respectively, $P = 0.1130$).

Finally, by multivariate analysis, Breslow thickness, but not ulceration, correlated with OS. Several reasons may justify these results including: (i) the relative small sample size, (ii) the subgroup comparisons, (iii) the influence of subsequent therapies on patient outcome.

Thirty-four patients received a BRAFi at some point after diagnosis of MM [25 treated with Vemurafenib (no restrictions on prior therapy), 9 with Dabrafenib (patients had to have completed treatment or experienced treatment failure with at least one prior standard systemic therapy)]. In the subgroup of patients treated with BRAFi median OS was 16 months in PD-L1$^-$ versus 9 in PD-L1$^+$ melanomas ($P = 0.0473$).

When OS was analyzed as the time from diagnosis of metastatic disease to death, membrane PD-L1 expression remained an independent prognostic factor as continuous variable or when a 5% cut off was considered (supplementary Table S11, available at *Annals of Oncology* online).

These results suggest that PD-L1 membrane expression is an independent marker of unfavorable prognosis for MMP.

morphological features of PD-L1$^+$ versus PD-L1$^-$ A375 variants

In the second part of the work, we asked whether PD-L1 expression was simply the result of microenvironmental pressures on the tumor cell or whether it was an intrinsic feature, marking a disease subset with specific characteristics. After testing PD-L1 expression in a panel of melanoma cell lines, the A375 cell line emerged as the only one with a constitutive PD-L1$^+$ subpopulation (supplementary Figure S6, available at *Annals of Oncology* online).

A375 variants homogeneously PD-L1$^+$ and PD-L1$^-$ were stabilized from the parental BRAF-mutated A375 cell line by repeated cycles of immunomagnetic bead separation and cell sorting (supplementary Figure S7A, available at *Annals of Oncology* online).

PD-L1$^+$ A375 cells grew loosely adherent to plastic and displayed an elongated shape, while the PD-L1$^-$ variant was made of polygonal cells tightly adherent to plastic (supplementary Figure S7B, available at *Annals of Oncology* online). The PD-L1$^-$ variant was also infected with lentiviruses carrying PD-L1

