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ORIGINAL ARTICLE

In vitro increased natural killer cell activity of metastatic melanoma patients with interferon-alpha alone as opposed to its combination with 13-cis retinoic acid is associated with modulation of NKG2D and CD161 activating receptor expression

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Summary

Purpose: *Considering tumor-induced suppression of natural killer (NK) cell activity the aim of this study was to investigate the in vitro effect of a standard immunotherapeutic cytokine, interferon (IFN)α, and a less investigated agent, 13-cis retinoic acid (RA) on the functional and receptor characteristics of CD16-defined NK cells and their functionally diverse dim and bright subsets in patients with metastatic melanoma (MM).*

Methods: *Peripheral blood lymphocytes (PBL) of patients with clinical stage IV MM were stimulated in vitro for 18 h in RPMI 1640 culture medium (CM) alone, CM supplemented with IFN-α (250 U/ml), RA (10^{-6}M) and their combination. NK cell activity was determined using standard 4 h radioactive cytotoxicity assay, while the expression of activating (NKG2D, CD161) and inhibitory (CD158a, CD158b) NK cell receptors on $CD3^{-}CD16^{+}$ NK cells and their functional bright and dim subsets were analyzed by flow cytometry.*

Results: *NK cell cytotoxic activity was increased after in vitro treatment with IFN-α alone and in combination with RA, while only IFN-α induced increase in NKG2D and CD161 activating NK cell receptor expression. Contrary to this, RA treatment increased the expression of inhibitory KIR CD158b. IFN-α-obtained increase in CD161 expression was due to its induction on both NK cell subsets, while for NKG2D only on $CD16^{bright}$ subset.*

Conclusion: *The favorable enhancement of NK cell activity of MM patients obtained with IFN-α is associated with upregulation of activating NKG2D and CD161 receptors, while the lack of RA-associated upregulation is probably due to the shown increased expression of inhibitory KIR receptor CD158b after in vitro treatment with this agent.*

Key words: IFN-α, melanoma, NK cells, NK cell receptors, retinoic acid

Introduction

MM patients with advanced disease show impairments in their immune response, including decreased NK cell activity [1,2]. As MM is an immunogenic tumor resistant to chemotherapy and irradiation, immunomodulating agents have been included in its treatment [3]. In this sense, IFN-α, as an innate cytokine showing antiproliferative and immunomodulatory properties, has been included in the immunotherapy of MM for several decades. On the other hand, considering that its therapeutic effect is limited, many other immunomodulating agents, alone or in combination with IFN-α, have been investigated in this disease. Therefore, taking into account the antiproliferative and differentiating effects on tumor cells and the immunomodulating effect on various aspects of immune response of RA, this biological agent has been used in the treatment of MM [4].

It is well known that IFN-α, a type I interferon, stimulates NK and T cytotoxic cells and induces the production of Th1 cytokines, i.e. interferon-γ (IFNγ), interleukin (IL)-2 and IL-12, that enhance favorable, cell-mediated, antitumor reactions [1,5]. The effect of IFN-α is realized through phosphorylation of STAT1 and STAT2 that induces transcription of various IFN-responsive genes that affect the cell cycle, induce cytokine secretion and perforin and FasL production [6]. In this sense, through interferon-regulating transcription

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factors 1 and 2 (IRF-1 and IRF-2), IFN-α also affects genes involved in cell cycle control, cytokine secretion and the synthesis of their receptors [6].

On the other hand, RA, a derivative of vitamin A, is a biological agent that has antiproliferative and differentiating properties on tumor cells, as well as immunomodulating effects on innate, as well as adaptive immune responses, including induction of innate cytokines (IFNγ, IL-12 and IL-15) and increase of NK cell activity [7]. ATRA, a 13-*cis* RA isomer, has been less investigated although it has shown clinical benefit in the treatment of solid tumors and MM [4]. The activity of RA depends on the presence of its nuclear retinoic acid receptors A and X (RAR and RXR, respectively) in target cells through which it regulates proliferation, differentiation, susceptibility to apoptosis and sensitizes tumor cells to cytotoxic lymphocytes by RA-inducible molecules, MICA and RA early transcripts, ULBP1-3 and RAET1, known to be ligands for NK cell activating receptor NKG2D [1,8]. Moreover, its antiproliferative effect on *in vitro* cultured tumor cells [9] shows synergy with IFN-α [7]. Nevertheless, the effect of RA alone or its combination with IFN-α has not been investigated with respect to modulation of newly defined NK cell receptors and their effect on cytotoxicity of this innate immune subset with antitumor activity.

NK cells have rarely been studied with respect to the expression of one of the most important NK cell cytotoxic receptors, CD16, the low affinity receptor for IgG (FcγRIII) (ADCC) engaged in direct cytotoxicity and antibody-dependent, cellular cytotoxicity [10], proliferation, cytokine production, as well as post-activational NK cell apoptotic death [11]. NK cells with respect to CD16 phenotypically and functionally comprise the $CD16^{bright}$ NK cell subset [10] (analogous to $CD56^{dim}$ subset), that aside from high expression of CD16 and inhibitory KIR receptors [12], expresses abundant perforin and granzyme granules and is involved in NK cell cytotoxicity. $CD16^{dim}$ NK cell subset (analogous to $CD56^{bright}$ subset) has low expression of CD16 and low to absent expression of KIR receptors, and displays a regulatory function owing to its abundant cytokine production and weak cytotoxicity [12]. It has been shown that NK cell subsets differ in IL-2 receptor expression [12], while there are no data related to the expression of IFN-α receptors.

