NKp30 isoforms and NKp46 transcripts in metastatic melanoma patients: Unique NKp30 pattern in rare melanoma patients with favorable evolution

Meriem Messaoudene, Giulia Fregni, David Enot, Nicolas Jacquenet, Emmanuelle Neves, Nathalie Germaud, Henrik Jean Garchon, Wahid Boukouaci, Ryad Tamouza, Johan Chanal, Marie-Françoise Avril, Antoine Toubert, Laurence Zitvogel, Sylvie Rusakiewicz, and Anne Caignard

INSERM U1160, Institut Universitaire d’Hématologie, Hôpital Saint Louis, Paris, France; Centre Hospitalier Universitaire Vaudois, Faculty of Biology and Medicine, University of Lausanne, Lausanne, Switzerland; U1015 INSERM-CIC, Institut Gustave Roussy, Villejuif, France; University of Paris Sud XI, Kremlin Bicêtre, France; Inserm U1173 and University of Versailles Saint-Quentin, Montigny-le-Bretonneux, France; APHP, Ambroise Paré Hospital, Division of Genetics, Boulogne-Billancourt, France; APHP, Department of Dermatology, Hospital Cochin, University Paris Descartes, Paris, France; Université Pierre et Marie Curie, Paris, France; Institut de Cancérologie Gustave Roussy Cancer Campus (GRCC), Villejuif, France; INSERM, U1015, GRCC, Villejuif, France; Center of Clinical Investigations in Biotherapies of Cancer, CICBT507, GRCC, Villejuif, France

ABSTRACT
Given the NK cell-based immunosurveillance of melanoma, we investigated the prognostic value of NKp46 transcript and NKp30 isoform (NKp30A, NKp30B and NKp30C) profiling in blood of 187 melanoma patients including 13 long survivors (LS), metastatic patients that have controlled the disease. Compared to healthy volunteers (HV), patients had reduced amounts of transcripts of the three NKp30 isoforms (NKp30A, B and C) but similar ratios between NKp30 isoforms (ΔAB, ΔAC, ΔBC). Stratification of patients according to disease stage showed higher NKp30C and lower NKp46 transcripts in stage IV patients. Furthermore, patients with previous history of conventional chemotherapy displayed reduced NKp30A transcripts. The expression levels of NKp30 isoforms failed to predict survival from sampling of patients, while NKp46 expression predicted melanoma outcome. LS patients displayed elevated NKp30A levels, accordingly high ΔAB and ΔBC ratios, and a unique pattern of rare allelic variants of NKp30 SNPs. Moreover, NK cells from LS displayed correlated NKp30/NKp46 membrane expression, high spontaneous and NKp30- or NKp46-triggered degranulation. These data outline the impact of NKp30 and NKp46 transcripts on melanoma evolution and identify unique genetic features of NKp30 associated with higher NK activation in rare LS melanoma patients that control a metastatic disease.

Introduction
Melanoma is a severe form of skin cancer with high metastatic potential. Metastatic stage IV melanoma patients have a short median survival (6–9 mo). In some patients, the immune system may control the evolution of the disease. The rare cases of spontaneous regression of metastatic melanoma have prolonged tumor-free survival (>24 mo) and present large particular infiltrate of immune cells comforted the role of the immune system in tumor control.1,2 Cytotoxic immune cells are key antitumor effectors releasing cytotoxic molecules in tumor cells. Among them, Natural Killer (NK) cells infiltrate several tumors. They are potent cytotoxic effectors, acquiring high lytic potential in response to cytokine activation. Their role was first demonstrated in myeloid leukemia and there is now growing evidence that NK cells are involved in the control of human solid tumors.3,4

NK cells have the natural ability to distinguish stress-induced molecules and alterations of MHC-I molecules on transformed targets. Activating and inhibitory receptors on NK cells are triggered during target cell recognition and the integration of these opposite signals determines NK activation.5,6 The main activating NK receptors are NKG2D and the natural cytotoxicity receptors (NCRs). NKp30 (also named NCR3), NKp46 (also named NCR1) expressed by resting NK cells and NKp44 (also named NCR2) induced on cytokine activated NK cells. Activating and co-receptors recognize various ligands on targets that are upregulated upon cellular stress.7 Most of the inhibitory receptors recognize different HLA class-I molecules and include the Killer Immunoglobulin Receptors (KIRs), NKG2A, CD85j and LAIRs.8-10 Accordingly, NK cells can kill target cells that have lost (or express low amounts of) HLA class I molecules which are frequent alterations of tumor cells including melanoma cells.

