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The circulating pool of functionally competent NK and CD8+ cells predicts the outcome of anti-PD1 treatment in advanced NSCLC

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ABSTRACT

Introduction: A prospective investigation of the circulating immune profile in NSCLC patients receiving nivolumab was performed to identify potentially predictive parameters.

Methods: Flow Cytometry of peripheral blood (PB) CD3+, CD8+, CD4+, NK, Treg and MDSCs was prospectively performed in 31 consecutive advanced NSCLC patients at baseline (T0) and after 2 (T1) and 4 (T2) cycles of bi-weekly nivolumab. Functional molecules (PD-1, CD3ζ, Granzyme B, Perforin), cell proliferation (Ki67) and NK receptors (NKG2 A, NKG2D, NKp30) were also explored. The immunohistochemical evaluation of PD-L1 and TILs was restricted to available tumor biopsies. Tissue and circulating parameters were correlated to clinico-pathological features and treatment outcomes.

Results: *KRAS* mutations, active smoking, COPD and steroid treatment conditioned a different distribution of circulating phenotypes. At baseline, clinical benefit (CB, n = 19) group displayed higher number of phenotypically active NK and PD-1+CD8+ cells ($p < 0.01$) compared to non-responders (NR, n = 12). Prolonged survival outcomes ($p < 0.01$) were recorded in cases with high baseline circulating NK and PD-1+CD8+ cells. At tissue level, low PD-1 expression in CD8 + TILs was a positive prognostic feature ($p < 0.001$). Strikingly, high circulating NK and PD-1+CD8+ cells combined with low PD-1/CD8+ ratio in TILs characterized a privileged context able to provide a significantly prolonged ($p < 0.01$) progression-free survival (PFS). During PD-1 blockade, NKs progressively raised in CB while declined in NR ($p < 0.05$) and this phenomenon was counterbalanced by parallel changes in Treg.

Conclusion: The functional pool of circulating NKs associated with a divergent PD-1 expression in blood and tissue CD8+ lymphocytes portrays an immune profile predictive of anti-PD1 treatment efficacy.

1. Introduction

Cancer development and progression are complex and dynamic processes regulated by non-random adaptations resulting from a continuous interaction between the tumor and its microenvironment. A delicate although active balance is established with the host in which cancer editing through immune checkpoints involvement ultimately triggers anti- or pro-tumorigenic contexts. In this scenario, specific

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inhibitors of several checkpoint related pathways have received particular attention due to an unparalleled efficacy in advanced hematologic and solid malignancies, including non-small cell lung cancer (NSCLC) [1–3].

The identification of NSCLC immune contextures with prognostic and predictive impact has been largely focused on tumor infiltrating lymphocytes (TILs) and/or on the expression of programmed death-ligand 1 (PD-L1) in neoplastic and, to a less extent, in immune cells [4–7]. Significant advances have been obtained by the introduction of standard criteria for PD-L1 evaluation and, more notably, by a comprehensive phenotypic and functional characterization of TILs, including the evaluation of programmed death-1 (PD-1) expression on specific cell subpopulations [8–10]. In addition, more advanced classifications of the broad spectrum of the tumor immune microenvironment (TIME) might proficiently predict the response to immunotherapy and will likely define new targetable therapeutic paths [11].

Although a local contribution by tumor draining lymph nodes and tertiary lymphoid structures (TLS) occurs, cancer immune editing and surveillance is largely accomplished systemically [12]. Tissue and circulating compartments enclose two contrasting pools of effector (CD8+, CD4+ and NK) and suppressor (Treg and Myeloid Derived Suppressor Cells, MDSCs) phenotypes, known to exert opposite effects on cancer development [13,14]. Interestingly, phenotypes operative at tissue level to shape the immune response are similarly present in the blood [15,16]. Indeed, following immune checkpoints blockade (ICB), an intense lymphocyte trafficking has been strongly suggested in NSCLC and melanoma patients by the documentation of cycling (Ki67+) CD8+ and PD-1+ lymphocytes shared by tissue and blood [17–19]. Importantly, this population was able to predict the response to ICB, indicating that a competent local anti-tumor immune activation is conveyed by a circulating phase. An additional relevant observation arose from these studies uncovers the double edged function of PD-1 receptor in lymphocytes, which upon PD-L1 pressure at tumor site displays an inhibitory activity, while following its in vivo cleavage is involved in reinvigoration of circulating cytotoxic cells [20].

The extraordinary success of ICB, still in a limited population of NSCLC patients, and the emerging incidence of immune related adverse events [21], underscore the urgent demand to define predictive parameters. A comprehensive characterization of both tissue and circulating immune cells compartments may conceivably increase the threshold for the identification of valid biomarkers. This approach has been successfully employed in melanoma [22,23], while similar analyses have been partly described in NSCLC, where counts of peripheral neutrophils and lymphocytes, usually combined in a ratio and possibly incorporating LDH values, represent prognostic and predictive factors [24–26].

Thus, the present work was addressed to determine whether specific subsets of circulating cells might predict the outcome of nivolumab treatment in advanced NSCLC. To provide an accurate immune profile, the number and activated phenotype of peripheral blood subpopulations were prospectively investigated and integrated in a restricted group of patients with the tissue immunophenotypic features. Based on our findings, the circulating number of functionally competent NK and CD8 cells may represent a potential predictive parameter of the benefit from anti-PD1 therapy.

2. Materials and methods

2.1. Patient population

The clinico-pathologic characteristics of our patient population are resumed in Table 1. According to RECIST criteria 1.1 [27], patients were categorized, in clinical benefit group (CB), including complete (CR) or partial (PR) response or stable disease (SD) lasting at least 6 months and non responders (NR), including patients with disease progression and stable disease lasting less than 6 months as best response.

Table 1
Patient Population.

Age, years (Mean ± St. Dev.)		Patients n: 31 68.2 ± 9.7 n (%)
Histotype	Squamous	17 (55)
	Non Squamous	14 (45)
Sex	Male	23 (74)
	Female	8 (26)
Smoking status	Smokers	6 (19)
	Ex-Smokers	21 (68)
	Non Smokers	4 (13)
ECOG PS	0-1	27 (87)
	2	4 (13)
Stage	IIIB	5 (16)
	IV	26 (84)
Metastatic Involvement	Lymph nodes	28 (90)
	Liver	3 (10)
	Bone	5 (16)
	Adrenal	4 (13)
	Brain	3 (10)
	Controlateral lung	12 (39)
	Pleura	13 (42)
	Others	9 (29)
Mutations	EGFR	0/16 (0)
	K-RAS	6/11 (54)
	ALK	0/13 (0)
	ROS-1	0/7 (0)
Steroids	Before	6 (19)
	During	11 (36)
	Never	14 (45)
Nivolumab Treatment	II line	22 (71)
	III line	6 (19)
	≥ III line	3 (10)

n: number of cases; ECOG: Eastern Cooperative Oncology Group.
PS: Performance Status.

Entry criteria and clinical protocol are detailed in Supplementary (Suppl.) Material and Methods.