The balance between NK cell activating and inhibitory signals mediated by a group of receptors that were originally described on NK cells, regulates NK cytotoxic activity [13]. These families of NK cell receptors include activating c-lectin-like receptors (NKG2D, CD161), other natural cytotoxicity receptors and inhibitory killer cell receptors (KIR). NKG2D, as the most prominent activating receptor, mediates immune responses in surveillance against cancer and its downregulation in MM has been shown in one study [1]. Upon binding stress-induced ligands, such as MICA/MICB [14] and ULBP1-4, NKG2D induces NK cell cytotoxicity. Another common NK cell receptor, CD161, one of the earliest markers in NK cell development [15], is primarily described as an activating receptor [13]. Contrary to this, the superfamily of KIR receptors, type I membrane glycoproteins, are classified into inhibitory or stimulating receptors, and can prevent NK cell mediated lysis of cells that express class I MHC [13]. Inhibitory KIR receptors, CD158a (KIR2DL1) and CD158b (KIR2DL2,3) belong to a more frequent group of KIR receptors and recognize HLA-C1 and HLA-C2 molecules, respectively, on tumor cells [16]. For this reason these KIR receptors are most frequently investigated since, by downregulating NK cell activity, they facilitate tumor escape [16].

The aim of this study was to investigate the immunomodulatory effect in predictive *in vitro* treatments of PBL of MM patients with a standard immunotherapeutic cytokine (IFN-α) and a much less investigated agent (RA) on NK cell activity and their influence on the expression of newly defined activating and inhibitory NK cell receptors. Moreover, we analyzed the receptor expression on the entire NK cell population and its two functionally diverse subsets, especially on the larger $CD16^{bright}$ subset engaged in tumor cytotoxicity and also on the smaller regulatory $CD16^{dim}$ NK cell subset. Unlike for IFN-α, there is no or only scarce data regarding modulation of NK cell cytotoxicity and receptor expression with RA, or its combination with IFN-α, since RA is a biological agent whose effect has been mostly investigated on tumor cells.

Methods

This study has been reviewed and approved by the Ethics Committee of the Institute for Oncology and Radiology of Serbia and all subjects gave written informed consent.

Patients

In this study included were 35 patients (median age 51 years) with histologically proven MM (stage IV), according to the modified AJCC/UICC staging system (Table 1).

PBL isolation

PBL were isolated using Lymphoprep (Nypacon, Oslo, Norway) density gradient, centrifuged at 500 g, 40 min, and washed 3 times in RPMI 1640 culture medium (CM, Gibco, Bristol, UK) supplemented with 10% FCS (Sigma, USA).

In vitro treatment of PBL with IFN-α and 13-cis retinoic acid

PBL isolated from MM patients were cultivated for 18 h in RPMI 1640 culture medium (CM) alone, CM supplemented with

Table 1. Patient characteristics

Characteristics	*N (%)*
Age (years)	
Range	28-69
Median	51
Gender	
Male	22 (53)
Female	13 (47)
Primary tumor localization	
Head and neck	5 (10)
Trunk	12 (26)
Upper limb	3 (6)
Lower limb	11 (23)
Unknown	4 (11)
Metastases	
Lung	12 (26)
Liver	13 (28)
Soft tissues	8 (17)
Bones	6 (13)
Adrenals	2 (4)
Spleen	2 (4)
Retroperitoneum	2 (4)
Lymph nodes	25 (54)

IFN-α (250 U/ml), 13-*cis* retinoic acid (10^{-6}M) and their combination in 6-well plates (Sigma, St Louis, MO) at 37° C and 5% CO_2 in humid atmosphere.

Flow cytometric analysis

Surface immunophenotype of *in vitro* treated PBL from 26 MM patients was identified using the following combinations of directly labeled monoclonal antibodies (mAbs): CD3PerCP/CD16FITC, CD3PerCP/CD16FITC/CD161PE, CD3PerCP/CD16PE/CD158aFITC, and CD3PerCP/CD16FITC/CD158bPE (Becton Dickinson, San Jose, CA, USA) and CD3PerCP/CD16FITC/NKG-2DPE (R&D, USA). The samples were prepared as previously described [17]. 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, San Jose, CA, USA). NK cells were defined and gated within the lymphocyte gate according to their expression of CD3 and CD16 ($CD3^-CD16^+$). In order to define the two NK cell subsets of low ($CD3^-CD16^{dim}$) or high ($CD3^-CD16^{bright}$) subsets, $CD3^-CD16^+$ NK cells were divided based on the density of CD16 antigen defined by mean fluorescence intensity (MFI) as previously reported [18]. NK cell receptors CD161, NKG2D, CD158a and CD158b on $CD3^-CD16^+$ NK cells were expressed as percents in PBL as well as percents in gated $CD3^-CD16^+$ NK cells, while the expression of these receptors on $CD3^-CD16^{bright}$ and $CD3^-CD16^{dim}$ NK cell subsets was estimated in PBL.

