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Soluble NKG2D ligands are biomarkers for checkpoint blockade-treated melanoma patients

Original Research

Soluble NKG2D ligands are biomarkers associated with the clinical outcome to immune checkpoint blockade therapy of metastatic melanoma patients

Cristina Maccalli[1,*], Diana Giannarelli[2], Carla Chiarucci[3,4], Ornella Cutaia [3,4], Gianluca Giacobini[3,4], Wouter Hendrickx[1], Giovanni Amato[3], Diego Annesi[3], Davide Bedognetti[1], Maresa Altomonte[3], Riccardo Danielli[3], Luana Calabrò[3], Anna Maria Di Giacomo[3], Francesco M. Marincola[5], Giorgio Parmiani[3,6], Michele Maio[3]

[1]Research Branch, Division of Translational Medicine, Sidra Medical and Research Center, Doha, Qatar;

[2]Unit of Statistics, Regina Elena National Cancer Institute, Rome, Italy;

[3]Medical Oncology and Immunotherapy, University Hospital of Siena, Istituto Toscano Tumori, Siena, Italy;

[4]University of Siena, Siena Italy;

[5]Office of the Chief Research Officer (CRO), Research Branch, Sidra Medical and Research Center, Doha;

[6]Italian Network for Bio-therapy of Tumors-(NIBIT)-Laboratory, Siena, Italy;

*Previous address: Italian Network for Bio-therapy of Tumors-(NIBIT)-Laboratory and Medical Oncology and Immunotherapy, University Hospital of Siena, Istituto Toscano Tumori, Siena, Italy,

Corresponding author: Cristina Maccalli, Department of Research, Division of Translational Medicine, Sidra Medical and Research Center, North Education Campus, Al Luqta Street, POBOX 26999 Doha, Qatar, Email: cmaccalli@sidra.org; phone: +974-4003 7421

Abstract

The introduction of immune checkpoint blockade into the clinical practice resulted in improvement of survival of a significant portion of melanoma patients. Consequently, predictive biomarkers of response are needed to optimize patient's stratification and the development of combination therapies. The aim of this study was to determine whether levels of soluble NKG2D ligands (MICA, MICB, ULBP1, 2 and 3; sNKG2DLs) in the serum of melanoma patients can serve as useful predictors of response to the treatment with immune checkpoint blockade. sNKG2DLs were measured by ELISA in baseline and post-treatment serum and these results were correlated with the clinical outcome of melanoma patients (N=194). The same determinations were performed also in a cohort of patients (N=65) treated with either chemotherapy, radiotherapy, or mutated BRAF inhibitors (BRAFi). Absence of soluble MICB and ULBP-1 in baseline serum correlated with improved survival (OS= 21.6 and 25.3 months and p= 0.02 and 0.01, respectively) of patients treated with immunological therapies while detectable levels of these molecules were found in poor survivors (OS= 8.8 and 12.1 months, respectively).

Multivariate analysis showed that LDH (p<0.0001), sULBP-1 (p=0.02) and sULBP-2 (p=0.02) were independent predictors of clinical outcome for the cohort of melanoma patients treated with immune checkpoint blockade. Only LDH but not sNKG2DLs was significantly associated with the clinical outcome of patients treated with standard or BRAFi regimens. These findings highlight the relevance of sNKG2DLs in the serum of melanoma patients as biomarkers for patients' stratification and optimization of immune checkpoint inhibition regimens.

Keyword

Metastatic Melanoma, NKG2D ligands, Cytotoxic T-lymphocyte antigen-4 (CTLA-4), Programmed cell death-1 (PD-1); T cell responses.

Introduction

In tumors with different histological origin the adaptive immune response can influence recurrence, metastatic spread and the overall survival. [1, 2, 3] This concept has been further characterized by the demonstration that the nature, location and density of tumor-infiltrating lymphocytes (TILs) is associated with the prognosis of cancer patients, allowing better staging of disease and consisting in a more reliable prognostic marker compared to traditional TNM staging. [4, 5, 6, 7]. Nevertheless, the immunesurveillance of tumors is often impaired by immunomodulatory mechanisms occurring at tumor site, such as regulatory immunological cell populations (T regulatory cells, Tregs and Myeloid Derived Suppressor Cells, MDSCs), the pro-tumor cross-talk between cancer cells and tumor microenvironment (TME) and the presence of negative regulatory factors in the TME (Indoleamine 2,3-dioxygenase; IDO, IL-10, IL-13, TGF-beta, etc.). [8, 9, 10] Immunotherapy aims at circumventing negative immunomodulatory pathways to induce potent systemic immunological responses against tumors. [10] Antibodies (Abs) that block immune checkpoints, such as the anti-cytotoxic T-lymphocyte antigen-4 (CTLA-4) and the anti-programmed cell death-1 (PD-1) or its ligand (PD-L1), can potentiate or rescue the effector functions of the anti-tumor cell-mediated immune responses. [11, 12] The clinical activity of immune checkpoints agents has been conclusively demonstrated for different types of tumors. [13, 14, 15, 16, 17, 18] The combination treatment with anti-CTLA-4 and anti-PD-1 mAbs for metastatic melanoma yielded striking clinical results, leading to the FDA approval. [19, 20] The efficacy of this combination is also under evaluation in other solid tumors such as lung cancer. [21] Nevertheless, similarly to single agent therapies, a

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proportion of patients do not respond to this combination, thus the optimization of these strategies is still under investigation. [22, 23, 23]

In this context, it is desirable to identify biomarkers to be used prospectively for the selection of patients more likely to respond to single agent or the combination therapy and for the optimization of treatment schedules. This information might also provide insights about how to prevent immune related adverse events (iAEs), in particular high grade toxicities (≥3) observed in about 10-20% of patients treated with single agents and in up to 50% of cases treated with the combination. [24]

We recently showed that the baseline serum levels of soluble NKG2D ligands (sNKG2DLs) can discriminate melanoma patients treated with the combination of ipilimumab plus chemotherapy who experience poor clinical outcome from those with long-term survival. [25]

The aim of this study was to assess the value of serum levels of sNKG2DLs as predictors of responsiveness in melanoma patients undergoing immunotherapy regimens. We determined sNKG2DLs levels in pre- and post-treatment sera of melanoma patients treated with immune checkpoint blockade (anti-CTLA-4 or anti-PD-1 mAb monotherapy or their combinations) and the results were correlated with patients' (N=194) clinical outcome. The same determinations were performed in a control group of melanoma patients treated with standard therapy or mutated BRAF inhibitors (N=65; BRAFi).