2.2. Peripheral blood flow cytometry

Peripheral blood (PB) was collected right before the first (baseline, T0), the third (T1, week 4) and the fifth (T2, week 8) nivolumab administration and analysed by FACSCanto II flow cytometer (BD Biosciences) using the FACSDiva Software.

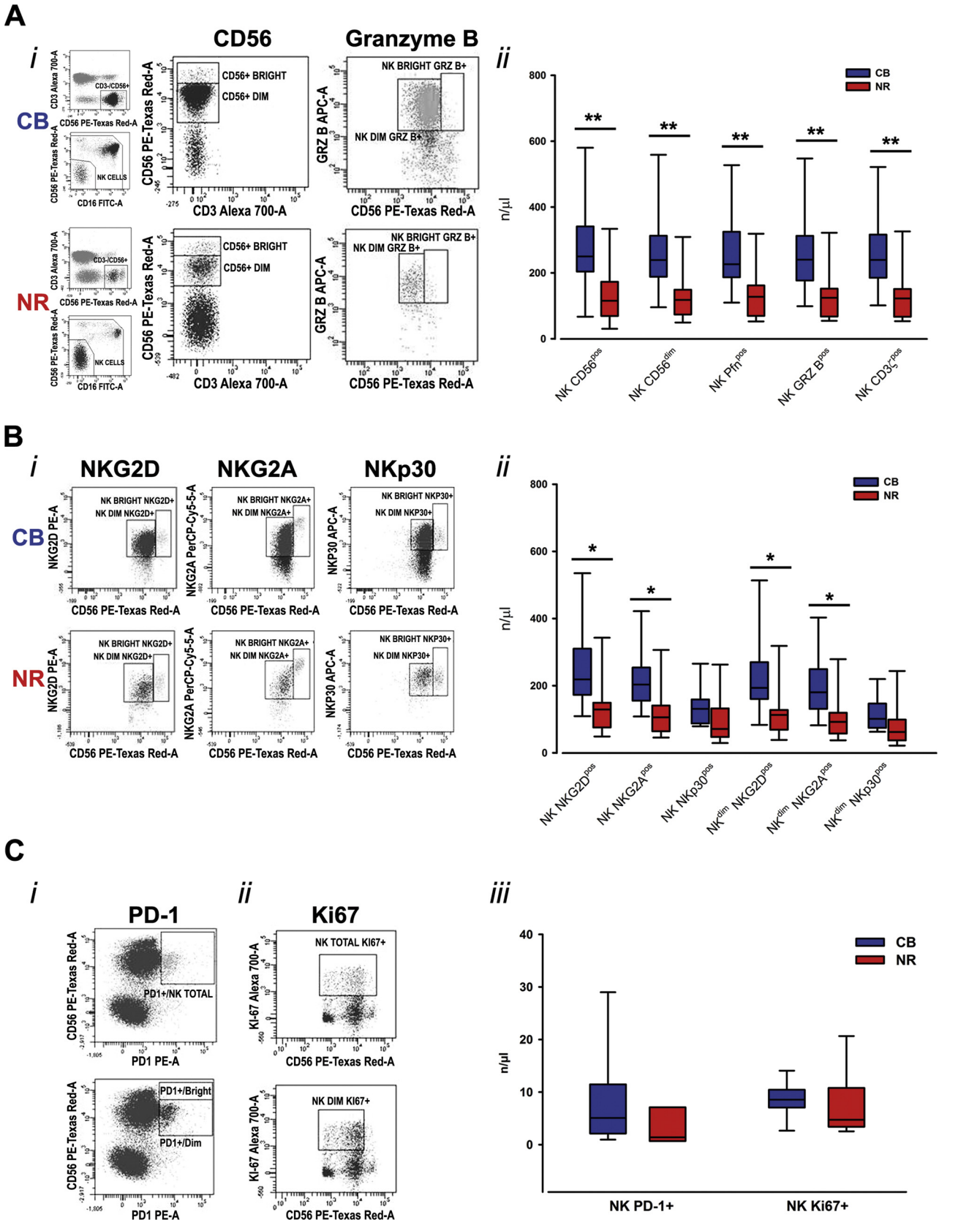
A previously employed gating strategy [28] to assess the frequency, absolute number, functional molecules, including PD-1, and proliferative index (Ki67) of circulating T lymphocytes, NK cells and MDSCs, is detailed in Suppl. Material and Methods and Suppl. Fig. S1-S2. Median, mean and range values of each measured phenotypes are reported in Supplementary Table S1.

2.3. Immunohistochemical analysis

Five μm thick sections from formalin fixed and paraffin embedded NSCLC biopsies were subjected to the immunohistochemical evaluation of PD-L1 and the incidence of TILs subpopulations (Suppl. Fig. S3). This methodology, previously described by our laboratory [29], is detailed in Supplementary Material and Methods.

2.4. Statistical analysis

The Fisher's exact test was used to examine the differences in categorical variables, and the Mann-Whitney test to detect differences in continuous variables between groups of patients, given that the distribution of data was not normal (Kolmogorov –Smirnov test). Overall survival (OS) and progression-free survival (PFS) were estimated by means of the Kaplan Meier method (see Suppl. Materials and Methods).



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ROC curves were used to test the sensitivity and specificity of a marker, and to find the cut-off values, with the area under the curve (AUC) being given with its 95% confidence interval (CI) (see Suppl. Materials and Methods).

Log-rank test was performed to determine the difference in survival between groups. PFS and OS data were then analysed through Cox

Fig. 1. The Number and Functional Phenotype of Baseline Circulating NKs Impact on the Response to Nivolumab. A*i*: sequential dot plots illustrating the Flow Mass Cytometry (FMC) strategy for the detection of CD3-CD16 + CD56 +, CD56^{dim} or CD56^{bright} and granzyme B + (GrzB) NKs in peripheral blood of a representative patient from Clinical Benefit (CB) and Non Responder (NR) groups. A*ii*: box plots graph documenting the difference between CB and NR in the absolute number (n/μL) of total (CD56 +), CD56^{dim}, perforin + (Pfn), GrzB + and CD3ζ + NKs. ** $p < 0.01$. B*i*: representative baseline peripheral blood FMC of a CB (upper panel) and NR (lower panel) patient to document activating NKG2D, inhibitory NKG2 A and natural cytotoxic NKp30 receptors on CD56^{dim} and CD56^{bright} NKs. B*ii*: box plots graph of the absolute circulating number of total and CD56^{dim} NKs carrying NKG2D, NKG2 A and NKp30 receptors in CB and NR patients. *$p < 0.05$. C: representative dot plots of flow cytometry showing the expression of PD-1 receptor (*i*) and Ki67 (*ii*) on total (top) and CD56$^{bright/dim}$ (bottom) NK cells. C*iii*: bar graphs of the quantification of the absolute circulating number of PD-1 + and cycling Ki67 + NK in CB and NR patients at baseline.

regression multivariate models. p value of 0.05 was always considered significant. IBM SPSS Statistics v 24.0 (IBM) was used to perform the computational analyses.

3. Results

Clinical benefit (CB) group included two CR (6.4%), five PR (16.2%) and twelve SD (all lasting at least 6 months, 38.7%), while 12 PD (38.7%) constituted the non-responders (NR) group.