NK cell assay

NK cell activity of 36 MM patients were determined using standard cytotoxicity assay [19]. One hundred µl of *in vitro* stimulated PBL, as effector cells, at 4.0×10^6/ml concentration of CM and two 1:1 dilutions, were mixed with 100 µl of the erythromyeloid cell line K562 as target cells, at 0.05×10^6/ml concentration, prelabelled with radioactive ^{51}Chromium (Na_2CrO_4, As = 3.7 MBq, Amersham, UK), to form triplicates of 3 effector cell (E) to target cell (T) ratios (E:T): 80:1, 40:1 and 20:1. The mean percent cytotoxicity was calculated using the following formula:

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

*counts per min

Maximal release was obtained by incubation of target K562 tumor cells at the same concentration in the presence of 5% Triton X-100, and spontaneous release was obtained by incubation of K562 cells in CM alone.

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from *in vitro* stimulated PBL using TRIZOL (Invitrogen, Madison, WI, USA) reagent. One µg of total RNA was reverse-transcribed by random priming while incubated with 1 µl reverse transcriptase MuLV, 10 mM deoxynucleotide triphosphate mix (dNTP), RNAse inhibitor and 2 µl 0.1 M dithiothreitol (DTT) for 1 h at 37° C and then 5 min at 99° C. Total cDNA was then subjected to PCR program 5 min at 95° C, and 30 cycles of amplification (15 sec at 95° C, 30 sec at 57° C, 45 sec at 72° C) and 7 min at 72° C. The sequence of primers used for amplification of 414 bp (base pair) fragment of IRF-1 was: GACCAGAG-CAGGAACAAG corresponding to bp 483-501 and the antisense was TAACTTCCCTTCCTCATCC corresponding to bp 881-889. β actin primer selected to amplify 685 bp fragment was: TGGGT-CAGAAGGATTCCTAT corresponding to 181-200 and the antisense AAGGAAGGCTGGAAGAGT corresponding to 821-838 from the published β actin sequence.

Western blot analysis

The protein content in whole-cell extracts was determined by means of Bradford assay. Equal amounts of proteins (20 µg/well) were resolved by 10% SDS-PAGE and transferred to nitrocellulose (Bio-Rad, USA). Blots were incubated overnight with Anti-Stat1(pY701) (BD Transduction laboratories, San Hose, CA), rabbit anti-human MEK1/2 and ERK1/2 (Sigma, St. Louise, USA) phospho specific antibodies. The blots were developed using enhanced chemiluminescence (ELC) detection system (Amersham, Arlington Heights, IL USA) and analyzed with Gel Doc BioRad system using Multi-Analyst 1.1 software.

Statistical analysis

The GraphPad Prism software was used for statistical analysis. The significance of differences between results obtained after treatments with IFN-α, RA and their combination was evaluated with respect to control culture medium treatments by using nonparametric Wilcoxon signed rank test. A p value below 0.5 was considered to be statistically significant.

Results

NK cell cytotoxicity of MM patients performed against standard sensitive erythromyeloid K562 tumor target cell line after *in vitro* PBL cultivation showed that NK cell cytotoxicity after treatment with 250 U of

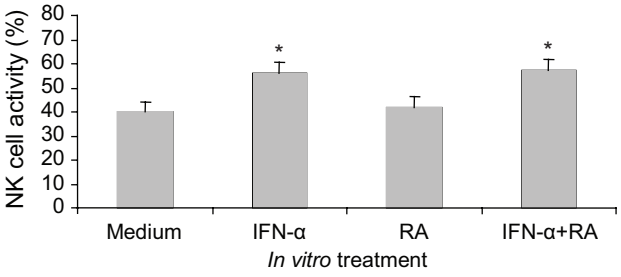

Figure 1. NK cell cytotoxic activity was evaluated against standard sensitive erythromyeloid K562 tumor target cell line for 80:1 effector to target cell ratio after 18h *in vitro* treatment of PBL of MM patients with 250 IU IFN-α, 10^{-6} M 13-*cis* RA and their combination. Significant (*p<0.05, Wilcoxon signed rank test) enhancement of NK cell cytotoxicity was obtained with IFN-α alone, as well as in combination with 13-*cis* RA. Results are expressed as percentage of specific lyses and are shown as mean ± SE.

IFN-α alone or in combination with RA (10^{-6} M) was significantly augmented (p<0.05, Wilcoxon signed rank test) compared to PBL treated in medium alone (values were 40.04% in CM, 56.15% with IFN-α and 56.92% with IFN-α and RA for an E:T ratio 80:1). Contrary to this, RA alone (10^{-6} M) did not potentiate NK cell activity of the investigated MM patients (Figure 1).

Expression of NKG2D, CD161, CD158a and CD158b NK cell receptors after *in vitro* treatment of PBL of these patients with IFN-α, RA and IFN-α together with RA was estimated on $CD3^{-}CD16^{+}$ NK cells in PBL (Figure 2a), as well as on gated $CD3^{-}CD16^{+}$ NK cells (Figure 2b). The obtained results showed that IFN-α treatment, as opposed to either RA alone or IFN-α and RA together, induced significant increase (p<0.05, Wilcoxon signed rank test) in activating NK cell receptor expression on $CD3^{-}CD16^{+}$ NK cells, with the increase being from 4.88±0.95% in CM to 5.52±0.89% for NKG2D (Figure 2a), and from 3.01±0.54% in CM to 3.73±0.54% for CD161. Analyses of activating receptor expression after IFN-α PBL treatment on gated $CD3^{-}CD16^{+}$ NK cells showed similar significant increase (Wilcoxon signed rank test, p<0.05) of both NKG2D (values were 0.52±8.04% in CM and 58.57±8.71% after IFN-α treatment) and CD161 expression (values were 25.91±3.38% in CM and 37.75±4.25% after IFN-α treatment) (Figure 2b).