Results

1. Detection of sNKG2DLs in the serum of patients

sNKG2DLs levels were recorded pre- and post-treatment (12 weeks following the first drug administration) in the sera of 162 melanoma patients undergoing treatment

with immune checkpoint blockade. The clinical and treatment characteristics of the patients are summarized in Table 1. MICA, MICB and ULBP-2 were detectable in a minority of patients at baseline (N=18, 42 and 38, respectively), Figure 1 A, B and D. In some patients (N=11, 19, and 10, respectively) these factors were detectable only in post-treatment samples. Few patients (N= 4, 8, and 2, respectively) showed ≥50% reduction in the concentration of these factors 12 weeks' post-treatment. Similarly, increased levels of sMICA, sMICB and ULBP-2 after treatment were observed in few cases (N=6, 11 and 17, respectively). Higher levels of sULBP-1 and -3, were observed as compared to the others NKG2DLs, in the sera of patients with peak concentrations of $1x10^5$-$1x10^6$ pg/ml (Fig. 1 C and E). Moreover, these molecules were most frequently found in the serum of patients both at pre- (N = 83, 62.5% and N= 65, 40%, respectively) and post-treatment (N= 85, 65% and N= 62, 38%) time points (Fig. 1 D and E). The presence of sULBP-1, due to the limiting amount of serum for some patients, was determined in N=131 patients. Modulation of the concentration of sULBPs according to treatment followed a trend like MICA and MICB (Fig. 1).

Baseline levels of sNKG2DLs were also analyzed in melanoma patients not undergoing any immunotherapy and rather treated either with standard therapy or BRAFi (see Table 1 for clinical details). As shown in Figure 2, all sNKG2DLs could be detected in the serum of these patients (MICA in N=19, MICB in N=25, ULBP-1 in N=32, ULBP-2 in N=20 and ULBP-3 in N=15 patients). The levels of sNKG2DLs were heterogeneous with peak of concentration, except for ULBP-2, lower as compared to patients treated with immunotherapy. Serum levels of sNKG2DLs from

patients treated with ipilimumab plus chemotherapy (NIBIT-M1 study; N=37) that have been previously described, [25] were also included in the subsequent analyses.

Soluble MICA and MICB were most commonly detected in stage III melanoma patients (p= 0.05 and 0.001, respectively); conversely the detection of sULBP-1 was most frequently associated (p= 0.02) with stage IV. No associations between stage of the disease and detection of soluble levels of ULBP-2 and 3 were observed (data not shown).

2. *Identification of biomarkers of clinical outcome in melanoma patients treated with immune checkpoint blockade.*

Association between the presence or absence of sNKG2DLs in baseline or post-treatment serum of melanoma patients receiving either anti-CTLA-4 or -PD-1 mAbs as monotherapy or their combination and clinical outcome was determined for N=194 melanoma patients (Tables 2 and 3). This analysis included also melanoma patients treated with the combination of anti-CTLA-4 and fotemustine (N=37, *see* Table 1) for which the levels of sNKG2DLs and the modulation during treatment have been previously reported. [25] Disease control (DC) and OS information were available for N=193 and 194 patients, respectively.

Absence of sULBP-1 in baseline serum of 77/162 evaluated patients correlated with better clinical outcome in terms of DC (DC rate 57.1%, p=0.002; Table 2). No relationship was found between the serum levels of sNKG2DLs and the clinical outcome of patients not treated with immunotherapy (control group; Table 2). Absence or presence at baseline of detectable sULBP1, respectively discriminated patients with improved (N= 78 patients; median OS= 25.3 months; p=0.01) from poor OS (N= 85 patients; median

OS=12.1 months; Table 3). Similarly, lack of sMICB in pre-treatment serum identified patients experiencing long-term survival (N= 151 patients; median OS= 21.6 months; p=0.02) compared to those with detectable soluble molecules (N= 42 patients; median OS=8.8 months; Table 3). No relationship between serum levels of sNKG2DLs and OS was observed in the control group was (Table 3).

Absent detection of sMICA and sMICB in post-treatment serum of melanoma patients undergoing immune checkpoint blockade correlated with improved survival (median OS= 20.2 and 22.8 months; p=0.02 and 0.01, respectively; Table 3) compared to cases in which the two factors could be detected (median OS= 12.4 and 10.4 months, respectively).

No significant association between post-treatment serum levels of sNKG2D and OS of melanoma patients treated with BRAFi was found (Table 3). Figure 3 represents the Kaplan-Meier analysis of OS for melanoma patients treated either with immunotherapy (Panels A and B), standard therapy or BRAFi (Panels C and D) according to detection in baseline serum of sMICB (Panels A and C) and sULBP-1 (Panels B and D). These findings highlight an inverse association between levels of sNKG2DLs and the OS specifically in patients treated with immunotherapy. No significant association was detected between levels of sMICB (p=0.40) and sULBP-1 (p=0.84) and OS of patients who did not undergo immunotherapy (Figure 3 Panels C and D). An inverse significant association (p=0.02 and 0.01, respectively) between levels of sMICA and sMICB in post-treatment serum and OS of melanoma patients treated with immunotherapy are portrayed by the Kaplan Meier curves in Figure 4 (Panels A and B).

The available cancer sets in the Cancer Genome Atlas (TCGA) were used to assess any relationship between the expression of NKG2DLs, that we found as candidate predictors for immunotherapy treatment of melanoma patients, and the clinical outcome of 30 cohorts of patients with different types of primary tumors. The hazard ratio through COX analysis of the highest versus the lowest tertile of expression of these molecules was calculated (Figure 1S). The lowest expression of MICB was significantly associated with favorable clinical outcome in lower grade glioma (LGG), pancreatic adenocarcinoma (PADD) and thymoma patients (THYM) (p=0.000369, 0.012; and 0.0399, respectively, Figure 1S). Low or negative expression of ULBP-1 inversely correlated with the risk of death for 8 different cohorts of patients with tumors with different histological origins, including LGG (p=0.000131), glioblastoma (GBM; p=0.014), breast cancer (BRCA; p=0.0166), mesothelioma (MESO; 0.00963) (Figure 1S). Scant information is available in these data sets regarding metastatic melanoma patients and the therapeutic regimens administered to cancer patients. Although our observations couldn't be confirmed through TCGA in the same setting of cancer patients, the data represented in Figure 1S corroborate the role of NKG2DLs as prognostic candidate biomarkers for the clinical outcome of cancer patients.

