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ORIGINAL ARTICLE

Effect of cytokines on NK cell activity and activating receptor expression in high-risk cutaneous melanoma patients

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ABSTRACT. ***Objective:*** **Stage II melanoma patients have high risk for regional and distant metastases and may benefit from novel therapeutic strategies. To clarify the role of NK cells in Stage II melanoma, we characterized the cytotoxic activity of NK cells and the expression of various activating and inhibitory receptors in high-risk cutaneous melanoma patients (Stages IIB and IIC) compared to low-risk patients (Stage IA).** ***Materials and Methods:*** **Native and cytokine-treated peripheral blood mononuclear cells were used for functional and phenotypical analyses.** ***Results:*** **Compared to Stage IA-B patients, Stage IIB-C patients showed significantly decreased NK cell activity, as well as decreased expression of the activating NKG2D and CD161 receptors, most likely due to increased serum levels of the immunosuppressive cytokine TGF-β1 in these patients. Interestingly, treatment of periperal blood mononuclear cells with IFN-α, IL-2, IL-12 or the combination of IL-12 and IL-18 significantly induced NK cell activity for both groups of melanoma patients. However, only low-risk patients had a significant increase in the expression of the NKG2D receptor after *in vitro* treatment with IFN-α, as well as an significant increase in the expression of CD161 after treatment with IFN-α or IL-12. Although IL-2 induced the expression of NKG2D in both groups of patients, this increase was significantly lower in high-risk melanoma.** ***Conclusion:*** **NK cell parameters may be useful as biomarkers of disease progression in localized melanoma patients. Our results further suggest that the use of NK cell-activating cytokines in combination with inhibitors of immunosuppressive factors like TGF-β1 could be a therapeutic option for the treatment of high-risk cutaneous melanoma patients.**

Key words: NK cells, cytotoxic activity, high-risk cutaneous melanoma patients, IFN-α/IL-2/IL-12/IL-18, NKG2D, TGF-β1/VEGFA

INTRODUCTION

Natural killer (NK) cells are innate lymphoid cells (ILC) that are able to eliminate malignant cells by cytotoxic mechanisms and the production of interferon gamma (IFN-γ) [1]. Tumor cell recognition by NK cells is regulated by a complex balance of inhibitory and activating signals delivered via different germline-encoded NK cell receptors [2, 3]. NK cell-mediated cytotoxicity toward cancer cells is mediated via binding of the activating NK cell receptor NKG2D to ligands on transformed cells including the MHC class I chain-related (MIC) proteins A and B, and the UL-16 binding proteins (ULBPs) [4]. The CD161 NK cell receptor contributes to the activation of NK cell cytotoxicity toward different human tumor cell lines [5, 6]. However, it has been reported that the binding of the CD161 receptor to lectin-like transcript 1 (LLT1) can inhibit NK cell function [7, 8]. Therefore, the role of CD161 receptor in the regulation of NK cell function in malignancies remains unclear. The killer immunoglobulin-like receptors (KIRs) belong to the immunoglobulin superfamily. According to the length of their cytoplasmic tail, KIRs are classified into long inhibitory (e.g., KIR2DL and KIR3DL) and short stimulating (e.g., KIR2DS and KIR3DS) receptors, although inhibitory KIRs are dominant [9]. Inhibitory KIR receptors prevent NK cell-mediated lysis of normal cells that express MHC-I molecules, the major ligands for KIRs. In this sense, according to the "missing-self" hypothesis, the activation of NK cells occurs in contact with malignant transformed cells that have low expression of MHC-I molecules and are thus susceptible to attack by NK cells [9, 10].

Melanoma is the most aggressive form of skin cancer and is characterized by a high metastatic potential [11]. Since melanoma is an immunologically reactive tumor, spontaneous regressions have been observed which

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may be associated with NK cell activity. In agreement, both experimental and clinical studies suggest that melanoma cells are susceptible to NK cell-mediated lysis [12]. However, more recently, we and others have shown that NK cells from melanoma patients with advanced metastatic disease have impairments in their immune response including decreased cytotoxic activity and receptor expression [13-15]. In this sense, immuno-modulating agents such as stimulating cytokines that enhance the cytotoxicity of NK cells may be beneficial for treatment of metastatic melanoma patients [16]. IFN-α and IL-2 have been approved as therapeutic agents for melanoma over the past several decades. However, considering their limited therapeutic activity, additional cytokines, such as IL-12 and IL-18, are currently under investigation for the treatment of this disease [17].

