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Original Research

Circulating innate immune markers and outcomes in treatment-naïve advanced non–small cell lung cancer patients

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Abstract ***Introduction:*** Innate immunity represents the first step of activation of the immune system and dictates the quality of adaptive immune responses. Studies have reported links between systemic inflammatory or innate immune markers and prognosis in patients with lung cancer. To our knowledge, the prospective and concomitant study of these systemic markers

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receptor;
NCR3/NKp30

has never been performed.
Methods: Advanced treatment-naive non–small cell lung cancer (NSCLC) patients eligible for first-line platinum-based chemotherapy were prospectively included from December 2012 to July 2015 ($N = 148$). Blood samples of patients were collected before the first cycle for fresh NK cell phenotyping. Peripheral blood mononuclear cells were cryopreserved for natural cytotoxicity receptor (NCR) genotyping as well as sera for NCR's ligand quantification. Data on leukocytes, neutrophils and monocyte counts and lactate dehydrogenase (LDH) levels were extracted from electronic medical records.
Results: Among all studied markers, monocytosis, neutrophilia, leucocytosis, high LDH and sBAG6 levels and reduced levels of NCR3 transcripts were associated with poor overall survival (OS) in univariate analysis. The levels of NCR3 transcripts was linked to age, number of metastatic sites, monocyte counts, LDH and sBAG6 levels. Neutrophilia was associated to high sBAG6 levels. NCR3 was the unique innate immune parameter that remained as an independent factor associated with both OS ($P = 0.003$) and progression-free survival ($P = 0.009$) in the multivariate analysis.
Conclusion: This study brought evidence that these biomarkers are entangled; parameters associated with an inflammatory process were related to reduced levels of NCR3 transcripts. Finally, the level of NCR3 transcripts was independently associated with outcomes in treatment-naive patients with advanced NSCLC.

1. Introduction

The innate immune system is considered the non-specific immune system in humans, representing the immediate response to defend the host against external agents, as several pathogens, or cancer but not providing long-lasting immunity to the host [1]. However, without proper activation of innate immunity, the adaptive response will not be effective, demonstrating the critical link between innate immunity and long-lasting protection.

In patients with cancer, inflammatory status at diagnosis can negatively affect prognosis of the patient and alter response to treatment [2]. Recently, it was shown in advanced non–small cell lung cancer (NSCLC) patients treated with immune checkpoints blockers (ICBs) that neutrophilia and high LDH levels could predict resistance to anti–programmed cell death protein 1 (PD-1) treatments, suggesting a link between inflammatory status and the capacity to activate or reactivate T cells properly during ICB therapy [3]. NK cells are the effector cells of the innate immune system, named after their ability to spontaneously kill transformed or infected cells, without prior immunisation [4]. Globally, NK cells cytotoxicity is determined by a balance between signalling of activating and inhibitory receptors, where the expression of major histocompatibility complex-I molecules and several NK receptors and ligands give them the ability to recognise target cells. The main activating receptors are the natural killer group 2 member D (NKG2D) and the natural cytotoxicity-triggering receptors (NCRs) commonly named NKp46/NCR1, NKp44/NCR2 and NKp30/NCR3. Previous evidence supports NK cell involvement in cancer development [5], The presence of tumour-infiltrating NK cells have often been associated with an improved disease-free survival and better overall survival (OS) in several cancer types [6–8]. In addition, both tumour-infiltrating and peripheral blood-NK cells of patients with cancer are endowed with decreased receptor expression and unsuitable cytotoxic function [9,10]. In NSCLC patients, tumour-infiltrating NK cells has been proposed as prognostic factors in the early stage of NSCLC [8,11–13], showing that an efficient NK effector function is associated with better outcomes, but so far there is no solid evidence regarding the role of NK cells or other innate immune cells in advanced NSCLC disease. Several immune cells related mainly to the adaptive response are being assessed as potential prognostic factors in advanced NSCLC in immunomonitoring studies, but so far no prior studies have evaluated the role of systemic innate immune markers in treatment-naïve advanced NSCLC population. Moreover and to our knowledge, the concomitant study of these systemic markers was never performed. In this report, we studied the impact of NK cell phenotyping, levels of NCRs transcripts, NCR3 isoforms, leukocytes, neutrophils and monocyte counts as well as levels of LDH and soluble BAG6 on patient's outcomes in a homogeneous prospective cohort of treatment-naïve advanced NSCLC patients.