Recently, polymorphisms of activating NCR3/NKp30 receptor controlling the receptor functionality and involved in the evolution of Gastrointestinal Stromal Tumor (GIST) were identified. Three major NKp30 isoforms were identified, each one with a specific intracytoplasmic domain i.e. NKp30A, NKp30B and NKp30C. NKp30A and NKp30B signaling mediates cytolytic and IFNγ/TNF-α production,
respectively, while the NKp30C isoform induces the production of the immunosuppressive cytokine IL-10.\(^9\)

Several recent studies correlated the NKp30 isoform expression pattern with the prognosis and evolution in different malignancies. Hence, a high level of the NKp30C or a low level of NKp30B isoforms was found to correlate with a negative impact on the prognosis of patients with GIST or neuroblastoma, respectively.\(^11-13\) Furthermore, Delahaye et al., have shown that the predominant transcription of the NKp30C isoform determined the prognosis of metastatic GIST patients treated with Imatinib (IM).\(^11\) The authors also showed a relation between a NKp30 single-nucleotide polymorphism (SNP) (rs986475) and the distribution of spliced RNA isoforms.

The aim of the present study was to explore further the impact of NCR transcripts and the genetic diversity of NCR3 in metastatic melanoma patients. We studied a series of melanoma patients at different stages of the disease including a small group of 13 LS patients whose metastatic tumor had regressed and who remained tumor free for at least 24 mo without treatment.

We demonstrate that (i) the expression levels of each NKp30 (NCR3) isoforms are lower in melanoma patients at all stages of the disease compared to HV, (ii) NKp46 (NCR1) transcripts were decreased in metastatic patients and predicted survival from sampling and finally we found, (iii) a unique pattern of expression of NKp30 isoforms and frequency of genetic variant in rare melanoma patients, associated with a higher NK cell cytotoxic activity, that controlled the disease.

**Materials and methods**

**Patients and specimens**

Patient characteristics are depicted in Table 1. The study was approved by the ethic committee and informed written consent was obtained from patients. Heparinized blood was drawn from patients at the time of clinical consultation from Gustave Roussy Cancer Campus and Cochin hospital. One hundred and six HV (sex and age-matched) were used as controls (EFS, Créteil and Besançon, France). The patients enrolled consisted of stage I to stage IV melanoma patients from Cochin hospital and from Institute Gustave Roussy.

---

**Table 1.** Characteristics of the melanoma patients included in the studies.

| Gander (male/female) | STAGE 0–IIIA (n = 27) | STAGE IIIB–IIC (n = 40) | STAGE IV (n = 107) | Long survival (LS) (n = 13) |
|---------------------|----------------------|-------------------------|--------------------|-----------------------------|
| (16/11)             | (20/20)              | (58/49)                 | (8/5)              |
| Age at diagnosis    | (59.7[25.2–88.34])   | (53.5[28.9–93.5])       | (49.1[13.22–84.44])| (52.68[36.15–73.45]) |
| Tumor thickness     | <2 mm                | 19                      | 32                 | 9                           |
|                     | >2 mm                | 11                      | 39                 | 1                           |
|                     | Unknown              | 10                      | 36                 | 3                           |
| Ulceration          | Present              | 7                       | 9                  | 2                           |
|                     | Absent               | 14                      | 12                 | 3                           |
|                     | Unknown              | 5                       | 19                 | 8                           |
| Conventional chemotherapy | Yes | 12                      | 56                 | 12                          |
|                     | No                   | 22                      | 29                 | 1                           |
|                     | Unknown              | 6                       | 22                 |                             |

---

**Peripheral blood mononuclear cells (PBMC) preparations**

PBMC were obtained by Ficoll-Hypaque density gradient (PAA Laboratories), washed twice in Dulbecco’s phosphate-buffered saline (PBS, GIBCO Invitrogen), re-suspended in RLT buffer containing β-2 mercaptoethanol (Qiagen) to preserve the RNA quality, and stored at −80°C.

**Analyses of transcripts by qRT-PCR**

Total cellular RNA was isolated from PBMC samples with the RNeasy kit (Qiagen) following the manufacturer’s recommendations. First strand cDNA was synthesized from 1 μg of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen) and random primers (Promega) according to the manufacturer’s recommendations. The NKp30 isoforms (NKp30A, B, C) and NKp46 expression was determined by qRT-PCR and normalized on β2M expression as previously described.\(^11,13\) The expression of the gene of interest was calculated with the 2^−ΔΔCt method \(^14\) normalized to the expression level of β2M, with the following formula: \(2^{−\Delta\text{Ct}} = 2^{(\text{Ct}_\text{reference} − \text{Ct}_\text{gene})}\).\(^10^\circ\) The level of expression of the distinct NKp30 isoforms compared with each other (delta, Δ) was determined using the following formula \(\Delta\text{NKp30}_x\) = \(\text{CqNKp30}_y − \text{CqNKp30}_x\) with \(\text{Cq} =\) Cycle threshold. The median of each NKp30 isoform pair delta (Δ) was used for the overall survival (OS) curve analysis.

**Analysis of SNPs in NKp30 gene**

NCR3 SNP genotyping: DNA was isolated from whole blood according to the DNeasy kit protocol (QIAGEN). After dilution in DNase free water at a concentration of 5 ng/mL, the NCR3 SNPs (rs986475 and rs11575842) were genotyped using a custom TaqMan® SNP Genotyping Assay (pre-designed primers and Taqman genotyping Master Mix) in 96-well plates that were read and analyzed using the 7,300 Real-time PCR system (Applied Biosystems; Life technologies™).