Contrary to this, the analyses of CD158a and CD158b KIR expression showed that only treatment with RA alone significantly increased (Wilcoxon signed rank test, p<0.05) the expression of CD158b on $CD3^{-}CD16^{+}$ NK cells in PBL (values were from 3.50±0.70% in CM to 3.88±0.7% after IFN-α treatment) (Figure 3a), whereas the analyses of KIR expression on gated $CD3^{-}CD16^{+}$ NK cells did not show any change (Figure 3b).

The evaluation of signaling pathways engaged in *in vitro* performed treatments indicated that IFN-α, unlike RA, increased 1.27-fold the level of pSTAT1 in PBL of MM patients after 4 h of *in vitro* treatment (Figure 4a). On the other hand the investigation of RA associated signaling molecules MEK1,2 and ERK1/2 showed an 1.28-fold increase in pMEK1,2 and 1.50-fold increase in pERK1,2 protein level after treatment with RA alone and also 1.57-fold increase in p-MEK1,2 and 1.50-fold increase in p-ERK1,2 after its combination with IFN-α (Figure 4c). Representative results for pSTAT1, MEK1,2 and ERK1,2 are displayed in Figure 4b and d, respectively. Our results also indicated a substantial IFN-α-mediated induction of IRF-1, a key transcription factor engaged by this cytokine (Figure 5a). Representative results for IRF-1 are shown in Figure 5b.

The analyses of NK cell receptor expression after these *in vitro* treatments on $CD16^{dim}$ and $CD16^{bright}$

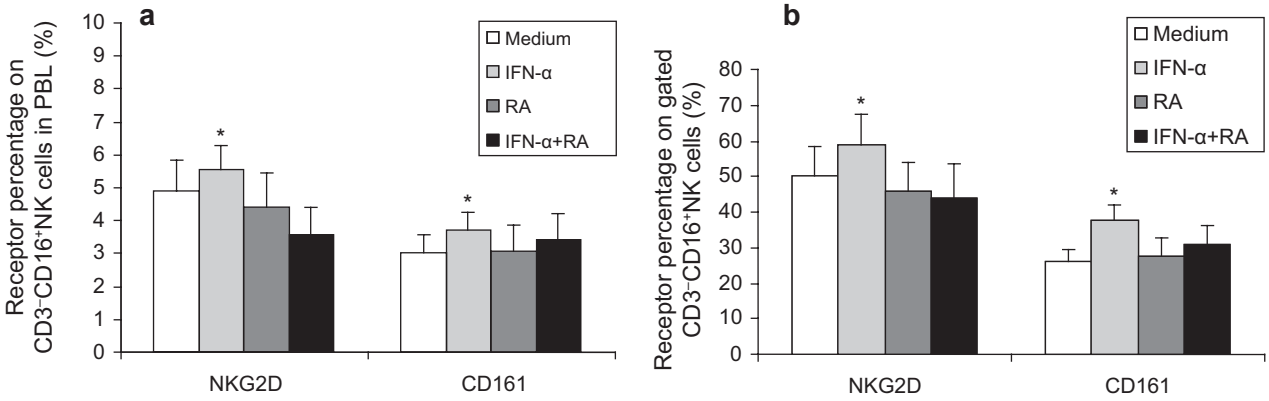

Figure 2. Expression of CD161 and NKG2D activating NK cell receptors after 18h in vitro treatment of PBL of MM patients with IFN-α, 13-*cis* RA and their combination was estimated **(a)** on $CD3^{-}CD16^{+}$ cells in PBL and **(b)** on gated $CD3^{-}CD16^{+}$ NK cells. Only treatment with IFN-α significantly (*p<0.05, Wilcoxon signed rank test) increased the percentage of CD161 and NKG2D activating receptors on NK cells in PBL, as well as on gated NK cells. Results are shown as mean ± SE.