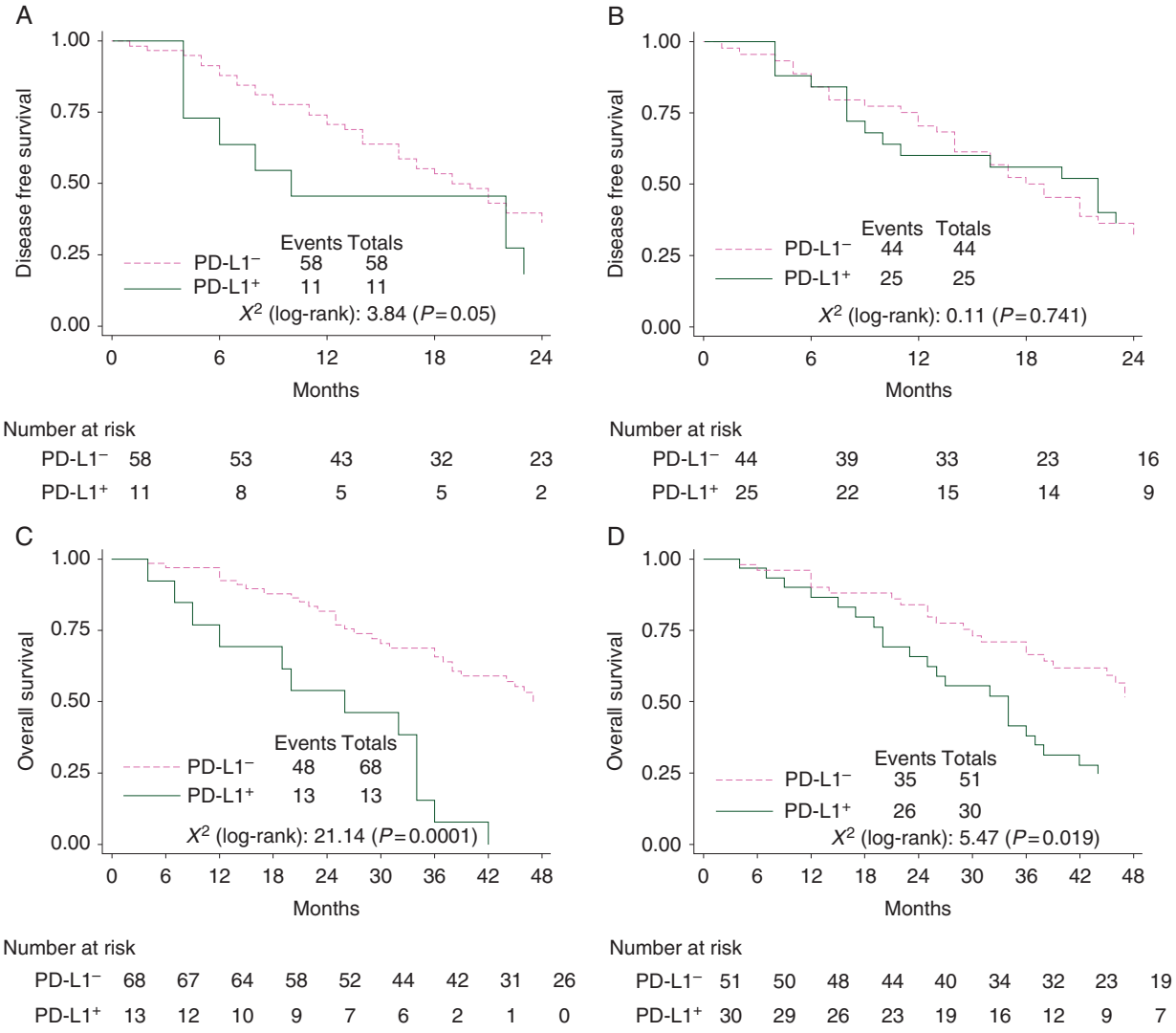

Figure 2. Kaplan–Meier curves showing DFS and OS of MMP according to PD-L1 expression. (A) DFS according to membrane PD-L1 immunohistochemical expression in melanoma tissues (cutoff 17.5%). (B) DFS according to membrane PD-L1 immunohistochemical expression in melanoma tissues (cutoff 5%). (C) OS according to membrane PD-L1 immunohistochemical expression in melanoma tissues (cutoff 17.5%). (D) OS according to membrane PD-L1 immunohistochemical expression in melanoma tissues (cutoff 5%).

genetic material to express high levels of PD-L1 (PD-L1^{INF}), but these cells could not be distinguished from PD-L1− cells (supplementary Figure S8, available at *Annals of Oncology* online). When cultured in 3D, PD-L1$^+$ A375 cells migrated through matrigel and grew with a spindle cell morphology at the bottom of the well. PD-L1$^-$ or PD-L1^{INF} cells on the contrary formed islet-like solid structures which did not leave matrigel (supplementary Figure S7C, available at *Annals of Oncology* online). The A375 unselected cell line behaved predominantly like the PD-L1$^-$ variant, with a subpopulation of infiltrating PD-L1$^+$ cells, consistent with basal levels of PD-L1 expression (supplementary Figure S7B and C, available at *Annals of Oncology* online).

These results suggest that PD-L1 expression defines a subset of A375 cells characterized by a fibroblast-like morphology and invasive properties. They also suggest that PD-L1 is a marker of a more activated status of the melanoma cell line and not mechanistically responsible for the observed phenotype.

PDL-1$^+$ A375 cells show a distinct gene profile with upregulation of genes regulating tumor growth and diffusion

Global gene expression profiles of PD-L1$^+$ and PD-L1$^-$ A375 cells were then compared. In unsupervised hierarchical clustering analysis, the gene expression profiles of A375/PD-L1$^+$ were clearly distinguishable from those of PD-L1$^-$ replicates. The characteristics of the genetic signature of PD-L1$^+$ cells are shown in Figure 3A and supplementary Table S12, available at *Annals of Oncology* online. The genes most differentially expressed by PD-L1$^+$ cells are connected with activation/adhesion/movement pathways (Figure 3A and B).

