Unique Functional Status of Natural Killer Cells in Metastatic Stage IV Melanoma Patients and Its Modulation by Chemotherapy

Giulia Fregni, Aurélie Perier, Gianfranco Pittari, Simon Jacobelli, Xavier Sastre, Nadine Gervois, Mathilde Allard, Nadège Bercovici, Marie Françoise Avril, and Anne Caignard

Abstract

**Purpose:** Immunotherapy is an alternative for metastatic melanoma patients resistant to chemotherapy. Natural killer (NK) cells are powerful antileukemia effectors and their role in solid tumors is suspected. NK cell activation is regulated by a balance between activating receptors, which detect stress molecules on tumor cells, and HLA-I specific inhibitory receptors. Here, we studied the phenotype and function of NK cells in stage IV metastatic melanoma patients.

**Experimental Design:** Circulating NK cells from 35 healthy donors and 51 patients were studied: 24 patients before chemotherapy (prechemotherapy), 17 patients 1 month after 1 to 4 lines of chemotherapy (postchemotherapy), and 10 patients analyzed pre- and postchemotherapy. NK functionality was carried out toward 2 primary metastatic melanoma cell lines, analyzed for the expression of NK receptor ligands.

**Results:** NK cells from prechemotherapy patients exhibit an NKp46dim/NKG2Adim phenotype. In contrast, NK cells from postchemotherapy patients display high expression of NKp46 and NKG2A receptors. Purified NK cells from patients are efficiently activated in response to melanoma cells. Melanoma cells express different level of NKG2D ligands and HLA-I molecules. In agreements with their phenotype, NK cells from pre- and postchemotherapy patients present distinct functional status toward these primary melanoma cells. A dynamic label free assay was used to determine the pathways involved in the lysis of melanoma cells by IL-2–activated NK cells. NKG2D, NCR (natural cytotoxicity receptor), and DNAM-1 are involved in the NK-mediated lysis of melanoma cells.

**Conclusions:** These results provide new arguments and clues to design NK cell–based immunotherapeutic strategies for melanoma patients.

Introduction

Melanomas are highly metastatic tumors for which the treatment is still unsatisfactory. The main way of dissemination is the lymph and the invasion of sentinel lymph node is correlated to prognosis. Metastatic melanomas are resistant to chemotherapy and radiotherapy, but immunotherapy remains an alternative for metastatic patients. However, most of the immunotherapeutic trials, aimed at boosting cytotoxic T cells specific for the melanoma antigens, led to overall limited benefit in terms of survival in several randomized phase III studies (1–3) as these effectors are frequently anergic in situ (4, 5).

Besides antigen-specific T cells, natural killer (NK) cells are potent cytolytic effectors that play a role in the innate and adaptive antitumor immune responses. As they can be efficiently expanded in vitro, NK cells can be used in autologous and allogeneic settings and constitute interesting reagents for adoptive cellular therapy. First identified as a distinct subpopulation of lymphocytes endowed with the capacity to kill tumor cells without prior sensitization, NK cells are now considered as highly sophisticated detectives of harmful changes in cellular self- and pivotal catalyzers of adaptive T-cell responses. For instance, NK cells are considered as the major source of IFN-γ in vivo and NK-derived IFN-γ is crucial in priming T helper 1 responses (6, 7).

Human NK cells are CD3−CD56+ lymphocytes that represent 5% to 20% of circulating lymphocytes. Two major NK cell subpopulations according to the expression level of the adhesion molecule CD56 (neural cell adhesion molecule) and the expression of CD16 (FcγR) are defined: CD56dim/CD16+ and CD56bright/CD16− subsets. The CD56dim population predominates in blood (90% of NK cells) and at site of inflammation, exhibits a high cytotoxic potential, and broadly expresses MHC-I specific inhibitory receptors. In contrast, CD56bright subset...
predominates in lymph nodes (95% of NK cells), produces cytokines on activation, displays a low cytotoxic potential, and is considered to be a precursor stage of terminally CD56dim NK cells (8, 9).