In this study, we wanted to establish NK cell-mediated cytotoxicity, as well as the expression of various activating and inhibitory NK cell receptors in high-risk cutaneous melanoma patients in Stage IIB and IIC compared with low-risk melanoma patients in Stages IA and IB. Our results suggest that certain NK cell parameters may serve as biomarkers of disease progression for localized melanoma patients. Our results further provide leads for the improvement of immunotherapy for this group of patients.

METHODS

Blood samples

In this study peripheral venous blood was obtained from 20 melanoma patients with clinical stages IA and IB (Stage IA-B group) and 20 melanoma patients with clinical stage IIB and IIC (Stage IIB-C group) according to the new 8th modified AJCC/UICC staging system [18]. The melanoma patients were age and gender matched, with no evidence of any other disease or infection. Blood was drawn at the time of diagnosis prior to any therapy. Before inclusion in the study, informed consent was signed by all the patients and approved by the Ethical committee of the Institute of Oncology and Radiology of Serbia. The characteristics of the melanoma patients enrolled in this study are listed in *table 1*.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from heparinized blood obtained from melanoma patients using Lymphoprep (Nypacon, Oslo, Norway) density gradient, centrifuged at 500 g for 40 min, and washed three times in RPMI 1640 culture medium (CM), (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Sigma). After washing, PBMCs were immediately used or treated *in vitro* for 18 h with various cytokines followed by functional, phenotypic, and molecular analysis.

In vitro treatment of PBMC with various cytokines

PBMCs isolated from patients were cultivated for 18 h in CM alone, CM supplemented with IFN-α (1000 U/ml) (Sigma), IL-2 (200U/ml) (Sigma), IL-12 (10 ng/ml) (Becton Dickinson, San Jose, USA) or IL-18 (100 ng/ml) (R&D, Minneapolis, USA) as well as with IL-12 and IL-18 in combination in six well plates at 37 °C and 5% CO_2 in a humid atmosphere.

NK cell assay

NK cell activity of freshly isolated or 18 h *in vitro* treated PBMCs was determined using a standard cytotoxicity assay [19]. One hundred microliters of PBMCs, as effector cells, at a concentration of 4.0×10^6/ml CM and two 1:1 dilutions were mixed with 100 µl of the erythromyeloid cell line K562, as target cells, at a concentration of 0.05×10^6/ml, prelabeled with radioactive ^{51}Chromium (Na2CrO4, As = 3.7 MBq, Amersham, UK), to form triplicates of effector cell (E) to target cell (T) ratio (E:T) of 80:1. The assay was performed in 96 round-bottom microwell plates (Falcon, USA), which were incubated in an incubator 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 the determination of the amount of ^{51}Chromium released from the lysed K562 cells in a gamma counter (Berthold, FRG) and expressed in counts per minute (cpm). The mean percent cytotoxicity was calculated using the following formula:

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

Maximal release was obtained by incubation of target K562 tumor cells at the same concentration in the presence of 5% TritonX-100, and spontaneous release was obtained by incubation of K562 cells in culture medium.

Table 1
Patient characteristics

Clinical stage	Pathological characteristics (thickness, ulceration)	N	Age: years	Gender: Male/Female
Stage IA	<0.8mm, without ulceration	5	58	3/2
	<0.8mm, with ulceration	3	60	2/1
Stage IB	0.8-1.0mm, with or without ulceration	6	61	3/3
	1.01-2.00mm, without ulceration	6	58	3/3
Stage IIB	2.01–4.00mm, with ulceration	8	61	4/4
	>4mm, without ulceration	5	63	3/2
Stage IIC	>4mm, with ulceration	7	65	4/3

Flow cytometry analysis

The surface immuno-phenotype of native or 18 h *in vitro* treated PBMC subsets were identified using the following combinations of directly labeled monoclonal antibodies (mAbs): CD3PerCP/CD56FITC/CD161PE, CD3PerCP/CD56FITC/CD158bPE, CD3PerCP/CD56PE/CD158aFITC (Becton Dickinson), and CD3PerCP/CD56FITC/NKG2DPE cells (R&D). The samples were prepared as previously described [20]. Briefly, 1.0×10^5 PBMCs in 100 µl RPMI 1640 supplemented with 10% FCS were incubated for 30 min at 4 °C with 20 µl of appropriate mAbs combination, washed twice with ice-cold phosphate-buffered saline (PBS), and fixed with 1% paraformaldehyde prior to FACS analyses. Surface marker expression was quantified on a FACSCalibur flow cytometer (Becton Dickinson). A total of 50 000 gated events, verified as peripheral blood lymphocytes (PBL) according to their physical characteristics (forward scatter (*FSC*) and side scatter (*SSC*)), were collected per sample and analyzed using CellQUEST software. Exclusion of nonspecific 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 CD56 ($CD3^{-}CD56^{+}$). In order to define the two NK cell subsets of CD56, CD56low, i.e., the $CD3^{-}CD56^{dim+}$ or CD56 high, i.e., the $CD3^{-}CD56^{bright+}$ subset, $CD3^{-}CD56^{+}$ NK cells were divided based on the density of the CD56 antigen defined by mean fluorescence intensity (MFI). The expression of NKG2D, CD161, CD158a, and CD158b receptors was given in percentage for gated $CD3^{-}CD56^{+}$ NK cells.