In a supervised analysis, a number of differentially expressed genes connected with cell growth and invasion were selected. Relevant examples of upmodulated genes include integrin α3 (ITGA3) and caveolin 1 (CAV1, Figure 3C). On the contrary, genes connected with antigen presentation and immune

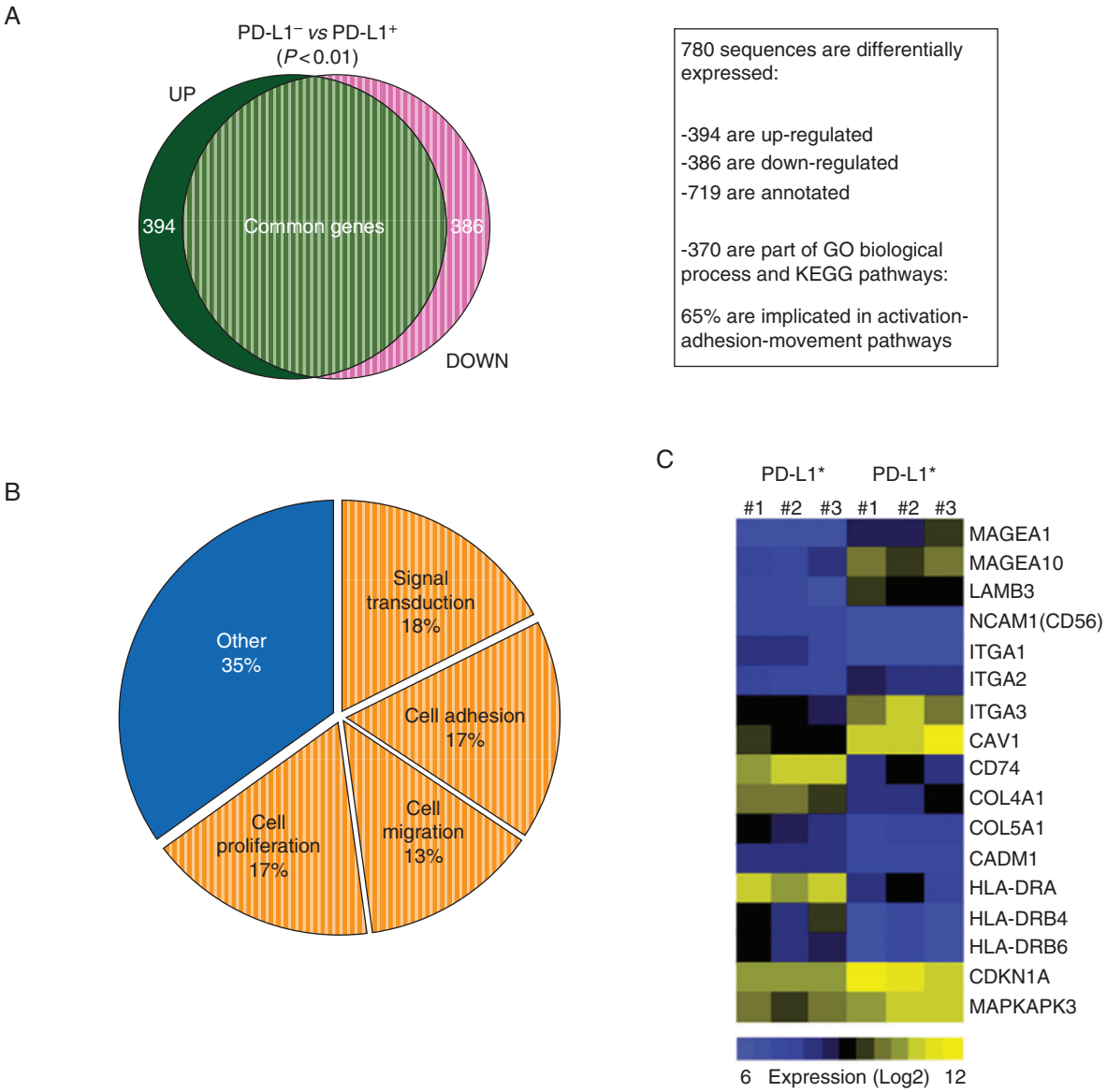

Figure 3. PD-L1$^+$ and PD-L1$^-$ A375 cells show different gene expression profiles. (A) Venn diagram showing 394 upregulated and 386 downregulated sequences in PD-L1$^-$ compared with PD-L1$^+$ A375 variants. (B) Functional annotations are reported in the pie chart and (C) most representative differentially expressed genes are shown in a heat map. (D) Differential expression of HLA Class II, CD74, CD56 and caveolin 1 in PD-L1$^+$ cells (full line) and PD-L1$^-$ cells (dashed line) was confirmed by flow cytometry. Isotype control is shown as filled histogram. (E) Western blot and (F) confocal microscopy analyses of α integrin subunits and caveolin 1 in PD-L1$^+$ versus PD-L1$^-$ cell lines.