NK activation depends on an intricate balance between activating and inhibitory signals that determines whether the target will be susceptible to NK-mediated lysis (10, 11). Three main natural cytotoxicity receptors (NCR) involved in NK cell activation were identified: NKp46 and NKp30, expressed by resting NK cells, and NKp44, induced after stimulation by cytokines. A specific ligand for NKp30, B7H6 was recently identified (12). However, the NCR ligands on tumor cells are not yet well defined and there is no specific mAbs to measure their expression. Recombinant NCR-Fc molecules are available for the detection of NCR ligands, although binding has low affinity. In addition, the use of blocking anti-NCR mAbs in functional assays confirms the implication of NCR in the lysis of various tumor cells (13). Activation of NK cells is triggered by additional receptors. NKG2D, expressed by a majority of peripheral NK cells, binds MHC-related antigens (MIC)-A/B molecules, and UL16-binding proteins (ULBP1-4), induced on membrane of stressed cells. DNAX accessory molecule 1 (DNAM-1), an adhesion molecule belonging to the immunoglobulin superfamily, promotes many of these functions in vitro. NK cells require DNAM-1 for the elimination of tumor cells that are comparatively resistant to NK cell-mediated cytotoxicity caused by the paucity of other NK cell-activating ligands (14). Simultaneous engagement of NKp46 and DNAM-1 induces the cytotoxicity and cytokine secretion by resting NK cells (15).

NK cell activation is controlled by inhibitory NK HLA-I specific NK receptors: KIR (killer Ig type receptor), present on NK CD56dim subset and the ubiquitous C lectin–type CD94/NKG2A receptor that binds to HLA-E molecules (16). KIR receptors, CD155a and b, recognize HLA-Cw4 (C2 type) and HLA-Cw3 (C1 type) molecules respectively (17, 18).

Reports in the literature indicate that melanoma cells express different molecules that trigger NK cells (19). The frequent alterations of HLA-I molecule expression by melanoma cells may also contribute to NK cell activation.

Moreover, activation of endogenous NK cells with interleukin (IL)-2 and adoptive transfer of in vitro activated autologous NK cells mediate antitumor activity in experimental and clinical settings (20). NK cells can cure human melanoma lung metastases in nude mice treated with chronic indomethacin therapy combined with multiple rounds of IL-2 (21). There is an inverse relationship between NK cell activity and magnitude of lymphocyte infiltration as well as partial regression of the primary tumor in melanoma patients in some cases (22), suggesting that NK cell activity may be an additional prognostic factor in melanoma patients.

A prerequisite for the intelligent implementation of NK cells in antitumor regimen is a thorough molecular understanding of how NK cells recognize malignant cells and how tumors manage to subvert NK cell–mediated recognition and elimination. Here we investigated the phenotype and function of peripheral NK cells in a series of metastatic melanoma patients that had or not received prior chemotherapy. We also characterized 2 melanoma cell lines derived from 2 different lymph node metastases for their susceptibility to activated NK-mediated recognition and lysis.

### Material and Methods

#### Collection of samples, peripheral blood mononuclear cell isolation, and NK cell immunoselection

Blood samples (20–25 mL in EDTA collection tubes) from metastatic stage IV melanoma patients were obtained before or after chemotherapy, after written informed consent. Blood samples from healthy donors (HD) were analyzed as controls. For phenotype analysis (HD, n = 22; prechemotherapy patients, n = 18; postchemotherapy patients, n = 17), peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB) density gradient centrifugation. For function analysis, a 1-step immunoselection kit was used to enrich NK cells (purity of 70 ± 10% of CD56+ cells) from patient (prechemotherapy, n = 13; postchemotherapy, n = 13) and healthy donor (n = 11) peripheral blood samples (RosetteSep; Stem Cell Technologies). For xCELLigence assay, donor NK cells were purified by negative immunoselection using the NK selection kit (Miltenyi Biotech). Purified NK cells (0.5–1 × 10^6/mL) were cultured in RPMI 1640 medium (GIBCO Invitrogen) supplemented with IL-2 (10 ng/mL; R&D System) and 10% human serum AB (Biowest) for 6 to 12 days. Primary melanoma cell lines were derived by enzymatic dissociation of regional metastatic lymph nodes from 2 stage III melanoma patients and maintained in DMEM medium supplemented with 10% FCS (GIBCO Invitrogen). K562 cells (0.3–1 × 10^6/mL) were maintained in RPMI 1640 supplemented with 10% FCS.