2. Materials and methods

2.1. Patients and healthy volunteers

Advanced treatment-naïve NSCLC patients (stage IIIB/IV) eligible to first-line platinum-based chemotherapy

were prospectively included from December 2012 to July 2015 in our institution ($N = 148$). All patients were included in the MSN study (NCT02105168) and signed an inform consent. A peripheral blood sample (5–15 ml) of each patient was collected before the first cycle of chemotherapy. Clinical, pathological and molecular features were collected. White blood cell count, LDH and albumin levels before treatment (from 2 days to the day of beginning chemotherapy) were also collected. Blood samples ($N = 48$) of healthy volunteers (HVs) were obtained after inform consent signature and were collected according to a procedure validated by the CCPSL UNT-N°12/EFS/079.

2.2. Statistical analyses

For immune parameters, statistical analyses were performed using Prism 6 software (GraphPad, San Diego, CA). P values < 0.05 were considered significant. Groups were compared using Mann–Whitney U test or Wilcoxon matched pairs test where appropriate. For correlation statistics, the Spearman's correlation test was used. OS was defined as the time from the first date of platinum-based chemotherapy use until death. Patients alive were censored at their date of last follow-up. Progression-free survival (PFS) was defined as the time from the first date of platinum-based chemotherapy use to first progression or death. Patients alive without progression were censored at their date of last follow-up. OS and PFS were estimated using Kaplan–Meier method. The median follow-up was estimated using the reverse Kaplan–Meier method [14]. As a first step, univariate analyses were performed using log-rank tests and univariate Cox models. Then, as a second step, multivariate analyses were performed with multivariate Cox models. Hazard ratios and their 95% confidence intervals (95% CI) were estimated through the Cox models to compare the risk of death or progression between the two categories. Statistical analyses were carried out using SAS, version 9.3 (SAS Institute, Cary, NC).

3. Results

3.1. Patient characteristics and survival

Characteristics of the 148 eligible patients are shown in Supplementary Table S1. The median duration of follow-up was 24.2 months (95% CI: 18.6–32.4 months). Patients were predominantly male (66%) with median age of 62 years (30–83 years). Eighty-nine percent of the patients presented a stage IV and 74% had adenocarcinoma histology. All patients were previously untreated. The median OS and PFS for platinum-based chemotherapy were 15.0 months (95% CI: 12.2; 16.2) and 5.9 months (95% CI: 5.0; 6.7), respectively. All patients received platinum-based chemotherapy, with standard antiemetic treatment, including intravenous and oral steroids.

3.2. Systemic NK cell-related markers in treatment-naïve advanced NSCLC patients

Extensive fresh whole blood phenotyping of NK cells (see Supplementary Material for methods) was realised in 70 advanced NSCLC patients and compared with 48 sex- and age-matched HVs. The absolute number of NK cells was similar between HVs and patients (Fig. 1A). The frequency of $CD56^{dim}$ subset was increased in patients ($P = 0.0016$) (Fig. 1B). As $CD56^{dim}$ NK cells also express the CD16 activating receptor it is not surprising to observe an increase in the proportion of CD16 among NK cells in patients compared to HVs (Fig. 1C). Percentages of $NKp44^{+}$ and $NKp46^{+}$ NK cells were higher in patients compared to HVs (Fig. 1C); the proportion of patients, with more than 70% $NKp30^{+}$ NK cells, was also increased in patients as compared to HVs (Fig. 1D). On the contrary, the surface NKG2D expression was similar to HVs (Fig. 1E). These different patterns of expression suggested a selective modified NCRs expression, but not for NKG2D, in advanced NSCLC patients. Because increased expression of NCR has been assigned to NK cell activation, we monitored activation-associated molecules (Human Leukocyte Antigen – DR isotype [HLA-DR] and CD69). We did not find any evidence of increase in HLA-DR (Fig. 1E) or CD69 (not shown) expressions compared with HVs. These results, therefore, suggest that peripheral NK cells in advanced NSCLC patients do have increased NCR expression, which might not be associated with cell activation.