response were downregulated, as highlighted by CD74 and HLA Class II (HLA-DR, Figure 3C). Flow cytometric analyses confirmed a marked downregulation in the expression of HLA Class II, CD74 and CD56 by PD-L1$^+$ cells, while caveolin 1 protein was increased (Figure 3D). All integrin α subunits were significantly upregulated in A375 PD-L1$^+$ variant, as confirmed by western blot (Figure 3E) and confocal microscopy (Figure 3F), indirectly supporting the morphologic observations and suggesting that PD-L1$^+$ cells may have a more aggressive behavior. The arrays were validated at the protein level, confirming highly significant results in PD-L1$^+$ versus PD-L1$^-$ variants for the expression of MHC-Class II, CD74, CD56, caveolin 1 and alpha1,3,5 integrins (supplementary Figure S9, available at *Annals of Oncology* online).

PD-L1$^+$ A375 cells show enhanced migration and invasion *in vitro*

The enrichment in the expression of genes connected to migration and invasion in PD-L1$^+$ A375 cells was then functionally confirmed. Chemotaxis assays indicated that the PD-L1$^+$ variant migrated in a significantly more efficient way than the PD-L1$^-$ variant, with the parental A375 cell line showing an intermediate behavior (Figure 4A). In line with the morphological data,

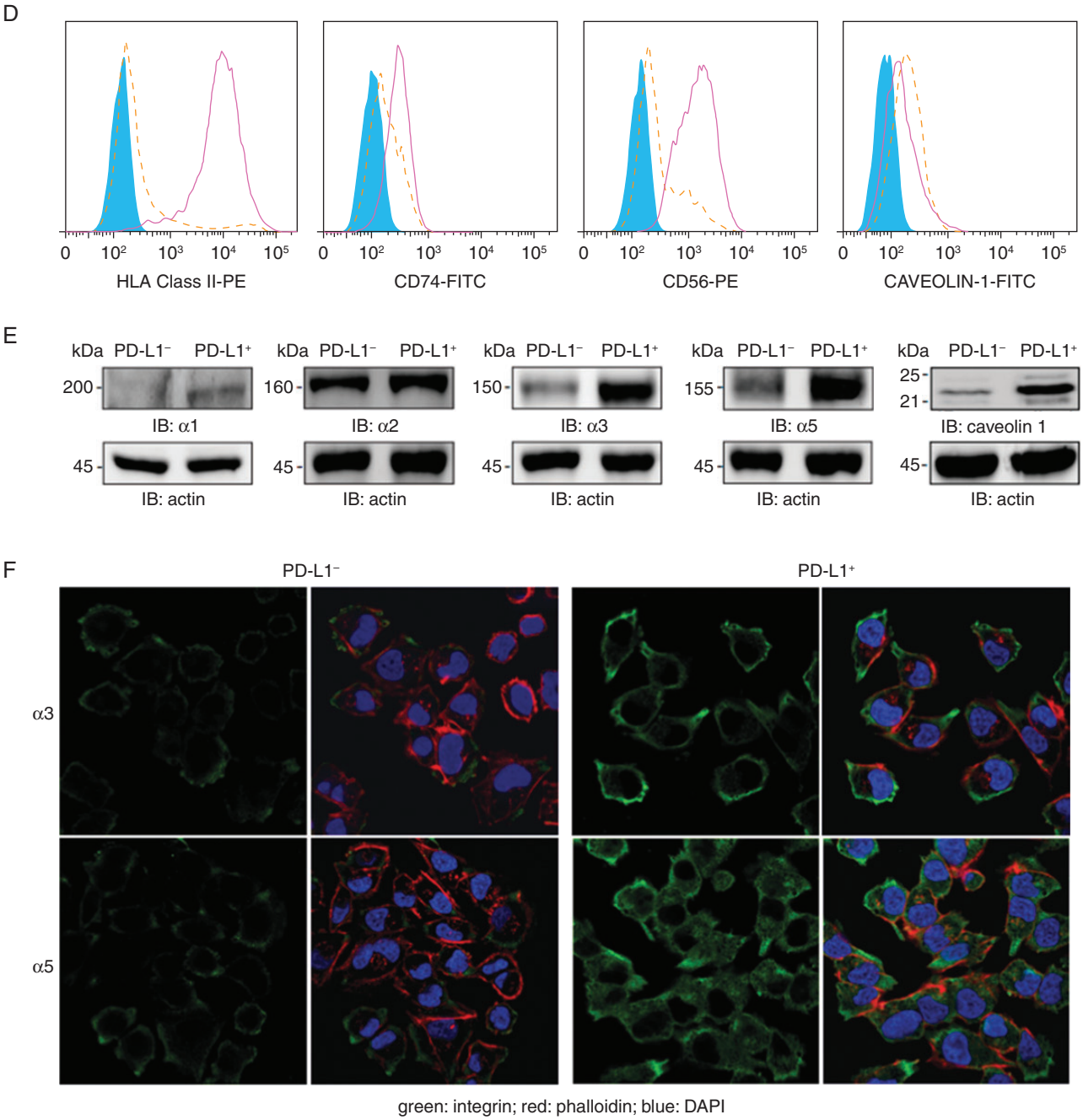

Fig. 3 *Continued*