3. *Multivariate analysis of biomarkers*

The impact of individual and clinical parameters, such as age, LDH, PS, stage etc. (Table 1) and the levels of sNKG2DLs in the serum of melanoma patients was evaluated by Cox regression analysis (Table 4). LDH was heterogeneously detected in melanoma patients with a range of 124-2190 IU/L and median= 302 IU/L. This molecule was reported as increment of 10 IU/L revealing to be the strongest (HR= 1.01, p<0.0001) prognostic

markers for OS survival for melanoma patients treated either with immune checkpoint blockade or with standard or BRAFi therapies (Table 4). PS and disease stage are associated with clinical benefit only for the control group (HR= 2 and 6.2 and p=0.03 and 0.01, respectively; Table 4). The latest two markers were not significantly associated (p=0.18 and 0.10, respectively) with clinical responses to immunotherapy. Interestingly, MICB, ULBP-1 and ULBP-2 significantly predicted clinical outcome of patients undergoing immunotherapy strategies (HR= 1.67 and 1.78 and p=0.02 and 0.01, respectively; Table 4). Multivariate COX regression analysis confirmed the role of LDH and ULBP-1 as independent prognostic biomarkers of the clinical outcome (HR= 1.02 and 1.72 and p=0.02 and 0.0001, respectively; Table 4) in melanoma patients treated with immune checkpoint blockade. Of note, ULBP-2 also resulted as a marker associated with the clinical responses of this cohort of melanoma patients (HR=1.91 and p=0.02; Table 3). The same multivariate analysis applied to the melanoma patients in the control group demonstrated that only LDH and age were associated with prognosis (HR= 1.03 and p<0.0001 and p=0.02, respectively; Table 4).

Discussion

In the present study, we assessed the levels of sNKG2DLs in the serum of melanoma patients to test whether they could represent baseline predictors of clinical outcome in response to treatment with immune checkpoint blockade. Absence of sMICB and sULBP-1 in patient's serum at baseline distinguished long-term from poor survivors (OS 21.6 and 25.3 vs. 8.8 and 12.1 months and p=0.02 and 0.01, respectively). In univariate analysis, the HR for patients with detectable levels of sMICB and/or sULBP-1 in baseline serum was 1.67 and 1.78, respectively (p=0.02 and 0.01, respectively). LDH was

confirmed as a predictive marker (HR=1.01 and p<0.0001) for the clinical outcome of melanoma patients independent on the type of therapeutic treatment. [26, 27] sULBP-1 and ULBP-2 were identified through multivariate analysis as candidate independent predictive markers (HR=1.72 and 1.91 respectively and p=0.02) of clinical response in patients treated with immune checkpoint inhibitors. Thus, the level of these biomarkers in baseline serum enables the distinction of melanoma patients with favorable clinical outcome from poor survivors to the treatment with immune checkpoint blockade.

NKG2D-mediated signaling plays a relevant role in tumor immunosurveillance. [28, 29, 30] This receptor is expressed by NK, T, NKT and γδ T cells, providing activating signal to NK and co-stimulation to T cells. [30] Both *in vitro* and *in vivo* studies have demonstrated that the expression of NKG2DLs by tumor cells can lead to the efficient development of anti-tumor immune responses. [30, 28, 29] NKG2DLs are expressed by tumor cells of different histological origin, although the surface expression of these ligands is strictly regulated by different mechanisms and by the interaction with the TME. [31, 30] The presence of sNKG2DLs in the serum of cancer patients has been widely documented in association with tumor progression. [32, 33, 28, 34, 35] sULBP-2 was identified as a prognostic factor, stronger than S100B, in early-stage (I-III) melanoma patients. [29] NKG2DLs can either promote anti-cancer immune responses or mediate immune evasion of cancer cells, depending upon their pattern of expression, e.g. membrane localization or proteolytic shedding in soluble form by tumor cells. [30, 36] sNKG2DLs can suppress anti-tumor immune responses through multiple mechanisms. The most common is binding of soluble ligands to the NKG2D receptors on T and NK cells facilitating their endocytosis and degradation thus impairing the anti-tumor activity of the lymphocyte populations. [28,37, 38]

The suppression of anti-tumor immune responses by tumor cells secreting sMICB was clearly shown in a prostate cancer model of humanized transgenic mice. [39] sMICA can interfere with NK homeostatic maintenance in the peripheral blood [39] and can also promote the expansion of MDSCs. [40] On the other hand, high expression of membrane ULBP-1 positively correlated with overall survival of pancreatic cancer patients while sULBP-2 was found as an independent marker of poor clinical outcome for these patients indicating that the molecular nature of NKG2DLs can affect positively or negatively the clinical outcome. [35] The phenomenon described above might explain our observation that the presence of sNKG2DLs in baseline serum is associated with poor clinical outcome of melanoma patients treated with immunotherapy strategies. sNKG2DLs could impair anti-tumor T cell-mediated responses thus counterbalancing the unleashing of immune responses by immune checkpoint blockade, such as anti-CTLA-4 or PD-1 mAbs. Similarly, sNKG2DLs can abolish the unlocking activity of anti-PD-1 mAbs on tumor reacting NK cells.