Measurement of serum TGF-β and VEGFA levels

Blood samples were obtained from melanoma patients in both the investigated groups by venipuncture and clotted at room temperature. The sera were collected following centrifugation and frozen immediately at -20 °C until analysis.

TGF-β and VEGFA was determined in serum samples using TGF-β1 and VEGFA Human ELISA Kits (ThermoFisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's instructions. To activate latent TGF-β1 to the immunoreactive form, the solutions for acid activation and neutralization were used (incubation with 1N HCl for 10 min, followed by addition of 1N NaOH).

STATISTICAL ANALYSIS

Significance of difference for obtained results was done by the nonparametric Mann-Whitney U test and the Wilcoxon signed rank test.

RESULTS

NK cell cytotoxic activity

Our results show significantly impaired ($p < 0.05$, Mann–Whitney U test) NK cell cytotoxicity in Stage IIB-C melanoma patients compared with patients in Stage IA-B (*figure 1A*) as evaluated by a standard assay against erythromyeloid K562 tumor target cells.

In contrast, there is no significant difference ($p > 0.05$, Mann Whitney U test) between the two groups of melanoma patients with respect to the overall percentages of NK cells as well as their dim and bright subsets in peripheral blood (*figure 1B*).

The expression of activating NKG2D and CD161 and inhibitory CD158a and CD158b NK cell receptors

The results show a significant downregulation ($p < 0.05$, Mann-Whitney U test) of the two major types of activating NK cell receptors, NKG2D and CD161, on NK cells in melanoma patients with Stage IIB-C compared to patients with Stage IA-B disease (*figure 2A, B*).

In contrast, we observed no difference ($p > 0.05$, Mann-Whitney U test) for the expression of the two inhibitory NK cell KIR receptors, CD158a and CD158bin, in Stage IA-B melanoma patients compared to Stage IIB-C patients (*figure 2C*).

The serum TGF-β1 and VEGFA levels

The serum TGF-β1 level shows statistically significant difference between the two groups of melanoma patients. Specifically, the level of TGF-β1 is significantly ($p < 0.05$, Mann–Whitney U test) higher in Stage IIB-C melanoma patients compared to patients in Stage IA-B (*figure 3a*). In contrast, there is no significant difference ($p > 0.05$, Mann Whitney U test)

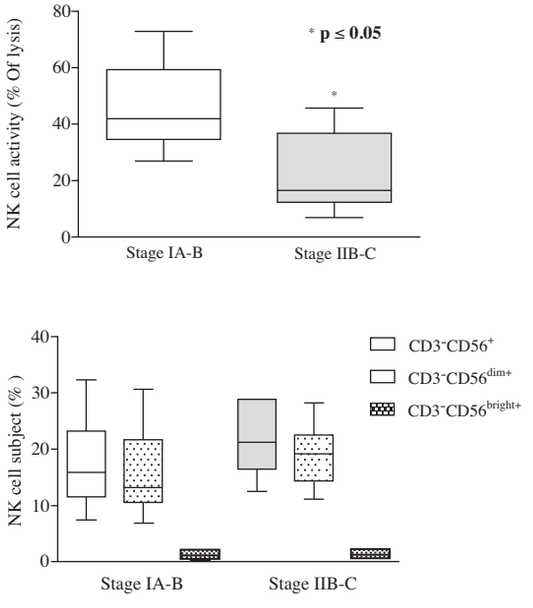

Figure 1

NK cell cytotoxic activity. A) NK cell activity evaluated against the sensitive erythromyeloid K562 tumor cells for effector to target cell ratio 80: 1 in Stage IIB-C melanoma patients is significantly impaired (*$p < 0.05$, Mann–Whitney U test) compared to the activity of NK cells in Stage IA-B patients; **B)** No significant difference in the percentages of NK cells and their dim and bright subsets in peripheral blood ($p > 0.05$, Mann Whitney U test) was observed between the two groups of patients. The results shown are as mean values ± SE for 20 patients in Stage IA-B and 20 patients in Stage IIB-C.