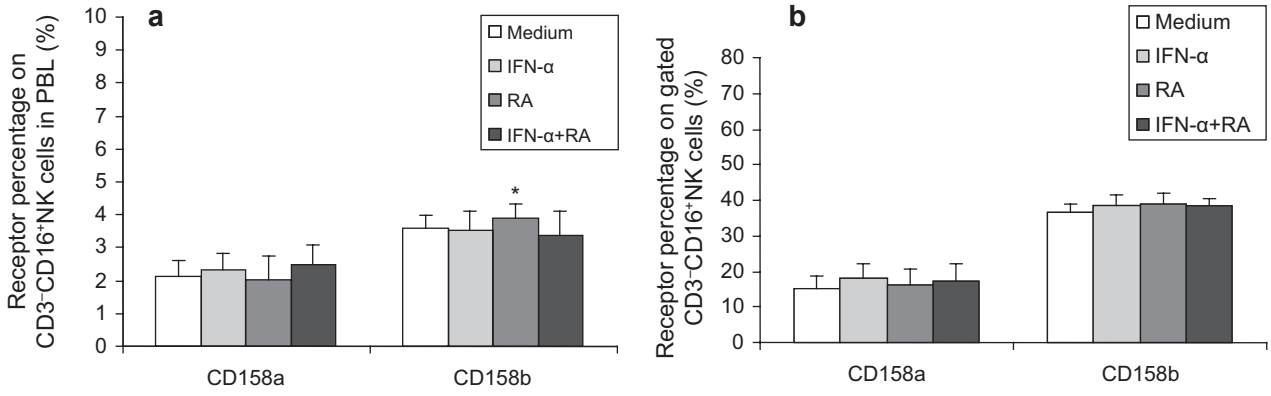

Figure 3. Expression of CD158a and CD158b inhibitory KIR receptors after 18h *in vitro* treatment of PBL of MM patients with IFN-α, 13-*cis* RA and their combination was estimated **(a)** on $CD3^{-}CD16^{+}$ cells in PBL and on **(b)** gated $CD3^{-}CD16^{+}$ NK cells. Treatment with 13-*cis* RA, alone, significantly (Wilcoxon's signed rank test, *$p<0.05$) increased the expression of CD158b on $CD3^{-}CD16^{+}$ NK cells in PBL. Results are shown as mean ± SE.

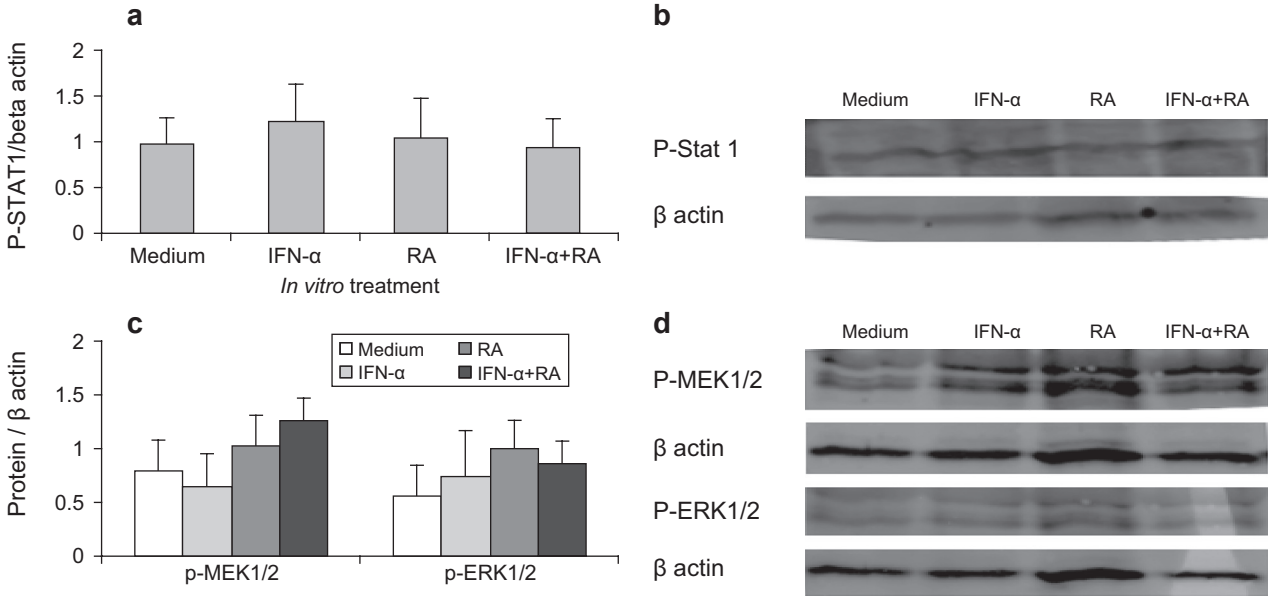

Figure 4. IFN-α substantially increased the level of pSTAT1 in PBL of MM patients after 4h of *in vitro* treatment, while 13-*cis* RA, either alone or in combination with IFN-α, gave an increase in molecules associated with the MAPK signaling pathway, i.e., pMEK1,2 and pERK1,2. The average level of induced **(a)** pSTAT1 and **(c)** pMEK1,2 and pERK1,2 protein level is given with respect to β-actin protein level. Representative Western blots are given for pSTAT1 **(b)** and pMEK1,2 and pERK1,2 proteins **(d)**.

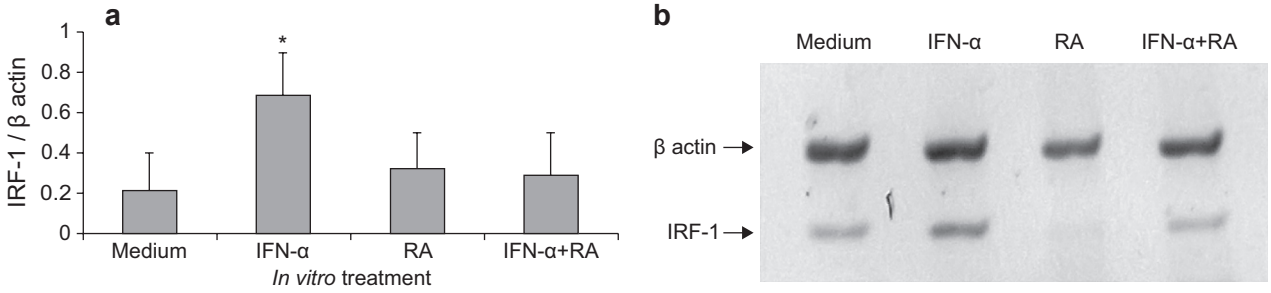

Figure 5. IFN-α significantly (*$p<0.05$, Wilcoxon signed rank test) induced IRF-1 transcription after 4h *in vitro* treatment of PBL of MM patients (results are shown as mean ± SE). **(a)** The average level of induced IRF-1 expressed with respect to β-actin level. **(b)** A representative RT-PCR result.

