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RESEARCH ARTICLE

Biomarkers of suppressed natural killer (NK) cell function in metastatic melanoma: Decreased NKG2D and increased CD158a receptors on CD3–CD16+ NK cells

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Abstract

In metastatic melanoma (MM) patients we evaluated natural killer (NK)-cell activity, distribution of several NK receptors and their correlation with NK function. Peripheral blood lymphocytes (PBL) of MM patients and controls were analysed for NK activity and expression of activating NKG2D, CD161 and KIR, CD158a and CD158b receptors on CD3–CD16+ NK cells. MM patients not only had significantly decreased NK activity and NK-cell interferon (IFN)-γ production, a redistribution of NK-cell subsets with an increase in $CD16^{dim}$ and a reduction in $CD16^{bright}$ NK subsets. There was a decreased CD161 and NKG2D and an increased CD158a NK-cell expression in MM patients, with a positive correlation between NKG2D expression and NK cytotoxicity and an inverse correlation between CD158b expression and NK-cell cytotoxicity in patients. Furthermore, patients' $CD3\text{-}CD16^{bright}$ NK subset showed lower expression of CD161 and CD158a. Therefore, NKG2D, CD158a and CD158b expression in MM patients may represent several clinically useful 'biomarkers' of suppressed NK function.

Keywords: *NK cells; melanoma; cytotoxicity; NKG2D; CD158a; CD158b*

Introduction

Natural killer (NK) cells, unlike tumour-specific T cells, are defined by their capacity to kill certain tumour-target cells without prior sensitization or major histocompatibility complex (MHC)-restriction (Kiessling et al. 1975, Robertson & Ritz 1990). They are characterized by a CD3–CD16+ phenotype, CD16 (FcγRIII) being the low-affinity receptor for IgG, expressed on the majority of NK cells, which is involved in direct (Nagler et al. 1989, Warren & Skipsey 1991, Mandelboim et al. 1999) and in antibody-dependent cell-mediated cytotoxicity (ADCC) (Lanier et al. 1986). In spite of the fact that CD16 is an important cytotoxic receptor, NK cell phenotype and function have been almost exclusively defined with respect to the expression of CD56, an adhesion molecule that is not directly involved in cytotoxicity (Cooper et al. 2001a, Konjevic et al. 2003). CD16 associates with two intracytoplasmic domains composed of FcεRI-γ or TCR-ζ chains (Warren & Skipsey 1991) that comprise the immunoreceptor tyrosine-based activation motifs (ITAM), which upon ligand binding become phosphorylated and induce signal transduction by activation of ZAP-70 and syk kinases (Vivier et al. 2004, Lanier 2005). Based on CD16 cell-surface expression, NK cells are divided into two subsets functionally analogous to CD56 subsets, a larger $CD16^{bright}$ NK-cell subset has high expression of CD16 and is composed of cytotoxic cells, while the smaller $CD16^{dim}$ NK subset has low expression of CD16 antigen and encompasses immunoregulatory NK cells that produce different cytokines including interferon (IFN)-γ, tumour necrosis factor (TNF)-α,

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interleukin (IL)-10, IL-13 and granulocyte macrophage–colony-stimulating factor (GM-CSF) (Nagler et al. 1989, Biron et al. 1999, Cooper et al. 2001b).

Recently numerous new activating and inhibitory NK-cell receptors have been identified and their engagement by cognate ligands on target tumour cells allows NK cells to discriminate between normal and transformed cells (Farag & Caligiuri 2006). Activating receptors include recently characterized members of the C-type lectin family, receptors NKG2D (Raulet 2003) and CD161 (Pozo et al. 2006) that cooperate, and with other cytotoxic receptors determine NK-cell cytotoxicity against transformed cells. NKG2D is a pivotal activating receptor (Raulet 2003) which upon binding stress-induced ligands on transformed cells, such as MHC-class-I-related molecules, MICA/MICB (Nausch & Cerwenka 2008) and UL16-binding protein (Diefenbach et al. 2003), induces cytotoxicity by recruiting (PI)-3 kinase after its intracellular domain associates with DAP10 adaptor protein (Wu et al. 1999). For this reason activating NKG2D receptor has a role in tumour immunosurveillance, as well as in immune-mediated rejection of tumour cells to prevent tumour progression (Hayakawa & Smyth 2006, Solana et al. 2007, Colonna 2008). The other member of the C-type lectin family, the CD161 receptor, is primarily designated as an activating receptor that plays a role in NK cell-mediated cytotoxicity (Lanier 1998, Azzoni et al. 1998), although recently, on identification of its lectin-like transcript 1 (LLT1) ligand, its inhibitory potential has been described; however, it still remains controversial (Rosen et al. 2005, Aldemir et al. 2005).

The killer cell inhibitory, i.e. immunoglobulin, receptors (KIR) are type I membrane glycoproteins responsible for the inhibition of NK cell-mediated lysis of normal cells that express MHC class I molecules (Farag & Caligiuri 2006). In this sense, activation of NK cells, according to the 'missing-self' hypothesis, occurs in contact with malignantly transformed cells that have lost MHC class I molecules, and have therefore become susceptible to lysis (Lanier 1998). However, even without loss of MHC class I molecules, NK cells can be activated if target cells abundantly express NK cell-stimulating ligands (Farag & Caligiuri 2006).

The KIR family is divided into haplotype A, which is more frequent, and haplotype B; in this study we investigated the expression of two frequent inhibitory receptors that belong to haplotype A, i.e. inhibitory receptors CD158a (KIR2DL1) and CD158b (KIR2DL2,3) that recognize HLA-Cw4,6,5 and HLA-Cw1,3,7 class I molecules, respectively (Uhrberg et al. 1997, Trowsdale & Parham 2004, Yawata et al. 2006). It has recently been reported that higher expression of CD158a and CD158b receptors, especially in patients that express their specific HLA-C ligands, has been associated not only with susceptibility to melanoma but also with disease progression, shown by their increase in NK cells in advanced stages of melanoma (Campillo et al. 2006, Naumova et al. 2007). Owing to the fact that the inhibitory signals that they initiate by dephosphorylating surrounding tyrosine kinases and adaptor proteins, including NKG2D associated DAP-10, can dampen or override activation of NK cells, these KIR receptors may facilitate tumour escape and progression (Naumova et al. 2007).

In spite of the importance of the functional dichotomy of NK cells, aside from several reports for healthy individuals (Cooper et al. 2001a, Lima et al. 2001, Takahashi et al. 2007), there are no reports for melanoma patients that deal with quantitative distribution of activating or inhibitory receptor on CD16-defined dim and bright NK-cell subsets.

As NK-cell activity is downregulated in advanced malignancies, including melanoma (Sibbitt et al. 1984, Seidel et al 1998, Jurisic et al. 2007, Konjevic et al. 2007), it is of interest to define, for the first time, the association of impaired NK cytotoxicity with the expression of a representative set of activating and inhibitory NK-cell receptors on less-investigated CD16-defined NK cells. Moreover, the differential expression of these receptors was investigated on CD3–CD16^{bright} cytotoxic and CD3–CD16^{dim} regulatory NK-cell subsets. As the NK cells belong to the first line of immune defence, the evaluation of these NK-cell receptors is of importance as they are indicators of impaired NK-cell function that is associated with increased susceptibility to tumour escape and metastatic progression.