Many reports have failed to correlate NCR transcript's level and NCR's protein level [15,16]; thus, we next monitored the expression levels of NCR transcripts (see Supplementary Material for methods). NCR3, NCR2 and NCR1 transcripts were decreased in patients compared to HVs (Fig. 2). These transcripts levels were highly correlated (Supplementary Fig. S1) as already described in previous works [17,18]. Thus, the higher level of NCR protein expression did not reflect the level of NCR transcripts in these patients. Several recent studies correlated the NKp30 isoform expression pattern with the prognosis and evolution in different malignancies [16,15,19]. The relative expression of each NCR3-encoded NKp30 isoforms (NKp30A, NKp30B and NKp30C) was significantly lower in patients than in HVs (Supplementary Fig. S1), in line with a global diminution of NCR3 transcripts; note that transcripts levels of each NKp30 isoforms were highly correlated to NCR3 transcripts levels (Supplementary Fig. S1). We assessed the relative expression of NKp30 isoforms compared with each other, using the "delta" formula as described elsewhere [15]. No difference between ΔAB, ΔAC and ΔBC was observed in patients with late-stage NSCLC compared to HVs (Supplementary Fig. S1),

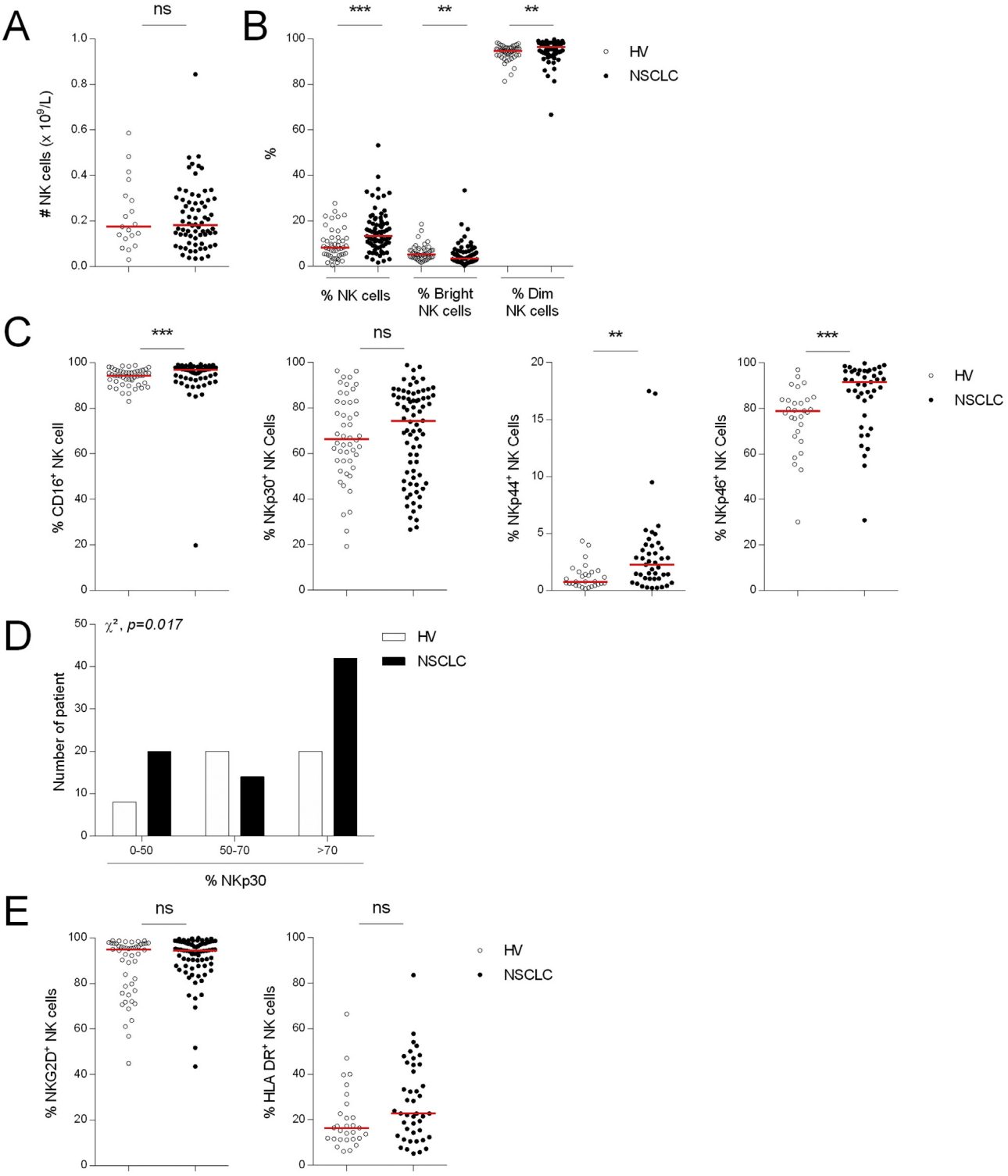

Fig. 1. Phenotype of circulating NK cells from donors and advanced NSCLC. (A) Absolute numbers and (B) proportion of circulating NK cells, $CD56^{bright}$ and $CD56^{dim}$ cells. (C) Levels of NK receptors (CD16, NKp30, NKp44 and NKp46) and (D) proportion of patients expressing high (>70%), normal (50–70%) and low levels (<50%) of NKp30 compared to healthy