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ORIGINAL ARTICLE

IL-2 And IL-15 Induced NKG2D, CD158a and CD158b Expression on T, NKT- like and NK Cell Lymphocyte Subsets from Regional Lymph Nodes of Melanoma Patients

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Abstract
Regional lymph nodes (LN)s represent important immunological barriers in spreading of malignant tumors. However, they are the most frequent early metastatic site in melanoma. Immunomodulatory agents including cytokines have been included in therapy of melanoma and have shown severe side effects and toxicity. In this sense, there is a growing need for bringing these agents to further in vitro testing that may enlighten aspects of their regional application. Therefore, the aim of this study was to investigate the effect of interleukin (IL)-2 and IL-15, the two cytokines with similar immune-enhancing effects, on the expression of activating NKG2D, inhibitory CD158a and CD158b receptors on $CD8^+$ T, NKT-like and NK cell lymphocyte subsets from regional LNs of melanoma patients. In this study, we showed significant effects of IL-2 and IL-15 cytokine treatments on the expression of activating NKG2D and on inhibitory CD158a and CD158b receptors on lymphocytes, $CD8^+$ T, NKT-like and NK cell lymphocyte subsets originating from regional LNs of melanoma patients. Furthermore, IL-2 and IL-15 by inducing the expression of NKG2D activating receptor on innate and on adaptive lymphocyte subsets and by augmenting NK cell antitumor cytotoxicity that correlated with the cytokine-induced NKG2D expression, increased antitumor potential of immune cells in regional LNs of melanoma patients irrespective of LN involvement. These findings indicate the importance of immune cell population from regional LNs of melanoma patients in the development of immune intervention strategies that may if applied locally increase antitumor potential to the level that controls tumor progressions.

Keywords Regional lymph nodes · IL-2 · IL-15 · T cells · NKT-like cells · NK cells

Introduction

Regional lymph nodes (LN)s are the most frequent early metastatic site in melanoma. Although melanoma is an immunogenic tumor, the metastatic stage of disease is often accompanied with multiple deflects in immune cell function and phenotype that favor tumor progression [1]. Considering that immunosuppression induced by tumor provides in adjacent LNs an environment prone to tumor infiltration [2], the efforts to revert immunosuppression in regional LNs may be of importance for the reduction of metastasis [3]. Immuno-enhancing effects of γc chain-receptor family cytokines have been shown initially for interleukin (IL)-2 and had led to therapeutic application of this cytokine in metastatic melanoma, while IL-15 has been investigated in clinical trials [4, 5]. Since severe side effects are associated with systemic cytokine treatment, there is a need for more targeted therapeutic approach. This represents a rationale for bringing these agents to further in vitro testing that may enlighten some aspects of regional application of these cytokines.

The first line of antitumor immune response is carried by natural killer (NK) cells. These innate effector cells possess the unique ability to directly kill tumor cells by cytotoxic mechanisms without prior antigen presentation by major

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histocompatibility complex (MHC) molecules [6]. Antitumor activity of NK cells is regulated by the balance of signals derived from engaged activating and inhibitory NK cell receptors. Although basically discovered on NK cells, NK cell receptors play an active role in regulating antitumor effector functions not only of NK, but also of $CD8^+$ cytotoxic T lymphocytes (CTL)s and NKT-like lymphocyte subsets.

NKG2D is the most prominent activating NK cell receptor that upon binding to stress-induced ligands MICA/MICB and ULBP1–4 on malignant cells induces antitumor cytotoxicity [7]. However, in $CD8^+$ T cells, the engagement of NKG2D receptor functions as a co-stimulatory signal that amplifies T cell activation mediated through the T-cell antigen receptor in antitumor immune response [8]. The superfamily of killer cell immunoglobulin-like receptors (KIR)s prevents NK cell-mediated cytotoxicity towards "healthy" cells that express MHC class I molecules. Therefore, 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 NK cell-mediated lysis. The role of KIR receptors on T cells is less clear, with some evidence suggesting that they may inhibit T cell receptor (TCR)-mediated T cell activation while other data suggest that they may function as costimulatory molecules facilitating T cell activation [9]. NKT-like cells, like NK and $CD8^+$ T cells, express KIR receptors at a frequency similar to KIR expressing conventional T cells. Thus, NKT-like cells can display alloreactivity, for which they use mechanisms characteristic of both NK and conventional T cells [10]. Considering the importance of these receptors for antitumor immune response, in this study we have investigated the association between their expression on innate (NK and NKT-like) and adaptive $CD8^+$ T effector cells in regional LNs with the number of tumor-involved regional LNs.