NK cell subsets showed only after IFN-α treatment a highly significant increase ($p<0.01$, Wilcoxon signed rank test) in CD161 expression on both NK cell subsets (0.49±0.09% in CM and 0.62±0.01% after treatment for $CD16^{dim}$ and from 2.04±0.92% in CM to 2.25±0.92% after treatment for $CD16^{bright}$ NK cell subset). Unlike this, the expression of NKG2D increased significantly after IFN-α treatment ($p<0.01$, Wilcoxon signed rank test) only on $CD16^{bright}$ NK cell subset (2.51±0.82% in CM and 2.92±0.72% after IFN-α treatment) (Figure 6a). Moreover, there was no change in the expression of inhibitory KIR NK cell receptors, CD158a and CD158b, on either $CD16^{dim}$ and $CD16^{bright}$ NK cells after *in vitro* performed treatments ($p>0.05$, Wilcoxon signed rank test) (Figure 6b).

The analyses after treatment with IFN-α, RA and their combination of the density of expression (mean fluorescence intensity, MFI) of activating NK cell receptors on the two NK cell subsets showed that receptor CD161 expression increased significantly ($p<0.05$, Wilcoxon signed rank test), on both NK cell subsets after IFN-α treatment compared to treatments in CM (from 5.19±1.10% to 6.96±1.65% on $CD16^{dim}$ and from 5.54±1.54% to 7.14±1.95% on $CD16^{bright}$), whereas treatment with RA and IFN-α combination significantly increased ($p<0.05$, Wilcoxon signed rank test) its density of expression only on $CD16^{dim}$ NK cell subset (from 5.19±1.10% in CM to 8.4±2.22% after treatment). However, IFN-α treatment increased the density of expression of activating NK cell NKG2D receptor (12.18±2.38% in CM and 20.22±6.98% after IFN-α treatment) only on $CD16^{bright}$ NK cell subset (Figure 7a). With respect to the density of expression for inhibitory CD158a and CD158b KIR receptors no significant change was obtained after treatments (Figure 7b).

Discussion

As it has been shown that MM patients have an impairment in innate immune response, especially in the activity of NK cells as its major effector subpopulation [1], in this study we investigated the *in vitro* effect of INF-α and RA on different NK cell parameters in these patients.

In the present study we demonstrated that NK cell cytotoxicity of the investigated MM patients is increased by the *in vitro* performed treatments with IFN-α and IFN-α together with RA, while RA alone does not provoke any enhancement of NK cell activity. Considering that it has been shown for various cytokines, including IFN-α, that increase of NK cell activity was due to upregulation of various cytotoxic secretory molecules, such as perforin, granzymes A and B, as well as FasL and TRAIL receptors [20], in this study we showed for the first time that the effects of IFN-α, as well as IFN-α in combination with RA, are associated with a change in expression of the newly described activating and inhibitory NK cell receptors. The results obtained in this study indicate that the investigated agents affect the balance of these receptors in favor of the activating NKG2D and CD161 receptors. In this sense, upregulation of NK cell cytotoxicity by IFN-α is not only due to the known upregulation of cytolytic molecules [20], but also to the shown upregulation of the main activating receptor, NKG2D, whose expression has been