Our preliminary analysis of cancer sets in TCGA indicated that the expression of either MICB or ULBP-1 was significantly associated with favorable clinical outcome of cancer patients with different type of tumors (e.g. LGG, PADD, THYM, GBM, BRCA, HNSC MESO, SARC, KIRC, KIRP, DLBC). Moreover, the highest expression of the activatory/co-stimulatory receptor, NKGD2, was associated with a reduced risk of death for cancer patients with LGG ($p=1.59 \times 10^{-6}$), HNSC ($p=0.00029$), BRCA ($p=0.011$), THYM ($p=0.0064$), bladder carcinoma (BLCA; $p=2.02 \times 10^{-8}$), cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC; $p=0.00094$), lung squamous cell carcinoma (LUSC; $p=0.0073$), stomach adenocarcinoma (STAD; $p=0.039$), uterine

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corpus endometrial carcinoma (UCEC; p=0.012) (Figure 1S). Interestingly, for some type of tumors, such as LGG, GBM, HNSC, BRCA, SARC, THYM, highest expression of NKG2D and lowest expression of either ULBP-1 or MICB were detected in patients with reduced risk of death (Figure 1S). Limited information regarding either metastatic melanoma patients or the therapeutic regimens received by cancer patients are included in the TCGA data sets, preventing us to perform any association analysis between gene expression of NKG2D and OS of patients treated with immune checkpoint blockade. Nevertheless, this exploratory analysis can corroborate the role of NKG2DLs as candidate predictors for the prognosis of cancer patients and the need to further investigate their role as biomarkers for the clinical responses to immunotherapy.

We have previously described the role of ULBP-1 and -2 as candidate predictive markers for the clinical outcome of melanoma patients with metastatic disease treated with ipilimumab and fotemustine (NIBIT-M1 study). [25] These findings are substantiated by the present study that evaluated patients treated with immune checkpoint blockade both as monotherapy and in combination. In this study, we also evaluated a cohort of melanoma patients treated with standard therapies or BRAFi. In this context, the levels of sNKG2DLs did not affect clinical outcome, indicating that these molecules represent useful tools predicting the clinical activity of immune checkpoint blockade (*see* Figure 3). We have also evaluated the relationship between the presence of sNKG2DLs in the serum after treatment and clinical outcome. Indeed, the absence of sMICA and sMICB in post-treatment serum was significantly associated (p=0.02 and 0.01, respectively) with improved OS (20.2 and 22.8 months, respectively) of melanoma patients undergoing anti-CTLA-4 or anti-PD-1 mAb therapy. These results confirm the relevance of levels of

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sNKG2DLs in pre- and post-treatment sera in predicting clinical responses in melanoma patient receiving immunotherapy.

The clinical activity of ipilimumab in combination with a vaccine composed by tumor cells secreting GM-CSF was observed in patients with high levels of autoantibodies directed to MICA. [41] The impairment mediated by sNKG2DLs on T and NK cell-mediated anti-tumor responses could be rescued by treatment with neutralizing antibodies. [42] Taken together these and our observations suggest a rationale to explore the therapeutic efficacy of the combination of immune checkpoint blockade with sNKG2DL neutralizing mAbs. [43] In addition, high sMICA levels in the serum were found to be associated with less frequency of immune related adverse events in a cohort of melanoma patients treated with ipilimumab [44] suggesting that sNKG2DLs can indeed play a relevant role in determining the fate of anti-tumor immune responses unleashed by immune checkpoint blocking agents.

Our findings demonstrate that sNKG2DLs can play a role as predictive biomarkers for OS of melanoma patients treated with immune checkpoint blocking mAbs (including ipilimumab, nivolumab, pembrolizumab and their combinations) and broaden the list of parameters that can be worthy of monitoring in melanoma patients. Of note, these findings identify candidate biomarkers determinable in the serum of patients through assays easily accessible in different clinical centers. Further prospective investigation of the role of these molecules as baseline biomarkers of clinical outcome of cancer patients treated with immune checkpoint blockade agents and their combinations are warranted. It might also be interesting to elucidate the relationship that exists among gene expression in tumor tissues and the soluble protein levels in the serum for NKG2DLs in cancer

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patients with different type of histology for which immunotherapy either represents a promising strategy or is currently under investigation. It will be worthy to assess these determinations in association with the extent of NKG2D expression, as a marker of lymphocyte infiltration, at tumor site to establish the most accurate possible biomarker immune signature(s) for patients undergoing immune-based therapies.

Material and Methods

Melanoma Patients

Patients (N=162) with measurable unresectable stage III or stage IV melanoma were included in this study; *see* Table 1 for detailed patient's characteristics and treatments. These melanoma patients have been treated with: *i.* ipilimumab at 3 or 10 mg/kg in the context of expanded access programs (EAP) or, more recently, as "on-label usage"; (N= 132); *ii.* pembrolizumab for patients previously treated with ipilimumab (N=15); *iii.* monotherapy with ipilimumab or nivolumab or their combination (N=15). Moreover, patients treated with ipilimumab plus chemotherapy (NIBIT-M1 study; N=37) that have been previously described [25], were included in this study to carry out a more extensive evaluation. A control group of melanoma patients included subjects with metastatic disease treated either with standard chemotherapy or radiotherapy regimens (N= 31) or with BRAF inhibitor (BRAFi; vemurafenib or dabrafenib) -based targeted therapies (N=34). These patients did not ever receive any immunotherapy regimen. The therapeutic treatment of melanoma patients that were performed in the context of clinical studies were conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice and have been approved by the Ethics Committee of the University Hospital of Siena. An informed consent for bio-

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banking and use of biological samples and clinical data for scientific research was obtained from all the patients enrolled in this study.

Response criteria were assessed according the proposed immune-related response criteria for immunotherapy treatments, where objective response included immune-related complete or partial response while disease control included immune-related confirmed complete, partial or stable disease. [45] [46] For patients not treated with immunotherapy, response criteria were assessed according WHO. Clinical responses evaluated as disease control (DC) and overall survival (OS) were available for from N= 193 and 194 patients, respectively.

Biological samples

Serum from melanoma patients was collected at pre-treatment (baseline) and 12 weeks (12W), post-treatment and then isolated by centrifugation and cryopreserved.