**Phenotypic and CD107a degranulation analyses**

PBMC were stained with conjugated antibodies (CD3, CD56, NKp30 and NKp46) for 20 min at 4°C, washed and
fixed with 1% paraformaldehyde and analyzed by a 9-color flow cytometer (CantoII, BD bioscience). Data were analyzed with DIVA software (BD bioscience). To evaluate the degranulation of NK cells, 10⁶ PBMC were stimulated with 10⁵ K562 targets (10/effectortarget ratio) in V-bottom plates for 5 h. Cells were then labeled for 20 min at 4°C with CD3, CD56 and CD107a (BD Biosciences). Cells were collected on a FACS Canto II flow cytometer. Results are expressed as the percentages of CD107a.

**Ex-vivo NKp30 and NKp46 stimulation**

1.05 PBMC cells were cultured 6 h in 96-flat bottom plates precoated with anti-NKp30 mAb or anti-NKp46 mAbs (5 μg/mL, R&D systems), in RPMI Medium 1640–GlutaMAX™-I (Life technologies) supplemented with 10% FCS, penicillin/streptomycin (Life technologies) with or without 10ng/mL rhIL-2 (Miltenyi) + PMA/ ionomycin at 37°C and 5% CO2. The control IgG2a isotype (5 μg/mL; clone 133304;

---

Figure 1. Relative expression levels of NKp30 isoforms in melanoma patients. (A) The expression of NKp30A (left panel), NKp30B (middle panel) and NKp30C (right panel), normalized to β-2microglobulin housekeeping gene, in Healthy Volunteers (HV) and in melanoma patients is shown. (B) NKp30 isoforms ratios (ΔAB, ΔAC, ΔBC) in HV and melanoma patients, non-parametric Mann–Whitney test was used compare groups, *p < or = 0.05, **p < 0.01, ***p < 0.001. ns = not significant. (C–D) Correlation between transcript levels of each NKp30 isoform with membrane expression of NKp30 by blood NK cells (C, n = 63) or with NK cells frequency (D, n = 33) from melanoma patients. Spearman test was used.
R&D systems) for the anti-NKp30 mAb was systematically included in control cultures. Then, the cells were harvested and stained with CD3, CD56 and CD107a (BD bioscience).

**Statistical analyses**

Data were analyzed with Prism 5 software (GraphPad San Diego, CA, USA). The unpaired t-test and the non-parametric Mann–Whitney test were used for comparison of the different groups, as indicated in the Figure Legends. Correlations were analyzed by Spearman test. Survival curves were plotted according to the Kaplan–Meier method and compared by Firth’s penalized-likelihood Cox regression. A p value of < 0.05 was considered statistically significant.

**Results**

**Definition of the cohort of patients**

The present study included 187 melanoma patients enrolled from two institutions, Cochin hospital and Gustave Roussy Cancer Center. The cohort included 79 non-treated patients (from stage I to stage IV patients, not treated at the time of sampling), 80 patients that had received conventional chemotherapy or multiple therapy and 28 patients whose treatment was unknown as detailed in Table 1. Characteristics of the primary melanoma as well as disease stage are indicated at the time of sampling: the cohorts gather 27 patients with localized disease (stage 0-IIIA), 40 stage IIIB/C, 107 stage IV patients (Table 1). The OS of the patients enrolled in the studies is as expected, correlated with the Breslow index (Fig. S1). For metastatic Stage IV patients, it is of note that sampling was done at least 8 weeks after the last course of chemotherapy. In addition, 13 patients with exceptionally favorable outcome defined as LS (Table 1) were analyzed separately, due to their particular and unique characteristics.

**Reduced expression levels of NKp30 isoforms in melanoma patients**

The expression of each NCR3-encoded NKp30 isoforms (NKp30A, NKp30B and NKp30C) normalized to β-2 microglobulin expression...
was assessed by qRT-PCR in PBMC from 164 melanoma patients. NKp30 transcript analyses of patients were compared with 106 age- and sex-matched HV, a control series included in previous studies performed in GIST patients. The relative expression of the three distinct isoforms (NKp30A, NKp30B and NKp30C) was significantly lower in melanoma patients than in HV (Fig. 1A). We then assessed the relative expression of NKp30 isoforms compared with each other, using the "delta" formula $\Delta$NKp30x NKp30y = NKp30y - NKp30x for $\Delta$AB, $\Delta$AC, $\Delta$BC as described elsewhere. Importantly, this NKp30 cell profiling is stable over time and was found similar in melanoma patients and in HV (Fig. 1B). There was no correlation between the transcripts levels of each NKp30 isoform and the percentage of NKp30 expression in circulating NK cells from patients (Fig. 1C). Furthermore, using a nonparametric Spearman test, we found that the relative expression of NKp30 isoforms did not reflect the percentages of circulating NK cells (Fig. 1D). In conclusion, NKp30 isoforms transcripts are lower in NK cells from melanoma patients but the ratios between the isoforms are comparable to those found in NK cells from healthy donors.