Given the unique position regional LNs that implies the immunosuppressive effects of tumor microenvironment and their role as immunological barriers, it may be of relevance to explore the effects of IL-2 and IL-15, on lymphocyte subsets in regional LNs. Specifically, the aim of this study was to investigate in vitro effects of these two cytokines with similar immune-enhancing effects on the expression of activating NKG2D, inhibitory CD158a and CD158b receptors on $CD8^+$ T, NKT-like and NK cell lymphocyte subsets originating from regional LNs of melanoma patients.

Materials and Methods

Patients

In this study 48 melanoma patients (25 women and 23 men) in clinical stage II–IV, according to modified American Joint Cancer Committee (AJCC)/Union for International Cancer Control (UICC) staging system that underwent regional LN dissection were included. For the purpose of this research one regional LN per patient was selected based on its largest diameter and subjected to further analysis. Immediately after surgical removal specimen taken from selected regional LN was processed in order to obtain single cell suspension, while the rest of the tissue was paraffin embedded for standard pathohistological examination. Tumor infiltration was evaluated by at least two independent examinations of hematoxylin/eosin stained sections per LN. This study has been reviewed and approved by Ethics Committee of Institute of Oncology and Radiology of Serbia, and all subjects had given written informed consent.

Mononuclear Cell Isolation

In order to form single cell suspension, LN tissue samples were minced with sterile scalpel and filtered through a 100 μm mesh. Mononuclear cells (MNC) were isolated using Histopaque (Sigma-Aldrich Chemie, Steinheim, Germany) density gradient, centrifuged at 500 g for 40 min and washed three times in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum (Sigma, St. Louis, USA).

In Vitro Treatments

MNC isolated from regional LNs of melanoma patients were cultivated for 7 days in RPMI 1640 culture medium (CM) alone, CM supplemented with IL-2 (200 U/ml) and IL-15 (25 ng/ml) in six-well plates (Sigma-Aldrich, Germany) at 37^0 C and 5% CO_2 in humid atmosphere. After 3 days of in vitro cultivation CM, as well as CM supplemented with these cytokines in proper concentrations, were added.

Flow Cytometry

In freshly isolated MNC population NK cells and T cells were identified using directly labeled monoclonal antibodies (mAbs): anti-CD3, anti-CD8, anti-CD56, anti-NKG2D, anti-CD158a, anti- CD158b (Becton Dickinson, San Jose, USA). The samples were prepared as previously described [11]. A total of 50,000–100,000 gated events verified as lymphocyte population according to their physical characteristics (Forward Scatter- FSC and Side Scatter- SSC), were collected per sample and analyzed using Cell Quest software. Exclusion of non-specific fluorescence was based on matched isotype mAb combinations conjugated with FITC, PE and PerCP (Becton Dickinson, San Jose, USA).

Statistical Analysis

Significance of differences between controls and cytokine treatments was tested by statistical non-parametric Wilcoxon signed rank test.

Spearman rank correlation coefficient has been evaluated to estimate statistical dependence between the investigated parameters and lymph node involvement.

Results

The prevalence of activating NKG2D and inhibitory CD158a and CD158b receptors on all lymphocytes, $CD8^+$ T and NKT-like lymphocyte subsets in regional LNs of melanoma patients was evaluated in gated lymphocyte population by flow cytometry. Obtained data showed that activating NKG2D receptor in lymphocyte population of investigated regional LNs was predominantly present on $CD8^+$ T lymphocyte subset (Fig. 1a). Similar distribution on lymphocyte subsets was found for CD158a, while for CD158b inhibitory receptor the similar prevalence on T, NKT-like subsets was shown (Fig. 1b).