infection of PD-L1 in A375 cells did not significantly modify chemotaxis (supplementary Figure S8D, available at *Annals of Oncology* online). Similar results were obtained when comparing chemotaxis toward FCS (supplementary Figure S10, available at *Annals of Oncology* online).

Wound-healing assays confirmed that the PD-L1^{+} cells were able to migrate to the site of the wound with marked repair after 48 h, while the counterpart was significantly less efficient. As observed above, A375 WT cells displayed an intermediate ability to heal the wound (Figure 4B). PD-L1^{INF} cells were not significantly different from the WT counterpart (supplementary Figure S8E, available at *Annals of Oncology* online).

PD-L1^{+} A375 cells show enhanced growth and diffusion *in vivo*

The second set of genes that were up-regulated in the PD-L1^{+} A375 variant was connected to growth and activation. Consistently, PD-L1^{+} A375 cells grew more than PD-L1^{-} ones in 3D cultures. PD-L1^{+} cells were characterized by a constitutively higher degree of phosphorylation of the MAP kinases, including ERK1/2, p38 and JNK both in 2D (Figure 4C) and 3D cultures (supplementary Figure S11, available at *Annals of Oncology* online).

By using a xenograft model, we could confirm the increased growth potential of PD-L1^{+} when compared with PD-L1^{-} A375 cells. In these experiments, cells were injected subcutaneously

