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RESEARCH

Decreased Interferon γ Production in $CD3^+$ and $CD3^-CD56^+$ Lymphocyte Subsets in Metastatic Regional Lymph Nodes of Melanoma Patients

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Abstract As lymphogenic dissemination is very common in melanoma, regional lymph nodes (LN)s represent first immunological barriers to tumor invasion and play a complex role in antitumor immune defense. In this sense, their most prominent role is the presentation of tumor-derived antigens to naïve T cells and generation of cell-mediated adaptive immune response. Since tumor micro-environment affects immune cell function in this study we have evaluated the ability of T cells and NK cells in metastatic (involved) and non-metastatic regional LNs to produce interferon γ (IFNγ), a pleiotropic cytokine that regulates adaptive antitumor immune response. Our results show reduced IFNγ production in both T and NK lymphocyte subsets and decreased prevalence of T cells in metastatic regional LNs of melanoma patients. The decrease of IFNγ production in T cells was more pronounced with increased number of involved regional LNs indicating tumor-induced functional impairment of both T and NK cell lymphocyte subsets in involved regional LNs. Therefore, shown low IFNγ production in metastatic LNs may represent an obstacle in adaptive cell-mediated antitumor immune response and hence may enable tumor progression.

Keywords Regional lymph nodes · Interferon γ · T cells · NK cells

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Introduction

As lymphogenic dissemination is the most common in melanoma, regional lymph nodes (LN)s represent the first immunological barrier for spreading this tumor into visceral organs. Among many complex roles of regional LNs in antitumor immunity the most prominent one is the presentation of tumor-derived antigens to naïve T cells and generation of adaptive immune response [1].

Interferon (IFN)γ is a type II interferon and its biological activity is associated with antitumor mechanisms during cell-mediated adaptive immune responses. The most prominent role of IFNγ in upregulation of major histocompatibility complex class I (MHC I) molecule expression that aids the priming and presentation of antigens by antigen presenting cells (APC)s [2]. IFNγ is as a pleiotropic cytokine involved in differentiation and regulation of function of many immune cell types. In this sense IFNγ stimulates Th-1, while inhibits Th2 T cell responses, activates macrophages and induces production of chemokines which recruit specific effector cells to the site of inflammation. IFNγ is produced mainly by $CD4^+$ T helper cell type 1 (Th1) lymphocytes, $CD8^+$ cytotoxic T lymphocytes (CTL)s and natural killer (NK) cells [3]. Both T and NK cells have been found to co-localize in T cell-dependent paracortical area of LN where naïve T cells are brought into contact with APCs [4]. Dendritic cells (DC)s are the most prominent APCs that patrol peripheral sites and upon tumor antigen-induced maturation migrate to draining LNs [5]. Mature DCs in LNs present tumor antigens and prime both $CD8^+$ and $CD4^+$ T cell responses, secrete interleukin (IL)-12 and IL-15 and subsequently activate NK cells [6]. In LNs activated NK cells by secreting IFNγ assist in Th1 polarization in DC-mediated T cell priming. Th1 polarized $CD4^+$ T cells exert antitumor response *via* both IFNγ and IL-2 secretion [7, 8] and provide helper signals to CTL-mediated cytolysis of

transformed cells. Furthermore, IFNγ can also induce the expression of membrane-bound IL-15 on DCs and further sustain T and NK cell survival and activation [9, 10]. Unfortunately, IFNγ may also exert tumor promoting functions by stimulating T regulatory (Treg) and myeloid derived suppressor cell (MDSC) development, by CTL suppression through indoleamine 2,3-dioxygenase expression induction in melanoma cells as well as by inducing tumor cell resistance to NK cell and CTL lysis [3].

Considering numerous beneficial biological effects of IFNγ in antitumor immune response, IFNγ production by T and NK cells may be evaluated as parameter that defines antitumor immune function of regional LNs. Therefore the aim of this study was to analyze the effect of tumor microenvironment on this aspect of immune function by comparing IFNγ production in metastatic and non-metastatic regional LNs.