Materials and methods

In this study 66 patients with histologically proven MM (clinical stage IV) according to the modified AJCC/UICC staging system, aged 22–71 years (41 males and 25 females) were investigated for NK-cell activity and immunophenotype. Performance status was evaluated from 0 to 4 by the ECOG scale. Fifty healthy volunteers, age and sex matched, with no evidence of any disease or infection were evaluated for NK-cell activity and other investigated immunological parameters (Table 1). Heparinized blood samples were obtained from patients before initiation of treatment. Before inclusion in the study informed consent was signed by each patient and approved by the ethical committee of Institute of Oncology and Radiology of Serbia.

Peripheral blood lymphocyte isolation

Peripheral blood lymphocytes (PBL) were isolated using Lymphoprep (Nypacon, Oslo, Norway) density gradient, centrifuged at 500*g*, 40 min, and washed three times in RPMI 1640 culture medium (CM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS, Sigma, St. Louis, MO, USA).

Table 1. The clinicopathological characteristics of metastatic melanoma (MM) patients and healthy controls.

Characteristic		Healthy	Melanoma patients
Age:			
	Range	21–65	22–71
	Median	48	51
Gender:			
	Male	37	41
	Female	13	25
Performance status:			
	0		17
	1		28
	2		10
	3		8
	4		3
Primary tumour localization:			
	Head and neck		12
	Trunk		21
	Upper limb		10
	Lower limb		15
	Unknown		8
Metastases			
	Lung		17
	Liver		22
	Soft tissues		8
	Bones		5
	Suprarenal		1
	Spleen		2
	Retroperitoneal tissue		3
	Lymph nodes		28

Flow cytometric analysis

Surface phenotype of freshly isolated PBL subsets were identified using the following combinations of directly labelled monoclonal antibodies (mAbs): CD3PerCP/CD16FITC, CD3PerCP/CD16FITC/CD161PE, CD3PerCP/CD16PE/CD158aFITC, CD3PerCP/CD16FITC/CD158bPE (Becton Dickinson, San Jose, CA, USA) and CD3PerCP/CD16FITC/NKG2DPE cells (R&D, Minneapolis, MN, USA). The samples were prepared as previously described (Jackson & Warner 1986). Briefly, 1.0×10^5 freshly isolated PBL in 100 µl RPMI 1640 supplemented with 10% FCS, were incubated for 30 min at 4°C with 20 µl of appropriate mAb combination, washed twice with ice-cold PBS and fixed with 1% paraformaldehyde prior to FACS analyses. Surface marker expression was quantified on FACSCalibur flow cytometer (Becton Dickinson). A total of 10 000–50 000 gated events, verified as PBL according to their physical characteristics (FSC and SSC), were collected per sample and analyzed using CellQUEST software. Exclusion of non-specific fluorescence was based on matched isotype mAb combinations conjugated with FITC, PE and PerCP (Becton Dickinson). NK cells were defined and gated within the lymphocyte gate according to their expression of CD3 and CD16 (CD3-CD16+). In order to define two NK-cell subsets of low, i.e. CD3–CD16^{dim} or high, i.e. CD3–CD16^{bright} subsets, CD3–CD16+ NK cells were divided based on density of CD16 antigen defined by mean fluorescence intensity (MFI). The NK-cell receptors, CD161, NKG2D, CD158a and CD158b on CD3-CD16+ NK cells were expressed as percentage in PBL as well as percentage in gated CD3–CD16+ NK cells. In order to define precisely the expression of any of these receptors on CD3–CD16^{dim} or CD3–CD16^{bright} NK-cell subsets for each individual the flow cytometry data of the percentage of the double-positive CD3–CD16^{dim} or CD3–CD16^{bright} NK-cell subset (e.g. CD3–CD16$^{dim/bright}$CD161+) was divided by the percentage of the same double-positive (e.g. CD3-CD16+CD161+) NK cells and multiplied by 100, according to the following formula:

$$\frac{\text{CD3}-\text{CD16dim/bright CD161+}}{\text{CD3}-\text{CD16}+\text{CD161+}} \times 100$$

for precise definition of the analyzed (e.g. CD3–CD16$^{dim/bright}$CD161+) subset.

For intracellular staining for IFN-γ 500 000 PBL were incubated with PMA (50 ng ml^{-1}) plus ionomycin (500 ng ml^{-1}) for 4 h at 37°C and with Brefeldin A (10 µg ml^{-1}) for the last 3 h (for flow cytometric analysis of intracellular cytokine staining). Cells were first stained for surface antigens with CD3PerCP and CD16PE antibodies, fixed and after permeabilization with BD FACS permeabilizing solution 2 (BD Biosciences, San Jose, CA, USA) stained for anti-IFN-γ FITC (Becton Dickinson).

Co-culture of PBL with tumour cell lines

Freshly isolated PBL were adjusted to 3×10^6 cells ml^{-1}. One hundred microlitres of the PBL suspension was mixed with 100 µl of the erythromyeloid cell line K562 (2×10^6 ml^{-1}) and 100 µl of human melanoma cell line FemX (2×10^6 ml^{-1}) as tumour target cells in 96-microwell plates. Cell mixture was centrifuged at 100*g* for 3 min and samples were incubated for 4 h at 37°C in a humidified atmosphere in a CO_2 incubator. Cells were precipitated, washed and stained afterwards as described above with CD3PerCP, CD16FITC, CD161PE and CD158aFITC mAbs for the analysis of the percentage of CD3–CD16^{dim} and CD3–CD16^{bright} NK-cell subsets in PBL, as well as the expression of CD161 and CD158a NK-cell receptors on dim and bright NK-cell subsets.

NK cell assay

NK cell-specific lysis was determined using standard cytotoxicity assay (Brown et al. 1985). One hundred microlitres of freshly isolated PBL, as effector cells, at concentration of 4.0×10^6 ml^{-1} of CM and two 1 : 1

dilutions, were mixed with 100 µl of K562 and FemX cell lines at concentration of $0.05 \times 10^6\,ml^{-1}$, (prelabelled with radioactive ^{51}Chromium (Na_2CrO_4, As = 3.7 MBq, Amersham, Buckinghamshire, UK)), to form triplicates of three effector cell (E) to target cell (T) ratios (E:T), 80:1, 40:1 and 20:1. The assay was performed in 96 round-bottomed microwell plates (Falcon, Irvine, CA, USA) which were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Plates were, then centrifuged for 3 min at 200*g* and the supernatant from each well was used for determination of the amount of released ^{51}Chromium from the lysed target tumour cells in a gamma counter (Berthold, FRG) and expressed in counts per min (cpm). The mean percentage cytotoxicity was calculated using the following formula:

$$\frac{\text{cpm(experimental release)} - \text{cpm(spontaneous release)}}{\text{cpm(maximal release)} - \text{cpm(spontaneous release)}} \times 100$$

Maximal release was obtained by incubation of K562 and/or FemX target tumour cell lines at the same concentration in the presence of 5% Triton X-100, and spontaneous release was obtained by incubation of the appropriate target tumour cell line in culture medium, alone.