Overall Response Rate (ORR) was 22.6%; median OS was 23 months (95% CI: 15.52–30.47) and median PFS 12 months (95% CI: 0.19–23.81) (Suppl. Fig. S4). At the time of data cut-off (30th December 2017), patients had received a median of 18 Nivolumab cycles (range 4–52). Three patients are still under treatment and six long-term responders have stopped nivolumab after 2 years.

Among the clinico-pathologic characteristics, only ECOG performance status (PS) 0-1 had a positive impact on clinical outcomes compared to PS 2 (Suppl. Tables S2 and S6).

3.1. Impact of clinico-pathological features on circulating phenotypes

3.1.1. Histotype

Compared to squamous cell carcinoma (SCC), baseline PB of adenocarcinoma (ADC) patients showed higher absolute number of CD3 + ($p = 0.015$) and CD8 + ($p = 0.005$) lymphocytes, which also displayed increased expression of Granzyme B (GrzB, $p = 0.014$) and Perforin (Perf, $p = 0.019$) (Suppl. Fig. S5 A).

3.1.2. Comorbities and smoking status

Active smoking (Suppl Fig. S5B) and Chronic Obstructive Pulmonary Disease (COPD, Suppl. Fig. S5C) appeared to condition a meaningful increased Ki67 labeling in T lymphocytes ($p = 0.034$) and NK ($p = 0.028$). COPD was also coupled with a significant reduction in CD14 + /CD33 + /DR- MDSCs ($p = 0.044$). Diabetes, cardiovascular, chronic kidney and hepatic diseases did not impact on PB parameters.

3.1.3. ECOG performance status (PS)

Patients with PS 2 showed reduced number of CD3, CD8 and NK cells, together with a 2-fold and 1.5-fold increase in MDSCs compared, respectively, to PS 0 ($p = 0.04$) and 1 ($p = 0.03$) (Suppl. Fig. S5D).

3.1.4. Steroids

NSCLC patients not receiving steroids displayed higher number of CD3 +, CD4 +, CD8 +, CD19 + lymphocytes and NK (absolute number, CD3ζ, dim, Perf and GrzB), and a significant reduction ($p < 0.05$) in MDSCs specifically when compared to patients on steroids before nivolumab (Suppl. Fig. S5E).

3.1.5. Number and location of involved metastatic sites

Lymph node involvement was linked with lower number of total ($p = 0.044$), GrzB + ($p < 0.01$) and Perf + ($p < 0.05$) CD8 lymphocytes (Suppl. Fig. S5F). Bone and liver metastases appeared to be associated with a reduction in CD3 +, CD8 +, CD4 + and NK cells as in their proliferative index (data not shown).

3.1.6. Mutational status

KRAS mutations conditioned higher number of CD3 + lymphocytes ($p = 0.043$) and a slight increase in CD8 +, CD4 +, CD19 + and NK cells including their functional activity. Intriguingly, in *KRAS* mutant cases PD-1 was highly expressed by CD8 lymphocytes reaching a 2.5-fold increase ($p = 0.039$) vs. wild-type. *KRAS* mutation was also associated with a 45% reduction ($p = 0.04$) in circulating MDSCs. See Supplementary Fig. S5G. Other mutations had no impact on PB parameters.

3.2. Baseline circulating and tissue immunophenotypic features and response to nivolumab

3.2.1. Circulating NKs and response to nivolumab

At baseline, the absolute number of circulating CD56 + NK cells resulted 2-fold higher in CB compared to NR ($p < 0.01$). Specifically, CD56^{dim} NK cells carrying Perf, GrzB, and CD3ζ, reflecting a more cytotoxic phenotype, were enhanced in CB vs. NR ($p < 0.01$) (Fig. 1A). Importantly, NK and CD56^{dim} NK carrying NKG2D, NKp30 and NKG2 A receptors were nearly 2-fold higher ($p < 0.05$) in peripheral blood of CB patients compared to NR (Fig. 1B). Fisher's exact test confirmed the striking impact of NK cells number and functional phenotype on the response to anti-PD-1 therapy (Suppl. Table S3).

A small fraction of NK cells also expressed PD-1 receptor (Fig. 1C*i*) and were cycling (Ki67 +) (Fig. 1C*ii*), however without statistical difference between CB and NR (Fig. 1C*iii)*. Interestingly, focusing our attention on CD56 dim or bright NK cells, we documented that PD-1 was differentially expressed in the two NK phenotypes. Specifically, the percentage of NK bright cells carrying PD-1 receptor resulted significantly higher compared to NK dim (Mean ± St.Err: 8.35 ± 1.74 vs 1.20 ± 0.25, $p < 0.001$, data not shown).

3.2.2. Circulating T lymphocytes and response to nivolumab

No distinctive features between NR and CB were observed at baseline in terms of number of circulating T and B lymphocytes, CD25^{pos} FOXP3^{high} Treg and MDSCs. The proliferative index (Ki67) and the expression of functional molecules (GrzB and Perf) in CD8 + and CD4 + cells were also similar in the two groups. See Supplementary Table S4.

However, a 2-fold increase ($p < 0.05$) in percentage and absolute number of circulating CD8 + lymphocytes expressing PD-1 receptor was detected in CB compared to NR. In line with these findings, the calculated PD-1/CD8 ratio within circulating T lymphocytes resulted 1.7-fold higher in clinical benefit group compared to non responder patients ($p < 0.05$) (Fig. 2A).

Interestingly, PD-1 tended to be more represented also in CD4 + lymphocytes from CB (Fig. 2A).

A 31% increase (ns, $p < 0.08$) in effector memory phenotype in CB vs. NR (Suppl. Table S4) was present while no differences were seen in naive or central memory cells as in their PD- 1 expression (data not shown).

Intriguingly, when the numerical ratio between the average pooled population of circulating effector (n/μl: NK CD56^{dim} + PD-1^{pos}CD8^{pos}Ki67^{pos}) and suppressor (n/μl: CD33^{pos}CD14^{pos}DR^{neg} MDSC + FOXP3^{high} Treg) cells was computed, a significantly higher value ($p = 0.042$) was measured in CB (ratio: 7.41) vs. NR (ratio: 4.64) patients.