**Impact of disease stage and of conventional chemotherapies on expression levels of NKp30 isoforms**

We next analyzed the NKp30 isoforms expression according to their melanoma stage at sampling. We found that patients at any stage of the disease exhibited low levels of NKp30 isoforms (NKp30A, NKp30B and NKp30C) compared to NK cells from HV (Fig. 2A). Among the patients bearing local, regional or metastatic disease, there was no difference in the levels of NKp30A or NKp30B transcripts whereas NKp30C (the immunosuppressive isoform) transcript levels were higher in NK cells from stage IV compared to stage 0-IIIA melanoma patients (Fig. 2A). In addition, we observed that the ratios between isoforms were not modified when HV and melanoma patients are compared (Fig. 2B), indicating a global decrease of NKp30 transcripts with a maintained hierarchy of each isoform in patients. Interestingly, in agreements with the high NKp30C transcripts in stage IV patients, the $\Delta$BC ratio value was slightly lower in NK cells from these patients compared to NK cells from less advanced patients (Fig. 2B).

In this study, most metastatic patients analyzed had received several previous courses of conventional chemotherapy followed or not by other agents at the time of sampling (Table 1). However, we also studied 37 stage IV patients that were untreated at the time of sampling, allowing the evaluation of the impact of chemotherapy treatment on NKp30 isoform expression. Thereby, when metastatic melanoma patients were analyzed according to a previous history of chemotherapy, we found lower NKp30A expression levels (Fig. 3A) and decreased $\Delta$AC (Fig. 3B) ratio in 60 chemotherapy treated patients (30 patients received dacarbazine, 26 patients received fotemustine or dacarbazine + fotemustine, three patients received cisplatin or dacarbazine + fotemustine + cisplatin and one patient received...
endoxan) compared to 37 non-treated patients (Fig. 3B), indicating that these conventional chemotherapies may modulate the relative expression levels of isoforms.

**Impact of expression of NKp30 isoforms and NKp46 transcripts in melanoma patients with disease evolution**

We analyzed the prognostic value of the relative expression of NKp30 isoforms by stratifying patients according to the median of the transcript level considered as a cut-off value. It is of note that expression of transcripts may change over time whereas ratio between isoforms remains constant, thus correlation with survival was assessed from day of sampling for transcripts levels and isoform ratios. Metastatic patients were included in the analyses.

Kaplan–Meier curves showed no impact of the relative expression of NKp30A, NKp30B and NKp30C on the survival from the day of sampling (Fig. 4A). We then analyzed the prognostic value of the ΔNKp30 ratios according to the median level of the cohort. There was no correlation of the three different ΔNKp30 ratios with better survival from day of sampling (Fig. 4B).

In addition, we analyzed the levels of another NK cell activating receptor, NKp46, on a subgroup of 47 melanoma patients including 36 inoperable stage III or stage IV metastatic melanoma patients. When patients were stratified according to the stage at sampling, NKp46 expression was lower in stage IV and certain stage III (three out of seven) metastatic patients (Fig. 4C, left panel). In contrast to NKp30, in 31 stage IV metastatic patients, the expression levels tend to predict the OS from the day of sampling (Fig. 4C right panel). These data are in accordance with our previous work describing a correlation between NKp46 expression and duration of

**Figure 4.** Prognostic values of NCR transcripts in melanoma patients. Survival from sampling day (OSS) of melanoma patients according to the median value of (A) the levels of NKp30A (left), NKp30B (middle) and NKp30C (right) and of (B) NKp30 ratio ΔAB (left), ΔAC (middle) and ΔBC ratios (right) were assessed by univariate analysis using the Kaplan–Meier method (n = 117). (C) Expression of NKp46/NCR1 transcripts in 47 melanoma patients according to their disease stage at sampling. (D) Kaplan–Meier curves of melanoma patient OSS based on the NCR1/NKp46 transcript levels from the day of sampling (n = 31), Log-rank (Mantel-Cox). *p < 0.05, **p < 0.01, ***p < 0.001. ns = not significant.
stage IV in metastatic patients\textsuperscript{15} indicating the importance of NKp46 in melanoma evolution.

**Long survivor (LS) patients display unique NKp30 transcripts and particular NKp30 polymorphisms**

Interestingly, LS patients display a distinct pattern of NKp30 isoforms. We found significantly higher expression of NKp30A transcript in LS patients compared to progressor melanoma patients (metastatic stage IV patients with a progressive disease at the time of sampling) (Fig. 5A). Expression levels of NKp30B and NKp30C were close to that of progressor patients. As a consequence of the high NKp30A transcripts, LS were characterized by high $\Delta$AB and $\Delta$AC ratios compared to HV (Fig. 5B), indicating a unique NKp30 regulation in these patients.