Statistical analysis

Significance of differences for obtained results was determined by non-parametric Mann–Whitney and Wilcoxon tests while the correlation between NK-cell activity and receptor expression was performed by Pearson's test.

Results

The evaluation of NK-cell activity of MM patients with metastatic, clinical stage IV, melanoma prior to therapy, showed significantly impaired NK-cell activity compared with healthy controls (24.80 ± 3.69% vs 49.5 ± 4.86%, respectively, $p < 0.01$, Mann–Whitney test) evaluated against a standard sensitive K562 tumour target cell line whereas there was no difference in NK-cell activity evaluated against the FemX tumour target cell line between MM patients and healthy controls (8.75 ± 2.34% vs 17.07 ± 3.88%, respectively, $p > 0.05$, Mann–Whitney test) (Figure 1A). Furthermore, analysis of intracellular IFN-γ production in CD3–CD16+ NK cells showed that MM patients have significantly lower IFN-γ level in comparison with healthy controls (19.57 ± 2.45% vs 26.14 ± 5.21%, respectively, $p < 0.05$, Mann–Whitney test) (Figure 1B). Analyses of NK-cell activity with respect to the primary localization of melanoma showed that NK-cell activity of patients with more favourable localization on the limbs had significantly higher activity than patients with primary

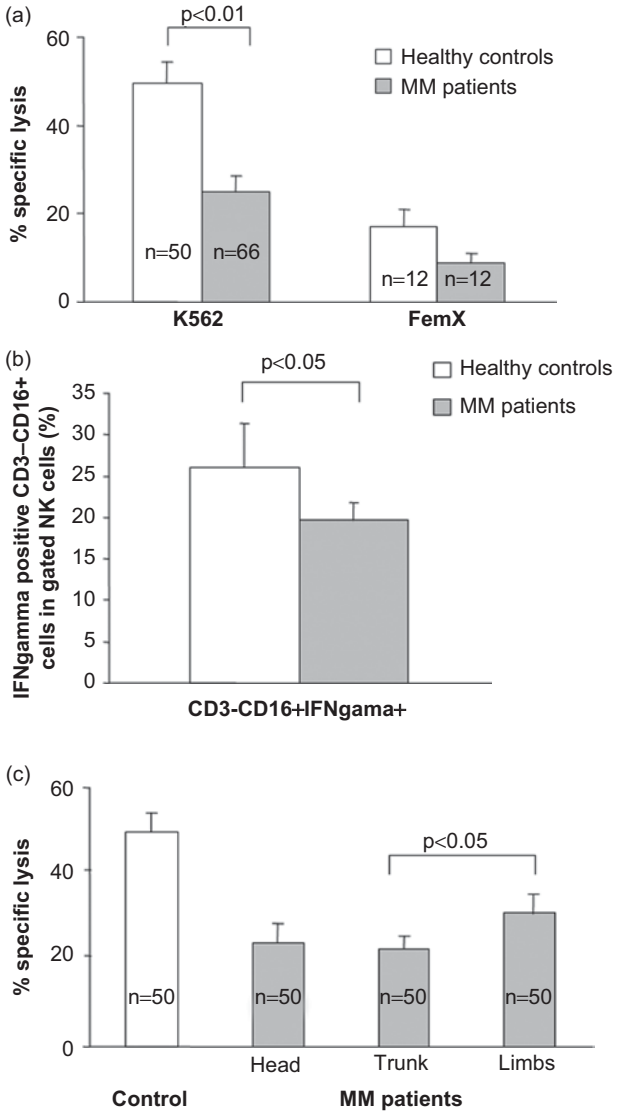

Figure 1. (A) Natural killer (NK)-cell activity evaluated against the K562 tumour target cell line (E:T, 80:1) of metastatic melanoma (MM) patients ($n = 66$) shows significant ($p < 0.01$) impairment compared with healthy controls ($n = 50$) whereas there is no difference ($p > 0.05$) in NK-cell activity evaluated against the FemX tumour target cell line (E:T, 80:1) between MM patients ($n = 12$) and healthy controls ($n = 12$). (B) The production of intracellular interferon (IFN)-γ in CD3–CD16+ NK cells is significantly ($p < 0.05$) lower in MM patients in comparison with healthy controls. Results are expressed as percentages of IFN-γ-positive cells in peripheral blood lymphocytes (PBL) and are shown as mean ± SE. (C) NK-cell activity of MM patients (E:T, 80:1) ($n = 50$) with respect to primary localization of melanoma shows a significant ($p < 0.05$) decrease in patients with localization on the trunk ($n = 20$) compared with localization on the limbs ($n = 24$). Results are expressed as percentages of specific lysis and are shown as mean ± SE.

localization on the trunk (30.83 ± 4.53% vs 22.56 ± 2.90%, respectively, $p < 0.05$, Mann–Whitney test) (Figure 1C).

The evaluation of the absolute number of lymphocytes per litre of blood and relative and absolute values for the CD3–CD16+ NK cells in peripheral blood did not show

significant differences between healthy controls and MM patients and MM patients with different primary localization, i.e. trunk or limbs ($p > 0.05$, Mann–Whitney test) (Table 2).

According to the density of expression of CD16+ there was a significant difference in the percentage of the CD3–CD16^{dim} and CD16^{bright} NK-cell subsets between MM patients and healthy controls. The percentage of CD3–CD16^{dim} NK cells was significantly higher ($p < 0.01$) in MM patients than in controls (33.19 ± 3.60% vs 21.03 ± 2.07%, respectively), while the percentage of CD3–CD16^{bright} NK cells was significantly lower ($p < 0.01$), in MM patients compared with controls (66.81 ± 3.60% vs 78.97 ± 2.07%, respectively), analyzed by the Mann–Whitney test (Figure 2A). Figure 2B shows representative dot plots for the expression of CD16 in the CD3–CD16^{dim} and CD3–CD16^{bright} NK-cell subsets in healthy controls and MM patients. Furthermore, in this study we showed a positive correlation between the percentage of the CD3–CD16^{bright} NK-cell subset and NK-cell activity in both healthy controls and MM patients ($p = 0.0073$ and $p = 0.0489$, respectively, Pearson's correlation). In addition, after 4 h of lymphocyte co-culture with target tumour cell lines, K562 and FemX, the percentage of the CD3–CD16^{dim} NK-cell subset significantly increased (0.88 ± 2.20% vs 2.25 ± 1.76% with K562 and 0.88 ± 2.20% vs 2.87 ± 2.07% with FemX), while the percentage of CD3–CD16^{bright} NK cells was significantly decreased (15.16 ± 0.88% vs 11.56 ± 1.10% with K562 and 15.16 ± 0.88% vs 12.18 ± 2.54% with FemX) ($p < 0.05$, Wilcoxon test) (Figure 2C).