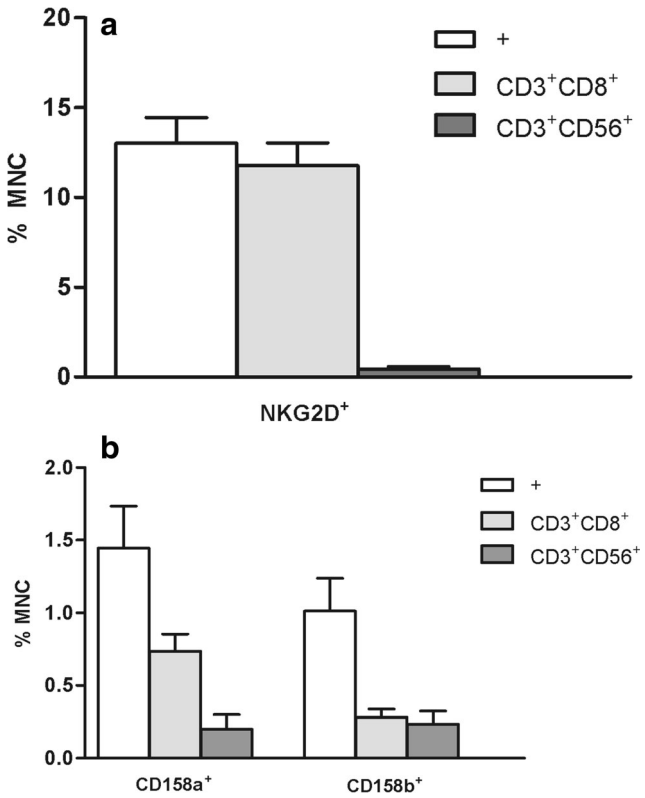


Fig. 1 Prevalence of lymphocytes and their $CD8^+$ T and $CD3^+CD56^+$ NKT-like cell subsets expressing (**a**) NKG2D activating receptor (**b**) inhibitory CD158a and CD158b receptors in regional lymph nodes of melanoma patients. Results are shown as mean ± SE

Spearman rank correlation coefficient was evaluated to estimate statistical dependence between the prevalence of receptor positive (Rc^+) lymphocyte subsets and lymph node involvement (N0- without tumor positive LNs-, N1 with a single positive LN, N2- with 2 to 3 positive LNs and N3 with 4 or more tumor-positive regional LNs). In this sense, for NKG2D as well as for CD158a and CD158b KIR receptors, there was no significant correlation ($p > 0.05$, Spearman's rank correlation) between LN involvement and Rc^+ lymphocyte subsets (data not shown).

We have further investigated the effect of 7 days of in vitro cultivation with IL-2 and IL-15 on prevalence of Rc^+ lymphocyte subsets from regional LNs of melanoma patients and compared them to the control cell culture media treatments. Our results show that IL-2 cytokine treatment significantly increased ($p < 0.05$, Wilcoxon signed rank test) the percentage of $NKG2D^+$ lymphocytes and $CD8^+$ T, NKT-like and NK cells while IL-15 cytokine treatment significantly increased ($p < 0.05$, Wilcoxon signed rank test) the percentage of $NKG2D^+$ lymphocytes and NKT-like and NK cells (Fig. 2a,b). However, the obtained cytokine-induced augmentation of the expression of NKG2D receptor on lymphocyte subsets did not show any significant correlation with LN involvement (data not shown).

Regarding the effect of cytokine treatments on KIR expression, for CD158a after IL-2 treatment we did not find any significant effect, while after IL-15 treatment the percentage of $CD158a^+$ lymphocytes and $CD8^+$ T and NK-T like lymphocyte subsets significantly increased ($p < 0.05$, Wilcoxon signed rank test). However, there was no significant effect of both cytokine treatments on CD158a expression on NK cells (Fig. 2c,d). Data obtained for CD158b show that IL-2 cytokine treatment significantly increased ($p < 0.05$, Wilcoxon signed rank test) the percentage of $CD158b^+$ lymphocytes and $CD8^+$ T and NK cells, while IL-15 treatment significantly increased ($p < 0.05$, Wilcoxon signed rank test) the percentage of $CD158b^+$ lymphocytes and all $CD158b^+$ investigated lymphocyte subsets (Fig. 2 e,f).

Considering that in vitro IL-2 and IL-15 cytokine 7-day treatments have been previously shown to strongly induce antitumor cytotoxicity of initially low cytotoxic NK cells from regional LNs of melanoma patients [12], we have performed correlation analysis between NK cell cytotoxic activity and the percentage of Rc^+ NK cells. In this sense, between the percentage of $NKG2D^+$ NK cells and NK cells cytotoxicity there was significant positive correlation after control culture media treatment ($p < 0.05$, Spearman rank test) and highly significant positive correlation ($p < 0.01$, Spearman rank test) after IL-2 and IL-15 cytokine treatments (Fig. 3a-c). Conversely, between the percentage of $CD158a^+$ NK cells and NK cell cytotoxic function there was no significant correlation either after cell culture media or cytokine treatments (data not shown). Furthermore, for the percentage of

CD158b$^+$ NK cells we showed after control cell culture media treatment negative correlation with NK cell cytotoxicity ($r = -0{,}5912$, $p < 0.05$, Spearman signed rank test) while after treatments with either IL-2 or IL-15 there was no significant correlation ($p > 0.05$, Spearman signed rank test) with NK cell cytotoxic function (Fig. 3d-f).