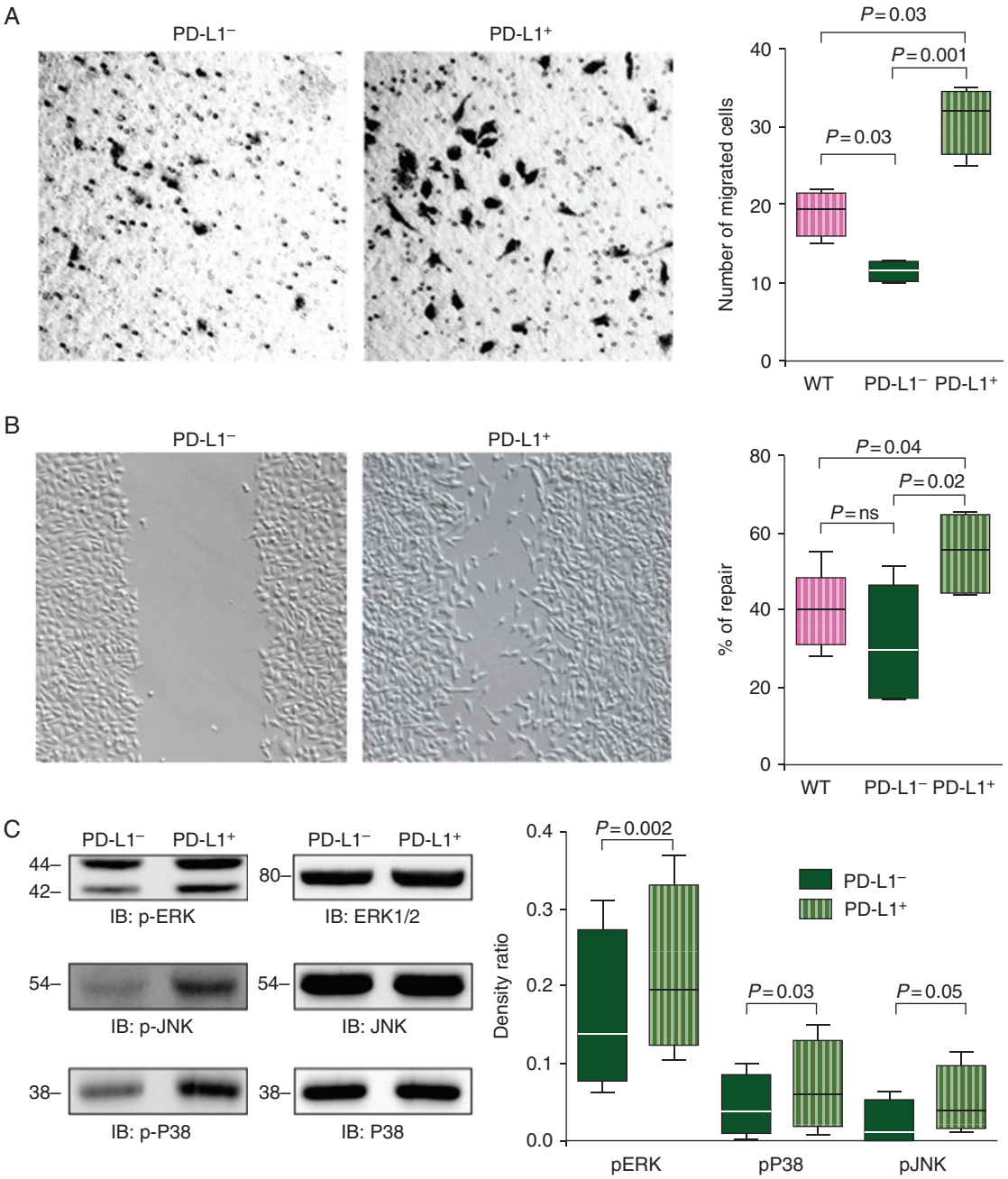

Figure 4. PD-L1$^+$ cells display increased aggressiveness *in vitro* and *in vivo*. (A) Representative pictures (×20 magnification) of Boyden chamber filters stained with hematoxylin/eosin showing increased invasion by PD-L1$^+$ A375 cell variant, when compared with PD-L1$^-$. Box plot on the right shows the absolute numbers of cells in the lower side of the chamber filter after 72 h. Results are reported as mean ± SD (standard deviation) of four independent experiments. (B) Representative pictures (×10 magnification) of a wound-healing assay at $t = 0$ h and after 48 h comparing PD-L1$^+$ and PD-L1$^-$ A375 variants. Box plot shows cell percentage of migration, determined by measuring wound widths ratio between the two time points. Values are representative of six independent experiments ± SD. (C) Lysates from PD-L1$^-$ and PD-L1$^+$ A375 variants were blotted for p-ERK, p-JNK and p-P38. Protein expression was normalized over the corresponding unphosphorylated protein. Box plot shows the mean density ratio ± SD of four independent experiments. (D) Representative images from PD-L1$^+$ or PD-L1$^-$ *in vivo* tumors injected subcutaneously in NOD/SCID mice. Box plot in the middle shows the mean tumor volume of 6 experiments. (E) Representative images from PD-L1$^-$ (upper panels) and PD-L1$^+$ (lower panels) A375 xenografts stained with hematoxylin/eosin (left), PD-L1 (middle) and Ki-67 (right) antibodies. The right panel in (D) shows a box plot with the mean % of Ki-67$^+$ areas in PD-L1$^-$ and PD-L1$^+$ tumors.

into the right and left flank of NOD/SCID mice, respectively and allowed to grow over a period of 2 weeks. Cells retained PD-L1 expression after *in vivo* growth. In all instances, PD-L1$^+$ cells formed larger masses (Figure 4D), with signs of local infiltration and showed a significantly higher expression of Ki-67 proliferation marker (Figure 4E).