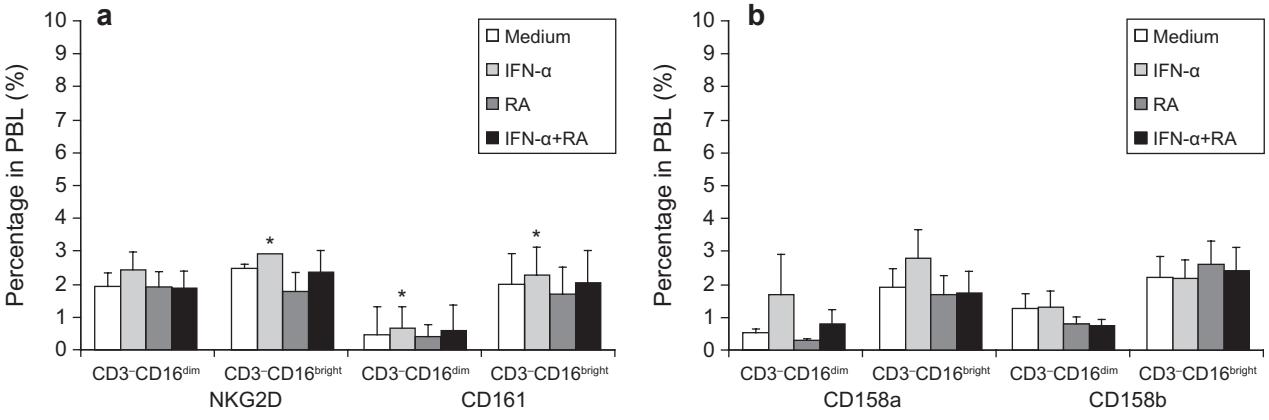

Figure 6. The expression of NKG2D and CD161 activating and CD158a and CD158b inhibitory KIR NK cell receptors was evaluated in PBL on $CD3^{-}CD16^{bright}$ and $CD3^{-}CD16^{dim}$ NK cell subsets after 18h *in vitro* treatments of PBL of MM patients with IFN-α, 13-*cis* RA and their combination. IFN-α treatment significantly (*$p<0.05$, Wilcoxon signed rank test) increased the expression of **(a)** NKG2D on $CD3^{-}CD16^{bright}$ NK cell subset, while the expression of CD161 increased significantly (*$p<0.05$, Wilcoxon signed rank test) on both $CD3^{-}CD16^{bright}$ and $CD3^{-}CD16^{dim}$ NK cell subsets. On the contrary there was no change in CD158a and CD158b NK cell receptor expression after treatments **(b)**. Results are shown as mean ± SE.

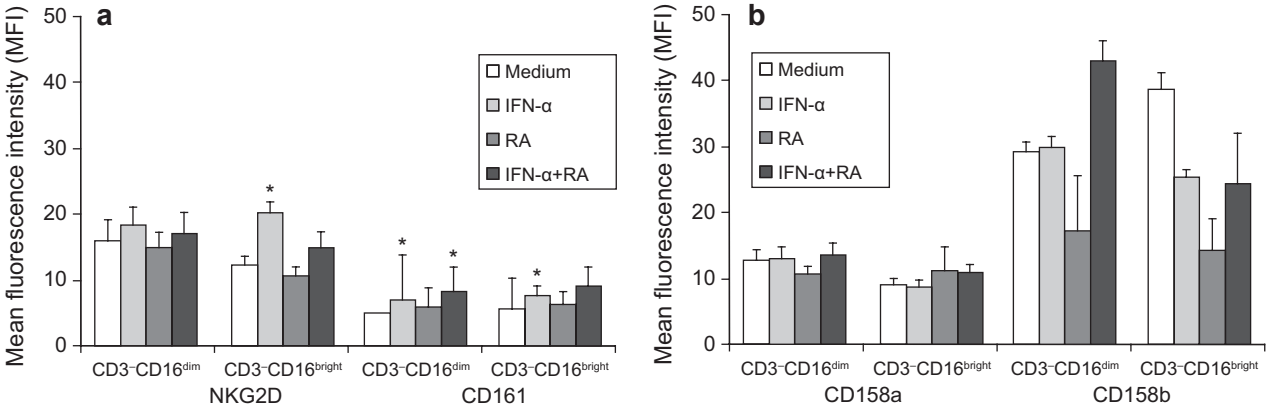

Figure 7. Mean fluorescence intensity (MFI) of expression of NKG2D and CD161 activating and CD158a and CD158b inhibitory KIR NK cell receptors was evaluated in PBL on $CD3^-CD16^{bright}$ and $CD3^-CD16^{dim}$ NK cell subsets after 18h *in vitro* treatments of PBL of MM patients with IFN-α, 13-*cis* RA and their combination. **(a)** IFN-α treatment significantly (*$p<0.05$, Wilcoxon signed rank test) increased the MFI of investigated activating NK cells receptors on $CD3^-CD16^{bright}$ NK cell subset for NKG2D receptor and on both $CD3^-CD16^{bright}$ and $CD3^-CD16^{dim}$ NK cell subsets for CD161 receptor (*$p<0.05$, Wilcoxon signed rank test). On the contrary, there was no change in CD158a and CD158b NK cell receptor MFI after treatments **(b)**. Results are shown as mean ± SE.

shown to be compromised in metastatic MM patients [1], as interaction of NKG2D and its ligands MICA or MICB on tumor cells facilitates NK cell cytotoxic antitumor mechanisms [14].

Our results showed that *in vitro* IFN-α and RA differentially affect the expression of the two newly investigated primary activating NK cell receptors NKG2D and CD161 and the newly investigated KIR receptors CD158a and CD158b on $CD3^-CD16^+$ NK cell population. Namely, we found that while IFN-α 18 h treatment significantly increased NKG2D and CD161 expression on NK cells, 18 h treatment with RA alone only increased the expression of the inhibitory CD158b KIR receptor. So far modulation of the expression of these NK cell receptors has been reported for IFN-α in one study for healthy controls [21] and in another for metastatic melanoma patients [22], while no such data has been available for RA. Therefore, by affecting the balance of the newly described activating and inhibitory NK cell receptors that shape NK cell activity, the investigated agents regulate NK cell cytotoxicity.