Detection of sNKG2DLs in the serum of melanoma patients

The concentration in the serum of melanoma patients (N= 162) of sNKG2DL (MICA, MICB, ULBP-2, ULBP-1), was assessed by the usage of ELISA kits (R&D Systems). Commercially available pair antibodies and related reagents (R&D Systems) were used to set up the ELISA assay to determine the concentration in the serum of sULBP-3. A standard curve with determined titrations of the recombinant human proteins allowed to measure sNKG2DL concentrations in the experimental samples. Data are means of duplicates and are represented as pg/ml. In some cases, N=100 patients, the amount of available serum allowed to repeat twice the Elisa assays; the inter-assay coefficient of variation has been calculated and was in the range of 2-8%. Statistical analysis of

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differences between means of concentration of NKG2DLs at different time points was performed using two-tailed t-test (p < 0.05). The concentration of sULBP-1 was determined in the baseline and W12 time points serum of N= 131 and 128 patients, respectively. The serum of N=10 HD was used as negative control as reported elsewhere. [25]

Gene expression analysis

Expression analysis for NKG2D, MICB and ULBP-1 were obtained from available RNA sequencing (RNA-Seq) cancer sets in TCGA research network (http://cancergenome.nih.gov/) for 30 cohorts of cancer patients with different type of tumors.

Statistical analysis

Data were analyzed in a descriptive way using mean and standard deviations. This study was aimed at the identification of variations in candidate biomarkers previously identified (*see ref. 25*) associated with DC and OS. Association between sNKG2DLs and DC was assessed by chi squared test. Survival curves were estimated by the Kaplan-Meier method and differences were evaluated with the log-rank test. Differences in OS according to sNKG2DLs (MICA, MICB, ULBP-1, 2, 3), gender, age, stage, LDH, PS were analyzed. A Cox regression analysis was implemented to investigate the role of each factor considered and of its relationship with the other variables in correlating with OS. A forward stepwise selection method was used based on Wald statistics, resulting models were confirmed by a backward procedure. Hazard ratio and their 95% confidence interval (95% CI) were reported. IBM SPSS v. 21 was used for statistical analysis. For TCGA data hazard ratio was calculated using R (v3.3.1) and survival package (v2.39-5).

The forest plot was generated using the forest plot package (v1.5.1). The Cox proportional hazards regression model was applied on the highest versus lowest tertiles of expression in each cancer cohort; p-values were calculated using pchisq function form the base stats package.

Disclosure statement

The authors have no conflict of interest to disclose.

List of abbreviations

Ab   Antibody

CTLA-4   Cytotoxic T-Lymphocyte Antigen-4

DCR   Disease Control Rate

FBS   Fetal Bovine Serum

HS   Human Serum

mAb   Monoclonal Antibody

MICA MHC (HLA) Class I Chain-Related Gene A

MICB MHC (HLA) Class I Chain-Related Gene B; MMA

NKG2D   the activating receptor NK cell group 2 member D (NKG2D); Rate

OS   Overall Survival

PD-1   Programmed Cell Death -1

PD-L1 Programmed Cell Death Ligand 1

ULBP-1 or -2 UL16-Binding Protein-1 or -2

Funding details

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Table 1. Clinical-pathological features of melanoma patients treated with immune checkpoint blockade agents or with standard/targeted therapies

Parameter	Control cohort (N=65)		Immune checkpoint Cohort (N=162)		Immune checkpoint plus chemotherapy Cohort [a] (N=37)	
	N	%	N	%	N	%
Men	42	65	92	57	24	65
Women	23	35	70	43	13	35
Median Age (years)	59		62		55	
PS						
0	24	36.9	42	25.9	33	89.2
1	38	58.5	118	72.8	4	10.8
2	3	4.6	2	1.2	0	0
Median LDH	268		290		332	

Pathological type						
Cutaneous melanoma	59	91	137	85	37	100
Ocular melanoma	0	0	9	6	0	0
Mucosal melanoma	2	3	12	7	0	0
Acral melanoma	4	6	4	2	0	0
Clinical stage						
III	12	18	7	4	1	3
IV	53	82	155	96	36	97
Therapeutic treatment						
ipilimumab	0	0	132	81	0	0
ipilimumab + pembrolizumab	0	0	15	9	0	0
Nivolumab, Ipilimumab monotherapy or combination	0	0	15	9	0	0
Ipilimumab plus chemotherapy	0	0	0	0	37	100

Standard therapies alone	31	48	0	0	0	0
BRAF inhibitors	34	52	0	0	0	0
Clinical Responses						
DCR	29	59	64	40	24	65
Median OS (95% C.I.)	13(7.7-18.3)		13.8(10.0-17.6)		24.3(18.0-29.7)	

[a]Patients were treated with the combination of ipilimumab plus fotemustine (*see ref.* [46]);

PS: Performance Status;

LDH: Lactate Dehydrogenase

DCR: disease control rate which includes complete responses (CR), partial responses (PR) and stable disease (SD) according the immune related response criteria assessment (*see refs.* [45,46]).

Table 2. **Association between the levels at baseline serum of sNKG2DLs and the disease control of melanoma patients**

sNKG2DLs [a]		Immune checkpoint treatment [b]	DCR [c]	p [d]	Control [e]	DCR	p
MICA	+	22	45.7	0.99	16	32.5	0.24
	-	171	45.6		42	45.2	
MICB	+	42	42.9	0.72	19	36.8	0.16
	-	151	46		39	56.4	
ULBP-1	+	85	32.9	**0.002**	35	54.3	0.42
	-	77	57.1		23	43.5	
ULBP-2	+	45	44.4	0.86	9	44.4	0.72
	-	148	45.9		49	51	
ULBP-3	+	77	49.4	0.39	23	43.5	0.42
	-	116	43.1		35	54.3	

[a]detection of sNKG2DLs (MICA, MICB, ULBP-1-3) in the pre-treatment serum of melanoma patients; positive (+) or negative (-) measurement (ng/ml) of sNKG2DLs in the patients' serum;

[b]Number of subjects from the cohort of patients treated with immune checkpoint blockade agents;

^cdisease control rate (DCR) expressed as the percentage of patients with complete responses (CR), partial responses (PR) and stable disease (SD) according the immune related response criteria assessment (*see refs.* [45,46]);

^dp value obtained from *Chi-squared test* analysis; ^e: number of patients from the control cohort.