Freshly isolated PBL of MM patients showed a significant decrease in the expression of both activating NK-cell receptors, CD161 and NKG2D, compared with healthy controls on CD3–CD16+ NK cells in PBL as well as on gated NK cells ($p < 0.05$, Mann–Whitney test) (Table 3). Figure 3 gives representative dot plots for the expression of CD161 and NKG2D on CD3–CD16+ NK cells in healthy controls and MM patients.

In this study we have provided new data related to KIRs, CD158a and CD158b, expression on CD3–CD16+ NK cells in PBL and on gated NK cells in MM patients, as well as in healthy controls. We showed that MM patients have a significant increase in expression, compared with healthy controls, of the CD158a receptor on CD3–CD16+ NK cells in PBL as well as on gated NK cells ($p < 0.01$, Mann–Whitney test). In contrast to the expression of the CD158a receptor, there was no significant difference

Table 2. The evaluation of the absolute number of lymphocytes and relative and absolute number of CD3–CD16+ natural killer cells in the peripheral blood of healthy controls and metastatic melanoma (MM) patients.

	CD3–CD16+ (%)	Lymphocytes ($\times 10^9$ l^{-1})	CD3–CD16+ ($\times 10^9$ l^{-1})
Healthy ($n = 35$)	15.99 ± 1.51	1.34 ± 0.11	0.19 ± 0.02
MM ($n = 47$)	13.85 ± 1.06*	1.40 ± 0.07*	0.21 ± 0.02*
Primary localization			
Trunk ($n = 18$)	13.87 ± 1.44	1.47 ± 0.11	0.21 ± 0.02
Limbs ($n = 18$)	14.07 ± 1.93	1.39 ± 0.12	0.20 ± 0.03

*$p > 0.05$, Mann–Whitney test.

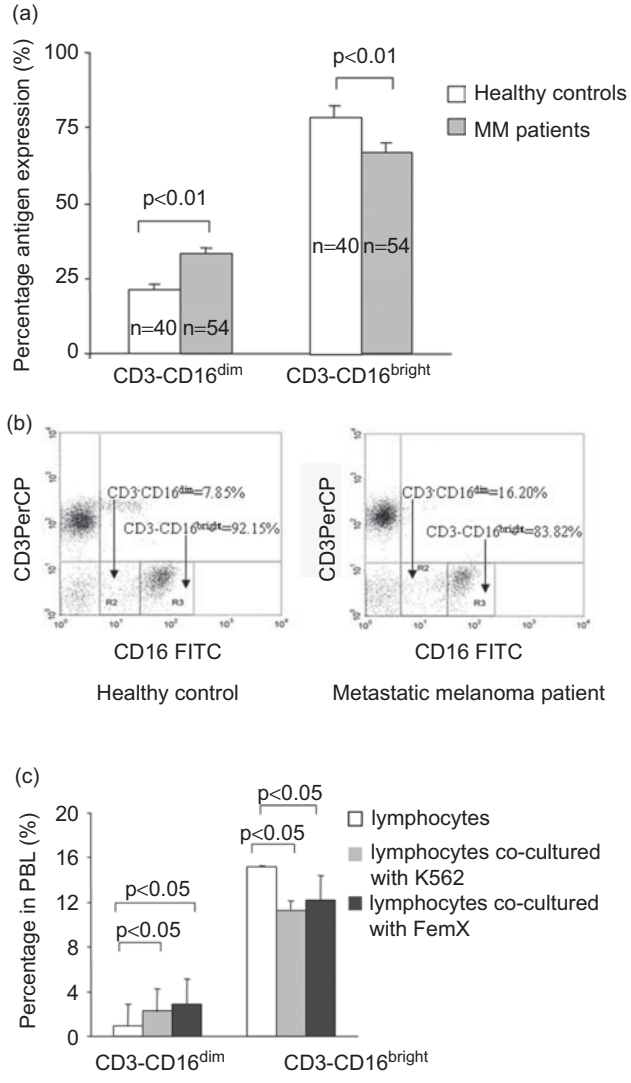

Figure 2. (A) The percentage of the CD16^{bright} subset is significantly ($p < 0.01$) lower, while the percentage of the CD16^{dim} natural killer (NK)-cell subset is significantly ($p < 0.01$) higher in metastatic melanoma (MM) patients ($n = 54$) compared with controls ($n = 40$). Results are presented as percentages in gated CD3–CD16+ NK cells and are shown as mean ± SE. (B) Representative flow cytometry dot plots show a significantly lower percentage of the CD3–CD16^{bright} and a significantly higher percentage of the CD3–CD16^{dim} NK-cell subset in an MM patients compared with controls. NK cells are defined within the lymphocyte gate according to their expression of CD3 and CD16 receptors (CD3–CD16+) and divided on CD3–CD16^{dim} and CD3–CD16^{bright} subsets based on the mean fluorescence intensity (MFI) of the CD16 receptor. (C) The percentage of the CD3–CD16^{dim} NK-cell subset is significantly ($p < 0.05$) increased while the percentage of the CD3–CD16^{bright} subset is significantly ($p < 0.05$) decreased after 4 h of lymphocyte co-culture with target tumour cell lines, K562 and FemX. Results are presented as percentages in peripheral blood lymphocytes (PBL) and are shown as mean ± SE.

Table 3. Activating natural killer (NK)-cell receptors on CD3–CD16+ peripheral blood lymphocytes (PBL) and gated NK cells in healthy controls and metastatic melanoma (MM) patients.

	CD3–CD16+CD161+ PBL (%)	CD3–CD16+CD161+ NK cells (%)	CD3–CD16+NKG2D+ PBL (%)	CD3–CD16+NKG2D+ NK cells (%)
Healthy controls ($n=30$)	5.45 ± 1.24	38.36 ± 5.06	13.09 ± 1.56	88.50 ± 3.10
Melanoma patients ($n=50$)	3.11 ± 0.53[a]	26.86 ± 3,96[a]	8.08 ± 1.30[a]	71.73 ± 6.05[a]

[a]The percentage of CD3–CD16+CD161+ and CD3–CD16+NKG2D+ cells in PBL and gated NK cells in MM patients is significantly below ($p<0.05$, Mann–Whitney test) that for healthy controls.