According to the fact that NCR3 genetic variants may inter-individually control (at least partly) NKp30 expression, we analyzed the frequencies of two NCR3 SNPs previously described to be associated with NKp30 function\textsuperscript{11} (and HJ Garchon unpublished data). Genotyping analyses revealed that the NKp30 SNP rs986475 (NKp30/NCR3$^\text{3790}$: T/C), associated with increased NKp30C transcript levels, was present in 32 of 108 (29.5%) progressor patients and in 25 of 109 HV (23%). In contrast, in the LS patients, the heterozygous CT genotype (associated with high NKp30C) was present in only 1 out of 13 patients and we detected the rare homozygous CC genotype in two patients (Fig. 6A). The SNP rs11575842 T minor allele associated with higher NKp30A isoform in HV (HJ Garchon unpublished data) was present in 7/25 (28%) of HV, 34 of 122 (27.9%) of progressors and 5/13 (46%) of LS melanoma patients (Fig. 6B). These results indicated particular frequencies of the two SNPs related to NKp30 isoforms in LS patients. There was no relation between presence of alleles and the percentages of membrane NKp30 on circulating NK cells (Fig. 6C–E). Of note, as expected from previous studies, the relative expression of NKp30C was significantly higher in patients bearing the rs986475 C/T allele and consequently slightly decreased $\Delta$AC and $\Delta$BC ratios (Fig. 6D). The rs11575842 C allele was not associated with higher NKp30A expression in progressor melanoma patients (Fig. 6F). In LS, the high levels of NKp30A was independent of the rs11575842 C/T allele (Fig. 6F). These data further indicate that higher NKp30A and the particular NKp30 isoform ratio values in LS are independent of the SNP alleles present in the patients (Fig. 6–F).

**Long survivor (LS) patients with unique NKp30 profile display activated NK cells**

Phenotypic analyses of blood NK cells in melanoma patients indicated that percentages of NKp30$^+$ NK cells from metastatic progressors and LS patients were comparable to that of

![Figure 5](image-url). Expression levels of NKp30 isoforms and NKp30 ratios in Long Survivors melanoma patients. (A–B) NKp30 isoforms transcripts (A) and $\Delta$NKp30 ratios (B) in 106 HV, 12 LS and 91 stage IV melanoma patients. Non-parametric Mann–Whitney test was used to compare groups.
NK cells from HV (Fig. 7A, left panel). Percentages of NKp46$^+$ NK cells were decreased in melanoma patients (which is significant in LS) compared to blood NK cells from HV (Fig. 7A, right panel). However, we found that in HV and LS patients, there was a correlation between NKp46 and NKp30 expression (Fig. 7B, left and right panels, respectively) whereas the two receptors were not correlated in progressor patients (Fig. 7B, middle panel). In addition, we found that NK cells from LS patients exhibited significantly higher degranulation potential than NK cells from HV or progressors (Fig. 7C).

We assessed the function of NK cells from LS and progressor patients by triggering NKp30 or NKp46 with or without IL-2 in a 5-h degranulation assay. NK cells derived from LS patients had a significantly higher capacity to degranulate upon NKp30 and NKp46 cross-linking than progressor patients or HV (Fig. 8). Thus, the particular NKp30 profile, namely high NKp30A transcripts in LS, conferred a higher capacity to respond to NKp30 stimulation.

Discussion

There is growing number of reports showing that the immunosurveillance of metastatic tumors involves NK cells. Indeed, immunophenotyping studies showed that NK infiltrating tumors cells, exhibited altered effector functions, as shown in breast and lung malignancies. NK cell relative anergy correlated with downregulation of their natural cytotoxic receptors. In addition to the numbers of tumor infiltrating NK cells, NK cell receptor expressions (NKp30 or KIRs) were found to strongly influence the prognosis in several malignancies such as in renal cell carcinoma, GIST, neuroblastoma and acute myeloid leukemia.

Our recent studies on NK cells in melanoma patients also indicate the involvement of NK cells in the natural history of the disease. We showed that NK cells infiltrated primary thick melanoma (more numerous in melanomas from aged patients) but their numbers did not impact survival. In blood NK cells, we observed a progressive decrease of NKp46 expression level.
in patients with advanced melanoma and this alteration correlated with shorter duration of stage IV.\textsuperscript{15} In addition, chemotherapy treated patients displayed distinct phenotypic modulations,\textsuperscript{24} indicating that tumor progression and conventional chemotherapy may interfere with NK cell status in melanoma patients.\textsuperscript{25}

In a recent study, comparing tumor positive and tumor-free Sentinel lymph nodes (SLN), we found that more numerous cytotoxic cells (CD8\textsuperscript{+} cells, Granzyme\textsuperscript{+} cells, NK cells) infiltrated tumor-free SLN. We also found that NK cells in SLN correlated with higher rate of relapses, supporting a tumor-promoting role of NK cells in SLN.\textsuperscript{23} However, we also showed that metastatic lymph nodes contained NK subset CD56\textsuperscript{bright}CD16\textsuperscript{+} endowed with high lytic potential following activation by cytokines.\textsuperscript{26} Depending of the disease stage localized or metastatic, lymph node NK cells may display different functions likely reflecting their recruitment from the tumor or their local activation.