Table 3. Association between the levels at baseline serum of sNKG2DLs and the OS of melanoma patients

	sNKG2DLs [a]	Immune checkpoint treatment [b]	OS [c]	P [d]	Control [e]	OS	P
Baseline							
MICA	+	22	12.5	0.45	19	15.6	0.94
	-	172	16.4		46	12.0	
MICB	+	43	8.8	**0.02**	25	13	0.4
	-	151	21.6		40	12	
ULBP-1	+	85	12.1	**0.01**	36	15.6	0.85
	-	78	25.3		29	8.5	
ULBP-2	+	45	9.8	0.11	9	15.6	0.77
	-	149	20.2		56	12	
ULBP-3	+	77	13.8	0.10	25	16	0.65
	-	117	16.7		40	11.2	
W12							
MICA	+	22	12.4	**0.02**	11	11.1	0.18
	-	159	20.2		13	n.r.	
MICB	+	54	10.4	**0.01**	11	n.r.	0.05
	-	127	22.8		12	11.0	

ULBP-1	+	80	14.7	0.17	13	11.1	0.14
	-	70	26.3		10	n.r.	
ULBP-2	+	49	14.7	0.27	5	15.6	0.82
	-	132	21.6		12	24.3	
ULBP-3	+	70	16.8	0.57	5	n.r.	0.49
	-	106	16.7		18	13.1	

[a]detection of sNKG2DLs (MICA, MICB, ULBP-1, 2, 3) in the pre- (baseline) or post- (W12) treatment serum of melanoma patients; positive (+) or negative (-) measurement (ng/ml) of sNKG2DLs are indicated;

[b]number of subjects from the cohort of patients treated with immune checkpoint blockade agents;

[c]OS: median overall survival expressed as months;

[d]P value obtained from *log-rank test* analysis; [e]: Number of patients from the control cohort.

[f]W12 post-treatment data from melanoma patients treated with BRAFi; n.r.= not reached. Post-treatment from patients treated with standard therapies was not available.

Table 4. COX regression analysis to identify biomarkers influencing the clinical outcome of melanoma patients

Marker	Patient							
	Immune checkpoint Cohort				Control cohort			
	Univariate analysis of factor		Multivariate analysis of factor		Univariate analysis of factor		Multivariate analysis of factor	
	HR (CI 95%)	P	HR (CI 95%)	P	HR (CI 95%)	P	HR (CI 95%)	P
Gender	0.96 (0.69-1.33)	0.80			1.26 (0.67-2.38)	0.47		
Age	1.00 (0.99-1.01)	P=0.39			1.02 (0.99-1.04)	0.16	1.03 (1.01-1.05)	**0.02**
LDH	1.01 (1.01-1.02)	**<0.0001**	1.02 (1.01-1.03)	**<0.0001**	1.01 (1.01-1.02)	**<0.0001**	1.02 (1.01-1.03)	**<0.0001**
PS	1.25 (0.90-1.72)	0.18			2.02 (1.07-3.82)	**0.03**		
Stage	2.63 (0.87-8.26)	0.10			6.20 (1.50-25.62)	**0.01**		
MICA	1.25 (0.70-2.24)	0.45			0.97 (0.50-1.91)	0.94		
MICB	1.67 (1.08-2.59)	**0.02**			0.75 (0.38-1.47)	0.40		
ULBP-1	1.78 (1.14-2.77)	**0.01**	1.72 (1.09-2.71)	**0.02**	0.94 (0.50-1.76)	0.84		
ULBP-2	1.42 (0.92-2.19)	0.11	1.91 (1.10-3.33)	**0.02**	0.88 (0.37-2.10)	0.77		
ULBP-3	1.38 (0.94-2.04)	0.10	1.38 (0.94-2.04)	0.10	0.87 (0.47-1.61)	0.65		