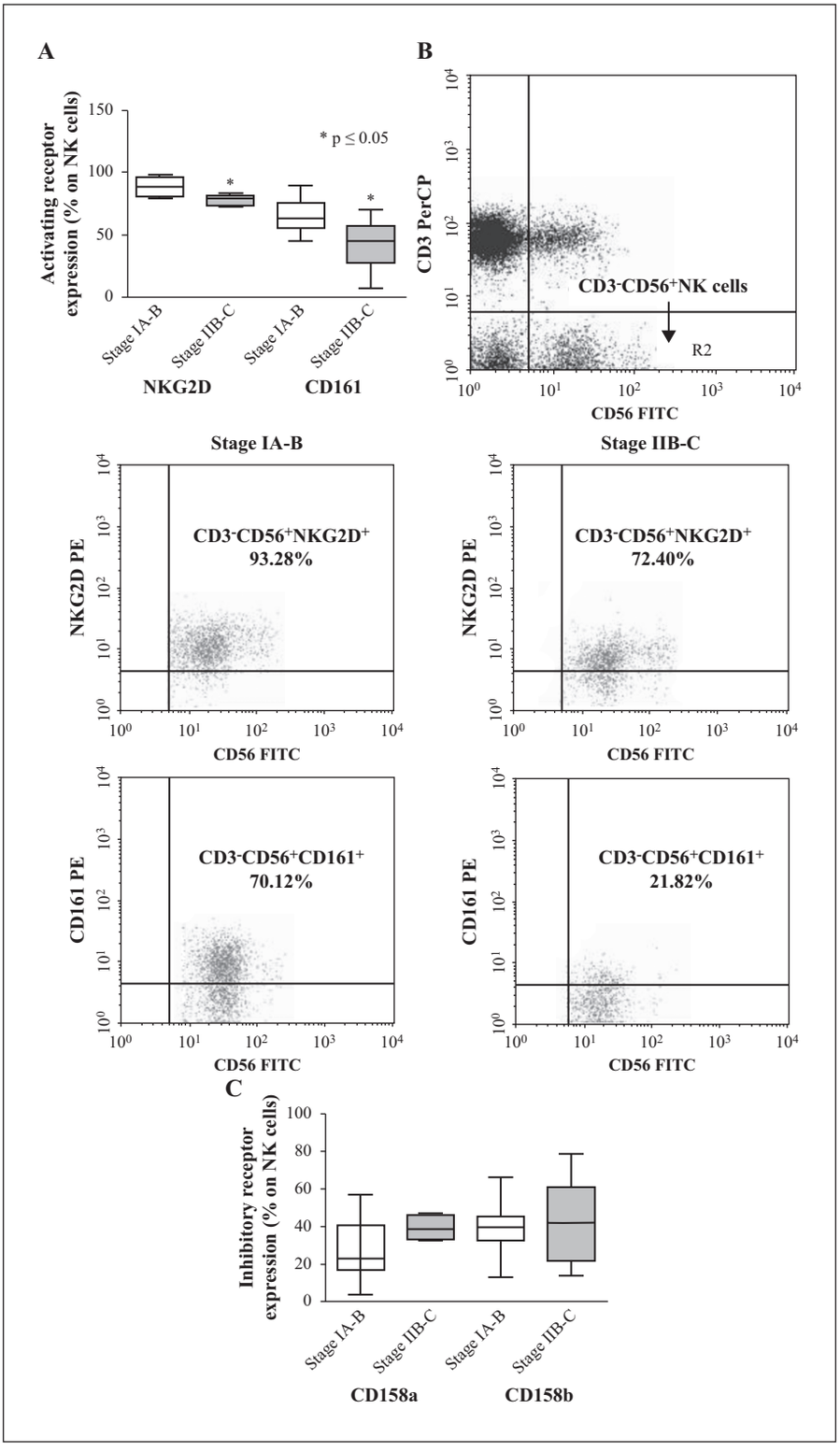

Figure 2

Expression of activating and inhibitory NK cell receptors. A) The percentage of NKG2D and CD161 activating receptors is significantly lower (*$p < 0.05$, Mann-Whitney U test) in Stage IIB-C patients compared to Stage IA-B patients; B) Representative Flow cytometry results; C) The percentage of CD158a and CD158b inhibitory KIR receptors on NK cells was comparable for the two groups of melanoma patients. The results shown are mean values ± SE for 20 patients in Stage IA-B and 20 patients in Stage IIB-C.

in the level of serum VEGFA between the two investigated groups of melanoma patients (*figure 3B*).