In the present report, we studied the mRNA expression levels of NKp46 and NKp30 isoforms (NKp30A, NKp30B and NKp30C) receptors and their ratio in PBMC of melanoma patients sampled at the different stages of the disease. Compared to blood NK cells from healthy donors, NK cells from melanoma patients with a cutaneous or metastatic melanoma displayed lower transcripts levels of each isoform of NKp30. Correlation with patient evolution showed that neither levels of NKp30A, NKp30B or NKp30C had a prognostic impact in melanoma patients. Among metastatic patients, the NKp30A transcripts were lower in patients with previous history of treatment with conventional chemotherapy compared to untreated patients, indicating that conventional chemotherapies may modify NK cell activation. It is of note that the splice variants of NCR (NKp30 and NKp44) were controlling the distinct functions of NK cells in response to cytokine environment was recently evidence in NK cells from decidua.\textsuperscript{27}

These results echo to recent report investigating the NKp30/B7H6 axis in other tumor types, gastrointestinal sarcoma (GIST), neuroblastoma and non-small cell lung carcinoma (NSCLC). The presence of high levels of NKp30C isoform in blood NK cells influences GIST patient’s prognosis\textsuperscript{11} and

Figure 7. Unique NCR expression in LS patients. (A) Analysis of NKp30 (left panel) and NKp46 (right panel) in 10 HV, 17 progressor and 13 LS melanoma patients. (B) Correlation between NKp30 and NK46 percentages of expression in HV (left panel), progressors (middle panel) and LS (right panel) melanoma patients. (C) K562-induced CD107a degranulation levels of NK cells from 15 HV, 21 progressor and 11LS melanoma patients. Non-parametric Mann–Whitney test was used to compare groups.
markedly impacted both event-free and OS, in two independent cohorts of metastatic GIST. Low DBC values and low expression levels of NKp30A were identified in one-third of patients with dismal prognosis across molecular subtypes. This low DBC value in PBMC was associated with a pro-inflammatory and immunosuppressive tumor microenvironment. In neuroblastoma patients, the expression levels of the distinct NKp30 isoforms correlated with 10-y event-free survival in three independent cohorts of patients in remission from metastases after induction chemotherapy. In NSCLC patients, the relative expression of NKp30 was lower than in HV as well as in all other analyzed malignancies and the low relative expression of NKp30 correlated with poor overall and progression-free survival (Fend L and Rusakiewicz S, personal communications). Moreover, a discrete proportion of NSCLC patients exhibited a particular low ratio between NKp30B and NKp30C (ΔBC).

In NSCLC patients, the relative expression of NKp30 was lower than in HV as well as in all other analyzed malignancies and the low relative expression of NKp30 correlated with poor overall and progression-free survival (Fend L and Rusakiewicz S, personal communications). Moreover, a discrete proportion of NSCLC patients exhibited a particular low ratio between NKp30B and NKp30C (ΔBC).

Previous and accompanying reports indicate that ΔBC ratios are important regulator of NCR3 gene in patients bearing GIST, Neuroblastoma and NSCLC. In Melanoma, decreased ΔBC ratios occur with disease progression. In addition, NKp30A expression levels are modulated in chemotherapy treated patients and high levels of NKp30A are present in LS patients. The data altogether outline that the mechanisms controlling total and isoforms of NCR3 mRNA expression are complex, yet not elucidated, and contribute to specific and distinct patterns of NKp30 isoform expression in blood NK cells from different tumor patients. There is no correlation between transcripts levels of isoforms and the membrane expression of NKp30 by blood NK cells, but isoform ratio interferes with functionality of NKp30 and subsequent NK cell activation. In melanoma and NSCLC patients, membrane expression of NKp30 by blood NK cells is close to that of donor NK cells. In NSCLC, NKp30 expression is altered in tumor NK cells, while maintained in NK cells from metastatic lymph nodes of melanoma patients.

Our data also indicated the diminution of NKp46 transcripts in metastatic melanoma and its prognostic value on OS. These results confirmed and further strengthened our previous data showing an impact of NKp46 expression at the surface of blood NK cells on the duration of stage IV in melanoma patients. Interestingly, our data show that NKp30A expression level isoform (NK cell fitness) was elevated in rare LS patients that have controlled a metastatic disease. These LS are characterized by unique polymorphisms and isoforms proportions of NKp30. In addition, strong correlations between NKp46 and NKp30 isoforms expression were found in LS patients (and donor NK cells) but not in progressor melanoma patients.

Despite history of chemotherapy, they express high levels of NKp30A. Blood NK cells from these patients display increased lytic potential in response to NKp30 triggering, favoring the involvement of NK cells in the disease control. In addition, NK cells of LS patients bear the genetic variant rs986475 associated

