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Original Article

Metformin promotes antitumor activity of NK cells via overexpression of miRNA-150 and miRNA-155

Andjela R Petrovic[1], Ivan P Jovanovic[1], Milena M Jurisevic[2], Marina Z Jovanovic[1], Marina M Jovanovic[5], Sladjana P Pavlovic[1], Nebojsa N Arsenijevic[1], Gordana M Supic[3,4], Danilo V Vojvodic[3,4], Milan M Jovanovic[6], Nevena M Gajovic[1]

[1]Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, Kragujevac 34000, Serbia; [2]Department of Pharmacy, Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, Kragujevac 34000, Serbia; [3]Institute for Medical Research, Military Medical Academy, Crnotravska 17, Belgrade 11000, Serbia; [4]Medical Faculty of Military Medical Academy, University of Defense, Crnotravska 17, Belgrade 11000, Serbia; [5]Department of Internal Medicine, Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, Kragujevac 34000, Serbia; [6]Department of Abdominal Surgery, Military Medical Academy, Crnotravska 17, Belgrade 11000, Serbia

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Abstract: Objectives: Metformin, an oral anti-diabetic drug, is known to possess a powerful antitumor effect by modulating the tumor-immune interaction. The precise influence of metformin on natural killer (NK) cells, a crucial innate immunity player, is not completely understood. In our study, analyses of the effect of metformin on the NK cell functional phenotype were performed, and the potential mechanisms underlying it were investigated. Methods: BALB/C wild type mice were treated with metformin, and the functional phenotype of splenocytes and potential underlying mechanisms were investigated. Results: Metformin significantly boosts NK cell cytotoxicity and the percentage of NKp46$^+$, FasL$^+$, and interferon (IFN)-γ$^+$ NK cells while decreasing interleukin (IL)-10 producing NK cells. Our research also demonstrated that the simultaneous administration of metformin and 1-methyl-DL-tryptophan (1-MT), a specific inhibitor of indoleamine 2,3-dioxygenase (IDO), significantly increased the NK cells synthesis of IFN-γ, IL-17, perforin, and FasL and NKp46 expression. These findings imply that metformin potentiates NK cell cytotoxicity through mechanisms other than IDO blockade. Metformin administration strongly increased the expression of immunostimulatory microRNA (miRNA)-150 and miRNA-155, while decreasing the expression of immunosuppressive miRNA-146a. Conclusions: These findings suggest that metformin can directly potentiate NK cell activation and cytotoxicity. This research may contribute to dissecting key mechanisms of metformin exerting antitumor activity to advance the use of metformin as an antitumor agent.

Keywords: Metformin, NK cell, indoleamine 2,3-dioxygenase, immunotherapy, microRNA, cancer surveillance

Introduction

Natural killer (NK) cells, a heterogeneous subpopulation of innate lymphoid cells (ILCs), represent an irreplaceable component of native immunity. They play a vital role in anti-tumor and antiviral immunity due to the exceptional potential of recognizing and eliminating infected and transformed cells without prior activation [1]. The main effector mechanisms of NK cells are engaging death receptors or perforin and granzyme secretion, but they also produce a variety of cytokines making a bridge that connect the innate and adaptive immune system [2]. NK cells are displaying an array of activating receptors, some of which are NKG2D, DNAM-1, NKp30, and cluster of differentiation 16 (CD16) and at least one inhibitory receptor, the most prominent of which are KIR, NKG2A, and TIM-3 [3]. Although NK cells are an efficient part of antitumor immunity, research indicates that cancer cells still manage to escape them, usually by decreasing the expression of activating receptors, while increasing inhibitory receptor expression [4, 5]. There is additional evidence that viruses utilize similar modes to suppress NK cells and avoid innate immunity [6]. Considering that findings suggest that inhibition

and/or deficiency of triggered NK cells usually leads to poor disease prognosis, the primary focus of the current study was identifying the possible methods for activating NK cells and amplifying their potential to kill [7].

It has been known for decades that metformin, an oral anti-diabetic drug, can inhibit tumor development and suppress tumor proliferation through the interference of several signaling pathways, such as AMPK/mTOR, JNK/p38, PI3K/Akt, and MAPK/p38 [8]. Lately, studies have revealed that metformin may also potentiate anti-tumor immunity [9]. One of the first described immunomodulating effects of metformin was its impact on T lymphocytes. Data confirmed that metformin potentiates the cytotoxic activity of $CD8^+$ T lymphocytes, an important component of antitumor defense while inhibiting the development of $CD4^+$ T lymphocytes into Tregs, a major source of immunosuppressive cytokines [10, 11]. Besides T lymphocytes, studies demonstrated that metformin affects tumor-associated macrophages (TAMs), one of the key regulators of the immune system within the tumor microenvironment (TME). According to a study by Ma *et al*. on BALB/c mice carrying 4T1 tumors, metformin administration stimulated the differentiation of TAMs towards the M1 functional phenotype characterized by the secretion of large amounts of the antitumor cytokine interferon γ (IFN-γ) [12].