The effect of cytokines on NK cell cytotoxicity

We next determined the cytotoxicity of patient-derived NK cells after *in vitro* exposure of PBMCs in the absence or presence of cytokines. The results show that after treatment with IFN-α, IL-2 or IL-12 alone or IL-12 and IL-18 in combination, NK cell cytotoxicity is significantly ($p < 0.05$ and $p < 0.01$, Wilcoxon signed rank test) enhanced compared to the control group for both groups of melanoma patients. In contrast, IL-18 alone had no significant ($p > 0.05$, Wilcoxon signed

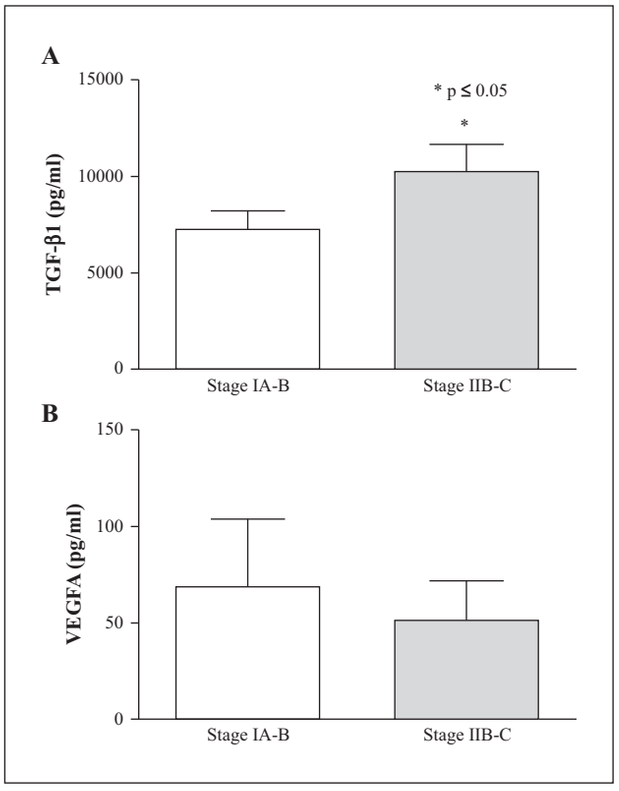

Figure 3
The level of *TGF-β1* and *VEGFA*. A) The serum level of TGF-β1 is significantly (*p < 0.05, Mann–Whitney U test) higher in Stage IIB-C patients compared to patients in Stage IA-B; **B)** There is no significant difference (p > 0.05, Mann Whitney U test) in the level of serum VEGFA between the two investigated groups of melanoma patients. The results shown are mean values ± SE for 20 patients with Stage IA-B and 20 patients with Stage IIB-C.