Figure 8. Superior NK cell activity in LS melanoma patients. Analysis of degranulation following mAb mediated triggering of NCR1/NKp46 or NCR3/NKp30 in one representative HV (A), one representative metastatic patients (B), and two LS melanoma patients (C). Resting NK cells (Med) or NK cells activated with (IL-2 + PMA/lonomycin) were triggered with isotopic control (ISO) anti-NKp30 or anti-NKp46 mAbs and percentages of degranulation (CD107a) were assessed by flow cytometry.
with low levels of NKp30C isoform and are endowed with high lytic potential, suggesting that this rare genetic variant of NCR3 gene may confer a unique immune status in patients developing sporadic melanoma. It is of note that LS patients developed melanomas that were not distinct in terms of oncogenic mutations from those developed by progressor patients, indicating that NKp30 isoforms and SNP are not related to specific oncogenic anomalies in melanoma patients. These data suggest the importance of immunogenetic tests (NKp30/NCR3 transcript and isoforms levels, SNPs) in melanoma patients to distinguish rare patients with particular evolution and to identify relevant immune pathway required for tumor control.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Deborah Rechard, Christelle Auger (URC Paris center) and Isabelle Scheer-Senyarich for their contribution to the data management. Drs Nathalie Franck, Nora Kramkimel, Elodie Regnier-Rosencher and Françoise Boitier for their contributions in patient care and sample collections. *co senior authors.

Funding
This work was supported by grants from the Institut National du Cancer (PLBIO-2011-6) and PAIR Melanome (2013-066203), an Association pour la Recherche contre le Cancer (ARC, 3964 to AC), the Ligue Nationale contre le Cancer (Comité Ile de France to AC, PhD grant for MM), l’Assistance Publique des Hôpitaux de Paris (APHP) (DRCD, Immunella program).

References
1. Ferradini L, Mackensen A, Genevee C, Bosq J, Duvillard P, Avril MF, Herend T. Analysis of T cell receptor variability in tumor-infiltrating lymphocytes from a human regressive melanoma. Evidence for in situ T cell clonal expansion. J Clin Invest 1993; 91:1183-90; PMID:8450047; http://dx.doi.org/10.1172/JCI116278
2. Movassagh M, Spatz A, Davoust J, Lebecque S, Romero P, Pittet M, Rimoldi D, Lienard D, Gugerli O, Ferradini L et al. Selective accumulation of mature DC-Lamp+ dendritic cells in tumor sites is associated with efficient T-cell-mediated antitumor response and control of metastatic dissemination in melanoma. Cancer Res 2004; 64:2192-8; PMID:15026362; http://dx.doi.org/10.1158/0008-5472.CAN-03-2969
3. Costello RT, Fauriat C, Sivori S, Marcenaro E, Olive D. NK cells: innate immunity against hematological malignancies? Trends Immunol 2004; 25:328-33; PMID:15145323; http://dx.doi.org/10.1016/j.it.2004.04.005
4. Marcus A, Gowen BG, Thompson TW, Iannello A, Ardolino M, Deng W, Wang L, Shifrin N, Raulet DH. Recognition of tumors by the innate immune system and natural killer cells. Adv Immunol 2014; 122:91-128; PMID:24507156; http://dx.doi.org/10.1016/B978-0-12-800267-4.00003-1
5. Caligiuri MA. Human natural killer cells. Blood 2008; 112:461-9; PMID:18650461; http://dx.doi.org/10.1182/blood-2007-09-077438
6. Moretta L, Moretta A. Unravelling natural killer cell function: triggering and inhibitory human NK receptors. EMBO J 2004; 23:255-9; PMID:14685277; http://dx.doi.org/10.1093/emboj/760009
7. Moretta L, Bottino C, Pende D, Castriconi R, Mingari MC, Moretta A. Surface NK receptors and their ligands on tumor cells. Semin Immunol 2006; 18:151-8; PMID:16730454; http://dx.doi.org/10.1016/j.smim.2006.03.002
8. Long EO. Regulation of immune responses through inhibitory receptors. Annu Rev Immunol 1999; 17:875-904; PMID:10358776; http://dx.doi.org/10.1146/annurev.immunol.17.1.875
9. Lopez-Botet M, Carretero M, Perez-Villar J, Bellon T, Llanos M, Navarro F. The CD94/NKG2 C-type lectin receptor complex. Curr Top Microbiol Immunol 1998; 230:41-52; PMID:9586349
10. Moretta L, Moretta A. Killer immunoglobulin-like receptors. Curr Opin Immunol 2004; 16:626-33; PMID:15342010; http://dx.doi.org/10.1016/j.coi.2004.07.010
11. Delahaye NF, Rusakiewicz S, Martins I, Menard C, Roux S, Lyonnet L, Paul P, Sarabi M, Chaput N, Semeraro M et al. Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. Nat Med 2011; 17:700-7; PMID:21552268; http://dx.doi.org/10.1038/nm.2366
12. Rusakiewicz S, Semeraro M, Sarabi M, Desbois A, Macchioni C, Merz R, Vimond N, Concha A, Garrido F, Isambert N et al. Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors. Cancer Res 2013; 73:3499-510; PMID:23592754; http://dx.doi.org/10.1158/0008-5472.CAN-13-0371
13. Semeraro M, Rusakiewicz S, Minard-Colin V, Delahaye NF, Enot D, Vely F, Marabelle A, Papoular B, Piperegou C, Ponzioni M et al. Clinical impact of the NKp30/B7-H6 axis in high-risk neuroblastoma patients. Sci Transl Med 2015; 7:283ra255; PMID:25877893; http://dx.doi.org/10.1126/scitranslmed.aad2327
14. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(ΔΔCt) Method. Methods 2001; 25:402-8; PMID:11846609; http://dx.doi.org/10.1016/S1046-2023(01)00033-4
15. Fregn G, Messaoudene M, Fourmentraux-Neves E, Mazouz-Dorval S, Chanal J, MuabeC. E, Marinho E, Scheer-Senyarich I, Cremer I, Avril MF et al. Phenotypic and functional characteristics of blood natural killer cells from melanoma patients at different clinical stages. PLoS One 2013; 8:e76928; PMID:24204708; http://dx.doi.org/10.1371/journal.pone.0076928
16. Lakshminath TK, Burke S, Ali TH, Kimpfler S, Ursini F, Ruggeri L, Capanni M, Unansky V, Paschen A, Sucker A et al. NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo. J Clin Invest 2009; 119(5):1251-63; PMID:19349689
17. Morgado S, Sanchez-Correa B, Casado JG, Duran E, Gayoso I,Labella F, Solana R, Tarazona R. NK cell recognition and killing of melanoma cells is controlled by multiple activating receptor-ligand interactions. J Innate Immun 2011; 3:365-73; PMID:21576932; http://dx.doi.org/10.1159/000328505
18. Mamessier E, Sylvain A, Thibult ML, Houvenaeghel G, Jacquemier J, Castellano R, Gonzáles A, Andrié P, Romagné T, Thibault G et al. Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. J Clin Invest 2011; 121:3609-22; PMID:21841316; http://dx.doi.org/10.1172/JCI45816
19. Platonova S, Cheriflis-Vicini J, Damotte D, Crozet L, Vieillard V, Validire P, Andrié P, Dieu-Nosjean MC, Alifano M, Régner JF et al. Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma. Cancer Res 2011; 71:5412-22; PMID:21708957; http://dx.doi.org/10.1158/0008-5472.CAN-10-4179
20. Carrega P, Morandi B, Costa R, Frumento G, Forte G, Altavilla G, Ratto GB, Mingari MC, Moretta L, Ferlazzo G. Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56 bright CD16(-) cells and display an impaired capability to kill tumor cells. Cancer 2008; 112:863-75; PMID:18203207; http://dx.doi.org/10.1002/cncr.23239
21. Remark M, Alifano M, Cremer I, Lupo A, Dieu-Nosjean MC, Riquet M, Crozet L, Ouakrim H, Goc J, Cazes A et al. Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin. Clin Cancer Res 2013; 19:4079-91; PMID:23785047; http://dx.doi.org/10.1158/1078-0432.CCR-12-3847
22. Ruggeri L, Capanni M, Urbani E, Perrucio K, Shlomchik M, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 2002; 295:2375-82; PMID:11896281; http://dx.doi.org/10.1126/science.1068440
23. Messaoudene M, Perier A, Fregni G, Neves E, Zitvogel L, Cremer I, Chanal J, Sastre-Garau X, Deschamps L, Marinho E et al. Characterization of the Microenvironment in Positive and Negative Sentinel Lymph Nodes from Melanoma Patients. PLoS One 2015;10:e0133363; PMID:26218530; http://dx.doi.org/10.1371/journal.pone.0133363