The markers were determined as the following: gender: male vs. female; LDH was categorized based on increment of 10 IU/L; PS: 1, 2 vs.0; Stage: IV vs. III; MICA, MICB, ULBP-1, 2 and 3: positive detection vs. absence in the serum.
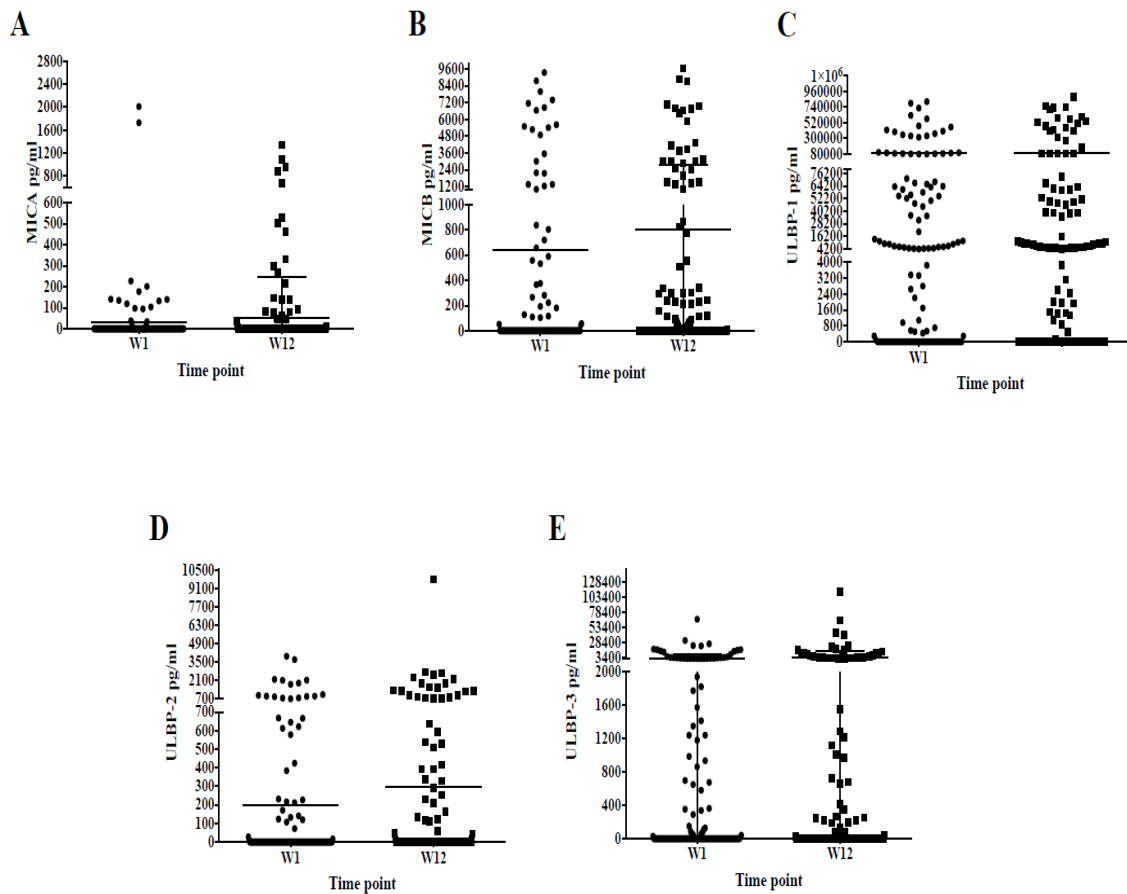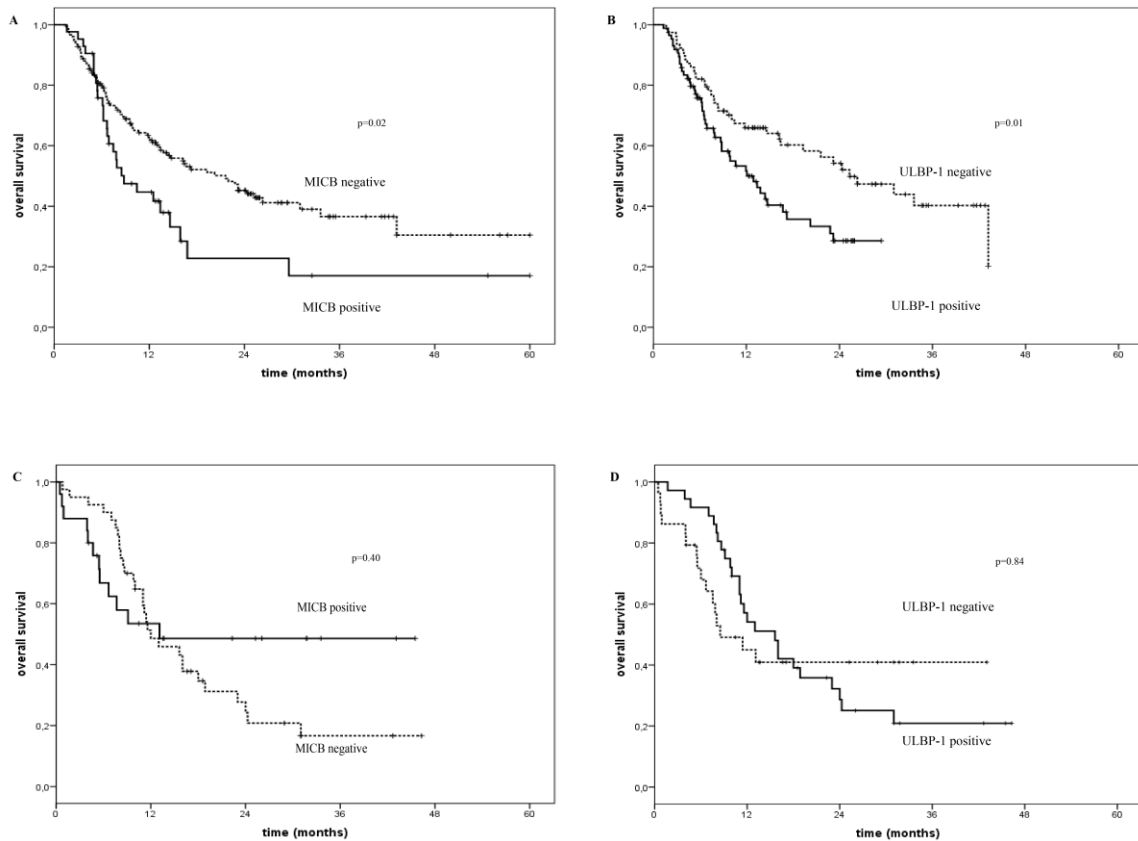
Figure 1. *sNKG2DLs in the serum of melanoma patients treated with immune checkpoint blockade agents.* The presence of sMICA (Panel A), sMICB (Panel B) and sULBP-2, 3 (Panels D and E) in the serum at baseline (black circle) and post-treatment (black square) of melanoma patients (N=162) treated with immune checkpoint blockade agents was measured by ELISA assay (*see Material and Methods*). ULBP-1 determinations at pre- and post-treatment were carried out in N= 131 and 128 patients, respectively (Panel C). Mean and error bars are shown in the graphs. As negative control the serum of N=10 HD was used in ELISA assays (*data not shown; see ref. [25]*).
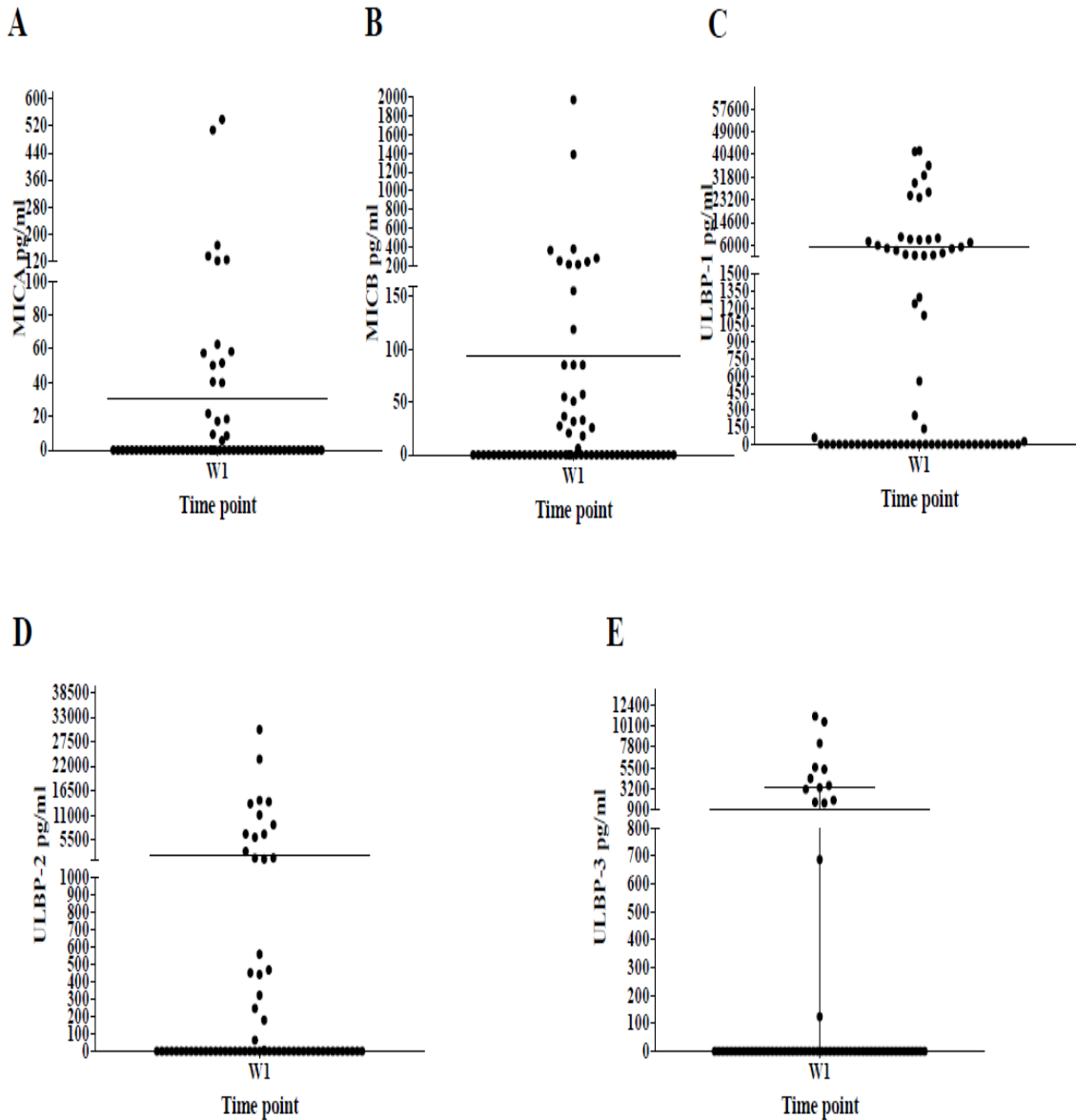
Figure 2: Detection of sNKG2DLs in the serum of control group melanoma patients.
The presence of soluble NKG2DLs (MICA, Panel A; MICB, Panel B; ULBP-1, Panel C; ULBP-2, Panel D; ULBP-3, Panel E) was measured by ELISA assay (*see Material and Methods*) in the baseline serum (black circle) of melanoma patients (N=65) treated with either standard or BRAFi therapies. Mean and error bars are shown in the graphs. As negative control the serum of N=10 HD was used in ELISA assays (*data not shown; see ref. [25]*).