Discussion

Immune cells in regional LNs have an important role in eradication of LN metastasis as well as in the control of spreading of malignant cells to distant organs. Among lymphocyte subsets that are able to express activating (NKG2D) and

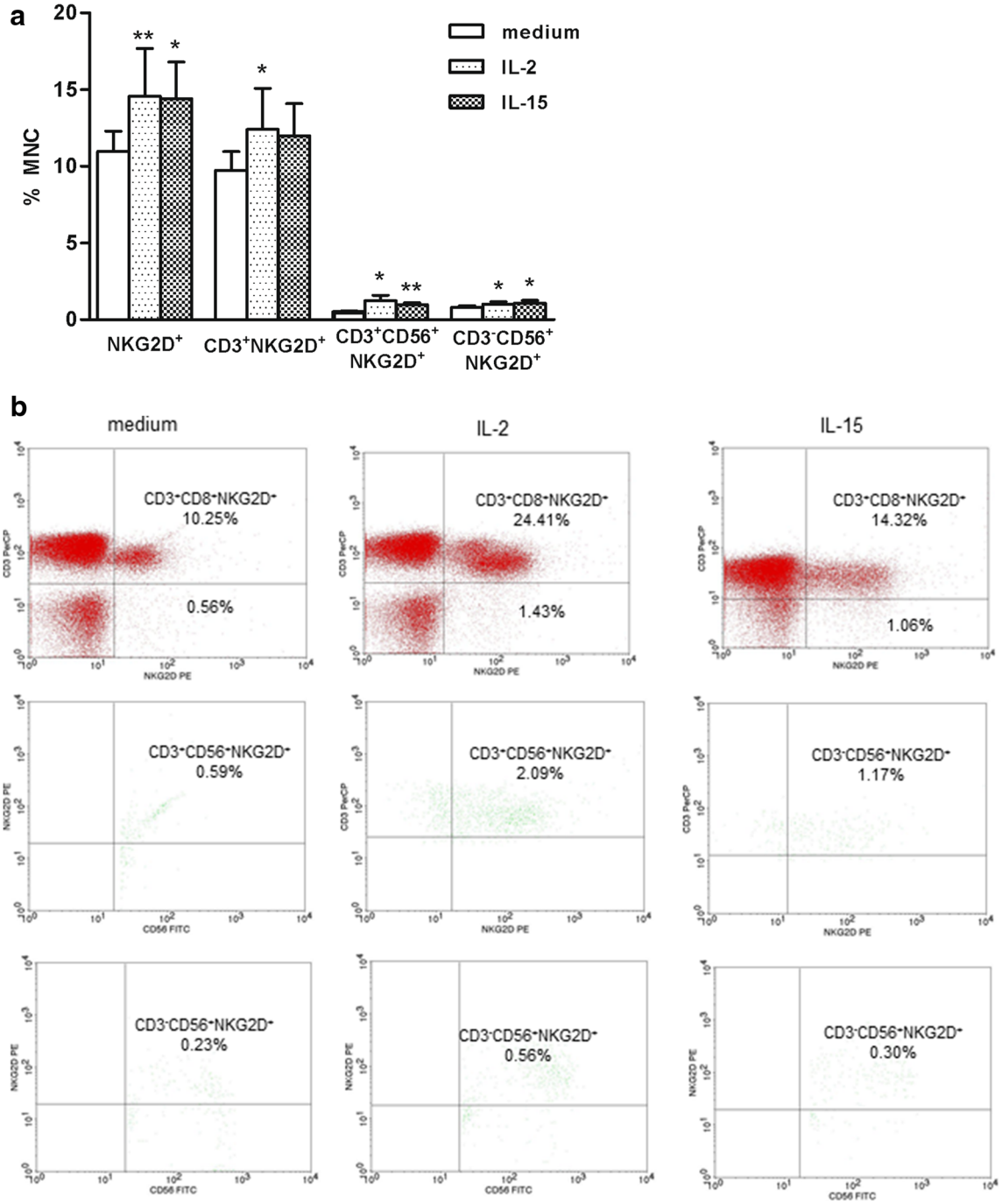
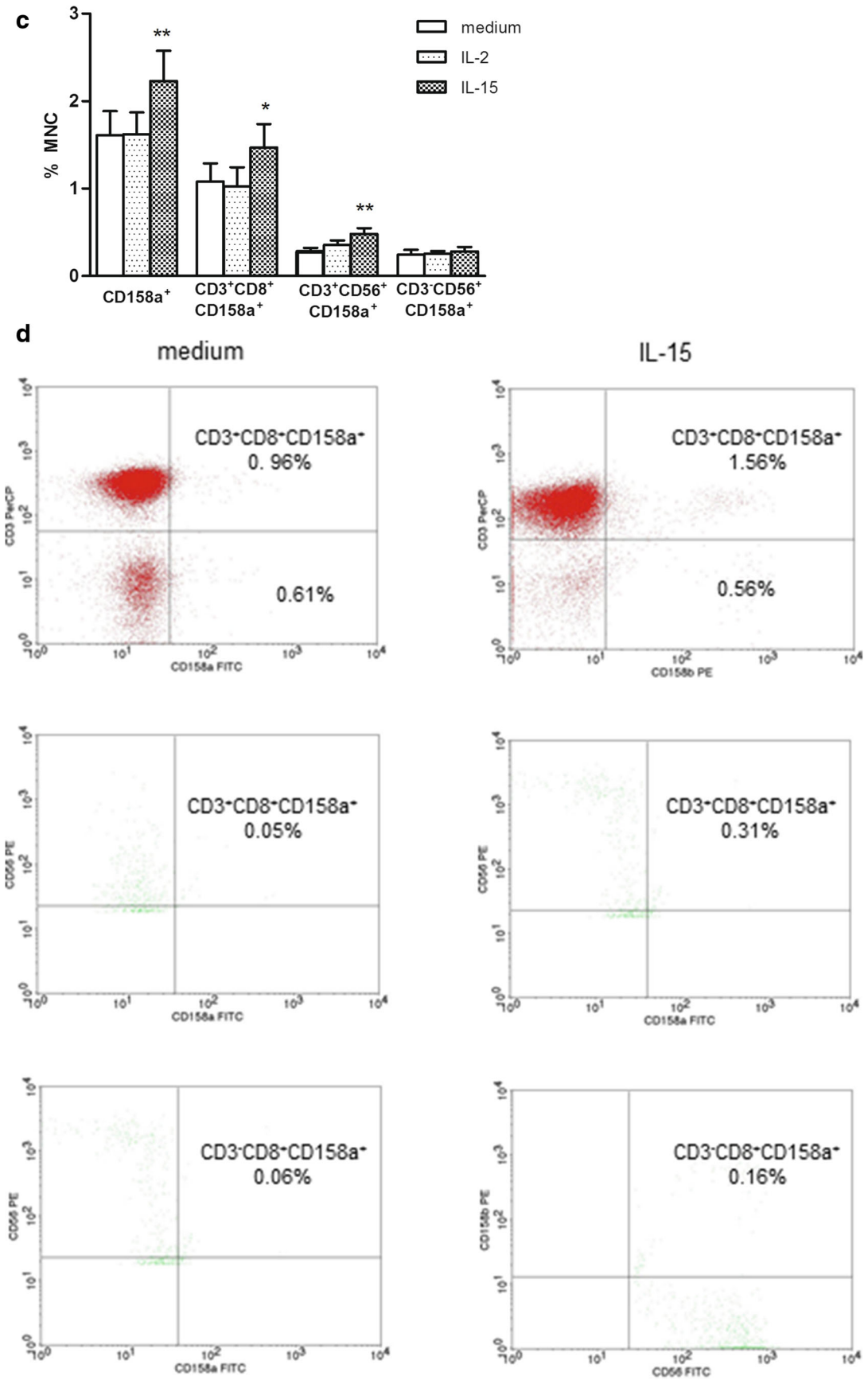