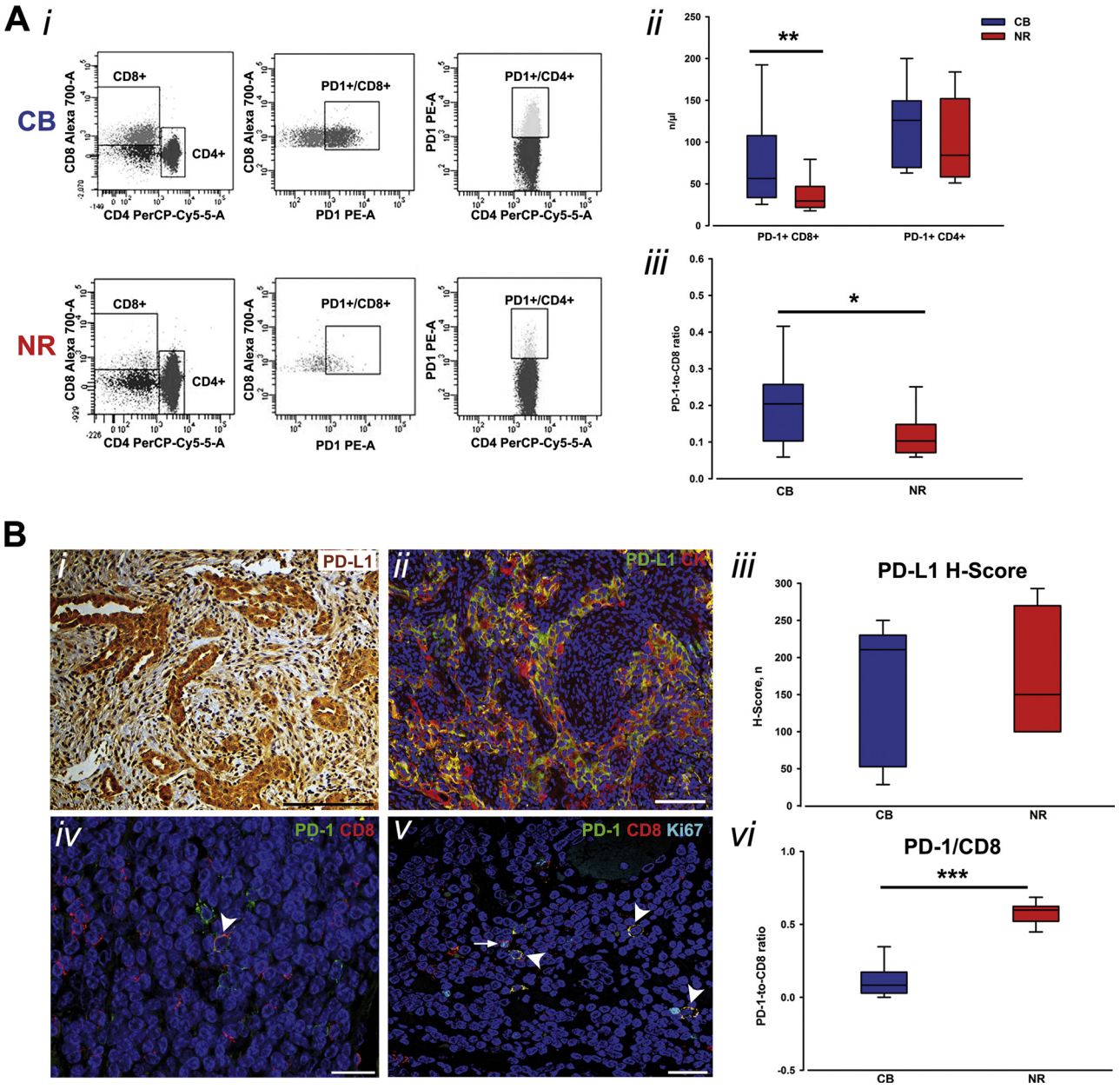

Fig. 2. PD-1 Expression in Baseline Circulating and Tissue CD8 + Cells Impacts on the Response to Nivolumab. A*i*: representative peripheral blood flow cytometry of a CB and NR patient for the detection of PD-1 receptor in baseline circulating CD8 + and CD4 + lymphocytes. A*ii*: box plots of the absolute number of circulating PD-1 + CD8 + and PD-1 + CD4 + lymphocytes in CB and NR patients. ** $p < 0.01$. A*iii:* box plot of the ratio of PD-1 + cells over the total number of PB CD8 + lymphocytes in CB and NR patients. * $p < 0.05$. B: sections from ADC (*i*) and SCC (*ii*) samples to illustrate PD-L1 expression by immunoperoxidase (*i,* brownish) or immunofluorescence (*ii*) in which yellowish fluorescence corresponds to double PD-L1 (green) and cytokeratin (CK, red) labelling of cancer cells. ***iii***: box plots of PD-L1 quantification by H-score in clinical benefit (CB) and non responders (NR) patients. *iv, v:* sections from NSCLC to document by immunofluorescence the surface expression of PD-1 (green) and CD8 (red) in two large clusters of TILs. Yellowish fluorescence corresponds to PD-1 + CD8 + lymphocytes (arrowheads). The nuclear expression of Ki67 (bright) is also shown in ***v*** to illustrate that the only cycling CD8 + lymphocyte (arrow) is PD-1 negative. Blue fluorescence in *ii, iv* and *v* corresponds to DAPI staining of nuclei. Scale Bars: *i* = 200 µm; *ii* = 100 µm; *iv, v* = 20 µm ***vi***: quantification of the rate of PD-1 expression on CD8 + TILs in CB and NR. *** $p < 0.001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2.3. *Tissue immunophenotypic features and response to nivolumab*

In a subset of patients, in which both tissue and PB samples were available (n = 15), a comprehensive characterization of the immune profile was performed (Suppl. Fig. S3). While samples from CB showed higher number of CD3 + and CD8 + TILs compared to NR (Suppl. Table S5), the density of CD4 +, CD57 + NK, GrzB + lymphocytes and CD68 + Tumor Associated Macrophages (TAM) did not display a significant impact on treatment response. In our limited patient population, PD-L1 score did not distinguish CB from NR (Fig. 2B*iii*, Suppl. Table S5), and no clear reciprocal correlations between the number of tissue and circulating T and NK cells were observed. However, low PD-1 expression in CD8 + TILs was present in 100% of CB patients (Suppl. Table S5, $p < 0.001$), confirming our previous findings [29] that PD-1 to CD8 ratio represents a distinctive feature of CB tissue immune microenvironment ($p < 0.001$, Fig. 2B*vi*).

3.2.4. *Impact of integrated immune profiles on the response to Nivolumab*

The statistical predictive power of circulating parameters associated to treatment response was then approached by the assessment of the Receiver Operating Characteristic (ROC) curve. High sensitivity and