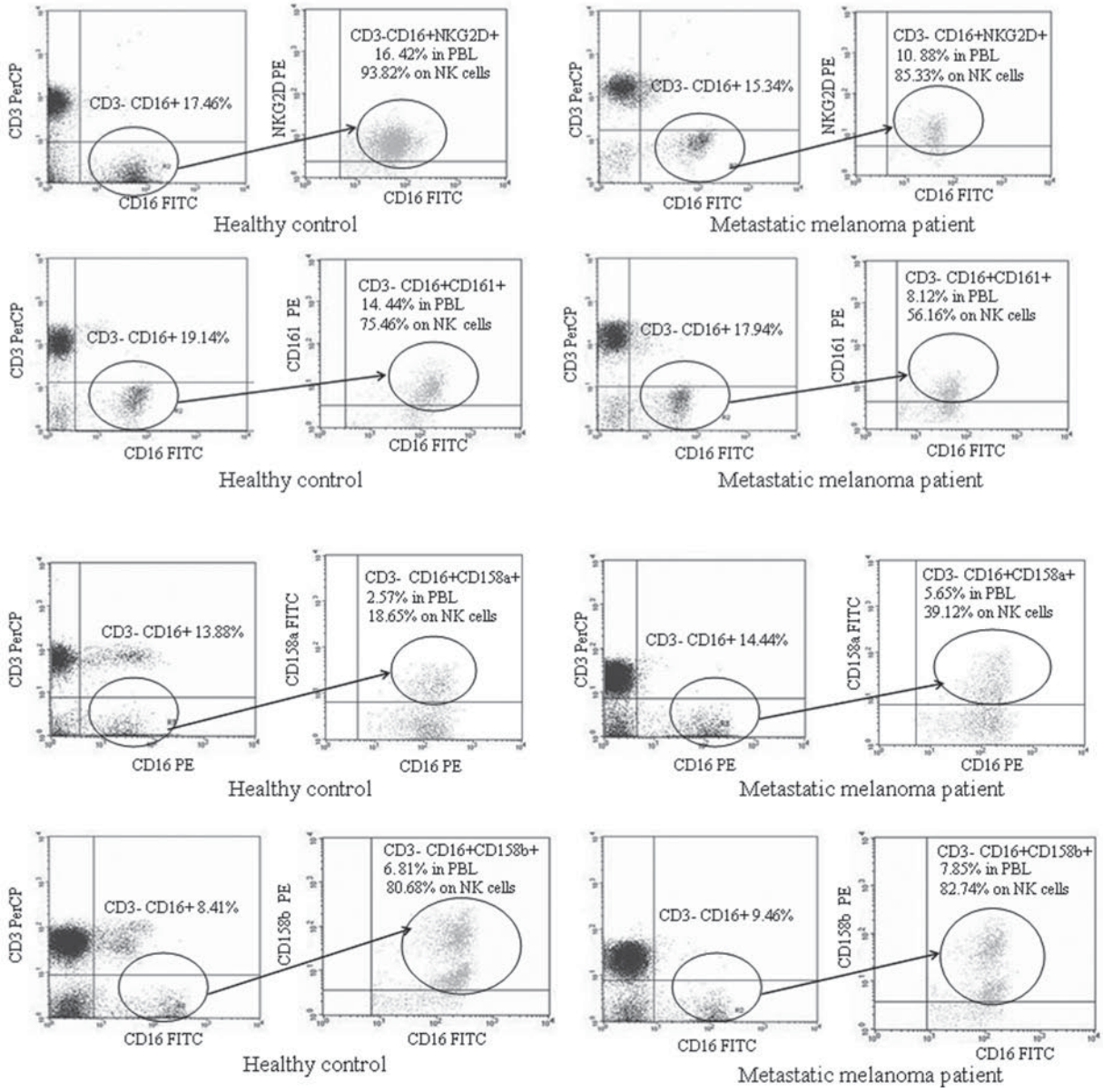

Figure 3. Representative flow cytometry dot plots of healthy individuals and metastatic melanoma (MM) patients show that MM patients have a significant decrease in the expression of both activating natural killer (NK)-cell receptors, NKG2D and CD161, as well as, an increase in the expression of the killer cell inhibitory receptor (KIR) CD158a, compared with healthy controls, on CD3-CD16+ NK cells in peripheral blood lymphocytes (PBL) and gated NK cells. The expression of the other KIR receptor, CD158b does not differ between a healthy individual and an MM patient. The first and the third columns represent dot plots of gated CD3-CD16+ NK cells of healthy controls and MM patients, while the second and fourth columns represent dot plots of the expression of NK-cell receptors, NKG2D, CD161, CD158a and CD158b derived from gated CD3-CD16+ NK cells. The values in columns two and four are given as the percentage of double-positive NK cells in total PBL, as well as in gated CD3-CD16+ NK cells for healthy controls and MM patients.

between MM patients and controls in the expression of the other KIR receptor, CD158b, on CD3–CD16+ NK cells in PBL as well as on gated NK cells ($p > 0.05$, Mann–Whitney test) (Table 4). Figure 3 gives representative dot plots for the expression of CD158a and CD158b on CD3–CD16+ NK cells in healthy controls and MM patients.

Correlation of NK cell-specific lysis of healthy controls and MM patients (E:T, 80:1) and the expression of the investigated NK-cell receptors showed that in contrast to the lack of correlation for the percentage of gated CD3–CD16+NKG2D+ NK cells with NK-cell activity in healthy controls ($p = 0.228$, Pearson's correlation) (Figure 4A), MM patients showed positive correlation of the percentage of gated CD3–CD16+NKG2D+ NK cells with NK-cell activity ($p = 0.019$, Pearson's correlation) (Figure 4B). Furthermore, there was no correlation in either healthy controls or MM patients of the percentage of gated CD3–CD16+CD158a+ NK cells and NK-cell activity ($p > 0.05$, Pearson's correlation) (Figure 4C, D). In contrast to this, the percentage of the more

Table 4. KIR inhibitory natural killer (NK)-cell receptors on CD3–CD16+ peripheral blood lymphocytes (PBL) and gated NK cells in healthy controls and metastatic melanoma (MM) patients.

	CD3–CD16+CD158a+ PBL (%)	CD3–CD16+CD158a+ NK cells (%)	CD3–CD16+CD158b+ PBL (%)	CD3–CD16+CD158b+ NK cells (%)
Healthy controls ($n = 30$)	0.95 ± 0.23	5.89 ± 1.47	4.84 ± 0.58	40.91 ± 3.75
Melanoma patients ($n = 50$)	2.07 ± 0.30[a]	14.66 ± 1.88[a]	4.02 ± 0.40	37.70 ± 2.87

[a]The percentage of CD3–CD16+CD158a+ cells in PBL and gated NK cells in MM patients is significantly higher ($p < 0.01$, Mann–Whitney test) than that for healthy controls.