volunteers (HVs). (E) Proportion of NKG2D and CD69 in the peripheral whole blood $CD3^{-}CD56^{+}$ NK cells of HVs and NSCLC patients. Each dot represents one patient. Groups were compared using Mann–Whitney U test. $^{**}P < 0.01$; $^{***}P < 0.001$. ns, not significant.

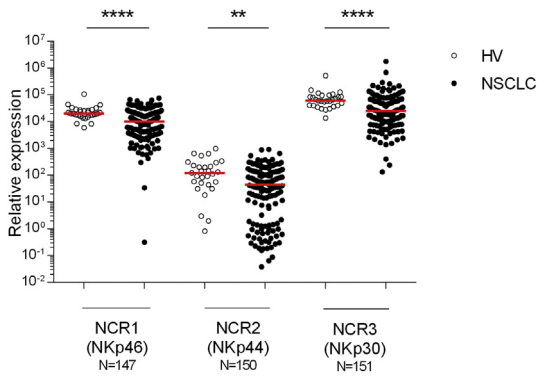

Fig. 2. Relative expression of NCR1, NCR2 and NCR3, by qRT-PCR in peripheral blood mononuclear cells (PBMCs) in advanced NSCLC patients and 29 healthy volunteers (HVs). Each dot represents one patient. Groups were compared using Mann–Whitney U test. $^{**}P < 0.01$; $^{****}P < 0.0001$. NSCLC, non–small cell lung cancer; qRT-PCR, quantitative real-time polymerase chain reaction.

indicating a global decrease of NCR3 transcripts with a maintained hierarchy of each isoform in patients with advanced-stage NSCLC. Altogether, patients with advanced-stage NSCLC patients have a global decrease in NCRs transcripts levels.

3.3. Systemic routine blood innate immune markers and NK cell–related markers and prognosis in treatment-naïve advanced NSCLC patients

We studied the routine blood parameters, mainly total leucocyte, neutrophil, lymphocyte, monocyte count and LDH, as well as NK cell phenotype and NCR transcripts. Univariate analyses were performed first and showed that monocytosis ($P = 0.011$), neutrophilia ($P < 0.001$), high LDH levels ($P = 0.008$) and derived neutrophil-to-lymphocyte ratio ($P = 0.033$) were associated with OS (Supplementary Table S2) while only neutrophilia ($P = 0.022$) remained associated with PFS in these patients who received platinum-based chemotherapy as first-line treatment (Supplementary Table S3).