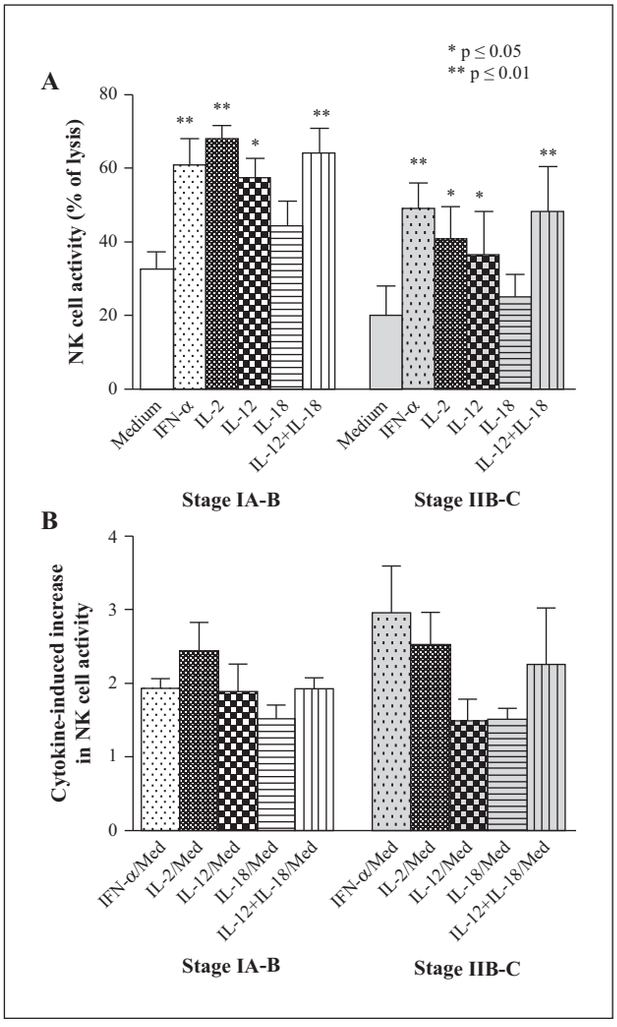

Figure 4
The effect of cytokines on NK cell cytotoxicity. A) NK cell activity after 18 h *in vitro* treatment of peripheral blood mononuclear cells with IFN-α, IL-2, and IL-12 alone or with IL-12 and IL-18 in combination against K562 tumor target cell line for effector to target cell ratio 80: 1 shows significant (*p < 0.05 and **p < 0.01, Wilcoxon signed rank test) increase compared to the respective untreated control cells in both groups of melanoma patients. **B)** There was no significant difference (p > 0.05, Mann-Whitney U test) in the cytokine-induced increase in NK cell cytotoxicity between the two groups of melanoma patients. The results shown are mean values ± SE for 20 patients with Stage IA-B and 20 patients with Stage IIB-C.

rank test) capacity to potentiate NK cell activity for none of the investigated melanoma patients (*figure 4A*).
When the two groups of melanoma patients were compared, no significant difference (p > 0.05, Mann-Whitney U test) for the relative increase in cytokine-induced NK cell cytotoxicity was observed for any of the cytokines (*figure 4B*).

The effect of cytokines on the expression of NKG2D and CD161 activating receptors

We next determined the influence of IFN-α and IL-2 on the expression of the NKG2D activating receptor on $CD3^{-}CD56^{+}$ NK cells from the two groups of patients. The results show that both IFN-α and IL-2 were able to significantly (p < 0.05, Wilcoxon signed rank test) increase the percentage of the receptor on NK cells derived from Stage IA-B patients. In comparison, only IL-2 was able to significantly (p < 0.05, Wilcoxon signed rank test) increase the percentage of the NKG2D receptor on NK cells from Stage IIB-C (*figure 5A, C*). Although IL-2 was able to increase the expression of the NKG2D receptor for both the groups of patients, the increase for Stage IIB-C patients was significantly (p < 0.05, Mann-Whitney U test) lower than the increase observed for NK cells from Stage IA-B patients (*figure 5B, C*).

We then determined the influence of investigated cytokines on the expression of the other activating receptor, CD161, on NK cells isolated from the two groups of patients. The results show that both IFN-α and IL-12 are able to significantly (p < 0.05, Wilcoxon signed rank test) increase the percentage of the CD161 receptor on cells derived from Stage IA-B patients. In contrast, none of the investigated cytokines had any significant (p > 0.05, Wilcoxon signed rank test) influence on the expression of CD161 on NK cells obtained from Stage IIB-C patients (*figure 5D, E*).

DISCUSSION

It is well established that patients with Stage IIB and IIC localized cutaneous melanoma are at high risk for both local recurrence and distant metastasis compared