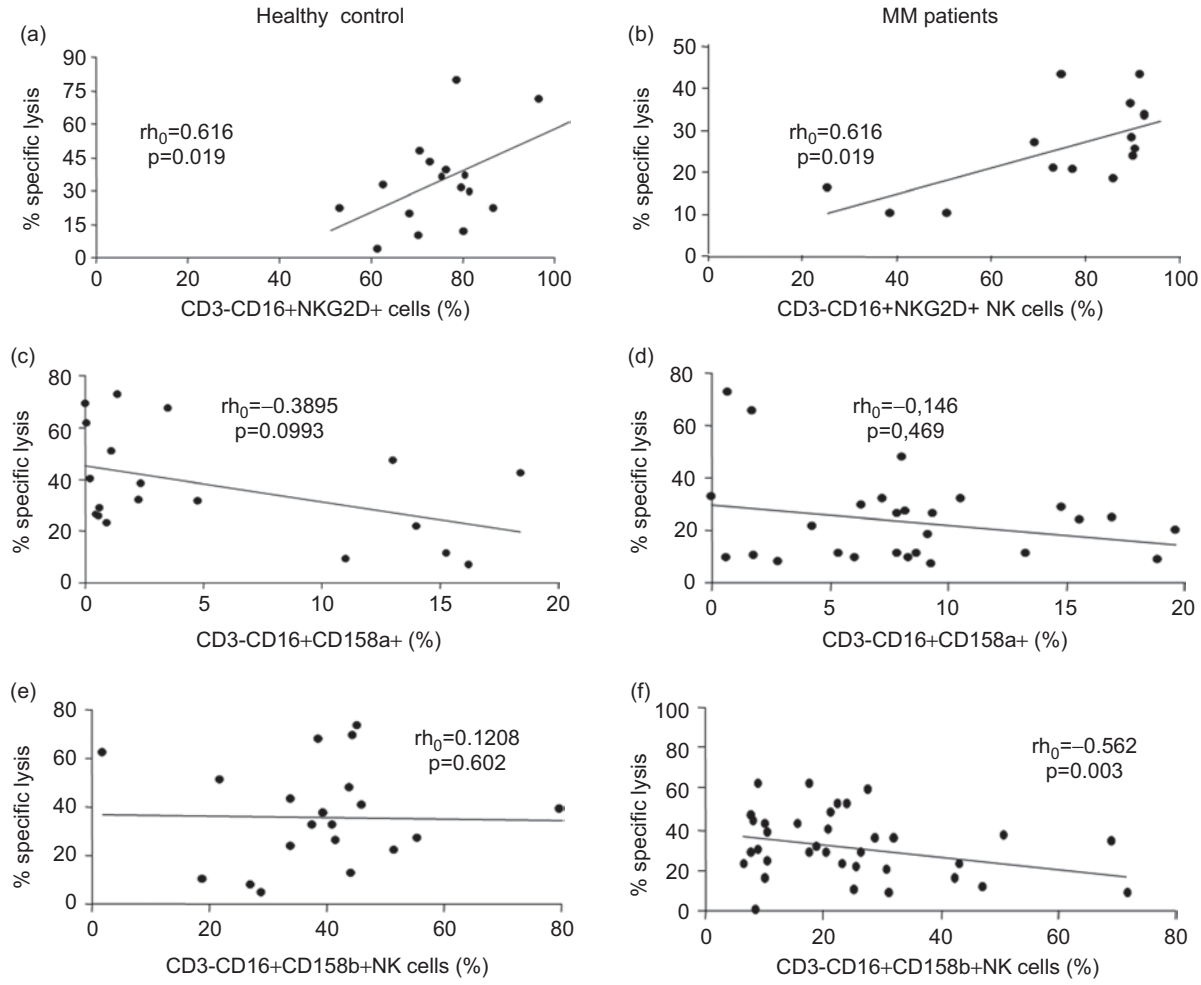

Figure 4. Correlation of the expression of natural killer (NK)-cell receptors on gated NK cells and NK-cell activity (E:T, 80:1) for CD3–CD16+NKG2D+ NK cells in (A) healthy controls (no correlation, $p = 0.228$ Pearson's correlation) and (B) metastatic melanoma (MM) patients (positive correlation, $p = 0.019$ Pearson's correlation), for CD3–CD16+CD158a+ NK cells in (C) healthy controls and (D) MM patients ($p > 0.05$, Pearson's correlation), and for CD3–CD16+CD158b+ NK cells in (E) healthy controls ($p = 0.602$, Pearson's correlation) and (F) MM patients ($p = 0.003$, Pearson's correlation).