Except these two studies, the association of *in vitro* IFN-α induced NK cell NKG2D receptor expression with enhanced NK cell activity has so far been shown only in NK92 cell line after treatment with higher concentrations (1000 U/ml) and after longer, 48 h, incubation [23]. Unlike IFN-α and RA combination, the lack of increase of NK cell activity after treatment with RA alone, may be the consequence of the newly shown increase in inhibitory KIR CD158b receptor expression on $CD3^-CD16^+$ NK cell population. Moreover, as in this study no decrease in KIR expression was observed after IFN-α treatment, although it has been shown that downregulation of inhibitory KIRs by IFN-α is needed for improvement of NK cell activity [23], we assume that upregulation of NKG2D expression leads to consequent increase of NK cell activity.

Considering that the level and inducibility of pSTAT1 by IFN-α in PBL determines its effects [44], evaluation of its level may be a useful parameter in pretherapy predictive assays of *in vivo* response to this cytokine [24]. The evaluation of signaling pathways engaged in PBL performed treatments indicates an increase in pSTAT1 level by IFN-α, while RA, either alone or in combination with IFN-α increases the molecules associated with MAPK signaling pathway, i.e. MEK1,2 and ERK1,2. These results support the obtained, or lack of, modulation of NK cell cytotoxicity by these agents. The semiquantitatively measured increase of the investigated STAT1 protein in IFN-α and RA-treated PBL reflects their upregulation in NK cells, since these cells have been identified in one flow cytometry study to express STAT1 after *in vitro* or *in vivo* IFN-α treatment [24]. It has been shown in different animal model systems or cell lines [25], and only scarcely in pathological conditions [48] that NK cell cytolysis of target cells by the effector molecules Fas-L and perforin, is STAT1-dependent; so far this pathway has been rarely investigated in human NK cells [26].

It has been shown that RA receptors can undergo posttranslational modifications that include phosphorylation at tyrosine and serine/threonine residues which affect RA activity and also allow "cross talk" with the MAPKinase signaling pathway [27]. In this sense, our results that show that, contrary to IFN-α alone, RA alone or in combination with IFN-α upregulates the expression of molecules associated with MAPK signaling pathway, i.e. MEK1,2 and ERK1,2, associated with

upregulation of NK cytotoxicity [28], supports the role of these signaling molecules in the obtained increase of NK cell activity of the investigated MM patients.

Furthermore, as IRF-1 is a STAT1-inducible key transcription factor involved in the synthesis of cytotoxic granules necessary for NK cytotoxicity [6], we showed that IRF-1 mRNA is promptly and significantly induced by IFN-α. This finding is consistent with one previous report on isolated NK cells of healthy individuals that also showed fast upregulated expression of IRF-1 after only 1 h of stimulation [29] with a peak level after 4 h treatment with 1000 U/ml of IFN-α [25]. In this regard, our results also indicate IFN-α-mediated induction of IRF-1, a key transcription factor engaged by this cytokine.

It is interesting that our new results indicate that the performed short-term *in vitro* treatments with IFN-α or RA in healthy individuals do not influence the size of either cytotoxic $CD16^{bright}$ or regulatory $CD16^{dim}$ NK cell subset [21], as a desirable shift would be in favor of the more mature, cytotoxic $CD16^{bright}$ subset [18,30]. Considering that there is no significant change in NK bright and dim subset distribution following the performed treatments (data not shown), as well as the recently shown NK receptor distribution in these subsets [30], the novel observed selective induction of activating NKG2D and CD161 receptor expression with IFN-α on NK cell population, are most likely the consequence of direct regulation on a single cell level by this cytokine rather than a reflection of the subset shift within the NK cell compartment.

In addition, the results obtained for IFN-α-mediated NKG2D and CD161 induction on the two NK cell subsets are in agreement not only with the found significant IFN-α upregulation of these receptors on the entire $CD3^{-}CD16^{+}$ NK cell population, but also with the increase of NK cell activity. Moreover, our findings also show, for the first time, how the investigated agents affect receptor expression on these two NK cell subsets. In this regard, the found IFN-α induction of NKG2D is the consequence of its increase mostly on cytotoxic $CD16^{bright}$ subset, while IFN-α induction of CD161 also on the entire NK cell population results from its increased expression on both NK cell subsets, which is in agreement with our previous finding that CD161, contrary to NKG2D, is more susceptible to IFN-α [21,22]. Contrary to this, RA does not display any effect on these two activating receptors when analyzed on these two NK cell subsets, while the trend of the observed RA-mediated induction of CD158b expression on the cytotoxic $CD16^{bright}$ NK cell subset is in agreement with the increase observed on the entire NK cell population that may underlie the found lack of induction of NK cell activity by this agent.

It is of interest that modulation of the density of expression (MFI) of the investigated NK cell receptors by these agents on the cytotoxic and regulatory NK cell subsets supports the obtained findings of their percentage in NK cell subsets, as these two parameters are now used interchangeably in the evaluation of flow cytometry data.

We conclude that our *in vitro* predictive pretherapy findings of favorable enhancing effect of IFN-α on NK cell activity of the investigated MM patients is associated with upregulation of activating NKG2D and CD161 NK cell receptors, while the lack of RA alone-associated upregulation is probably due to the newly shown increased expression of the inhibitory KIR CD158b NK cell receptor after *in vitro* treatment with this agent alone. However, in combination with IFN-α, RA enhanced NK cell activity probably originates from upregulated proteolytic enzymes that mediate cytotoxicity. In this sense, the obtained data, especially for RA, give novel insight of the involvement of the newer generation of NK cell receptors in increasing the antitumor NK cell activity of MM patients.

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