Fig. 2 Expression of (**a**) NKG2D, (**c**) CD158a and (**e**) CD158b inhibitory receptors on lymphocytes, CD8$^+$ T, CD3$^+$CD56$^+$ NKT-like and CD3$^-$CD56$^+$ NK cell lymphocyte subsets after 7 days of in vitro cultivation with interleukin (IL)-2 (200 IU/ml) and IL-15 (25 ng/ml) compared with the control treatment with cell culture medium (*$p < 0.05$, **$p < 0.01$, Wilcoxon signed-rank test). Results are shown as mean ± SE. Representative flow cytometry dot plot diagrams show increase in (**b**) NKG2D (**d**) CD158a and f) CD158b expression on lymphocyte subsets after 7 days of in vitro cultivation



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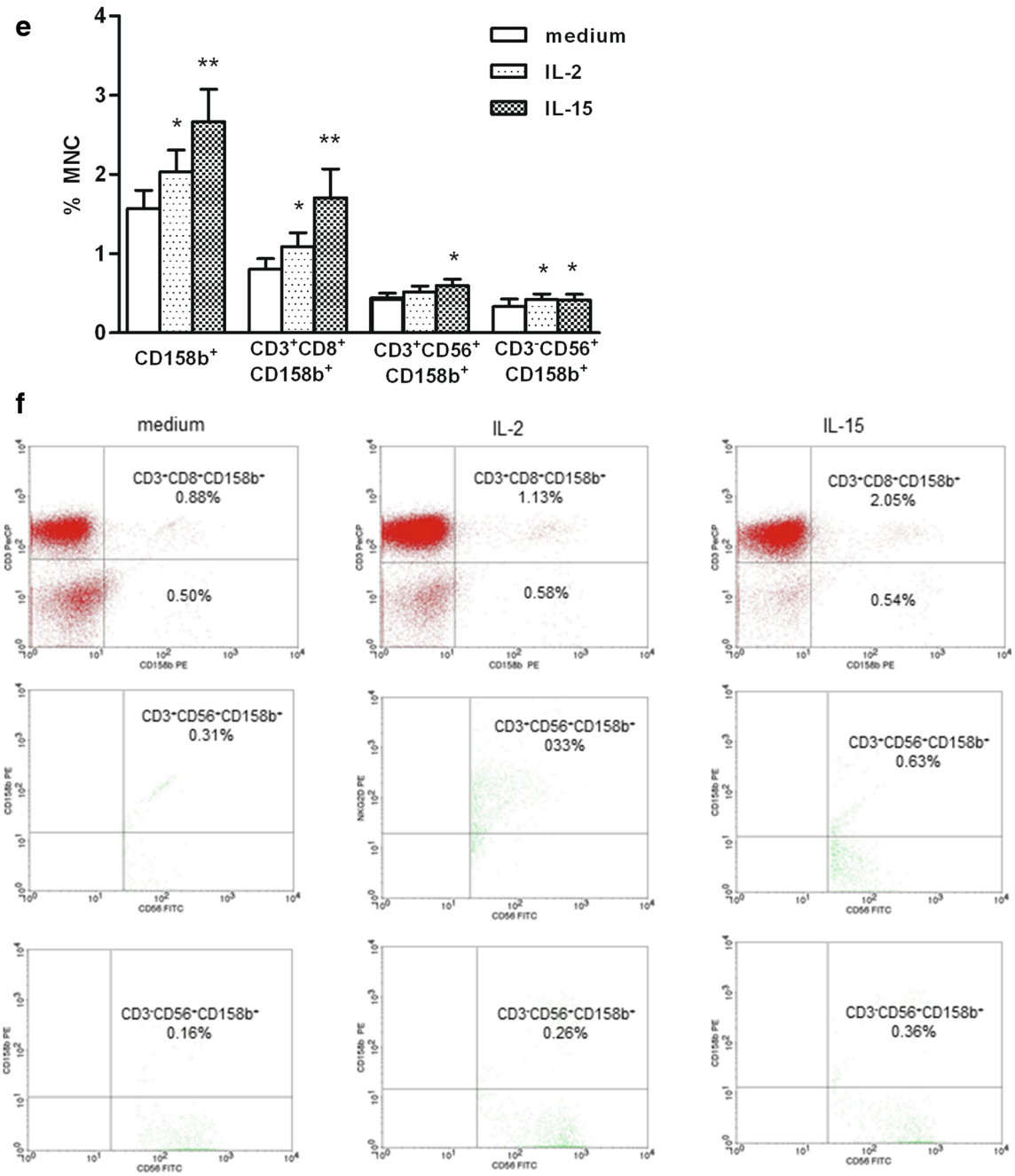

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inhibitory (KIR) NK cell receptors in human LNs, CTLs have been found to be more abundant compared to NK and NKT-like cells [13–15]. The number of involved regional LNs represents an important clinical parameter in melanoma [16]. Our study showed that LN involvement did not correlate with the expression of activating NKG2D receptor on NKT-like and CTL lymphocytes subsets that is similar to the finding reported for NKG2D expression on NK cells from regional LNs of melanoma patients [17]. Our data regarding the two most frequent KIR receptors CD158a and CD158b [18] showed the predominance of KIR^+ T cells in lymphocyte population of investigated regional LNs. Previous studies in melanoma patients have shown the positive correlation between the expression of CD158b inhibitory receptor on NK cells in regional LNs and the LN involvement [17], as well as the enrichment of KIR^+ NK cell population in tumor infiltrated LNs [19] that may be attributed to the effect of TGF-β secreted by tumor and suppressive immune cells [20].