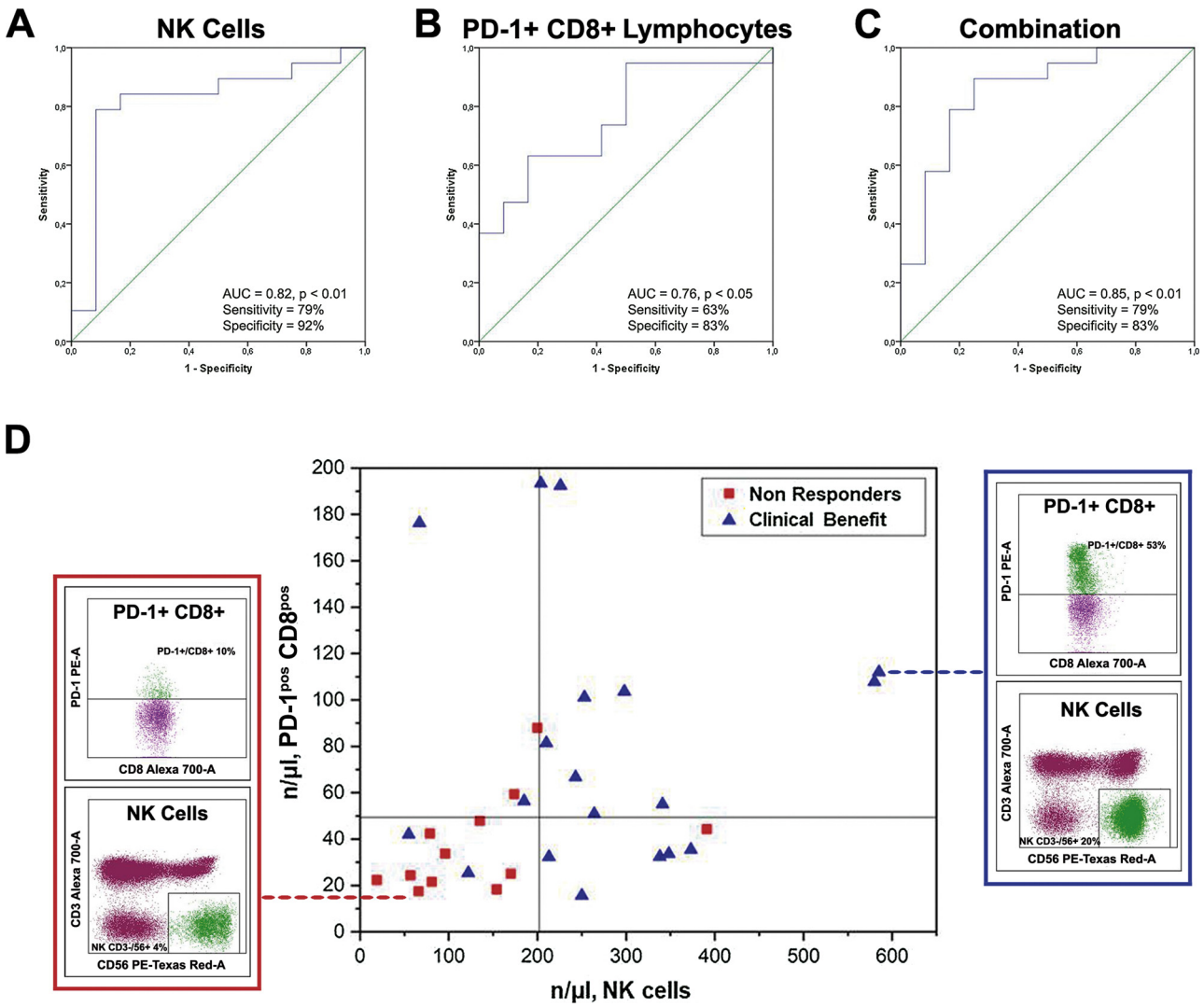

Fig. 3. Predictive Role of Baseline Number of Circulating NK and PD-1 + CD8 + cells. A: Receiver Operating Characteristic (ROC) curves of the number of baseline circulating NK (A), PD-1 + CD8 + (B) cells and their combination (C) to assess cut off values and sensitivity and specificity of each parameter. The area under the curve (AUC), p values and % specificity and sensitivity are reported. D: plotting on a Cartesian plane the absolute number of baseline circulating NK and PD-1 + CD8 + cells of the entire study population (n = 31) and applying a gate according to their respective cut off values, four different subsets of patients were defined. All cases falling into the upper right square belonged to the CB group while 75% of NR cases segregated in the lower left. Dotted lines connect the plotted value of a representative CB (blue) and NR (red) case to the corresponding FMC analysis of NK and PD-1 + CD8 + cells (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

specificity were documented testing NK cells number, reaching 0.82 AUC (0.65-0.99, $p < 0.01$) (Fig. 3A). Considering PD-1 + CD8 + lymphocytes, AUC was 0.76 (0.59-0.93, $p < 0.05$) (Fig. 3B). Although within a relatively limited number of observations (n = 15), testing tissue PD-1 to CD8 ratio, AUC intriguingly resulted 1.00 ($p < 0.01$) with 100% sensitivity and specificity (not shown).

Based on our findings, circulating NK and PD-1 + CD8 + lymphocytes were entered together in a multivariate logistic regression model in order to assess whether their combination had more predictive power than that of individual parameters. The probabilities calculated by the bivariate model were used to create the corresponding ROC curve. Nevertheless, at established cut-off, no relevant improvement over the predictive power of NK cells alone was obtained (AUC = 0.85, CI 0.70-0.99, $p < 0.01$) (Fig. 3C).

Conversely, as graphically represented in Fig. 3D, combining the cut-off values calculated for the two candidate biomarkers, NK and PD-1 + CD8 + cells, all the cases (10/10) with both parameters above their respective cut-off belonged to the CB group while 75% of NR had both parameters below cut-offs.

3.3. Impact of immune profiles on survival outcome

Although not reaching statistical significance on multivariate analysis (data not shown), peripheral blood NK subpopulations, PD-1 + CD8 + lymphocytes, and tissue PD-1-to-CD8 ratio paralleled the prognostic role reported for the response to treatment on univariate analysis (Suppl. Table S6). Circulating levels of B, T and MDSCs cells had no impact on survival outcome.

Kaplan-Meier survival curves (Fig. 5) documented longer OS ($p = 0.014$) and PFS ($p = 0.025$) in patients with high NK cells (> 202/µL) compared to their counterpart (Fig. 4A). Importantly, high number of circulating PD-1 + CD8 + lymphocytes (> 49/µL) was associated with prolonged ($p < 0.01$) OS and PFS (Fig. 4B). According to tissue parameters, patients with low ratio of PD-1 to CD8 TILs (< 0.45) displayed a significant increase ($p = 0.001$) in OS and PFS (Suppl. Fig. S6).

Finally, to provide a prognostic score, we explored the statistical power of combining the circulating levels of NK cells (< 202/µL vs. ≥ 202/µL) and PD-1 + CD8 + lymphocytes (< 49/µL vs. ≥ 49/µL)