Materials and Methods

Patients

In this study 35 melanoma patients in clinical stage II- IV (Patient's characteristics are show in Table 1) 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 histological examination. Excised regional LNs were subjected to standard pathohistological and cytological examination performed in the Department of pathology in the Institute of Oncology and Radiology of Serbia. 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 gave written informed consent.

Table 1 Patients' characteristics

	Number of patients
Total number	35
Gender	23
Male	12
Female	
Median age (years)	58 (range 33–84)
Clinical stage (AJCC)	
I-II	15
III	19
IV	1
Primary tumor site	
Head&neck	1
Limbs	18
Trunk	16
Lymph node involvement	
N0	15
N1	5
N2	4
N3	11
Histology	
Nodular	3
Non- nodular	32
Clark invasion	
I	2
II	1
III	11
IV	8
V	4
Unknown	9
Breslow	
≤1 mm	0
1.01–2 mm	5
2.01–4 mm	16
≥4 mm	5
Unknown	9
Ulceration	
Present	8
Absent	20
Unknown	7

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 to exclude undissociated fragments. 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-Aldrich).

Flow Cytometric Analysis

In freshly isolated MNC population NK cells and T cells were identified using the following combinations of directly labeled monoclonal antibodies (mAbs): CD3PerCP/ CD56FITC, CD3PerCP/CD4FITC and CD3PerCP/CD8FITC. 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 CellQuest software. Exclusion of non-specific fluorescence was based on matched isotype mAb combinations conjugated with FITC, PE and PerCP (Becton Dickinson, San Jose, USA). NK cells and T cells were defined. For intracellular staining of IFNγ 500,000 MNC were incubated 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 CD56PE antibodies, fixed and permeabilized with BD FACS permeabilizing solution 2 (BD Biosciences, San Jose, USA) according to standard Becton Dickinson procedure and subsequently stained with anti-IFNγ FITC (Becton Dickinson).

Statistical Analysis

Significance of differences between metastatic and non-metastatic LN groups was tested using statistical non-parametric Mann-Whitney test.

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

Results

Using flow cytometry we have first analyzed the prevalence of T and NK lymphocyte subsets in MNC population in regional LNs. Flow cytometry data show that metastatic LNs contained significantly lower ($p<0.05$, Mann-Whitney test) percentage of $CD3^+$ T cells compared to non-metastatic LNs of melanoma patients mostly due to lower abundance of helper $CD4^+$ T cell subset, while the prevalence of cytotoxic $CD8^+$ T cell subset was significantly higher ($p<0.05$, Mann-Whitney test) in metastatic compared to non-metastatic LNs (Fig. 1a). Consequently, metastatic LNs also showed lower CD4/CD8 T cell ratio compared to non-metastatic LNs (Fig. 1b). Furthermore, metastatic LNs showed significantly higher ($p<0.05$, Mann-Whitney test) prevalence of $CD3^-$ lymphocytes with higher percentage of $CD3^-CD56^+$ NK cell population compared to investigated non-metastatic LNs (Fig. 1c).

In order to evaluate the influence of the degree of tumor spread to regional lymph nodes on the prevalence of IFNγ producing lymphocyte subsets, we have performed the correlation analysis between the prevalence of 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 LNs). The Spearman rank correlation coefficient (r) obtained for the percentage of $CD3^+$ and $CD3^+CD4^+$ T cells indicated their negative correlation, while for $CD8^+$ T cell subset indicated positive correlation with lymph node involvement (Table 2). Moreover, for the percentage of complementary $CD3^-$ population and $CD3^-CD56^+$ NK cell population we showed significant positive correlation with lymph node involvement (Table 2).