NCR3 transcripts and its three isoforms A/B/C, NCR2, NCR1 and percentNKp30 have been categorised based on the tertiles (T1 = 33.3% and T2 = 66.6%) of their distribution. Only NCR3 tertiles were significantly associated with OS (trend test $P = 0.019$) and a trend for PFS (trend test $P = 0.057$). Other biomarkers related to NK cells were not associated with OS in univariate analyses (Fig. 3).

3.4. NCR3 relative expression based on other clinical or systemic innate immune parameters

Age at inclusion (Chi-square test, $P = 0.047$) and the number of metastatic sites (Chi-square test, $P = 0.022$) were significantly associated with levels of NCR3 transcripts (Supplementary Table S4). Monocytosis was strongly linked to levels of NCR3 transcripts (Chi-square test, $P = 0.005$) and to lower LDH levels (Chi-square test, $P = 0.033$) (Supplementary Table S5). Endogenous soluble forms of NKp30 ligands known to date are sBAG6 and sB7-H6. These soluble ligands were described to engage the NKp30 receptor and dampen its activity when shed in the serum or in supernatants [15,20,21]. No significant levels of sB7-H6 were found in these patients. For sBAG6 (see Supplementary Material for methods), a large majority of patients have detectable serum levels of sBAG6 (>0.5 ng/ml), and 36% presented with important levels of sBAG6 (>5 ng/ml). Levels sBAG6 were associated with low levels of NCR3 transcripts (Fig. 4) and neutrophilia (Chi-square test, $P = 0.016$) (Supplementary Table S6).

3.5. The level of NCR3 transcripts and neutrophils is independently associated with outcomes in patients with treatment-naive advanced NSCLC

Among blood measures, which were significantly associated with OS or PFS, monocytosis, neutrophilia, LDH and level of NCR3 transcripts were assessed in the

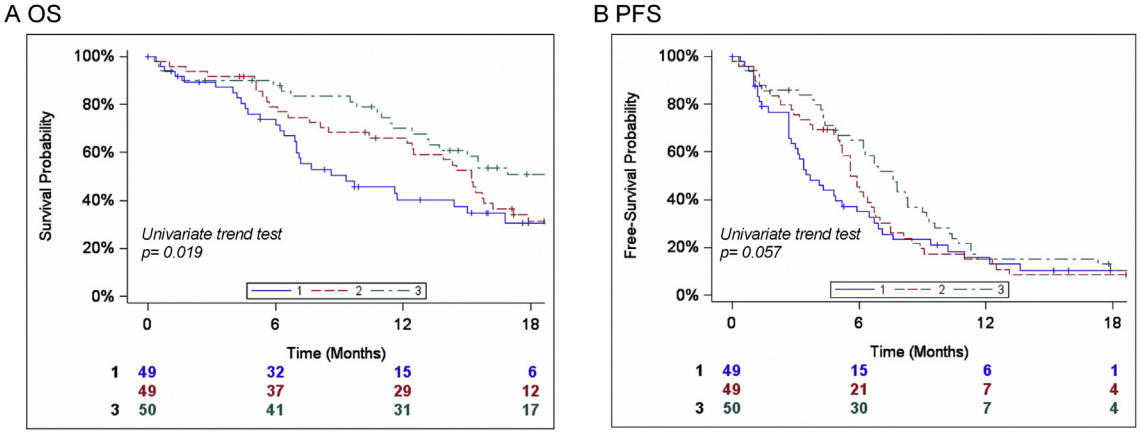

Fig. 3. (A) Overall survival (OS) and (B) progression-free survival of treatment-naïve advanced NSCLC patients according to NCR3 transcripts. NSCLC, non–small cell lung cancer.