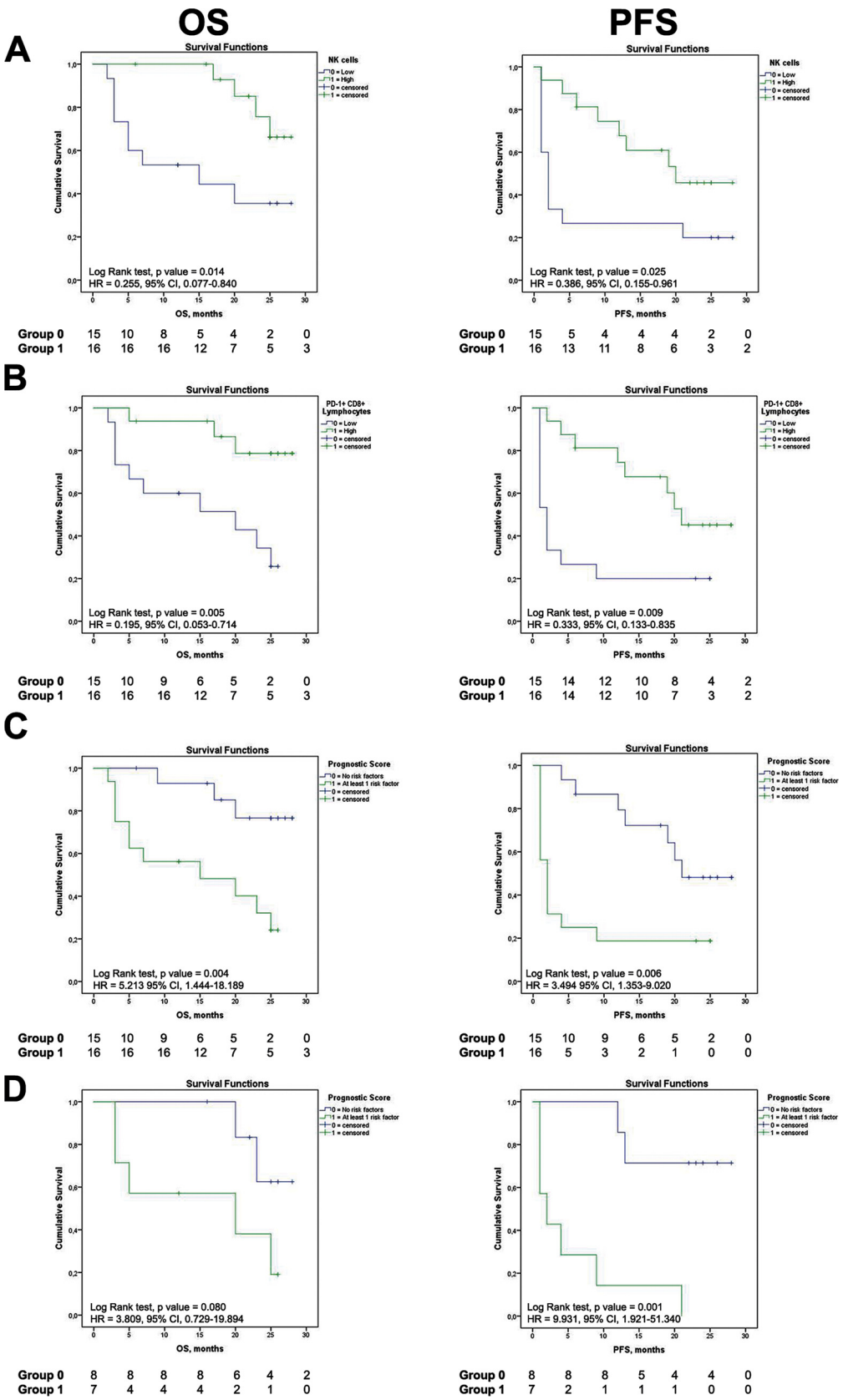

Fig. 4. Prognostic Parameters. Kaplan Meier survival curves showing the impact on overall survival (OS) and progression free survival (PFS) of the absolute number of baseline circulating NK (A) and PD-1 + CD8 + (B) cells. C, D: two prognostic scores were generated based on our three risk factors represented by low NK and low PD-1 + CD8 + cells in peripheral blood, and high PD-1-to-CD8 ratio at tissue level. C: Kaplan Meier analysis documenting the impact on OS and PFS of the combination of circulating NK and PD1 + CD8 + cells. The survival outcome resulting from the integration of peripheral blood NK and PD-1 + CD8 + with tissue PD-1-to-CD8 ratio is shown in D. The number of observations for each analysis is reported below corresponding curves.

(Fig. 4C) as well as their integration with tissue PD-1 to CD8 ratio (< 0.45 vs. ≥ 0.45) (Fig. 4D). Significantly reduced PFS was documented in the presence of at least one of the risk factors, while only the combination of peripheral blood NK with PD-1 + CD8 + had a significant impact on OS. Specifically, the comprehensive analysis of circulating and tissue parameters revealed that patients carrying one of the risk factors displayed a median PFS of 2.0 months vs. not reached (HR: 9.931, 95% CI, 1.921–51.340, p = 0.001).