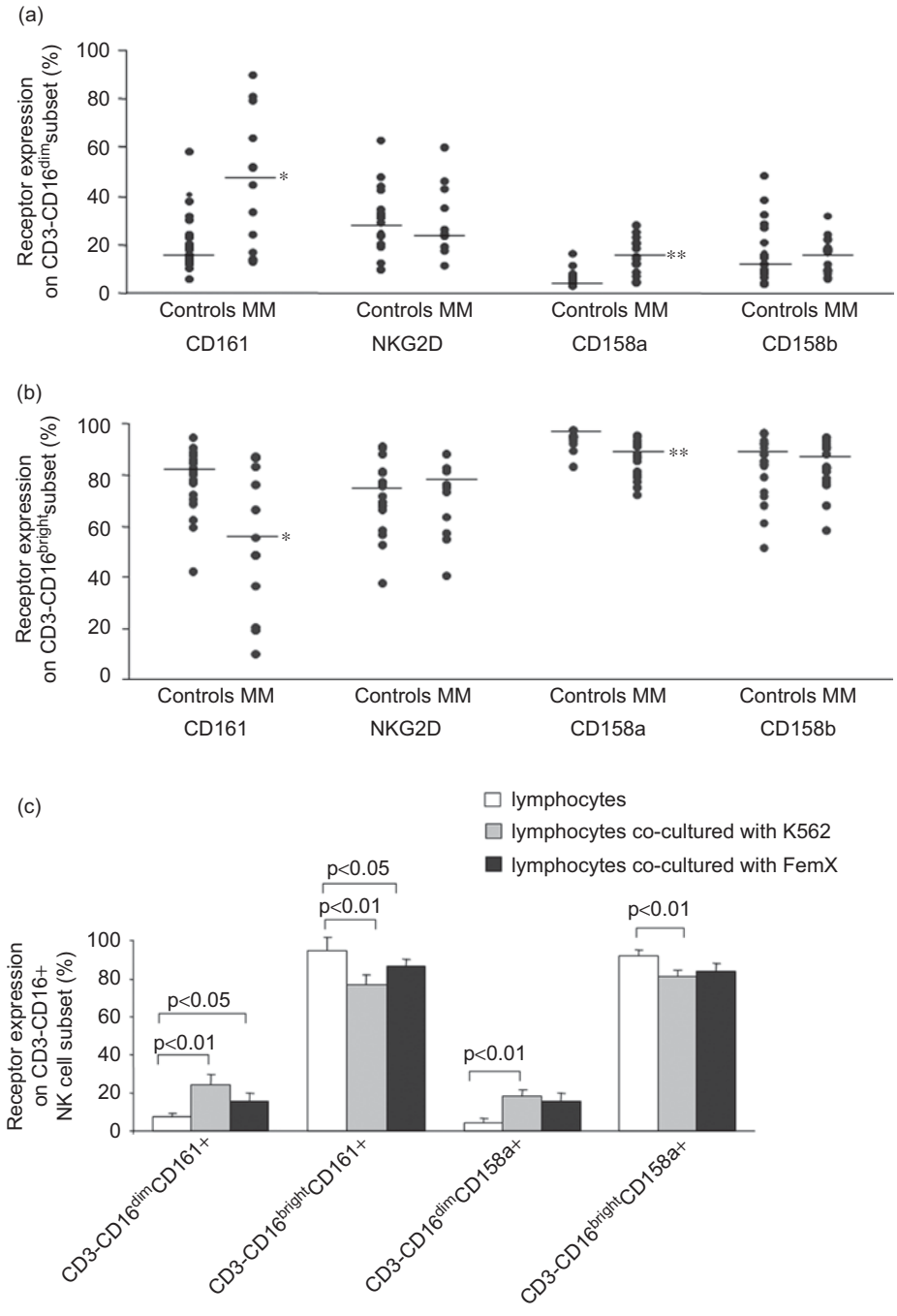

Figure 5. (A) The expression of CD161, NKG2D, CD158a and CD158b receptors on CD3–CD16^{dim} natural killer (NK)-cell subsets shows a significant increase of CD161 (*$p<0.05$) and CD158a (**$p<0.01$) in metastatic melanoma (MM) patients ($n=25$) compared with healthy controls ($n=30$) (B) In contrast to the expression on the CD3–CD16^{dim} NK-cell subset, the expression of CD161, NKG2D, CD158a and CD158b receptors on the CD3–CD16^{bright} NK-cell subset shows a significant decrease of CD161 (*$p<0.05$) and CD158a (**$p<0.01$) in MM patients ($n=25$) compared with healthy controls ($n=30$). The results are expressed as individual values of percentage of expression for each receptor, while horizontal lines represent median values for each receptor. (C) The expression of activating CD161 and killer cell inhibitory receptor (KIR) CD158a NK-cell receptors after 4 h of co-culture of peripheral blood lymphocytes (PBL) with the K562 and FemX tumour cell lines shows on the CD3–CD16^{dim} NK-cell subset a significant increase in the expression CD161 after co-culture with both tumour cell lines ($p<0.01$ and $p<0.05$, respectively), whereas the expression of CD158a significantly ($p<0.01$) increases only after co-culture with the K562 tumour cell line. In contrast to this, after 4 h of co-culture of PBL with the K562 and FemX tumour cell lines there is a significant reciprocal decrease in the expression of these two receptors on the CD3–CD16^{bright} NK-cell subset. The results are expressed as mean percentages ± SE.

abundant CD158b+ receptor on gated CD3–CD16+ NK cells was negatively correlated with NK-cell activity in MM patients ($p = 0.003$, Pearson's correlation) (Figure 4F), while there was no correlation in healthy controls ($p = 0.602$, Pearson's correlation) (Figure 4E).

The analyses of the expression of different NK-cell receptors on CD3–CD16^{dim} and CD3–CD16^{bright} NK-cell subsets showed a significantly higher expression of CD161 on the CD3–CD16^{dim} subset (Figure 5A) and a significantly lower expression on the CD3–CD16^{bright} NK-cell subset (Figure 5B) in MM patients compared with controls (median 44.08% vs 17.86%, $p < 0.05$ and 55.92% vs 82.14%, $p < 0.05$, respectively, Mann–Whitney test). Further analyses of the expression of the investigated activating NK-cell receptor, NKG2D, showed that there was no difference between MM patients and controls. Median for NKG2D on CD3–CD16^{dim} was 25.27% for MM patients versus 29.93% for controls and 74.73% for MM patients versus 70.06% for controls on the CD3–CD16^{bright} NK-cell subset ($p > 0.05$, Mann–Whitney exact test) (Figure 5A, B).

The expression of the investigated KIR receptors, CD158a and CD158b, showed that MM patients had a significantly higher expression of CD158a on the CD3–CD16^{dim} subset (Figure 5A) and a significantly lower expression on the CD3–CD16^{bright} NK-cell subset (Figure 5B) compared with controls (median 13.73% vs 5.88% and 86.27% vs 94.12%, respectively, $p < 0.01$, Mann–Whitney test). In contrast to this, there was no difference in the expression of CD158b between MM patients and controls on the CD3–CD16^{dim} (median 17.14% vs 23.57%, respectively) and CD3–CD16^{bright} NK-cell subsets (median 82.86% vs 78.43%, respectively, $p > 0.05$, Mann–Whitney test) (Figure 5A, B).

The analysis of the expression of the two investigated NK-cell receptors, activating CD161 and inhibitory KIR CD158a on CD3–CD16^{dim} and CD3–CD16^{bright} NK-cell subsets after 4 h of co-culture of PBL with K562 and FemX tumour cell lines showed a significant increase in the expression of CD161 on CD3–CD16^{dim} NK-cell subset after co-culture with both tumour cell lines, while CD158a expression also increased on the CD3–CD16^{dim} NK-cell subset, however, only after co-culture with the K562 tumour cell line and not with FemX. The expression of these two receptors showed a reciprocal decrease after co-culture with the two tumour cell lines on CD3–CD16^{bright} NK-cell subset (Figure 5C).

Discussion

Impairment in NK-cell cytotoxic activity is associated with advanced malignancies (Konjevic et al. 1999, Costello et al. 2004, Kiladjian et al. 2006) and has been observed to date in a few studies in MM patients (Sibbitt et al. 1984, Seidel et al 1998, Konjevic et al. 2007). We show that MM patients with advanced, clinical stage IV of the disease have a significant decrease in NK-cell activity compared with controls when evaluated against the standard NK-sensitive K562 tumour cell line, although there was no difference in relative or absolute NK-cell number. Moreover, even lower NK-cell cytotoxicity was obtained against a human melanoma, FemX, cell line that could be the consequence of either lack of expression on this tumour cell line of activating tumour-associated stress proteins, such as MICA/B, or persistent expression of HLA class I molecules that by engaging KIR receptors downregulate NK-cell lysis (Pende et al. 2001, Schrambach et al. 2007). As found in other advanced malignancies, this NK-cell dysfunction in MM patients is probably the consequence of cytokine dysbalance due to the prevalence of immunosuppressive cytokines such as, IL-10 and TGF-β (Lee et al. 2004), as well as tumour-produced inhibitory factors (Konjević & Spuzić 1992). Also in support of suppressed NK-cell function is our novel finding of decreased NK-cell IFN-γ production in MM patients compared with controls, considering that secretion of cytokines, especially IFN-γ, constitutes the other major, immunoregulatory, function of NK cells (Zwirner et al. 2007, Ciszak et al. 2009). We show, moreover, for the first time that patients with less favourable primary localization of melanoma, i.e. on the trunk, have significantly lower NK-cell activity compared with patients with more clinically favourable primary localization on the limbs associated with slower disease progression (Slingluff & Reintgen 1993, Schuchter et al. 1996, Måsbäck et al. 2001). Our results also indicate that the observed localization-associated impairment of NK-cell activity is not based on the difference in relative or absolute NK-cell numbers in these groups and controls.

Aside from giving detailed analyses of the CD16+ subset expression for healthy controls that are in accord with a previous report (Nagler et al. 1989), we show for the first time that MM patients, compared with controls, have a significantly lower percentage of the cytotoxic CD16^{bright} and a significantly higher percentage of the regulatory CD16^{dim} NK-cell subset. These interesting novel results indicate that a decrease in the cytotoxic CD16^{bright} NK-cell subset may contribute to the impaired NK cytotoxicity found in these patients (Sibbitt et al. 1984, Nagler et al. 1989, Konjević et al. 2007). To confirm this hypothesis we correlated NK-cell cytotoxicity with the percentage of CD16^{bright} NK cells and obtained new data that show positive correlation of these two parameters. Furthermore, we show that the decrease in CD16^{bright} NK-cell subset is significantly greater after co-culture of NK cells with tumour cells, suggesting, as reported in a few previous studies (Azzoni et al. 1995, Gryzwacz et al. 2007), that this interaction leads to a loss in CD16

expression and a consequent change in the dim and bright subset distribution. This finding in MM patients has broader implications as NK-cell subset redistribution has been investigated in the pathogenesis of HIV, autoimmunity and in connection to aging (Borrego et al. 1999, Tarazona et al. 2002, Pridgeon et al. 2003).