While metformin's effects on the components of immunity mentioned above have been the focus of extensive research for some time, its effects on innate immunity components, such as NK cells, remain poorly understood. A recent study by Xia *et al.* has begun to reveal ways in which metformin modulates NK cell antitumor functions. Their research demonstrated that metformin administration potentiates NK cell cytotoxicity by altering its functional phenotype [13].

Small non-coding RNAs called microRNAs (miRNAs) have been demonstrated to limit protein translation and consequently regulate a variety of cellular processes [14]. A study by Pesce *et al.* highlighted the significance of the expression of miRNAs for NK cell maturation and function, especially for anticancer therapy [15]. However, the direct influence of metformin on miRNAs in NK cells and therefore NK cell gene expression has not yet been described.

In the present study, it has been revealed that metformin administration potentiates the antitumor phenotype of NK cells. Moreover, metformin enhanced the expression of immunostimulating miRNA-150 and miRNA-155 and decreased the expression of immunosuppressive miRNA-146a. These findings could provide a promising basis for developing novel strategies for the future treatment of patients with cancer.

Material and methods

Mice

All animal work was performed on BALB/C wild type (WT) mice. Mice (six to eight weeks of age) were housed in an animal facility of the Faculty of Medical Sciences in an environment with a 12-hour light/12-hour dark cycle, 22 ± 2°C temperature and relative humidity of 51 ± 5%. Animals were given *ad libitum* access to the standard laboratory food and water before and throughout the whole study. Before the experiment, mice were randomly assigned to control and experimental groups. The following two groups were formed: 1) WT BALB/C mice, which were given metformin daily for 24 days, and 2) Control group with healthy untreated WT BALB/C mice.

Ethical statement

All experiments were reviewed and approved by the Animal Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia. The study was conducted according to the criteria of the ARRIVE guidelines and EU Directive 2010/63/EU for animal experiments.

Purification of NK cells

NK cells were enriched from spleens that were removed from metformin-treated mice and control mice by magnetic cell separation kit (MiltenyiBiotec, BergischGladbach, Germany). Initially, cells were labeled with FlowComp™ Mouse CD49b antibody (Ab) (Invitrogen, USA). Furthermore, they were captured by the Dynabeads (Invitrogen) and finally positively selected by magnetic beads (Invitrogen). Finally, beads were eliminated using FlowComp™ Release buffer (Invitrogen). The collected cells were highly enriched with $NK49b^+$ cells.

Cytotoxicity assay

Cytotoxicity assay was measured by the Roche xCELLigence Real-Time Cell Analyzer (RTCA), Dual Purpose (DP) Instrument (ACEA Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. 96-well plates were loaded with 100 µl of complete medium (Invitrogen) each, and background impedance was measured on the xCELLigence RTCA DP instrument at 37°C and with 5% carbon dioxide (CO_2). 4T1 cells were seeded in plates at a density of 1×10^4 cells/100 µl (per well) and used as target cells. After incubating for 36 hours under standard conditions, NK cells were added to the plates as effector cells (ratio of target to effector cells [T:E = 1:10]). NK cells were isolated from spleens. Cell index was evaluated during the next 72 hours. Data were analyzed with RTCA Software 1.2 (ACEA Biosciences).

Flow cytometric analysis of splenocytes

Splenocytes were isolated from experimental and control mice and analyzed using flow cytometry. Single-cell suspensions of splenocytes were obtained by mechanical dispersion through cellular meshes (BD Pharmingen, USA) in DMEM.

Cells were treated with fluorochrome-conjugated anti-mouse antibodies specific for CD45, CD3, CD4, CD8, CD11b, CD11c, CD19, CD25, F4/80, Foxp3, Ly6G, Ly6C, Lin coct, Sca-1, MHC II, CD86, Gr1, or isotype matched controls (BD Pharmingen, NJ/Invitrogen, Carlsbad, CA). For staining of intracellular cytokines, cells were stimulated with 50 ng/mL of phorbol 12-myristate 13-acetate(Sigma-Aldrich), 500 ng/mL of ionomycin (Sigma-Aldrich) and Golgi Stop (BD Pharmingen, NJ) for 4 hours at 37°C, 5% CO_2, and stained with perforin (APC), granzyme (PE), CD107a (FITC), Foxp3 (APC), IFN-γ (PE) interleukin (IL)-10 (APC), IL-17 (PercpCY5.5), tumor necrosis factor (TNF) α (PercpCY5.5); STAT-3 (PE), Ki67 (FITC), indoleamine 2,3-dioxygenase (IDO) (FITC), and inducible nitric oxide synthase (iNOS) (PE) (BD Pharmingen/BioLegend/eBiosciences). For staining NF-κB transcription factor, cells were additionally incubated with rabbit anti-NF-κB p65 antibody (Abcam) followed by the application of secondary FITC-labeled DNK anti-rabbit IgG antibody. The analysis was performed on a FACS Calibur flow cytometer (BD Biosciences, San Jose, USA). FlowJo software was used for data analyses (Tree Star).