Sera were collected from 5 mL of blood in 24 patients (prechemotherapy, n = 14; postchemotherapy, n = 10)
and 20 healthy donors. Tumor cell supernatants were collected from $10^6$ cells cultured for 48 hours in 6-well plates in serum free medium.

**Flow cytometry analyses**

Peripheral NK cells were characterized *ex vivo* by multicolor flow cytometry analyses using CD3-PerCP/CD56-APC mAbs combinations (BD Pharmingen). NK cells (gated on CD3$^+$CD56$^+$ in lymphocyte FSC/SSC subset) were labeled with PE conjugated mAbs: Nkp46, Nkp30, Nkp44, NKG2D (BD Pharmingen), CD16, CD158a, CD158b, NKG2A (Beckman Coulter). DNAM-1 was analyzed by a 2-step staining with primary anti-DNAM-1 mAb (R&D System) and secondary goat anti-mouse IgG-FITC (Beckman Coulter). The percentages of positive cells and the expression levels (ratio between the specific and control isotype staining; MFI ratio) were determined on more than 3,000 NK-gated events.

Melanoma cells were labeled with HLA-ABC-FITC (Beckman Coulter), CD112-PE (BD Pharmingen), and CD155-PE (R&D System). Indirect staining was carried out with anti-MICA, -MICB, -ULBP1, -ULBP2, -ULBP3 (R&D System), -HLA-E (MEM/E-08), -HLA-G (87G; Exbio) mAbs followed by incubation with a secondary anti-mouse IgG-FITC (Beckman Coulter). The percentages of positive cells and the expression levels were assessed by the system with a measure every 5 minutes for up to 20 hours. In some experiments, cocultures were carried out at 2:1 E/T ratio and in presence of 10 ng/mL of mAbs: NKG2D, Nkp30, Nkp46, DNAM-1, and NKG2A or isotype controls (R&D Systems). NK cells were incubated with mAbs 20 minutes before the coculture. Results are expressed as percentages of quadruplicate of lysis determined from Cellular Index (CI) normalized with RTCA Software (nCI): % of lysis = nCI (no effector) – nCI (effector))/nCI (no effector) × 100. Modulations of lysis by blocking mAbs are calculated as: (% of lysis in blocking mAbs condition) × 100/(% of lysis in isotype control mAb condition) – 100.

**Statistics**

Statistical tests and graphics were generated by Prism version 5 (GraphPad Software Inc.). Nonparametric Mann–Whitney (M–W) test was used to compare medians of absolute numbers of NK and CD3$^+$ cells between unpaired pre- and postchemotherapy patients (*, $P \leq 0.05$; **, $P < 0.01$). The nonparametric Kruskal–Wallis (K–W) test was used to compare the medians of percentages of lymphocyte subsets and antigen expression levels on NK cells in the 3 groups (HD and prechemotherapy/postchemotherapy patients). Equal variances between groups were compared using Bartlett’s test. Degranulation percentages and cytokine concentrations were determined in the 3 coculture conditions (toward MelC, MelS, or K562 cell lines) for each individual group (HD and prechemotherapy/postchemotherapy patients) with the nonparametric repeated-measures Friedman (Fr) test. For K–W and Fr tests the differences between any pair of the 3 groups were assessed by Dunn’s multiple comparison test and, in accord with their degree/level of significance, indicated in the graphs as *, **, and *** respectively.