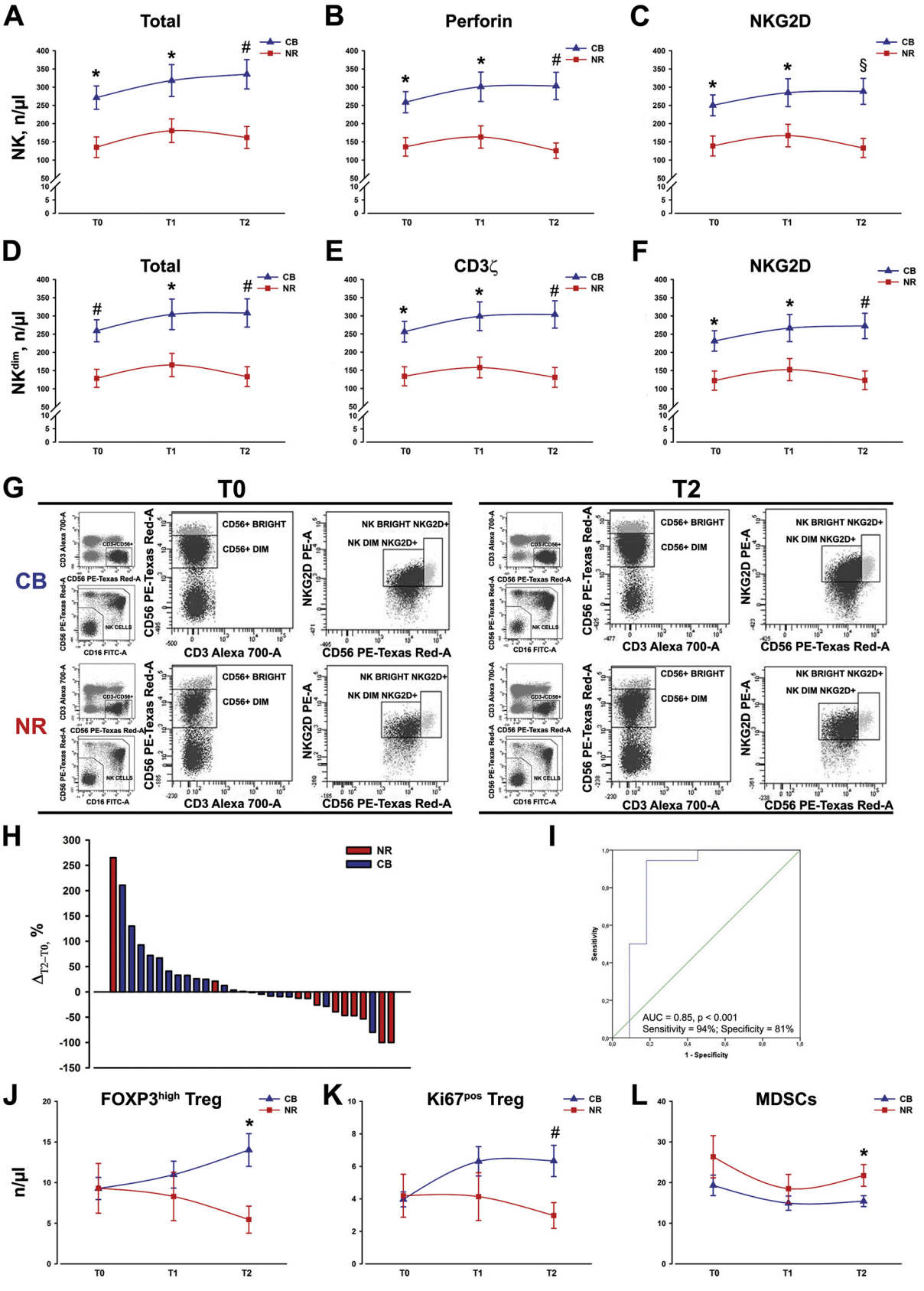

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Fig. 5. Kinetic of Circulating NK, Treg and MDSC During Nivolumab. Line plots of the absolute number of overall (A), perforin + (Pfn, B) and NKG2D + (C) NKs at baseline (T0) and after 2 (T1) and 4 (T2) nivolumab cycles in Clinical Benefit (CB, blue) and Non Responder (NR, red) patients. The corresponding changes with time in the number of overall, CD3ζ + and NKG2D + $CD56^{dim}$ NKs are reported, respectively in D, E and F. *$p < 0.05$; # $p < 0.01$; § $p < 0.001$. G: gating strategy for the quantification of CD3-CD16 + CD56 + and $CD56^{dim/bright}$ NKs carrying NKG2D + receptor at baseline (T0) and following 4 nivolumab administrations (T2) in representative CB and NR blood samples. H: waterfall plot graph representing the Δ % variation of NKs from T0 to T2 in CB (blue) and NR (red) patients. Noticeably, the NR outlier corresponds to a case that displayed the lowest NK count at baseline. I: ROC curve generated by Δ % values of NKs from T0 to T2. AUC, p values and % specificity and sensitivity are reported. J-L: line graphs showing changes in $FOXP3^{high}$ (J) and proliferating (K) Tregs, and CD33 + CD14 + DR- MDSCs (L) in CB and NR during nivolumab treatment. * $p < 0.05$ vs NR; # $p < 0.01$ vs NR (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.4. Kinetic of circulating immune cells during nivolumab

3.4.1. NK cells

The observed higher number of baseline circulating NK cells in CB vs. NR was sustained during treatment (Fig. 5). Specifically, a progressive rise in this population occurred in CB after 2 and 4 nivolumab administrations, while NK remained stable in NR to slightly decrease at T2 (Fig. 5A). Similarly, as shown in Fig. 5B-F, the difference between CB and NR in terms of $CD56^{dim}$ and cytotoxic (GrzB, Perf and CD3ζ) NK persisted at all the investigated time points. Moreover, NK carrying NKG2 A, NKG2D and NKp30 progressively declined in NR while increasing in CB in a time dependent manner. No differences were observed in NK proliferation (Ki67).

Additionally, to explore whether the lack of NK cell expansion following ICB in non responders patients could be attributed to steroid therapy, a further statistical analysis was performed. According to Mann-Whitney test no significant variation in NK subpopulations were observed between patients receiving steroids and those steroid free ($p = 0.615$).

The significant clinical impact of NK number and function at baseline together with their differential circulating dynamic in CB and NR following nivolumab, prompted us to determine whether their variation from baseline to T2 (Δ) could be associated with therapeutic benefit. As shown by the waterfall plot graph (Fig. 5H), NK number increased from T0 to T2 in 68% of CB while dropped in 85% of NR and similar results were obtained testing different NK phenotypes and receptors (data not shown). Importantly, focusing on percent changes in the total number of NK, the AUC of ROC curve generated from Δ values reached 0.85 ($p < 0.001$) (Fig. 5I). Furthermore, values above the established cutoff were able to segregate patients with markedly prolonged OS and PFS ($p < 0.001$) (data not shown).

3.4.2. Lymphocytes and MDSCs

As a result of PD-1 blockade, a progressive increase in CD3 ($p = 0.06$) and CD4 lymphocytes was observed in CB, reaching statistical difference vs. NR at T2 ($p < 0.05$). Conversely, no statistically significant changes were observed in both groups in terms of absolute number of CD8 + and CD19 + lymphocytes. See Supplementary Fig. S7.

Treg ($FOXP3^{high}$) and MDSCs number, although not different at baseline, displayed a distinctive profile in NR and CB during nivolumab. Specifically, the percentage and absolute number of Treg and activated CD45RA-CD25 + $CD127^{low}$ $FOXP3^{high}$ Treg progressively rose in CB while decreasing in NR, to reach a statistically significant difference at T2 ($p < 0.05$; Fig. 5J). Ki67 labelling in $FOXP3^{high}$ lymphocytes was also higher in CB vs. NR after 4 nivolumab courses ($p < 0.01$; Fig. 5K). Conversely, CD33 + CD14 + DR- MDSCs tended to be slightly higher in NR however reaching significant difference vs. CB only at T2 ($p < 0.05$; Fig. 5L).

When we tested the expression of PD-1 on peripheral blood CD8, CD4 and NK cells during treatment, a full saturation of the receptor was observed hampering the detection of its time dependent variation in circulating cell populations (Supplementary Fig. S8).

4. Discussion

Controversial results have been reported on the quantitative and qualitative assessment of clinically relevant immune profiles able to predict the efficacy of ICB [5,7,30,31]. Our extensive immunophenotypic analysis, prospectively undertaken on advanced NSCLC patients treated with nivolumab, documented that baseline levels of circulating NK carrying cytotoxic molecules and functional receptors together with the size population of effector cytotoxic lymphocytes expressing PD-1 were able to identify patients who benefit from anti-PD1 therapy. Based on our promising findings, in series cytometric assay of the number of peripheral blood NK and PD-1 + CD8 + cells might be proposed as a simple and clinically feasible approach to predict the response to PD-1 blockade.

The functional state of cytotoxic cells is finely regulated by a series of activating and inhibitory molecules. Specifically, the expression of PD-1, Perf and GrzB on circulating CD4 +, CD8 + and NK cells, respectively, outlines the effector cells population. Additionally, the tumor killing ability of NK has been ascribed to the expression levels of CD56 and CD3ζ and is adjusted by inhibitory (NKG2 A) or activating (NKG2D and NKp30) receptors, which ultimately define the efficiency of the innate support to cancer immune surveillance [32–34]. All these aspects together with the proliferative index have been investigated here indicating that NSCLC patients provided by an active pool of circulating cells with anti-tumor potential are prone to respond to anti-PD1 therapy.

The positive clinical impact of NK activation is conceivable and it has been widely documented in several tumors following chemotherapy [35–37]. However, robust data on the specific contribution of NK cells to the response to anti PD-1/PD-L1 drugs are limited. In support of our findings are recent evidences that human NKs expressing PD-1 can be enhanced by ICB [13] and innate-like subsets of NK carrying an invariant T cell receptor (iNKT), lead to the activation of NK effector subpopulations upon PD-1/PD-L1 blockade [38]. In peripheral blood from melanoma patients undergoing PD-1 blockade, the number of baseline functionally active NK cells expressing either the activation marker CD69 or the chemokine MIP-1β correlated with disease response [39]. These observations, together with the discovery that NKs recruit dendritic cells at tumor sites [40], highlight the leading role of these cells in cancer immune surveillance.