Function and phenotype of immune cells in tumor-draining LNs, due to their proximity to primary tumor, are directly influenced by tumor microenvironment [21]. Immune enhancing effects of IL-2 and IL-15 have been thoroughly

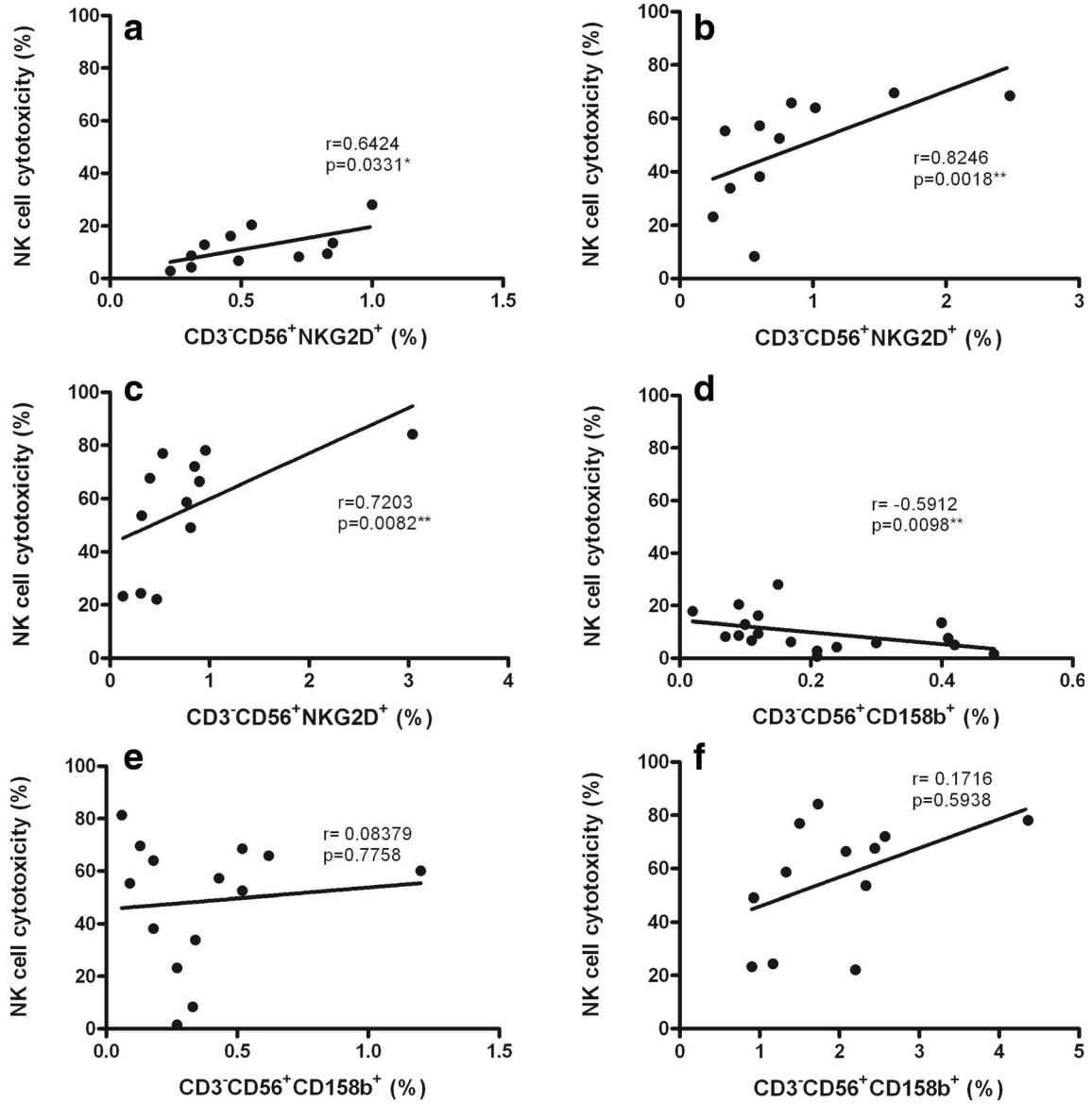

Fig. 3 Correlation between NK cell cytotoxic activity with: the percentage of NKG2D$^+$ and CD158b$^+$ NK cells following 7 days of in vitro cultivation in culture medium (medium) **(a,d),** interleukin (IL)-2 (200 IU/ml) **(b, e)** and IL-15 (25 ng/ml) **(c, f)** (*$p < 0.05$, **$p < 0.01$, Spearman's rank correlation)