24. Fregni G, Perier A, Pittari G, Jacobelli S, Sastre X, Gervois N, Allard M, Bercovic N, Avril MF, Caignard A. Unique functional status of natural killer cells in metastatic stage IV melanoma patients and its modulation by chemotherapy. Clin Cancer Res 2011; 17:2628-37; PMID:21224372; http://dx.doi.org/10.1158/1078-0432.CCR-10-2084

25. Fregni G, Perier A, Avril MF, Caignard A. NK cells sense tumors, course of disease and treatments: Consequences for NK-based therapies. Onco Immunol 2012; 1:38-47; PMID:22720210; http://dx.doi.org/10.4161/onci.1.1.18312

26. Messaoudene M, Fregni G, Fourmentraux-Neves E, Chanal J, Maubec E, Mazouz-Dorval S, Couturaud B, Girod A, Sastre-Garau X, Albert S et al. Mature Cytotoxic CD56bright/CD16+ Natural Killer Cells Can Infiltrate Lymph Nodes Adjacent to Metastatic Melanoma. Cancer Res 2014; 74:81-92; PMID:24225017; http://dx.doi.org/10.1158/0008-5472.CAN-13-1303

27. Siewiera J, Gouilly J, Hocine HR, Cartron G, Levy C, Al-Daccak R, Jabrane-Ferrat N. Natural cytotoxicity receptor splice variants orchestrate the distinct functions of human natural killer cell subtypes. Nat Commun 2015; 6:10183; PMID:26666685; http://dx.doi.org/10.1038/ncomms10183

28. Rusakiewicz S, Perier A, Semeraro M, Pitt JM, Pogge von Strandmann E, et al. NKp30 isoforms and NKp30 ligands are predictive biomarkers of response to imatinib mesylate in metastatic GIST patients. Oncoimmunol in press.2016; PMID:1137418; http://dx.doi.org/10.1080/2162402X.2015.1137418