The percentage of IFNγ producing lymphocyte population as well as gated $CD3^+$ (T cells) and $CD3^-CD56^+$ (NK cells) was significantly lower ($p<0.05$, Mann-Whitney test) in metastatic compared to non-metastatic regional LNs (Fig. 2a and b). In NK cell population, IFNγ production was lower in metastatic compared to non-metastatic LNs only in immunoregulatory

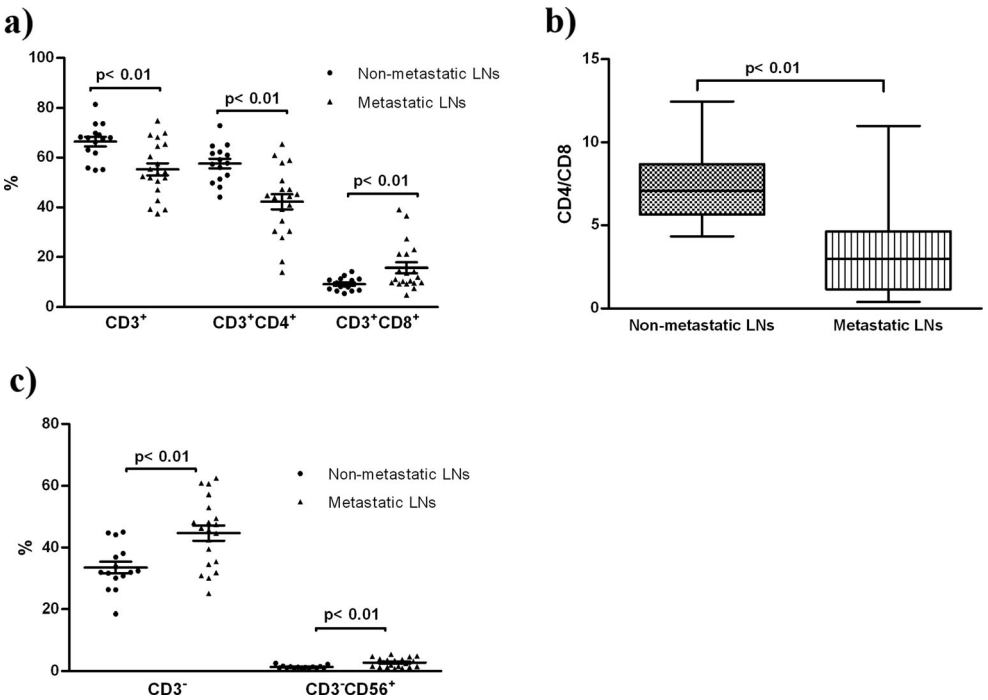

Fig. 1 Lymphocyte subset prevalence in regional lymph nodes (LN)s of melanoma patients: **a** metastatic LNs contain significantly lower percentage of $CD3^+$ T cells and $CD4^+$ helper T cells, while significantly higher percentage of $CD8^+$ cytotoxic T cells ($p<0.01$, Mann-Whitney test) compared to non-metastatic LNs. **b** significantly higher prevalence of $CD3^-$ cells and $CD3^-$ $CD56^+$ NK cells ($p<0.01$, Mann-Whitney test) in metastatic compared to non-metastatic LNs. **c** significantly lower CD4/CD8 T cells ratio ($p<0.01$, Mann-Whitney test) in metastatic compared to non-metastatic LNs. Results are presented as mean value±standard error of 15 non-metastatic and 20 metastatic regional LNs

Table 2 Correlation between lymph node (LN) involvement with percentage of lymphocyte subsets and gated IFNγ$^+$ lymphocyte subsets in regional LNs of melanoma patients

	r[a]	*p*
CD3$^+$	−0.4462	0.0099
CD3$^+$CD4$^+$	−0.4986	0.0023
CD3$^+$CD8$^+$	0.3395	0.0460
CD3$^-$	0.4462	0.0093
CD3$^-$CD56$^+$ [12]	0.4375	0.0060
IFNγ$^+$	−0.6157	0.0111
CD3$^+$IFNγ$^+$	−0.6935	0.0059
CD3$^-$CD56$^+$IFNγ$^+$	−0.4607	0.0974