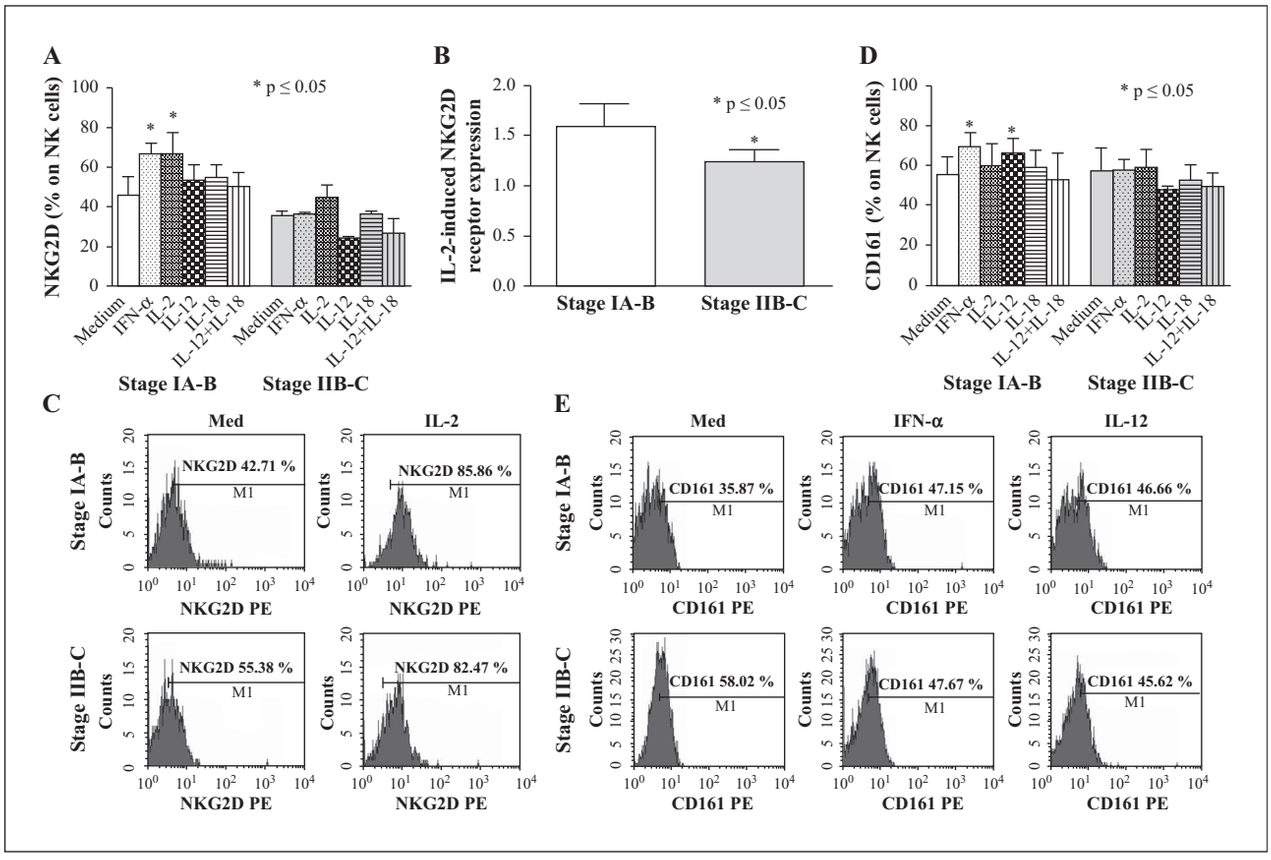

Figure 5

The effect of cytokines on the expression of activating receptors. A) Treatment of NK cells with both IFN-α and IL-2 significantly (*p < 0.05, Wilcoxon signed rank test) increased the percentage of NKG2D receptor in NK cells from Stage IA-B patients, whereas NK cells from patients with Stage IIB-C were only significantly (*p < 0.05, Wilcoxon signed rank test) increased by IL-2; **B)** NK cells from patients with Stage IIB-C have significantly (*p < 0.05, Mann-Whitney exact test) less increase in the expression of the NKG2D receptor after *in vitro* treatment with IL-2 compared to what is observed for NK cells from Stage IA-B patients; **C)** Representative flow cytometry histograms for NKG2D receptor; **D)** IFN-α and IL-12 significantly (*p < 0.05, Wilcoxon signed rank test) increase the percentage of CD161 activating receptor on NK cells from Stage IA-B patients but not for NK cells from Stage IIB-C; **E)** Representative flow cytometry histograms for CD161 receptor. The results shown are mean values ± SE for 20 patients in Stage IA-B and 20 patients in Stage IIB-C.

to patients with Stage IA and IB melanoma. We here determine the differences in NK cell activity between the two groups of melanoma patients. Interestingly, the results show that melanoma patients in Stage IIB-C have significant decrease in NK cell activity compared to Stage IA-B patients. These results are, to the best of our knowledge, the first results regarding high-risk localized melanoma patients and are in agreement with previous results obtained for metastatic melanoma patients as reported by us [13, 15] and others [14].

Further analysis revealed that there is no difference between the two groups of melanoma patients with respect to the percentage of NK cells and their subsets in peripheral blood. Therefore, the impairment in NK cell cytotoxicity is not simply a consequence of a decreased number of NK cells in Stage IIB-C melanoma patients.

Further studies revealed no differences in the expression of the inhibitory KIR receptors, CD158a and CD158b, on NK cells obtained from the two groups of melanoma patients. In clear contrast, NK cells from Stage IIB-C melanoma patients showed a significant decrease in the percentage of NKG2D and CD161 activating receptors compared to NK cells obtained from Stage IA-B patients. Since NKG2D is a major determinant of NK cell cytotoxic capacity against tumor cells [21], downregulation of this receptor together with the reduced expression of the CD161 activating receptor suggests that attenuation of NK-cell cytotoxicity is likely an evasion mechanism associated with tumor progression. In agreement, NK cells from high-risk patients exhibited impaired cytotoxicity and an altered phenotype [22]. These results indicate that the presence of NK cell receptors may be useful to identify high-risk cutaneous melanoma patients.