**Results**

**Altered distribution of peripheral NK and T cells in melanoma patients treated or not with chemotherapy**

Here, we conducted extensive studies to compare the circulating lymphocyte cell subsets (NK and T cells) between healthy donors and metastatic stage IV melanoma patients treated or not with chemotherapy. The characteristics of the patients (detailed in Supplementary Tables S1 and S2) are summarized in Table 1. Ten patients detailed in Supplementary Table S3 were studied pre- and postchemotherapy. Lymphocyte absolute numbers (AN) were
reduced in postchemotherapy patients compared to pre-chemotherapy patients (M–W test, \( P < 0.0001 \), Table 1) and to a previously studied series of healthy donors where the median value of lymphocytes was of 2,150 counts/mm\(^3\) (range = 1,100–3,400; ref. 23).

Percentages of CD3\(^+\) T cells were reduced in the 2 groups of patients compared to healthy donors (K–W test, \( P = 0.0006 \), Fig. 1A). The percentages and AN of CD3\(^+\) T cells were comparable between pre- and postchemotherapy patients. The activated CD3\(^+\)/CD56\(^+\) T cell subset was higher in prechemotherapy than in postchemotherapy patients and healthy donors (median values of 9% vs. 3% and 5% respectively; K–W test, \( P = 0.0076 \), data not shown).

In contrast to CD3\(^+\) T cells, the proportion of NK cells in lymphocyte population was larger in prechemotherapy patients compared to healthy donors (increased variance, Bartlett’s test, \( P = 0.0002 \)). Postchemotherapy patients displayed reduced percentage and AN of NK cells compared to prechemotherapy patients (M–W test, \( P = 0.0035 \), Fig. 1B). Regarding the CD56\(^{dim}\)/CD56\(^{bright}\) subsets, there is an increased proportion of CD56\(^{dim}\) NK subset in prechemotherapy patients compared to healthy donors (K–W test, \( P < 0.0001 \), Fig. 1C). Thus, the reduced AN of NK cell in postchemotherapy patients is likely the consequence of the decreased numbers of CD56\(^{dim}\) NK cells compared to prechemotherapy patients (M–W test, \( P = 0.0028 \) ) as both groups display similar AN of CD56\(^{bright}\) cells (data not shown).

In autologous setting, there was a decreased percentage of NK cells and CD56\(^{dim}\) subset after treatment, while the changes of CD3\(^+\) T cells (%) appeared not related to treatment (Fig. 1, right columns).

### Table 1. Summary of patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>Prechemo median (min–max)</th>
<th>Postchemo median (min–max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>M 24</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>F 10</td>
<td>7</td>
</tr>
<tr>
<td>Age, y</td>
<td>62 (22–84)</td>
<td>65 (34–90)</td>
</tr>
<tr>
<td>Lymphocyte count, ( \mu L )</td>
<td>1,620 (740–4,640)</td>
<td>995 (340–2,538)</td>
</tr>
<tr>
<td>Delay PM(^*) – stage IV, mo</td>
<td>14 (0–84)</td>
<td>24 (0–444)</td>
</tr>
<tr>
<td>LDH</td>
<td>375 (134–3,525)</td>
<td>433 (115–5,000)</td>
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<tr>
<td>Chemotherapy treatment</td>
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<td>34</td>
</tr>
<tr>
<td>(number of chemotherapy line)</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Stage IV (TNM classification(^*))</td>
<td>M1a 4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>M1b 6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>M1c 24</td>
<td>18</td>
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PM = Primary melanoma. *Chemotherapy treatments: 1, Dacarbazine; in case of brain metastases, Fotemustine, \( 2^{nd} \) line: Fotemustine, \( 3^{rd} \) line: Cisplatin and \( 4^{th} \) line: Vincristine and CCNU or Cyclophosphamide. \(^*\)TNM Classification of Malignant Tumors.

NK Cell Activation in Metastatic Melanoma

NK cells were characterized \( \text{ex vivo} \) for the expression of activating receptors. The percentages of NKp46\(^+\) NK cells were lower in prechemotherapy patients compared to healthy donors (K–W test, \( P = 0.0153 \)), and the MFI ratio reduced compared to postchemotherapy patients and healthy donors (K–W test, \( P = 0.0043 \); Fig. 2A). There was a trend for lower percentage of NKp30\(^+\) NK cells in prechemotherapy patients but the difference with the 2 other groups was not statistically significant (Supplementary Fig. S1). In the 3 groups, NKp44 was faintly expressed \( \text{ex vivo} \) by NK cells (data not shown). NKG2D, CD16, and DNAM-1 were expressed by 80% to 100% of NK cells from healthy donors and patients (Supplementary Fig. S1).