In vitro inhibition of IDO

Splenocytes derived from healthy, untreated mice were seeded in 24-well plates at a rate of 2×10^6 cells per well. Additionally, each well was added with the following: 1) RPMI 1640 medium with 1 mM of 1-methyl-DL tryptophan (pharmacological inhibitor of IDO) and 10 mmol/l of metformin; 2) RPMI 1640 medium with 10 mmol/l of metformin; 3) RPMI 1640 medium with 1 mM of 1-methyl-DL tryptophan; and 4) RPMI 1640 medium only. After incubating for 24 hours, cells were centrifuged and stained with primary conjugated monoclonal antibodies specific for FasL, Nkp46, IFN, IL17, and perforin. After staining, the analysis of cells was performed on a flow cytometer.

Analysis of miRNA expression

For the analysis of miRNA expression, NK cells from WT mice and WT mice that were given metformin daily were isolated. The sample consisted of approximately 10^4-10^5 cells. Total RNA, including small RNAs, from NK cells of both experimental groups were isolated using Mirvana miRNA Isolation Kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. The relative miRNA expression was measured using TaqMan MicroRNA Assays, which are based on the reverse transcriptase-polymerase chain reaction (RT-PCR) method of detection. Quantitative PCR determined the expression of miRNA-146a, miRNA-150, and miRNA-155 using TaqMan microRNA Assay Kits and TaqMan Universal Master Mix No AmpUNG in the 7500 Real-Time PCR system (Applied Biosystems). The assays were performed in triplicate using samples from two independent experiments.

Statistical analysis

The obtained data were analyzed using commercially available software (SPSS version 23.0). All results were analyzed using the Student's t-test or Mann-Whitney U test, where appropriate. Data are expressed as the mean ± standard error of the mean. Values of $P<0.05$ were considered to indicate a statistically significant difference.

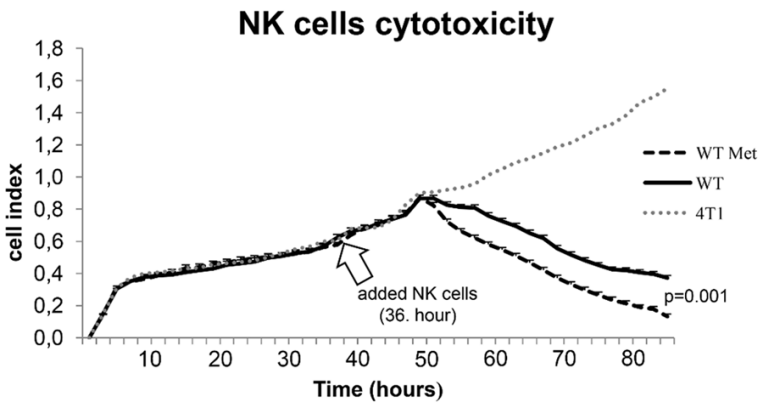

Figure 1. Metformin treatment enhanced splenic NK cell cytotoxicity. The cytotoxicity assay was performed on NK cells isolated from WT mice and compared to NK cells isolated from WT mice treated with metformin. The graph illustrates the ability of metformin to enhance the cytotoxicity of NK cells. Data was assessed by xCELLigence system. Results are presented as means ± SEM of two individual experiments, each carried out with six mice per group. Statistical significance was tested by a Student's t-test.

Results

Administration of metformin enhances NK cell cytotoxicity

To determine whether metformin can modulate the cytotoxicity of NK cells, we analyzed $CD49b^+$ spleen NK cells toward 4T1 tumor cells in groups defined before. Using the xCELLigence system we demonstrated that metformin treatment significantly increased NK cell cytotoxicity compared with non-treated NK cells ($P<0.01$; **Figure 1**).

Metformin alters the functional phenotype of splenic NK cells

The functional phenotype of NK cells isolated from the spleens of mice with and without metformin treatment was further examined. Percentages of $NKp46^+CD3^-CD49b^+$ ($P=0.018$) and $FasL^+CD3^-CD49b^+$ ($P=0.004$) NK cells were significantly higher in mice treated with metformin in comparison with mice in the non-treated group (**Figure 2A**, **2B**). In addition, although there were no differences in the number of IFN-γ producing $CD3^-CD49b^+$ NK cells, intracellular staining revealed significantly increased expression of IFN-γ in NK cells after metformin treatment ($P=0.032$; **Figure 2C**). Finally, the percentage of $CD3^-CD49b^+$ NK cells that produce IL-10 ($P=0.001$) was decreased in metformin-treated mice when compared with the control mice (**Figure 2D**). We also examined whether metformin influences the expression of other surface markers of interest, such as KLRG1, Foxp3, programed death ligand 1 (PD1) and IL-17. We noticed a trend in differences between previously defined groups of mice where the expression levels of inhibitory molecules KLRG1, Foxp3, and PD1 were lower in NK cells isolated from an experimental group, whereas the number of IL-17-producing $CD3^-CD49b^+$ NK cells in the same group were higher. Although the results were consistent with the potentiating role of metformin, the values did not reach statistical significance (data not shown).