The low expression of activating NK cell receptors could be a result of tumor-mediated suppression since an important number of cytokines (transforming growth factor-β (TGF-β), IL-10), growth factors (vascular endothelial growth factor (VEGF)) and enzymes (indoleamine 2,3-deoxygenase (IDO)), synthesized by tumor cells or by immunosuppressive cells in the tumor microenvironment (regulatory T cells (Tregs), myeloid derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs)) are able to decrease the expression of activating NK cell receptors [23]. Therefore, we determined the serum level of immunosuppressive molecules, TGF-β1 and VEGFA, in the two groups of patients. Our results

show that Stage IIB-C melanoma patients have increased serum level of TGF-β1 compared to Stage IA-B patients. TGF-β1 is well-established immunosuppressive cytokine produced by tumor cells of different histotypes, including melanoma, as well as by immunosuppressive cells such as Tregs, MDSCs, and TAM [24]. In particular, several studies in cancer patients have shown that TGF-β1 can allow escape from immune surveillance by its ability to decrease NK cell cytotoxicity and the expression of activating NKG2D receptors on NK cells [25, 26]. Thus, blocking of TGF-β not only prevented NKG2D downregulation but also restored NK cell antitumor cytotoxicity [24].

In addition to its major role in promoting the growth of tumor vessels, VEGF has recently been identified as an important factor in tumor-induced immunosuppression. VEGFA released by tumor cells, tumor associated fibroblasts (TAFs), TAMs, Tregs, MDSCs can inhibit the function of T cells, increase the development and recruitment of inhibitory Tregs, MDSCs and TAM to the tumor microenvironment, and impair the differentiation and maturation of dendritic cells (DCs) toward an immature, tolerogenic phenotype. Tolerogenic DCs by the production of TGF-β, IL-10, and IDO inhibit T cell activation [27]. However, we observed no difference in the level of serum VEGFA between the two investigated groups of melanoma patients that is in accord with previous results. Thus, in some studies, this difference was only seen between the melanoma patients and healthy individuals, but not between patients in stages I, II, and III [28, 29].

Next, we established the capacity of various cytokines to activate the cytotoxic activity of NK cells. Our results show that IFN-α, IL-2 and IL-12 alone or IL-12 and IL-18 in combination are able to enhance NK cell cytotoxic activity for both groups of melanoma patients in agreement with a previous report [14]. It is well established that these cytokines are able to directly or indirectly increase the cytotoxicity of NK cells by inducing secretory molecules such as perforin and granzymes, as well as FasL and TRAIL receptors [30]. Interestingly, IL-18 alone had no significant effect on the cytotoxicity of NK cells. A possible explanation is that IL-18 produced by tumor cells may convert conventional Kit^{-} into Kit^{+} NK cells that overexpress PD-L1, a ligand for the negative immunoregulatory molecule PD-1 [31].

The relative increase in NK cell cytotoxicity is similar for both groups of melanoma patients following treatment with all of the cytokines or cytokine combination. Importantly, although patients in Stage IIB-C have low NK cell activity and high risk for melanoma progression, IFN-α, IL-2 and IL-12 alone or IL-12 and IL-18 in combination are able to enhance the cytotoxic activity of their NK cells. These findings suggest that adjuvant therapy including cytokine immunotherapy applied postoperatively may provide better opportunity for cure of melanoma patients before the tumor relapses into an advanced inoperable stage [32]. At present, IFN-α is the only approved therapy for patients with high-risk Stage II or Stage III melanoma [33].

We also show for the first time that, in contrast to high-risk melanoma patients, patients with Stage IA-B show a significant increase in the expression of activating NKG2D receptor on their NK cells after *in vitro* treatment with IFN-α, as well as an significant increase in the expression of the other activating receptor, CD161, after *in vitro* treatment with IFN-α and IL-12. Although IL-2 was able to increase the expression of NKG2D on NK cells from both the groups, this increase was significantly lower for high-risk Stage IIB-C patients. An explanation could be that the inhibitory effect of TGF-β abundantly produced in high-risk melanoma patients is greater than the stimulatory effect of the investigated cytokines, IFN-α, IL-2, and IL-12. In agreement, Lee *et al.* [34] previously observed that IL-2 treatment alone could not block the down-regulation of the NKG2D receptor by TGF-β and was not associated with the recovery of the NKG2D expression on NK cells. These observations suggest that a more expansive cytokine treatment approach might be required to eliminate the effect of immunosuppressive molecules.

Since IFN-α, IL-2, and IL-12 alone or IL-12 and IL-18 in combination significantly improved NK cell cytotoxicity for Stage IIB and IIC patients, these cytokines might be useful as adjuvant therapy for high-risk cutaneous melanoma patients. However, according to the results from most preclinical and clinical studies, combination therapies are expected to have a better antitumor activity. Therefore based on the results presented here, we suggest that the combination of inhibitors of immunosuppressive factors such as anti-TGF-β together with NK cell activating cytokines is likely to show superior antitumor activity in high-risk melanoma patients.

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