Interestingly, we showed that NK cells from prechemotherapy patients displayed low percentage of NKG2A compared to healthy donors (Bartlett’s test, \( P = 0.0252 \), Fig. 2B). In contrast, the postchemotherapy patient NK cells exhibited high percentage (K–W test, \( P = 0.0070 \)) and MFI values of NKG2A (K–W test, \( P < 0.0001 \) ) that were increased compared to prechemotherapy patients and healthy donors (Fig. 2B). There was a high positive correlation between the expression levels (MFI ratio) of NKp46 and NKG2A in patients (Fig. 2C). Moreover, the expression levels of NKp46 receptor were negatively correlated to the lymphocyte counts that are reduced posttreatment (data not shown). The percentages of CD158a\(^{+}\) NK cells were...
reduced in postchemotherapy patients (K–W test, \( P = 0.0081 \), Supplementary Fig. S1) compared to prechemotherapy and healthy donors, while CD158b expression was similar in the 3 groups (Supplementary Fig. S1). In autologous setting, chemotherapy affected the expression level of NKp46, the percentage and expression levels of NKG2A, and the percentage of CD158a, thus strengthening our conclusions from unpaired patients (Fig. 3).

We show that NK cells from nontreated metastatic stage IV melanoma patients display a unique NKp46dim/NKG2Adim phenotype, while a NKp46high/NKG2Ahigh/CD158alow phenotype, compatible with a less mature phenotype of NK cells in these patients, characterized NK cells from postchemotherapy patients.

**Prechemotherapy patient NK cells display a unique functional pattern**

NK cells were immunoselected from PBMC from 26 melanoma patients (13 prechemotherapy and 13 postchemotherapy) and 11 healthy donors. Enriched NK cells were stimulated by primary melanoma cell lines (MelC, MelS), derived from metastatic lymph nodes and the classic NK target, K562 cell line (HLA-I negative). The percentages of degranulating NK cells were assessed by flow cytometry, and IFN-\( \gamma \) secretion measured by ELISA (Fig. 4A). Resting NK cells from healthy donors, pre- and postchemotherapy patients displayed distinct functional status. Healthy donor NK cells degranulated with a similar efficiency in response to the 3 targets (Fig. 4A). Prechemotherapy patient NK cells were endowed with a high lytic potential toward MelC cells and degranulated with lower efficiency in response to MelS and K562 (Fr test, \( P = 0.0023 \), Fig. 4A). Postchemotherapy patient NK cells also efficiently degranulated toward MelC and less toward MelS (Fr test, \( P = 0.0036 \)). The degranulation level in response to K562 was comparable in the 3 series analyzed (mean 20%–25%). Thus, resting NK cells from melanoma patients efficiently degranulated in response to melanoma cells. MelC actively triggered degranulation of NK cells from prechemotherapy patients: this response was mediated by the prominent CD56dim NK cell subset (Supplementary Fig. S2). IL-2–activated NK cells exhibited higher percentage of degranulation. IL-2–activated NK cells from healthy donors and patients displayed comparable functionality: the percentages of degranulation in response to K562 and MelC were similar, lower toward MelS. However, higher basal degranulation of NK cells without targets accounted for this increase, particularly in donor NK cells (data not shown).