Figure 3. Overall survival of melanoma patients treated with immunotherapy in relation with the presence or not of sNKG2DLs in serum. Kaplan-Meier plots of overall survival of melanoma patients treated with immune checkpoint blockade agents (Panels A and B) or with standard or BRAFi therapies (Panels C and D) in relation with the detection (black line) or not (dotted line) at baseline of sMICB (Panels A and C) and sULBP-1 (Panels B and D). The baseline serum levels of sMICB (Panel A) and ULBP-1 (Panel B) could discriminate melanoma patients with long term survival (median OS= 21.6 and 25.3 months, p=0.02 and 0.01, respectively) from poor survivors (median OS = 8.8 and 12.1 months, respectively) for the cohort of patients treated with immune checkpoint blockade agents. Panels C and D show the absence of association between

the serum levels of these ligands and OS in the control group of patients (median OS=

12.0 vs 13.1 and 8.5 vs.15.6 and p=0.4 and 0.85, respectively).
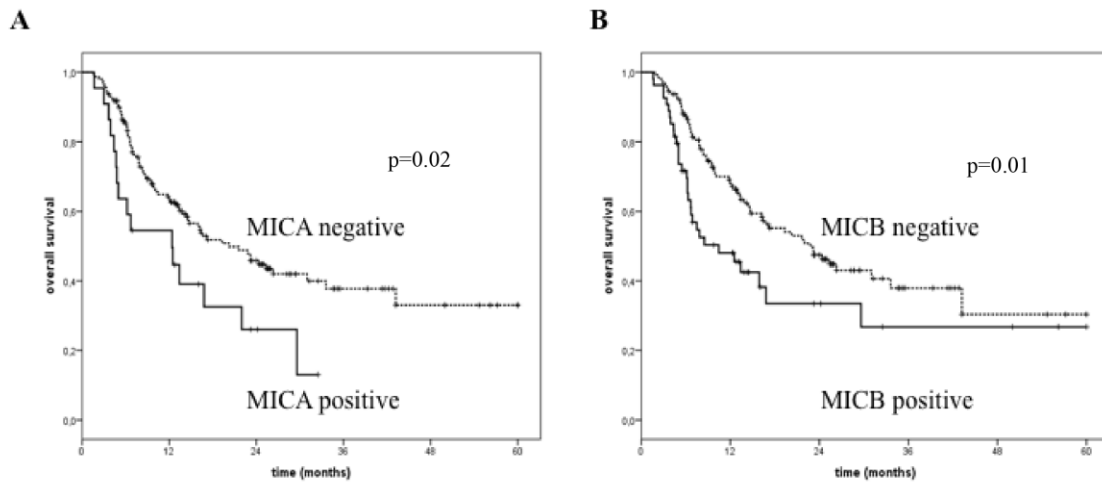
Figure 4. Overall survival of melanoma patients treated with immunotherapy in association with the levels of sNKG2DLs in post-treatment serum. The absence (dotted line) in the post-treatment serum of sMICA (Panel A) and sMICB (Panel B) correlated with improved OS (median OS= 20.2 and 22.8 vs. 10.4 months, p=0.02 and 0.01, respectively) while detectable levels of these molecules were found in the serum of poor survivor patients (median OS=12.4 and 10.4 months, respectively) (black line).