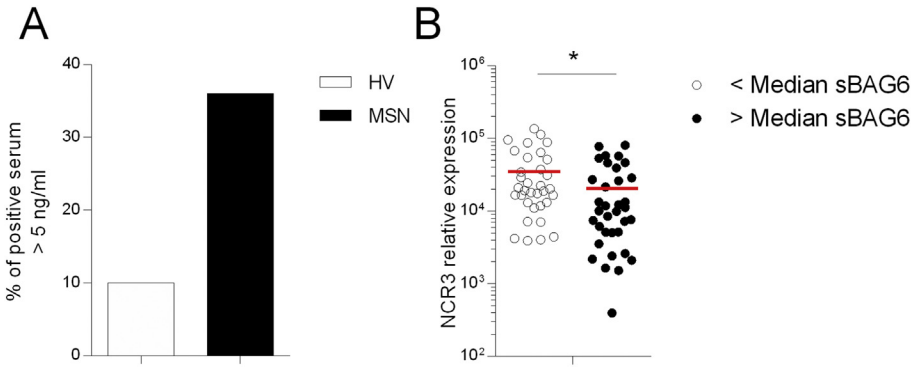

Fig. 4. Relative expression of NCR and NKp30 isoform according to sBAG6. (A) Percentage of advanced NSCLC patients and HVs with high serum levels of sBAG6 (>5 ng/ml). (B) NCR3 transcripts according to sBAG6 median. Each dot represents one patient. Groups were compared using Mann–Whitney U test. *$P < 0.05$. HVs, healthy volunteers ; NSCLC, non–small cell lung cancer.

multivariate Cox model. As neutrophilia and leucocytosis were highly correlated, neutrophilia was chosen above leucocytosis. For sBAG6 significantly related to OS in univariate analysis, the small number of patients with sBAG6 quantification ($N = 65$) led us to skip this systemic biomarker. After adjustment in the final Cox model, NCR3 transcripts (trend test, $P = 0.003$; Table 1) and neutrophilia ($P = 0.028$; Table 1) remained associated with OS, and NCR3 transcripts remained the unique innate immune marker associated with PFS (trend test, $P = 0.009$; Table 2).

At the time of the cutoff date (December 2016), only 15 (10%) patients received anti–PD-1 as second-line therapy or beyond. Among them, only 4 events for OS (death) were registered. Considering the small sample size, no statistical analysis was performed.

4. Discussion

In our study, we demonstrated that the innate immune system, particularly NK cell–related markers, can have a prognostic impact on treatment-naïve advanced NSCLC. Neutrophils and NCR3 transcript levels were independent prognostic factors in this population. To our knowledge, this is the largest study that has specifically assessed the prognostic impact of systemic immune innate biomarkers simultaneously in a homogeneous and prospective cohort of treatment-naïve advanced NSCLC patients.

In our cohort, we did not detect any difference in absolute number of NK cells compared with sex- and age-matched healthy donors. Considering NK cell phenotype, an increased frequency of $CD56^{dim}$ subset while no difference could be revealed for NKG2D as already described in very old healthy donors (>80 years) [22]. Increased cell surface expression of NCR proteins with global decrease in NCRs transcripts may seem contradictory. However, NKp30 receptor is associated with CD3ζ, and FcεRIγNKp30 receptor is associated with CD3ζ and FcεRIγ [23], being necessary for its cell surface expression. Thus, a discrepancy between the level of NCR3 transcripts and NKp30 surface expression can be observed, as already described [24]. In our cohort, we observed a coordinated diminution of NCRs, but only the level of NCR3 transcripts has been associated with prognosis. Cell surface NKp30 expression on NK cells was not related to the outcome. This seems consistent with previous observations demonstrating that diminution of NCRs transcripts can be associated with the decrease in cytotoxic NK cell functions while no association between protein expression and NCR3 transcript levels could be detected [19,25]. In our cohort, we assessed NK cell effector functions on *ex vivo*–purified NK cells from eight patients with advanced NSCLC. Granzyme B and perforin secretions after NKp30 engagement correlated with levels of NCR3 transcripts (data not shown). Thus, the low level of NCR3 transcripts was associated with diminished NK cell cytotoxic functions. Coordinated diminution of NCR1, NCR2 and NCR3 has been described by Venton et al. [18] in humans, ETS-1 transcription factor has been shown to bind to an *in silico*–predicted region of NCR promoters. ETS-1 gene expression was decreased in the presence of tumour cells, and this was associated with the diminution of NCR1, NCR2 and NCR3.