The IFN-\( \gamma \) secretion patterns by resting NK cells from the 3 groups toward melanoma and K562 cell lines were distinct (Fig. 4A). Donor NK cells secreted IFN-\( \gamma \) in response to MelC (mean of 44 pg/mL) and K562 cells (42 pg/mL). MelS did not induce secretion superior to background kit level (10 pg/mL; Fr test, \( P = 0.0377 \)). The prechemotherapy patient NK cells secreted IFN-\( \gamma \) in response to MelC (48 pg/mL), while responses to MelS and K562 were not significant (3 and 12 pg/mL respectively; Fr test, \( P = 0.0023 \)). Postchemotherapy patient NK cells secreted low IFN-\( \gamma \) amounts toward MelC (28 pg/mL) and K562 (19 pg/mL). MelS did not induced IFN-\( \gamma \) secretion (3 pg/mL; Fr test, \( P = 0.0231 \)). IL-2–activated NK cells from healthy donors, pre- and
postchemotherapy patients secreted high levels of IFN-γ in response to K562 (359, 163, and 113 pg/mL, respectively) while no secretion in response to MelC or MelS cells was induced (data not shown).

In autologous settings, degranulation and IFN-γ secretion by NK cells toward melanoma cells were decreased after chemotherapy (Fig. 4B).

Soluble seric molecules in melanoma patients

Seric soluble MICA molecules (sMICA) measured by Elisa (detection threshold 0.1 ng/mL; ref. 24) were detected in 6/20 healthy donors (0.2–32 pg/mL) and in 5/24 melanoma patients (0.5–24 ng/mL). There was no detectable (<5 ng/mL) soluble HLA-G molecules in the sera of healthy donors and patients. Soluble HLA-E molecules (>20 pg/mL) measured by ELISA (25) were detected in 5/20 healthy donors (22–81 pg/mL) and 6/24 patients (24–378 pg/mL). Low concentrations of inflammatory cytokines (IL-1β, IL-6, TNF-α, IL-8, IL-10, and IL-12) were detected in the sera from patients and healthy donors (data not shown). Thus, melanoma patients did not exhibit increased inflammatory seric cytokines and some patients may display soluble HLA-E molecules.

Receptors involved in the NK-mediated lysis of melanoma cells

The expression of the ligands for NK receptors was analyzed in melanoma cells (Fig. 5A). MelC cells expressed MICA and MICB molecules as well as ULBP2 and 3 but not ULBP1 (data not shown). MelS cells only expressed low levels of MICA, MICB, and ULBP3. MelC and MelS cells did not secrete sMICA (data not shown). Regarding the ligands of DNAM-1, CD112 was expressed by MelC not by MelS; a weak expression of CD155 was present on the 2 cell lines. No NKp30-Fc and NKp46-Fc binding was detected on MelC or MelS cells: transcripts for B7H6 (1 NKp30 ligand) were detected in MelS cells (data not shown). K562 expresses NCR and NKG2D ligands and displays low HLA-I expression (26). MelC and MelS cells expressed classical HLA-I (HLA-A/B/C) molecules, with a higher MFI on MelC than on MelS. MelC cells faintly expressed membrane HLA-E and secreted low concentrations of soluble HLA-E molecules (25 pg/mL). MelC and MelS did not express HLA-G molecules (not shown) nor secrete soluble HLA-G molecules.

The NK-mediated lysis of melanoma cells was assessed by xCELLigence, a label-free, real-time monitoring assay of adherent cell lysis by measure of impedance, with a high sensitivity comparable to Cr51 release (27). Figure 5B shows the dynamic lysis (expressed as percentages) of MelC and MelS efficiently lysed by IL-2–activated NK cells at low E/T ratio.

To determine the receptors involved in melanoma lysis, NK cells were incubated for 20 minutes with single or combined anti-NK receptor mAbs. Treated NK cells were added to adherent targets into the plates and the lysis was monitored. The results of the blocking experiments are expressed as percentages of modulation of the lysis after 5 hours and are summarized in Figure 5C. Anti-NKG2D decreased the lysis of MelC and MelS cells (40%). Anti-NKp30 decreased the lysis of MelS in agreements with the high transcript level of the B7H6 ligand in these cells.
Anti-NKp46 decreased the lysis of MelC. Single blockage by anti-DNAM-1 exerted a modest effect in both cell lines. However, simultaneous blockage of NKG2D with DNAM-1 or Nkp30 and NKp46 efficiently inhibited the lysis of MelC. Blockage of Nkp30 with NKG2D or with DNAM-1 and NKp46 abrogated the lysis of MelS. The effects of activating receptors and inhibitory NKG2A mAbs used separately on the dynamic measure of melanoma cell lysis are depicted in Supplementary Figure S3: NKG2A induced a modest increase of melanoma cell lysis by IL-2–activated NK cells.