Metformin treatment stimulated an antitumor phenotype of NK cells independently of blockade of IDO, in vitro

Furthermore, we evaluated the impact of concurrent treatment with metformin and IDO inhibitors on the functional phenotype of NK cells. Splenocytes extracted from healthy mice were separated into three groups and placed for 24 hours in a medium with and without added metformin and selective blocker of IDO-1-MT. $CD3^-CD49^+$ NK cells were analyzed within a cultivated population of splenocytes after 24 hours. Results revealed that the addition of metformin significantly increased the percentage of $FasL^+$ ($P=0.08$) and $NKp46^+$ ($P=0.016$), $perforin^+$ ($P=0.016$), IFN^+ ($P=0.016$), and $IL\text{-}17^+$ ($P=0.008$) $CD3^-CD49^+$ NK cells (**Figure 3A-E**). More interestingly, dual treatment with metformin and 1-MT showed a significant increment in the percentage of $FasL^+$ ($P=0.01$), $NKp46^+$ ($P=0.001$), $perforin^+$ ($P=0.0001$), IFN^+ ($P=0.0001$), and $IL\text{-}17^+$ ($P=0.0001$) $CD3^-CD49^+$ NK cells compared with metformin treatment only (**Figure 3A-E**).

Metformin-treated WT mice demonstrated altered miRNA expression in NK cells

Finally, the expression of miRNA-155, miRNA-150, and miRNA-146a in NK cells derived from healthy, untreated WT mice and NK cells derived from metformin-treated WT mice were measured. NK cells derived from metformin-

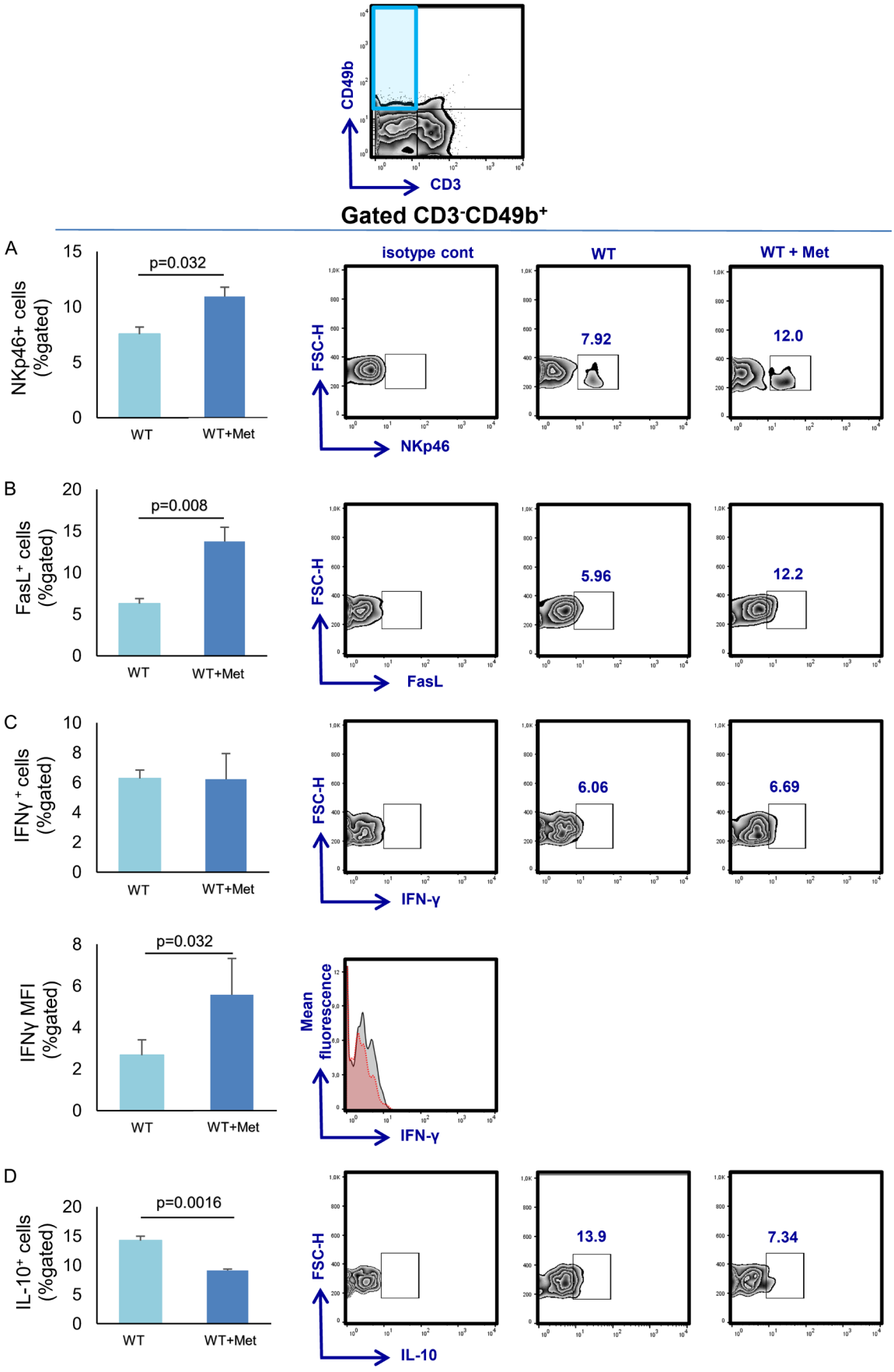
CD49b
CD3
Gated CD3-CD49b+
A
NKp46+ cells (%gated)
p=0.032
WT
WT+Met
isotype cont
WT
WT + Met
FSC-H
NKp46
7.92
12.0
B
FasL+ cells (%gated)
p=0.008
FasL
5.96
12.2
C
IFNγ+ cells (%gated)
IFN-γ
6.06
6.69
IFNγ MFI (%gated)
p=0.032
Mean fluorescence
D
IL-10+ cells (%gated)
p=0.0016
IL-10
13.9
7.34