As in melanoma [16], no differences between NKp30 isoforms from patients and HVs were observed, suggesting that balance between isoforms might not play a significant role in both populations. These data differ from the findings by Fend *et al.* [26], in which low level of NCR3 transcripts and a switch on the relative proportion of NKp30 isoforms were observed compared to HVs. At that time, they compared patients with advanced NSCLC with young HVs (median age 38 years). As the relative proportion of NKp30 isoforms is altered in elderly patients, a more reliable comparison with sex- and age-matched HVs is required. In our study, comparing patients with sex- and age-matched HVs led us to conclude that the relative expression of NKp30 isoforms was maintained in our cohort.

Systemic inflammatory status can impact the function of innate/adaptive immune responses [2] and has been

Table 1
Multivariate analysis of overall survival ($N = 107$).

Variable	HR (95% CI)	*P*-value	Overall *P*-value (trend test)
NKp30			
1	1 (ref)		
2	0.50 (0.27, 0.95)	*0.034*	*0.008*
3	0.32 (0.15, 0.66)	*0.002*	*(0.003)*
Age at inclusion			
<60 years	1.08 (0.48; 2.47)	0.851	0.963
60–69 year	1.12 (0.50; 2.48)	0.784	
≥70 years	1 (ref)		
Smoking status			
Non-smoker	1 (ref)		0.113
Former smoker	3.59 (1.06; 12.13)	*0.039*	
Current smoker	3.02 (0.84; 10.79)	0.090	
Histology			
Adenocarcinoma	0.72 (0.39; 1.36)	0.315	0.315
Non-adenocarcinoma	1 (ref)		
Stage			
Stage IIIb	0.35 (0.12; 1.02)	0.053	0.053
Stage IV	1 (ref)		
Number of metastatic sites			
<3	0.50 (0.26; 0.95)	*0.033*	*0.033*
≥3	1 (ref)		
Monocytosis			
$<0.8 \times 10^9$/L	1 (ref)		0.191
≥0.8	1.46 (0.83; 2.56)	0.191	
Neutrophilia			
$<7.5 \times 10^9$/L	1 (ref)		*0.028*
≥7.5	1.94 (1.07; 3.50)	*0.028*	
LDH			
<248 U/L (ULN)	1 (ref)		0.314
≥248 U/L	1.41 (0.72; 2.75)	0.314	

CI, confidence interval; LDH, lactate dehydrogenase; HR, hazard ratio; Italic values are for significant *P* values (<0.05); ULN, upper limit of normal.

Table 2
Multivariate analysis of progression-free survival ($N = 107$).

Variable	HR (95% CI)	*P*-value	Overall *P*-value (trend test)
NKp30			
1	1 (ref)		
2	0.66 (0.36; 1.21)	0.181	*0.034*
3	0.43 (0.23; 0.82)	*0.010*	*(0.009)*
Age at inclusion			
<60 years	0.84 (0.42; 1.69)	0.627	0.551
60–69 year	1.11 (0.57; 2.14)	0.767	
≥70 year	1 (ref)		
Smoking status			
Non-smoker	1 (ref)		
Former smoker	3.92 (1.54; 9.96)	*0.004*	*0.016*
Current smoker	3.60 (1.31; 9.87)	*0.013*	
Histology			
Adenocarcinoma	0.68 (0.39; 1.19)	0.174	0.174
Non-adenocarcinoma	1 (ref)		
Stage			
Stage III	0.29 (0.13; 0.65)	*0.003*	*0.003*
Stage IV	1 (ref)		
No. of metastatic sites			
<3	0.69 (0.38; 1.25)	0.217	0.217
≥3	1 (ref)		
Monocytosis			
$<0.8 \times 10^9$/L	1 (ref)		
≥0.8	1.32 (0.78; 2.23)	0.308	0.308
Neutrophilia			
$<7.5 \times 10^9$/L	1 (ref)		
≥7.5	1.06 (0.64; 1.76)	0.811	0.811
LDH			
<248 U/L (ULN)	1 (ref)		
≥248 U/L	0.80 (0.45; 1.44)	0.459	0.459