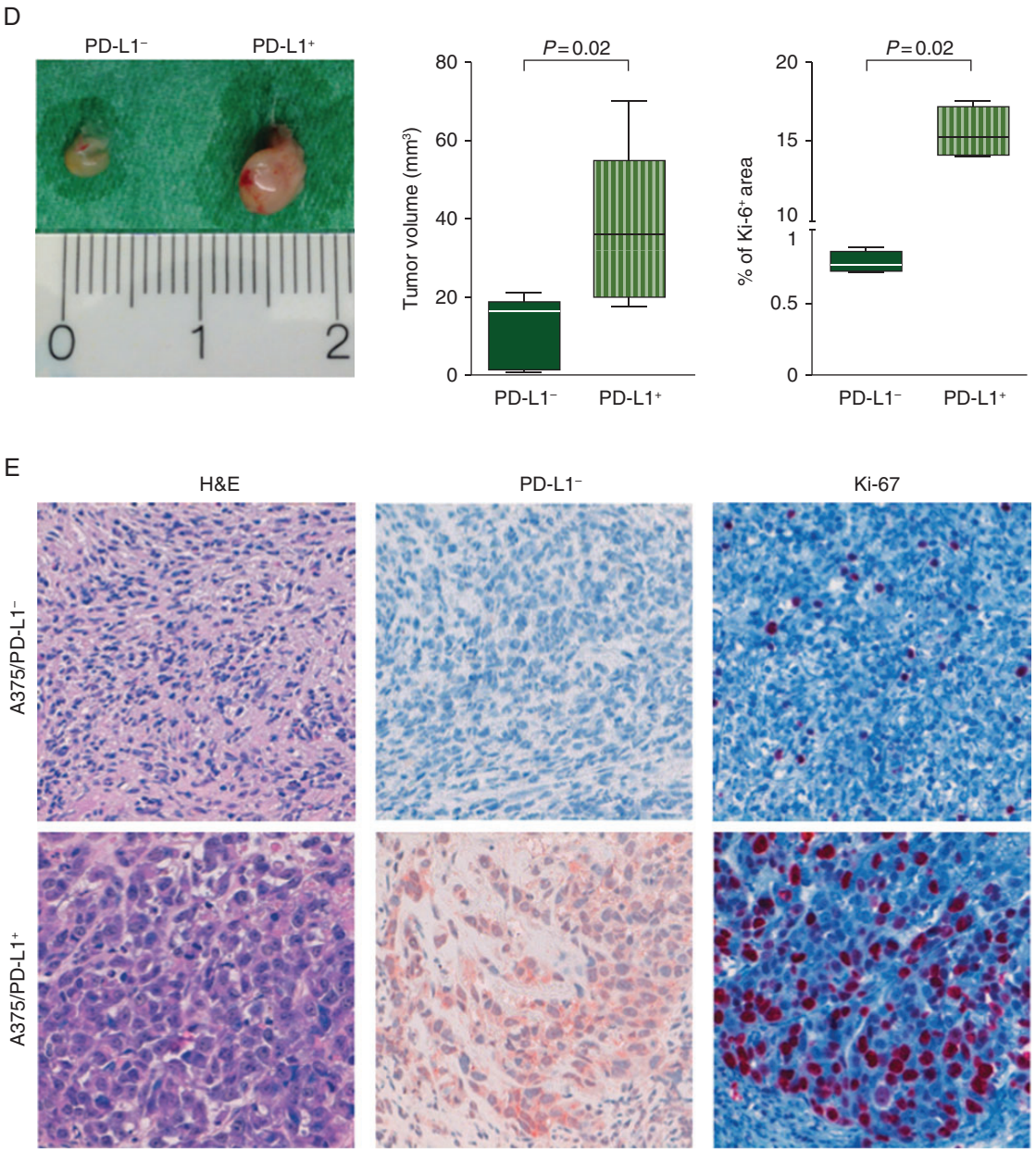

Fig. 4 *Continued*

Considered together, these results indicate that the PD-L1 expression marks a subset of the A375 cell line, which is intrinsically characterized by increased growth and motility.

discussion

This study shows that PD-L1 is an independent negative prognostic factor in melanoma patients. This conclusion was reached upon testing PD-L1 expression in a cohort of 81 consecutive MMP treated at a single institution, and comparing two antibodies specific for PD-L1. In our series, the mouse monoclonal 5H1 antibody yielded the most reliable results. The second indication is that PD-L1 cytoplasmic staining is not suitable for prognostic purposes. The third indication is that PD-L1 mRNA levels are not predictive of protein expression, in agreement with previous studies [17]. The lack of correlation between PD-L1 mRNA levels and protein expression may be explained on the basis of two hypotheses: (i) PD-L1 expression is controlled by posttranscriptional mechanisms, (ii) proinflammatory stimuli such as IFN-γ, IL-4 and GM-CSF are potent activators for inducing B7-H1 protein expression [9, 11].