[a] Spearman's rank correlation coefficient

CD56^{bright} NK cell subset, while similar in both LN groups in cytotoxic CD56^{dim} subset. Moreover, the percentage of overall IFNγ producing lymphocytes in regional LNs of melanoma patients negatively correlated with lymph node involvement due to its significant negative correlation with the percentage of IFNγ producing CD3$^+$ lymphocyte subset (Table 2). Contrary to T cells, the percentage of IFNγ producing NK cells showed no significant correlation with lymph node involvement (Table 2).

Discussion

As the most common dissemination route for melanoma is lymphatic, the ability of immune cells in regional LNs to produce IFNγ is crucial for both local and systemic cell-mediated adaptive antitumor immune responses.

In this study we have evaluated the effect of tumor microenvironment in regional LNs on immune function of IFNγ producing cells by comparing IFNγ production between metastatic and non-metastatic regional LNs. Our analysis on the prevalence of IFNγ producing lymphocyte subsets showed that metastatic compared to non-metastatic LNs contained lower percentage of T cells and its helper CD4$^+$ population and accordingly lower CD4/CD8 ratio. Contrary to T cells the percentage of NK cells was increased in metastatic LNs mostly, as we have previously reported, due to the CD56^{dim} NK cell infiltration into the tumor bearing regional LNs [13]. The finding of decreased prevalence of helper T and increased prevalence of NK cells was more pronounced with increased number of involved LNs. This is in agreement with the early reports obtained on regional LNs of melanoma and breast cancer patients in which these findings have been related to advanced clinical stage of disease [12, 14].

Flow cytometry data indicate that IFNγ production was lower in metastatic compared to non-metastatic LNs of melanoma patients due to its lower production in both T cells and NK cell lymphocyte subsets. Despite the higher prevalence of NK cells in metastatic LNs, NK cells in metastatic LNs similarly to T cells produced less IFNγ. It is of importance to emphasize that during initiation of antitumor immune response in tumor-draining LNs, NK cell activation often occurs upstream of T cell activation providing an early IFNγ production which is critical for subsequent development of adaptive immune response [15, 16]. Although CTL response needs helper signals provided by CD4$^+$ T cells, interactions