Discussion

In the present studies, we provide several original findings on the phenotypic and functional status of circulating resting NK cells in metastatic stage IV melanoma patients. First, we show that, before treatment, NK cells from these patients exhibit a unique NKp46dimNKG2Adim phenotype. Following treatment with chemotherapy, there is a high expression of NKp46 and NKG2A by melanoma patient NK cells. Thus, the balance between the activating (NKp46, Nkp30, NKG2D) and the inhibitory NKG2A receptors on NK cells and the expression level of NK ligands by melanoma cells likely explain the distinct ex vivo functional patterns of pre- and postchemotherapy patient NK cells toward melanoma cells. In addition, the functional assays with blocking antireceptors mAbs corroborate the ex vivo data on the hierarchy of activating receptors. Compared to healthy donors, NK cells from prechemotherapy melanoma patients are efficiently activated by MelC that likely trigger NKG2D with concomitant weak engagement of inhibitory NKG2A. The reduced degranulation toward MelC and MelS by postchemotherapy NK cells is likely due to the higher NKG2A/HLA-I interactions.

Several recent studies have reported various alterations in NK cells from melanoma patients. Low expression of CD161 and NKG2D activating NK receptors was previously associated with impaired NK cell cytotoxicity in metastatic melanoma patients (28). In metastatic melanoma patients with evidence of disease, a decrease expression of CD16, Nkp30, and NKp46 and no alteration of NKG2D were reported (29). In these studies, reference to previous treatments was not documented and may thus explain the discrepancies with our results. Thus, the peculiar phenotype of circulating NK cells in stage IV
melanoma patients was never described earlier, in melanoma or other solid tumors (26, 30). We hypothesized that, in stage IV melanoma patients, the invasion of various organs by tumor cells may interfere with the activation of NK. Particularly, the presence of tumor cells in lymph nodes may alter the differentiation of NK cells. In addition, soluble factors produced by circulating tumor cells may induce modulation of the NK cell phenotype (tumor editing of NKp46 and NKG2A). It was previously reported that NK cell activity changes in stage I and II melanoma lymph nodes (31, 32) indicating the involvement of NK cells in early phase of melanoma development. Further investigations to study these NK cells in stage I to III patients are required.

In contrast, NK cells from postchemotherapy melanoma patients are characterized by a high NKG2A expression and a restored NKp46, indicating a clear impact of chemotherapy on the phenotype and functions of NK cells. Moreover, no other tested variable (age: <55, >55, <70, and >70; gender; stage: M1a, M1b, or M1c; lymphocyte counts: <1,000, >1,000, <2,000, and >2,000; tumor evolution: interval between primary melanoma and stage IV: < or >12 months; and survival) apart from chemotherapy was found to differentially affect the expression (% and level) of NKG2A and NKp46 receptors. It is worth noting that, even if the tumor stage parameter did not affect significantly the NK phenotype, the effect of chemotherapy on NKp46 appeared pronounced in advanced M1c patients (Supplementary Fig. S4).

The comparable and low proportions of CD3+ NKG2A+ T cells (>5%, data not shown) in patients indicate that the induction of NKG2A is specific to NK cells. No seric inflammatory cytokines were detected in patients before or after chemotherapy. Moreover, postchemotherapy patients were analyzed 4 to 6 weeks after the last course of chemotherapy, excluding a direct effect of drugs on peripheral NK cells, but compatible with changes in the differentiation and/or maturation of NK cells. The autologous paired pre/post analyses confirm the observation on unpaired series of patients on the effect of chemotherapy on NK status.