Figure 2. Metformin alters the functional phenotype of NK cells in metformin-treated mice. The data on the Figure summarize the percentage of NKp46$^+$ (A), FasL$^+$ (B), IFNγ, and IFNγ MFI (C) and IL-10$^+$ (D) NK cells derived from the spleens of WT mice in comparison to NK cells derived from WT mice treated with metformin. Results were assessed by flow cytometry, and data is presented on graphs and representative FACS plots as mean ± SEM of six mice per group. Statistical significance was tested by the Man-Whitney U test.

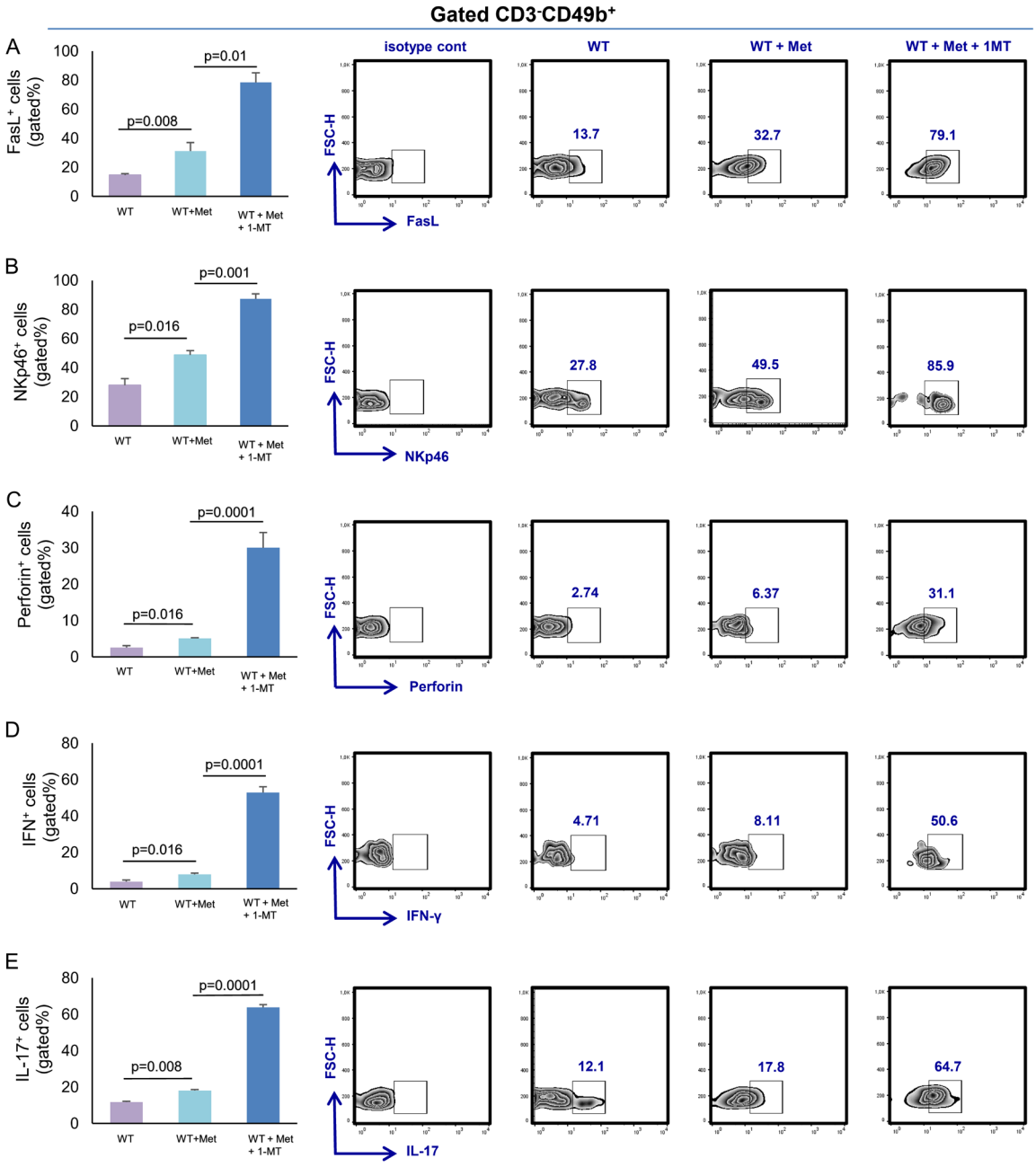

Figure 3. Functional phenotype of splenic NK cell is significantly altered by synergistic administration of metformin and IDO inhibitor. The graphs and representative FACS plots show the percentage of NK cells that are FasL$^+$ (A), NKp46$^+$ (B), perforin$^+$ (C), IFN$^+$ (D) and IL-17$^+$ (E). Splenocytes derived from healthy, untreated mice were cultivated in medium with added 1-MT and metformin, medium with added metformin, medium with added 1-MT and medium only. The percentages of FasL$^+$ (A), NKp46$^+$ (B), perforin (C), IFN$^+$ (D), and IL-17$^+$ (E) CD3$^-$CD49b$^+$ cells were determined by flow cytometry. The data are shown as mean ± S.E.M. of six mice per group and are representative of three separate experiments. Statistical significance was determined using the Mann-Whitney U test.