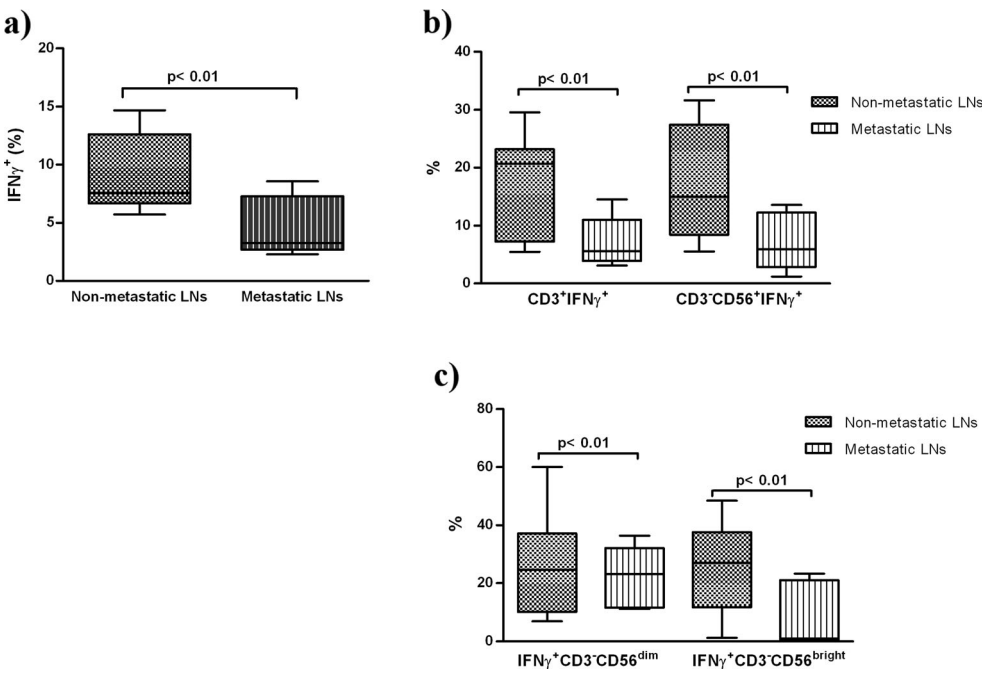

Fig. 2 Metastatic compared to non-metastatic regional lymph nodes (LN)s contain significantly lower ($p<0.01$, Mann-Whitney test) percentage of: **a** IFNγ producing lymphocytes. **b** gated IFNγ producing T cells and gated IFNγ producing CD3$^-$CD56$^+$ NK cells. **c** gated IFNγ producing CD3$^-$CD56^{briht} NK cell subset while similar percentage of gated CD3$^-$CD56^{dim} NK cell subset. Results are presented as box and whisker plots (showing minimum to maximum) with median value ±standard error of seven non-metastatic and nine metastatic regional LNs

occurring between DCs and NK cells can bypass these helper signals as NK cells via IFNγ secretion stimulate IL-12 production by DCs and eventually lead to a protective CTL response [6]. Our findings indicate that IFNγ production by NK cells in metastatic LNs was lower most probably due to its lower production in $CD56^{bright}$ NK cell subset responsible for sustained IFNγ production. Unlike $CD56^{bright}$ subset, $CD56^{dim}$ NK cell subset which has been recently characterized as an immediate and rapid IFNγ producer [17] showed similar ability to produce IFNγ in investigated metastatic and non-metastatic LNs. It appears that tumor infiltration into investigated regional LNs impaired mostly $CD56^{bright}$ subset previously reported to migrate from neoplastic tissues to secondary lymphoid organs via afferent lymph [18]. Although lower in metastatic compared to non-metastatic regional LNs, the IFNγ production in NK cells did not correlate with number of involved regional LNs. Conversely, IFNγ production in T cells was more impaired with increased number of involved regional LNs in investigated melanoma patients.

Decreased IFNγ production in T and NK cells in metastatic regional LNs may be a consequence of micro-environmental factors including intensive secretion of immunosuppressive mediators such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF) β, IL-10, NO and prostaglandins by melanoma cells [19] as well as expansion and accumulation of immunosuppressive cells in local microenvironment (Tregs, MDSCs, tumor associated macrophages, N2 polarized subsets of neutrophils, tolerogenic DCs). Furthermore, melanoma cells often express constitutively activated Ras/Raf signaling pathway that by inducing NF-κB transcriptional factor enhances synthesis of proinflammatory cytokines (tumor necrosis factor- TNF, IL-1, IL-6) and chemokines that attract immunosuppressive cells and in this sense facilitate melanoma growth and metastasis [19, 20].

Analogously to decreased IFNγ production in metastatic LNs obtained in our study it has been reported that metastatic melanoma patients show evidence of systemic Th2 cytokine-driven inflammation accompanied with low IFNγ which probably resulted from VEGF overproduction by malignant cells [21].

Our study on melanoma patients shows that lymph node involvement reduces IFNγ production in both T and NK lymphocyte subsets and decreases the prevalence of T cells. Impairment of IFNγ production in T cells was more pronounced with increased number of involved regional LNs in investigated melanoma patients. Our findings indicate tumor-induced functional impairment of both T and NK cell lymphocyte subsets in involved regional LNs. Therefore, low IFNγ production in metastatic LNs may represent an obstacle in adaptive cell-mediated antitumor immune response and hence may enable tumor progression.

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