Figure 5. A, phenotypic analyses of primary melanoma MelC and MelS cell lines, derived from metastatic lymph nodes. B, MelC and MelS lysis (%) by IL-2–activated NK cells from healthy donor (1 of 2 experiments is shown), assessed with a dynamic label free assay (xCELLigence) that monitor by cell impedance. Lysis was measured at different E/T ratios (black to white scale). Mean of quadruplicate values and standard error (SD) bars for each experiment are shown. C, the involvement of NK receptors in the lysis of MelC and MelS targets was determined in the presence of 10 μg/mL control isotype (IgG), or specific mAbs. IL-2–activated NK cells were added to adherent 10,000 MelC or MelS, at an E/T ratio of 2:1. Percentages of lysis inhibition are calculated at T = 300 minutes in presence of anti-NKG2D, anti-NKp30, anti-NKp46, anti-DNAM-1, alone or combined as indicated in the legends (mean values and SD of 2 independent experiments).
Interestingly, these unique NK populations in patients are endowed with peculiar *ex vivo* functions: resting NK cells from pre- and postchemotherapy patients are able to efficiently lyse primary lymph node–derived melanoma cells. Furthermore, we show that certain melanoma cells may trigger IFN-γ secretion by resting NK cells in patients.

The above studies on resting NK cells outline that the pattern of NK ligands expressed by primary metastatic melanoma cells may modulate their susceptibility to NK-mediated lysis. The recent collaborative studies (33) outline that lymph node metastases are highly susceptible to NK cell lysis and that NCR and DNAM-1 are involved in the lysis, whereas NKG2D is not. They showed that all human metastatic lymph node–derived melanoma cells express ligands for Nkp46 and DNAM-1, while NKG2D ligand expression was variable among the cell lines studied. Expression of DNAM-1 and NKG2D ligands by a large panel of melanoma cell lines was also reported (19) and correlation between NKG2D function and expression of ligands on melanoma cells was shown (34). Moreover, Markel and colleagues (35) reported that NKG2D plays a major role in the lysis of melanoma cells derived from metastatic lesions. NKP30 also contributes to the lysis of melanoma cells. To determine the activating receptors involved in melanoma lysis by IL-2–activated donor NK cells, we used a sensitive dynamic label free assay. Our blocking experiments show that simultaneous triggering of NKG2D and DNAM-1 is crucial for melanoma lysis whereas NCR involvement may vary from 1 cell line to another. The strong expression of NKG2D and DNAM-1 by prechemotherapy NK cells may account for their efficiency toward MelC and MelS. In IL-2–activated NK cells, NKG2A participates in the control of melanoma cell lysis. NK cells are not typically found in large numbers in advanced human neoplasms. However, their numbers increase following activation *in vivo* (20) or adoptive transfer (36). In addition, the level of NK infiltration positively correlates with prognosis in squamous cell lung carcinoma (37), and colorectal tumors (38). NK cells have been detected in melanoma after tumor-infiltrating lymphocyte (TIL) isolation, and these NK cells were much more potent than CD3+ TILs to kill melanoma cell lines (39). Previously reported correlation between histologic features and NK activity suggests that NK cells may represent an additional prognosis factor (22).

The presence of unique melanoma-reactive NK cells in stage IV patients and their modulation by chemotherapy are strong arguments for the involvement of these immune cells in melanoma, and widen their interest in immunotherapy strategies for melanoma patients.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

This work was supported by grants from INSERM, la Fondation pour la Recherche Médicale (FRM), la Société Française de Dermatologie (SFD), and la Ligue Nationale contre le Cancer (Comité Ile de France). C. Fregni had a grant from Cancereurope IdF and A. Perier from FRM. The authors thank Drs. F. Boitier, N. Franch, I. Gorin, N. Wallet-Faber, and Prof. N. Dupin for including patients in the studies and Dr. V. Fourchotte, surgeon in Institut Curie for providing samples of lymph node metastases. They also thank Dr. N. Freiss-Rouaiss for measurement of seric HLA-G, Dr. S. Caillat-Zucmann for seric MICA molecules, C. Faustin for B7H6 transcript analysis in melanoma cell lines, Dr. S. Grabar for helpful discussion and advices for statistical analysis, and the immunobiology platform of Institut Cochin. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 10, 2010; revised December 7, 2010; accepted January 2, 2011; published OnlineFirst January 11, 2011.

**References**


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