CI, confidence interval; LDH, lactate dehydrogenase; HR, hazard ratio; Italic values are for significant *P* values (<0.05); ULN, upper limit of normal.

correlated with worse prognosis in lung cancer, mainly in early-stage NSCLC [27,28]. Neutrophils, monocyte counts and LDH levels were associated with the level of NCR3 transcripts or concentration of sBAG6 in the serum of patients. Neutrophils, but not LDH and monocytes, remained independently associated with OS in our population. The presence of ≥7.5 G/L neutrophils before treatment was an independent prognostic factor, similar to prior studies, and may reflect the proinflammatory status in cancer. Interestingly, sBAG6 was correlated with the absolute number of neutrophils. These observations led us to the conclusion that increased concentration of sBAG6 and diminished levels of NCR3 transcripts could be the result of cancer-related inflammation.

Low levels of NCR3 transcripts were related to higher number of metastatic sites, reminiscent of the already published concept of an antimetastatic role of NK cells [29,30]. Secretion of ligands such as sBAG6 that can decrease NCR3 transcripts could disrupt NK cell functions and might represent an escape mechanism linked to metastatic spread in these patients. Altogether, NK cells and particularly NCR3 could represent good targets in patients with advanced NSCLC. NK cells have the ability to modulate the adaptive immune system through cytokine production or direct cell-to-cell interactions [31,32]. An alteration of NK functions could be a mechanism associated with impairment of adaptive immunity in these patients with cancer. We recently suggested that neutrophils and LDH may predict response to ICB [3]. More recently, a study demonstrated that among patients who achieved clinical responses after treatment with nivolumab, a significantly higher baseline transcriptional signature of NK cells, γδT cells and interleukin-15 was observed compared with patients with progressive disease [33]. Altogether, these data suggest that innate immunity might represent an important counterpart to reach a clinical response during anti-PD-1/PD-L1 treatments. The small size of this population treated subsequently with anti–PD-1 (N = 19) did not allow drawing solid conclusions, and larger studies should evaluate the role of Natural Killer cells (NK) in patients treated with anti-PD-1/PD-L1 treatments. Thereby, we propose that dampening cancer-related inflammation to restore NK cell functions might represent a good therapeutic strategy to boost efficacy of immunotherapy.

5. Conclusion

Prospective and concomitant evaluation of several systemic innate markers in never treated patients with advanced NSCLC brought evidence that parameters usually associated with an inflammatory process as monocytosis, neutrophilia and important levels of sBAG6 and LDH were related to reduced levels of NCR3 transcripts. Only diminished levels of NCR3 remained independently associated with both PFS and OS. Recent studies suggested that innate immunity might be associated with response to ICBs targeting PD-1/PD-L1. Thus, the predictive value of these systemic innate/inflammatory markers before the introduction of anti–PD-1/PD-L1 should be realised prospectively.

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Author contributions

NC and BB were involved in the study concept and design, analysis and interpretation of data, drafting of the manuscript and supervision of the study and obtained funding. MC and LM were involved in acquisition analysis and interpretation of immunological data and drafting of the manuscript. LD, DP, JRM, LC, LB, RF, BD and MN were involved in the acquisition of clinical or immunological data. KR and EPvS were involved in acquisition analysis and interpretation of BAG-6 dosage. SR was involved in acquisition analysis and interpretation of NKp30 isoforms. BL and JPP were involved in collection, analysis and interpretation of survival data and drafting of the manuscript.

Conflict of interest statement

The authors declare that they have no potential conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejca.2018.12.017.

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