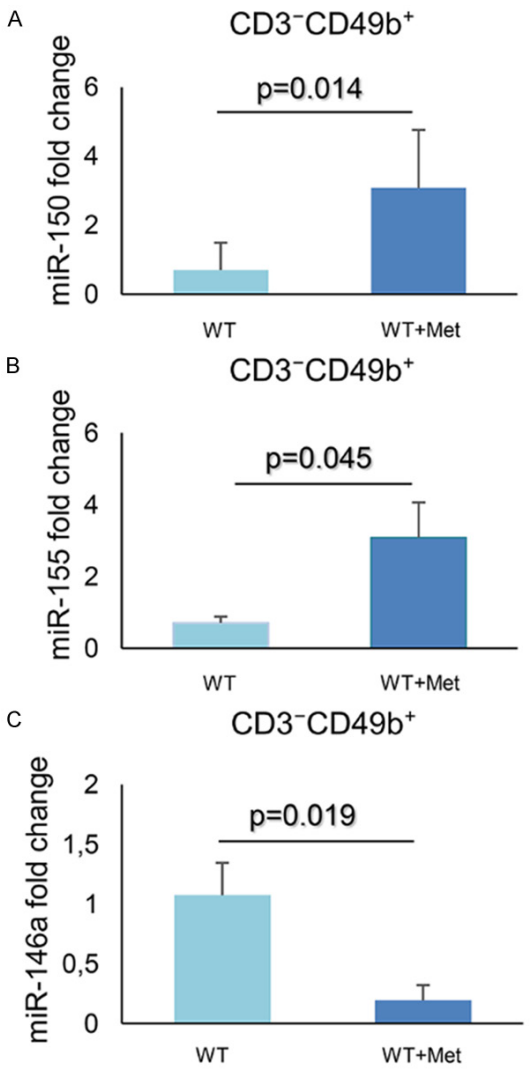

Figure 4. Expression of miRNA-150 and miRNA-155 was upregulated in NK cells isolated from WT mice treated with metformin while expression of miRNA-146a was downregulated. Mice were randomly divided into two groups, experimental group which were given metformin daily for 24 days and control group which consisted of healthy untreated mice. The expression of miRNA-146a, miRNA-150 and miRNA-155 was determined in NK cells isolated from both experimental and control group using quantitative PCR. A and B. Illustrate that miRNA-150 and miRNA-155 have significantly higher expression in NK cells derived from metformin-treated WT mice. C. Demonstrates significantly lower expression of miRNA-146a in NK cells isolated from WT mice treated with metformin in comparison to untreated WT mice. The data are shown as mean ± SEM of six mice per group and are representative of three separate experiments. For evaluation of statistical significance, we used Student's T-test.

treated mice had a considerably higher expression of miRNA-155 (P=0.014) and miRNA-150 (P=0.045) in comparison with NK cells derived from WT mice (**Figure 4A**, **4B**). Furthermore, it was found that miRNA-146a expression was significantly lower (P=0.019) in NK cells obtained from WT mice treated with metformin compared with NK cells derived from WT mice (**Figure 4C**).

Discussion

Several decades of research have provided compelling evidence that metformin has a major influence on the components of the immune system. However, most studies so far have focused on metformin's effect on adaptive immunity, leaving its effect on innate immunity relatively unexplored. Xia *et al*. studied NK cells *in vivo* and *in vitro*. Xia *et al*.'s *in vitro* results showed that metformin potentiates NK cell cytotoxicity by increasing the synthesis of IFNγ, perforin, and granzyme B. Xia *et al*.'s *in vivo* study in melanoma and lymphoma mouse models confirmed that metformin significantly boosted the NK cells cytotoxicity and thus cancer immune surveillance [13]. In this study, the aim was to investigate ways metformin modulates the cytotoxicity and phenotype of NK cells. To explore whether and how metformin alters NK cell cytotoxic capacity, NK cells derived from metformin-treated WT mice were analyzed and the findings were compared with the cytotoxicity levels of NK cells isolated from untreated WT mice. Data have shown that metformin administration significantly enhanced NK cell-based cytotoxicity toward 4T1 tumor cells (**Figure 1**). The current results are in line with the study by Xia *et al*. thus confirming the stimulative effect of metformin on NK cells. A study by Crist *et al*. revealed that metformin increases both the number and activity of peripheral NK cells in patients with head and neck squamous cell carcinoma [16].