investigated on immune cells from peripheral blood of patients with malignant tumors [22]. Exploring the effect of these two cytokines on the expression of receptors involved in antitumor immune response on T, NKT-like and NK lymphocyte subsets from regional LNs of melanoma patients may be of relevance considering the specific localization of these immune cells. In this sense, we showed that after long term 7-day in vitro cultivation, both cytokines increased the percentage of NKG2D$^+$ lymphocytes and CD8$^+$ T, NKT-like and NK cell lymphocyte subsets. Induction of NKG2D expression by IL-2 and IL-15 has been previously shown on NK, CD8$^+$ T and NKT-like cells from peripheral blood of healthy individuals [23–26] and patients with malignancies [27]. However, the augmented NKG2D expression on lymphocyte subsets originating from regional LNs shown in this study as well as data from similar study on NK cells [12], did not show any significant correlation with LN involvement. These findings may indicate the inducibility of NKG2D activating receptor expression by investigated cytokines irrespective of LN involvement.

Furthermore, IL-2 and IL-15 in vitro treatments have been reported to induce acquisition of inhibitory KIRs on peripheral blood CD8$^+$ T and NK T-like cells [26, 27]. In this study on lymphocytes derived from regional LNs of melanoma patients, IL-15 treatment increased the percentage of CD158a$^+$ cells due to augmentation of CD158a expression on T and NKT-like subsets. The percentage of CD158b KIR expression was increased with both cytokine treatments with significant effect on CTLs, NKT-like and NK cells. Our finding of cytokine-induced expression of CD158a and CD158b is in

agreement with previously reported upregulation of a group of several inhibitory KIRs upon cytokine stimulation on NK cells in healthy human LNs [28, 29] and in regional LNs of melanoma patients [12]. This effect may have resulted from facilitated KIR transcription, which IL-2 and IL-15 promote by inducing binding of the c-Myc transcription factor to the KIR gene promoter [30] and by induction of promoter demethylation [31].

Numerous studies have shown, initially for IL-2 [32] and subsequently for IL-15, pronounced stimulatory effect of these two cytokines on antitumor cytotoxicity of peripheral blood NK cells in patients with malignancies and tumor-infiltrating NK cells [33–36]. NK cell population in LNs has been reported to exhibit negligible cytotoxic activity without cytokine stimulation [29]. Stimulatory effects of IL-2 and IL-15 on NK cell cytotoxic function have been reported for NK cells originating from LNs of healthy individuals [29] and also for NK cells from regional LNs of melanoma patients [12, 29]. Based on stimulating effects of these cytokines on NK cell cytotoxicity and on the expression of NK cell receptors, we investigated the correlation between obtained NK cell cytotoxicity and percentage of Rc^+ NK cells from regional LNs of melanoma patients after 7 days of in vitro cultivation. We showed positive correlation between the percentage of $NKG2D^+$ NK cells and NK cell cytotoxicity after control cell culture medium treatment and showed that this correlation became even more pronounced after IL-2 and IL-15 cytokine treatments. This finding indicates the importance of NKG2D receptor expression in the induction of cytotoxicity of NK cells in investigated regional LNs. In this sense the increased expression of activating receptors alleviates NK cell to target cell contact and concomitantly with the induction of cytotoxic effector molecules (perforin and granzymes), contributes to augmented NK cell cytotoxic activity [37]. Regarding inhibitory KIRs we showed that the percentage of $CD158b^+$ NK cells negatively correlated with NK cell cytotoxic function after control cell culture media treatment that is consistent to the role of KIRs in shifting the balance of NK cell receptor-mediated signals towards negative regulation of NK cell antitumor cytotoxicity. According to more recent studies KIR binding to "self"-MHC class I ligands induces mobilization of activating receptors to nanodomains of the plasma membrane that are favorable for activating cellular signaling that subsequently contributes to NK cell cytotoxic function [38]. However, the KIR expression induced with cytokine treatments in this study did not significantly correlate with antitumor cytotoxicity of LN-derived NK cell.

In this study, we showed significant effects of IL-2 and IL-15 cytokine treatments on the expression of activating NKG2D and inhibitory CD158a and CD158b receptors on $CD8^+$ T, NKT-like and NK cell lymphocyte subsets originating from regional LNs of melanoma patients. Furthermore, IL-2 and IL-15 by inducing the expression of NKG2D activating receptor on innate and on adaptive lymphocyte subsets and by augmenting NK cell antitumor cytotoxicity, facilitated the antitumor potential of immune cells in regional LNs of melanoma patients regardless of LN involvement. Therefore, the augmented antitumor potential of immune cells from regional LNs of melanoma patients induced by investigated cytokines indicates the importance of regional LNs as potential sites of cytokine application. Development of therapeutic approaches that include local application of cytokines may eliminate some adverse effects that are associated with systemic administration of cytokines and increase antitumor immune response to the level that controls tumor progression.

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