An important aspect still requiring further insights is related to the double edge sword of PD-1 receptor. Although the PD-1 pathway has been generally ascribed to T cell exhaustion and tumor immunosuppression, PD-1 receptor is not an exhaustion-specific molecule as all effector T lymphocytes express PD-1 upon activation [17–20]. PD-1 + CD8 + cells can also be found in the circulation of healthy humans where do not always resemble exhausted T cells [41]. Several other cell types express PD-1, including B cells, myeloid cells, cancer cells [20,42] and, as confirmed by the present study, natural killer (NK) cells and memory T cells. With regard to this latter population, we registered a trend towards an increase of the effector memory phenotype in patients responsive to nivolumab when compared to non-responders, whereas neither baseline nor longitudinal differences were observed for naive or central memory cells. The progressive increase of a subset of central memory CD4 + T cells have nevertheless be reported as predicting durable clinical benefit in melanoma patients receiving anti-PD-1 agents [43]. Thus, the overall impact of the systemic neutralization of PD-1 by ICB is far from being elucidated because receptor timing and location in addition to antigen burden and metabolic states, all affect

the functional outcome of PD-1 engagement [16].

Along with these contentions is our observation that the diverging PD-1 expression in CD8 lymphocytes at tissue and peripheral blood sites equally results in positive clinical outcome. This potentially confounding issue requires an interpretation. In peripheral blood, PD-1, being mainly involved in activation of CD8+ lymphocytes, identifies a population of circulating effectors cells feasibly rescued by its therapeutic cleavage; conversely, at tissue level, the observed low expression of PD-1 may allow CD8+ cytotoxic TILs to elude PD-L1 pressure, thereby eliciting tumor killing potential. According to this view, the activating and inhibitory PD-1 pathways, respectively translated in high peripheral blood PD-1+CD8+ Ki67+ and low tissue PD-1+CD8+ TILs, were able to identify an immune profile highly predictive of nivolumab efficacy. The potential role of the circulating PD-1+CD8+Ki67+ population has been deeply studied by Huang and collaborators, analyzing repeated blood samples in melanoma patients undergoing pembrolizumab [44]. The dynamic increase of the mentioned phenotype, normalized for each patient tumor burden, correlated with clinical benefit. Of note, further characterization of the PD-1+CD8+Ki67+ population revealed their exhausted phenotype suitable of clinically convenient invigoration [44].

Although the tumor mutational burden (TMB) was not investigated here, an additional finding of our study was the correlation between *KRAS* mutations and immunophenotypic features. This mutation may be considered a poor prognostic and predictive factor, because of the recent discoveries that oncogenic RAS stabilizes PD-L1 mRNA [45] and confers an immune-suppressive tissue context specifically when the loss of *STK11/LKB1* is concurrently present [3,46,47]. However, the negative outcome of PD-L1 stabilization by *KRAS* is questionable and according to the positive impact of TMB on tumor immunogenicity and sensitivity to ICB, it has been shown that NSCLC patients carrying *KRAS* and/or *TP53* mutations display a better response to PD-1 blockade [48]. Finally, a comprehensive immune profile according to *KRAS* mutational status in NSCLC patients receiving immunotherapy has yet to be defined. We have previously shown that *KRAS* mutation conditions a different distribution of TILs in NSCLC tissue samples [29]. In the present study, *KRAS* mutated cases had higher circulating T cells and lower incidence of MDSCs while displaying an increased number of PD-1+CD8+ lymphocytes, which represented a positive predictor of the response to PD-1 blockade. Whether mutant *KRAS* itself or other associated pathways such as *STK11/LKB1* or *TP53* condition a different response to immunotherapy is under intense scrutiny.

Additional clinico-pathological features were associated with changes in specific circulating phenotypes. According to the documented inflamed “smoking signature”, increased proliferation of NK and T cells was present in current smokers and COPD, while steroids conditioned a reduction in T and NK cells together with a slight increase in MDSCs. Lymph nodes involvement was inversely correlated with the number and cytotoxic phenotype of CD8+ lymphocytes. All these observations may be relevant in clinical settings for the selection of patients to receive immunotherapy.

Although on-treatment changes of immune cells cannot be properly used to predict the clinical outcome, our data on NK and Treg kinetic strongly suggest that specific dynamic immune features might be associated with benefit from immunotherapy. The role played by Treg-NK interaction has been clearly highlighted in tumor models [49] and in patients with cancer [50]. Accordingly, we observed that, following PD-1 blockade, the rise in NK cells in CB patients was accompanied by a significant increase in number and proliferation of Tregs. Conversely, in patients who did not respond to ICB the number of circulating Tregs progressively declined. This observation is in line with the homeostatic control exerted by Tregs in attenuating NK cell immune reactions [49–51]. Tregs promptly reply to immune dynamic by proliferation reaching in mice and humans up to 50% in 10 days [52]. Indeed, as shown here, adaptive responses require a physiologic counterbalance largely exerted by Tregs, whose number and function are critical to prevent the onset and progression of malignant diseases in addition to patrol the advent of autoimmune and immunodeficient conditions.

Limitations of our study have to be acknowledged. The relatively low number of patients and the absence of a validation cohort might not allow a conclusive definition of peripheral blood parameters as biomarkers. Indeed, a validation study is undergoing to confirm the predictive significance of our proposed in series determination of NK and PD-1+CD8+ number by Flow Mass Cytometry (FMC). Additionally, in our cohort of advanced nivolumab treated NSCLC, the survival outcome was higher than that reported by other studies. This finding may reside in an unbiased positive selection according to the availability of PB samples at all the investigated time points that implied the exclusion of patients in which nivolumab was withdrawn before 8 weeks. Moreover, a comprehensive analysis of non synchronously collected PB and tissue samples was possible in only 50% of our consecutive NSCLC cases, potentially affecting the results. On the other end, this represents a common limitation in real life when studying advanced cases, in which most transbronchial biopsies do not allow adequate sampling to define TIME. Moreover we acknowledge that performing TMB analyses would have been meaningful, considering the current relevancy of this biomarker in addressing ICB activity [53] and the interest derivable by the correlations between immune parameters and TMB. Nevertheless, TMB has not entered clinical practice yet. Because of biological, technological, financial and practical (as the mentioned limitations intrinsic to the small biopsies in NSCLC) issues, we do not foresee its routine utilization in the very next future [54].

Finally, although representing our present achievements, the lack of in vitro functional characterization of lymphocytes and the incomplete analysis of the wide spectrum of circulating immune cells must be considered.

In conclusion, the present study indicates that the likelihood to benefit from immunotherapy is determined by the availability of an efficient pool of circulating cells (PD-1+CD8+ and NK cells) and strongly supports the notion that cancer immune surveillance is regulated by an intense reciprocal tumor-peripheral blood cross talk. Although circulating biomarkers are more accessible and clinically suitable, the road map to personalized immunotherapy entails a multidisciplinary approach aimed at decoding the multifaceted cancer immune landscape.

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Conflict of interest

All Authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.lungcan.2018.11.038.

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