In order to fully explain the collected evidence of increased NK cytotoxicity, the functional phenotype of splenic NK cells was further investigated. Several studies explored whether metformin alters the functional properties of NK cells. Studies by Xia *et al.* and Allende-Vega *et al*. investigated if metformin could potentiate the expression of NKG2D, NKp44, and NKp40-activating receptors [13, 17]. Here, the authors chose to assess metformin's effect on the functional phenotype of NK cells by focusing on molecules that have not yet been investigated.

The results revealed that NK cells isolated from metformin-treated mice had a higher expression of tumoricidal molecules, such as NKp46 and FasL (**Figure 2A**, **2B**). Fas ligand (FasL or CD95L or CD178) is a type II membrane-bound molecule and a member of the TNF superfamily [18]. It was thought for a long time that FasL was expressed only by lymphoid or myeloid cells, but additional studies have shown that FasL could also be expressed by nonlymphoid cells and even some tumor cells [19]. By binding to its receptor, FasL causes apoptotic cell death, and FasL-FasR interaction has been shown to be an important part of the elimination of tumor cells [20]. Hashimoto *et al.* were the first to propose that the FasL-FasR interaction is important in NK-cell-mediated tumor surveillance [21]. Since then, it has been demonstrated that increased expression of FasL strongly correlates with NK cell activation and enhanced antitumor activity [22]. A study by Bi *et al*. even suggested that lower expression of FasL is strongly related to diminished NK cell cytotoxic activity and NK cell exhaustion [23]. The current research demonstrated a significant difference in FasL expression on the surface of metformin-treated NK cells versus the control cells. While most of the data were restricted to cytokines that directly demonstrate NK cell activation, the authors found a lower percentage of IL-10$^+$ NK cells (**Figure 2D**) in metformin-treated mice. Given that IL-10, a macrophage-secreted cytokine, is a prominent immunosuppressive agent that suppresses NK cell cytotoxicity [24], it was hypothesized that reduced levels of IL-10 will additionally potentiate NK cell activity and provide an immunostimulating antitumor environment. To further validate the enhanced activation of NK cells, the authors also confirmed metformin's influence on IFN-γ production, as shown in **Figure 2C**. The data of the current study are in line with previous observations that metformin treatment can directly alter the splenic cytokine milieu and induce a stronger immune response [13]. The study by Nishida *et al*. demonstrates that metformin, in synergy with anti-PD-1 Ab treatment, induces IFN-γ production in CD8$^+$ T cells [25].

The TME is a dynamic area that surrounds proliferating tumor cells with the primary goal of promoting tumor growth and survival. TME, besides tumor cells, is composed of a great number of non-tumor cells, including stromal cells, immune cells, endothelial cells, and extracellular matrix (ECM). To support tumor growth, TME components produce vast amounts of extracellular proteins, chemokines, and growth factors. These mediators are well-known for either facilitating the differentiation of various regulatory or immunosuppressive cells or decreasing the proliferation and activation of antitumor immune cells, including NK cells [26]. One of the most frequently detected enzymes in the TME, is IDO. This enzyme is expressed by a variety of cells, including macrophages, dendritic cells, and placental cells, and new data suggest that it can even be induced in tumor cells [27]. The primary function of this enzyme is to convert L-tryptophan, an essential amino acid, to L-kynurenine, a metabolite whose synthesis is known to be increased in various diseases [28, 29]. It has been discovered that kynurenine and its metabolites create an immunosuppressive microenvironment for both innate and adaptive immune system components. Adaptive immunity is suppressed mostly through the inhibition of T cells and stimulation of Treg cells, whereas innate immunity is silenced through skewing macrophage and dendritic cell polarization toward immunosuppressive phenotypes accompanied by the suppression of NK cells [30]. In order to find out whether metformin can indirectly act via inhibition of IDO activity, an *in vitro* experiment was conducted using the pharmacological inhibitor of IDO, 1-MT while administering metformin. Administration of 1-MT alongside metformin to NK cells significantly enhanced the percentage of FasL$^+$, NKp46$^+$, perforin$^+$, IFN$^+$, and IL-17$^+$ NK cells in comparison with the sole metformin administration (**Figure 3A-E**). Based on these findings, it can be concluded that the mechanism of action of metformin is not based on IDO inhibition but on other signaling pathways, yet to be fully explained.

The development of new methods for gene analysis has revealed a number of mechanisms by which epigenetics can and does affect the functional phenotype of immune cells. RNA molecules extracted from introns, regions of DNA that were previously believed to be non-functional, have recently attracted the attention of genetic researchers. One of those molecules is miRNA, small non-coding RNA mole-