Even though an increasing number of NK-cell receptors has been recently characterized, we show for the first time that the expression of the activating receptor NKG2D is decreased on NK cells of MM patients, compared with controls. In other malignancies the presence of NKG2D^{low} NK cells has been associated with elevated plasma levels of transforming growth factor (TGF)-β (Lee et al. 2004), soluble NKG2D tumour-derived ligands (Groh et al. 2002), as well as with post-activational downregulation due to chronic NKG2D–MICA interaction (Coudert & Held 2006, Maccalli et al. 2007). Furthermore, as shown during disease progression in melanoma, due to proteasomal degradation tumour cells may become low in NKG2D ligands (NKG2DL^{low}) with consequent poor recognition by NKG2D^{low} NK cells (Zwirner et al. 2007, Fuertes et al. 2008). NKG2D-induced impairment in NK-cell activity has so far been implicated in patients with lung and colorectal cancer (Lee et al. 2004, Le Maux Chansac et al. 2005). However, our finding that significantly impaired NK-cell activity of these patients shows positive correlation with the percentage of NKG2D+ NK cells, emphasizes the activating role of this receptor and is in agreement with the only other study showing its activating role in myelodysplastic patients (Epling-Burnette et al. 2007). Furthermore, additional new data that we give of significantly reduced expression of CD161 (Azzoni et al. 1998), an activating receptor, on NK cells in these patients, together with significantly reduced expression of NKG2D, may underlie the serious impairment in their cytotoxic function.

With respect to the expression of KIR receptors we found an increase in the CD158a receptor, with no change in CD158b, on NK cells of MM patients. However, considering that the NK-cell KIR repertoire is determined by the expression of specific HLA-ligands by an individual (Kogure et al. 1999, 2003, Parham 2005a), increase in CD158a+ NK cells should reflect the fact, as shown in one report, that a greater number of melanoma patients bear corresponding HLA-C haplotype (Campillo et al. 2006). Aside from the genetic determination of KIR repertoire (Parham 2005b), the increase in CD158a may also be epigenetically influenced by cytokines such as TGF-β, which has been shown, unlike IL-4 and IL-6, to upregulate KIR expression (Pan et al. 2003, Zhang et al. 2005).

Furthermore, the negative correlation of CD158b+, unlike CD158a+ NK cells, with NK-cell activity of the studied patients reflects the inhibitory effect of previous *in vivo* KIR engagement, through CD158b–HLA-C interactions, considering that CD158b is more abundantly expressed on NK cells (37%), compared with CD158a (14%). Furthermore, our novel data show that the presence of KIR receptors affects NK cytotoxicity, as shown by low NK-cell lysis of the HLA-positive FemX tumour cell line, compared with the HLA-negative K562. These KIR receptors may be responsible for downregulation of NK-cell activity by inactivation of various signalling molecules in NK cells, which is supported by the newly shown basic data of dephosphorylation of the guanine exchange factor, Vav1 (Peterson & Long 2008). The unfavourable association of decreased NK-cell cytotoxicity and increased KIR expression may influence tumour escape and even consequent disease progression (Naumova et al. 2005, Raulet & Vance 2006, Campillo et al. 2006, Solana et al. 2007).

Considering that there are very few reports that deal with CD16-based NK cell dichotomy, we also give, for the first time, distribution of a set of activating and inhibitory NK-cell receptors on these two subsets. Unlike the shown equal expression of CD161 on CD56^{dim} and CD56^{bright} NK cells in the only two existing reports for healthy individuals (Jacobs et al. 2001, Lima et al. 2001), we show that the expression of CD161 is significantly lower on the cytotoxic CD16^{bright} and significantly higher on CD16^{dim} NK-cell subset in MM patients compared with controls. Considering that CD161 is a lectin-like, activating receptor (Azzoni et al. 1998, Pozo et al. 2006), this decreased novel finding on the more mature, cytotoxic CD16^{bright} NK-cell subset, may contribute to the shown impaired NK-cell cytotoxicity in MM patients.

Regarding KIR expression on NK-cell subsets, our data shed new light on the scarce and qualitative data of their expression in healthy controls (Colonna 1997, Cooper et al. 2001a, Lima et al. 2001, Takahashi et al. 2007) and in MM patients. In this sense, aside from confirming that KIR are abundantly present on the CD56^{dim}, i.e. the CD16^{bright} subset, and that they are low to absent on CD56^{bright}, i.e. the CD16^{dim} NK-cell subset in healthy controls (Cooper et al. 2001a), our analyses further show significantly lower expression of CD158a, and a less pronounced decrease in the expression of CD158b, on the CD16^{bright} subset, i.e. increased expression of CD158a and CD158b on the CD16^{dim} NK-cell subset, in MM patients.

Considering that KIR expression is the hallmark of a mature NK-cell phenotype (Held 2008, Caligiuri 2008), the decrease in CD158a on the CD16^{bright} subset may suggest the beginning of a shift to an immature NK phenotype in MM patients, considering that CD16^{bright} and CD16^{dim} NK subsets may switch from one to the other, depending on the microenvironment, as postulated in the novel 'linear differentiation model' of human NK development (Romagnani et al. 2007, Chan et al. 2007, Colonna 2008). As this model in normal conditions suggests that growth factors, such as fibroblast growth factor

(FGF), can influence the NK-cell subset switch (Chan et al. 2007), it would be expected that in tumour-bearing individuals numerous aberrant growth factors together with a cytokine dysbalance (Krasagakis et al. 1995, Lee et al. 2004) may be responsible for this detected unfavourable tip of the balance towards the less mature, $CD16^{bright}$ $158a^{low}$ phenotype. Moreover, our additional data, after co-culture of NK cells with K562 and FemX tumour cells induces the observed change in CD161 as well as CD158a expression in these subsets, supports the tumour-related nature of this phenomenon.

In this study we provide new data for metastatic melanoma patients showing distribution of a set of activating and inhibitory NK-cell receptors on CD16-defined NK cells and $CD3–CD16^{bright}$ and $CD3–CD16^{dim}$ subsets. These obtained novel results suggest that NKG2D surface down-modulation, together with increased expression of CD158a on NK cells, and negative CD158b correlation with NK-cell activity of metastatic melanoma patients may represent several clinically useful 'biomarkers' of suppressed NK-cell function. Moreover, these data indicate that increase in KIR+ NK cells, especially in the light of more frequent corresponding KIR ligand (i.e. HLA) expression in metastatic melanoma, may be important in evaluating the risk of disease progression.

Therefore, aside from elucidating some conditions that may underlie reduced cytotoxicity by unfavourably 'tumour-immunoedited' NK cells in MM patients, these results support the new therapeutic procedures based on blocking overexpressed inhibitory KIRs and enhancing the expression of NKG2D, together with its ligands, that may promote NK cell-mediated tumour cytolysis. The superior antineoplastic effect of these procedures in different solid tumours is encouraging and gives desired practical aspects to the research of NK cell receptors.

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