In agreement with a negative prognostic role for PD-L1, our study shows that MM express PD-L1 in significantly higher proportions than primary lesions (40.3% versus 14%). In 17/22 patients, the metastatic site resulted positive while primary melanomas were negative, suggesting that PD-L1 expression is acquired during disease progression. PD-L1 expression has been reported as neither a fixed characteristic of tumor cells, nor homogeneously expressed. Hence, in 10 PD-L1⁻ patients multiple metastatic tumor samples from the same patient were analyzed,

with concordant results. In two further PD-L1$^-$ patients that were treated with a BRAFi, resistance acquisition was accompanied by a transition of the lesion from PD-L1$^-$ to PD-L1$^+$.

The second result of this work is that PD-L1 expression appears to correlate with shorter OS during BRAFi treatment. If confirmed in larger cohorts, this observation may be useful to better stratify patients within clinical trials.

The impact of PD-L1 expression in terms of clinical behavior was addressed by prior studies with controversial results, which may be attributed partly to the absence of a validated staining method and partly to the relatively small size of the cohorts studied. While our study used a relatively large cohort and a validated staining method, its main limitation is the retrospective nature, suggesting that these results should be confirmed with a prospective study.

The functional significance of PD-L1 expression is classically attributed to the inhibition of T cell responses, obtained through binding of the PD-1 receptor [11]. Consistently, a recent paper reported a positive correlation between PD-L1 expression by melanocytes and lymphocyte infiltration in both nevi and melanomas, with PD-L1$^+$ melanocytes, being frequently adjacent to infiltrating lymphocytes [12]. In our series and in an additional independent cohort of 17 primary melanoma samples, no significant association was observed between PD-L1 expression and the degree of TILs. It should be noted that, in our series, most of patients received chemotherapy or BRAFi while in the study reported by Taube et al. patients were receiving immunotherapy [12]. This suggests that the type of treatment received should be considered when correlating PD-L1 expression with survival outcomes.

Nevertheless, the functional significance of constitutive and targeted therapy-modulated PD-L1 expression has not been entirely elucidated. A recent study evaluating PD-L1 expression in a large panel of melanoma cell lines with or without exposure to MAPK inhibitors showed that there is no association between MAPK/PI3 K activation and PD-L1 expression, suggesting that the constitutive PD-L1 expression or PD-L1 expression upon alterations of signaling pathways, in the absence of a T-cell infiltration, may not serve as a biomarker [18].

Besides its role as an immunomodulatory molecule, recent data in ovarian tumor models suggest that PD-L1 might *per se* determine a more aggressive clinical course [19, 20]. To clarify whether this may be confirmed in melanoma models, in the second part of the paper, we explored the functional significance of PD-L1 expression by melanoma cells. To do so, we used the A375 line, which constitutively presents distinct PD-L1$^+$ and a PD-L1$^-$ subpopulations, a unique situation in the melanoma lines tested. Stable PD-L1$^+$ and PD-L1$^-$ variants of the A375 cell line were thus stabilized and comparatively studied. Gene profiling indicated that the PD-L1$^+$ cell subset is characterized by a unique genetic signature, with enhanced expression of genes connected to growth, activation and invasion. A functional comparison confirmed that: (i) the PD-L1$^+$ variant showed signs of increased growth and invasion *in vitro*, (ii) these features were enhanced when using 3D cultures and maintained after xenografting in immunocompromised mice. Forced expression of PD-L1 molecules in the PD-L1- variant of the A375 line, however, was not followed by the acquisition of increased growth and motility properties. This observation suggests that PD-L1 expression might be a downstream marker of the activation of an oncogenic pathway characterizing a genetically different cell subset, which shows enrichment in genes that control cell differentiation and movement. It also argues against a mechanistic involvement of PD-L1 in determining increased aggressiveness of the disease.

In conclusion, from the clinical standpoint, the present results suggest PD-L1 expression as a negative prognostic factor in melanoma patients. From the translational standpoint, they indicate that beside the known effects on immune response modulation, PD-L1 expression marks a subset of melanoma cells characterized by a specific gene expression profile and by increased growth and aggressiveness. Our results suggest that PD-L1 is not mechanistically responsible for the more aggressive phenotype in melanoma cells. Future studies will tell whether PD-L1 expression is also a marker of resistance to selected therapies and whether it may be successfully exploited alone or in combination as a target for specific subsets of melanoma patients.

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disclosure

The authors have declared no conflicts of interest.

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