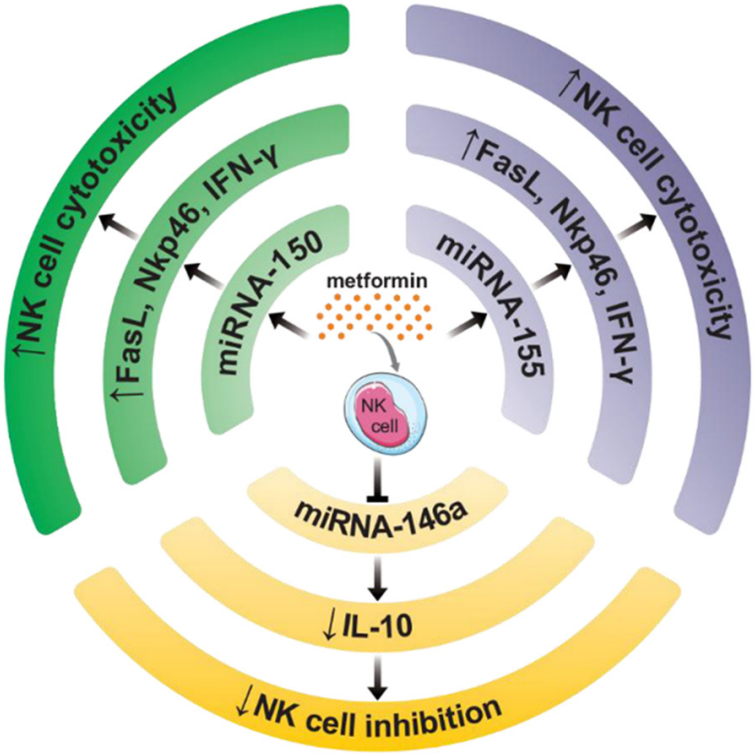

Figure 5. Metformin changes the expression of several microRNAs, and consequently modulates the function of the NK cell. Metformin increases the expression of miRNA-150 (green) and miRNA-155 (blue), which increase the expression of FasL, NKp46, and IFN-γ and thus the cytotoxicity levels of NK cells. Conversely, the level of miRNA-146a (yellow) decreases, which lowers levels of IL-10 and further amplifies the NK cell cytotoxicity.

cules which have a substantial ability to target messenger RNA (mRNA) and thus change gene expression. Considering that influencing miRNAs can alter various cellular processes, it is not surprising that miRNAs are the subject of a vast number of studies [31]. Genetic studies confirmed that miRNAs regulate a broad range of processes in NK cells, some of them in control of NK cell development and maturation (miRNA-150) and some in control of NK cell activation and cytotoxicity (miRNA-155) [14]. There is also emerging evidence that miRNAs could be involved in the suppression of NK cells, and one of the miRNAs that are being investigated in line with that effect is miRNA-146a. Studies by Xu *et al*. and Wang *et al.* not only confirmed that upregulation of miRNA-146a expression decreases NK cell-mediated cytotoxicity and production of IFN-γ, but they also demonstrated that inhibition of miRNA-146a restored levels of IFN-γ [32, 33].

Until now, to the best of our knowledge, no research on the link between metformin administration and NK cell-specific miRNAs has been reported. In this study, the goal was to elucidate the influence metformin administration has on miRNAs profile in NK cells because miRNAs could be promising targets against viral pathogens and tumors. The current results showed that NK cells isolated from metformin-treated WT mice have remarkably altered expression of previously described miRNAs compared with NK cells isolated from untreated, healthy WT mice. It was found that the expression of miRNA-155 and miRNA-150 was significantly higher in mice treated with metformin compared with those in the control group (**Figure 4A**, **4B**). These results are in line with the previous findings that metformin-treated NK cells demonstrate higher cytotoxicity levels. Accordingly, the current study also showed significant downregulation of miRNA-146a expression (**Figure 4C**). In line with the current findings, a recent study revealed significant downregulation of miR-146a, following treatment of metformin in patients with type 2 diabetes [34]. A study by Gou *et al.* showed that metformin, in combination with abelmoschus esculentus powder, attenuates endothelial inflammation via upregulation of miR-146a and miR-155 [35]. Taking into consideration that current research assigns miRNA-146a a mostly immunosuppressive role, downregulation of miRNA-146a could be an additional factor by which metformin potentiates the NK cell cytotoxic phenotype. The upregulation of miRNA-155 and miRNA-150 as well as the downregulation of miRNA-146a expression and their roles in modulating NK cell activity are schematically shown in **Figure 5**. The current findings have the potential to pave the way for finding novel approach-

es for amplifying NK cell-based immune responses.

Conclusion

Cumulatively, it can be concluded that metformin treatment increases NK cell cytotoxicity and alters the functional phenotype of NK cells. Given the importance of NK cells in innate anti-tumor and anti-viral immunity, the current findings imply that by potentiating the NK cell cytotoxic phenotype, metformin can play an important role in improving the elimination of transformed and infected cells. Additionally, the simultaneous administration of metformin and an inhibitor of the IDO enzyme demonstrated an independent mechanism of action of metformin apart from IDO inhibition. Finally, this study found that metformin administration modulates the expression of miRNAs in NK cells, which suggests that metformin-induced NK cell activation is, at least partly, accomplished through the alteration of protein synthesis on a post-transcriptional level. The current findings could be helpful for future studies regarding NK cell-based immunotherapies.

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Disclosure of conflict of interest

None.

Address correspondence to: Milena M Jurisevic, Department of Pharmacy, Faculty of Medical Sciences, University of Kragujevac, Svetozara Markovica 69, Kragujevac 34000, Serbia. Tel: +381-34-306-800; E-mail: milena.jurisevic@medf.kg.ac.rs

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