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INFLUENCE OF *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* BB-12 AND *LACTOBACILLUS RHAMNOSUS* GG ON POLARIZATION OF TUMOR-ASSOCIATED MACROPHAGES

Background. The microbiota has a significant impact on the host's immune system. However, the influence of the microbiome is heavily dependent on species, strain, and context. **The aim** of this study was to evaluate the influence of *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) and *Lactobacillus rhamnosus* GG (LGG) supplementation on the polarization of tumor-associated macrophages (TAMs) in mice with Ehrlich carcinoma. **Materials and Methods.** Female Balb/c mice bearing solid Ehrlich carcinoma were administered via gavage with BB-12, LGG, or 0.9% NaCl. On days 14, 21, and 28 of tumor growth, macrophages from tumor tissue were isolated and subjected to functional analysis. The nitric oxide (NO) production was measured using the standard Griess reaction. The arginase activity was determined based on the urea measurements. The reactive oxygen species (ROS) production was checked using flow cytometry. The cytotoxic activity was estimated by an MTT assay. **Results.** The application of different probiotic bacteria elicited different TAM polarization states. The TAMs in the group supplemented with LGG demonstrated M1 polarization with low arginase activity but high production of NO and ROS, as well as cytotoxic activity toward Ehrlich carcinoma cells. The TAMs of BB-12-treated mice exhibited M2 (supposedly M2b) polarization, characterized by high arginase activity alongside the preserved cytotoxic activity toward Ehrlich carcinoma cells in vitro. **Conclusion.** The results indicate that the consumption of two probiotics, BB-12 and LGG, affects the polarization of TAMs in distantly located experimental tumors.

Keywords: *Bifidobacterium animalis* subsp. *lactis* BB-12; *Lactobacillus rhamnosus* GG, Ehrlich carcinoma, tumor-associated macrophages, polarization.

The role of the microbiota in the host's health and diseases, including cancer, is well recognized [1]. Nowadays, microbiota is considered both as a target and as a tool in cancer treatment. The easiest way to manipulate with microbiota is through probiotics supplementation. *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) is one of the most studied probiotic strains from the *Bifidobacterium* genus [2]. It was extensively studied in both preclinical and clinical trials and has been documented in over 400 scientific publications [3]. Similarly, *Lactobacillus rhamnosus* GG (LGG) is considered one

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of the best clinically assessed and most commercialized probiotic microorganisms, with documented health benefits and immune-modulatory effects [4]. The immune-modulatory effects of BB-12 and LGG are well supported by a robust body of evidence from in vitro, animal, and clinical studies [3]. Both abovementioned strains have been shown to activate the maturation of dendritic cells, upregulating the expression of CD40, CD80, CD86, and HLA-DR molecules, as well as the production of proinflammatory cytokines [5]. Furthermore, several studies have reported increased levels of proinflammatory cytokines, including interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α, following BB-12 or LGG stimulation in vitro [5, 6], indicating the activation of a Th1-biased immune response, which is considered very desirable in the context of cancer treatment. However, several other studies have shown that LGG and especially BB-12 are more likely to activate T-regulatory cells (Treg) and IL-10 release, which calls into question their potential impact on cancer development. It is well known that the effects of probiotic application vary greatly depending on the species and even the strain. Furthermore, it appears that the results can be disease-specific [7]. Therefore, the practical utility and effects of individual strains in certain conditions should be thoroughly elucidated.

Macrophages are among the immune cells capable of interacting with bacteria in the intestinal lumen through the extension of transepithelial dendrites. Moreover, macrophages are a highly diverse, multifunctional, and widespread population of immune cells. They represent majority of immune cells in the lamina propria of the intestine and are able to interact with microbiota, enterocytes, and other immune cells. Macrophages are also potent immunoregulatory cells that can initiate and terminate immune responses, depending on the surrounding signals. Furthermore, signals generated by macrophages not only influence the activity of other immune cells and the outcome of the immune response but also play a key role in shaping the tumor microenvironment, predominantly paving the way for cancer progression and dissemination. On the other hand, properly activated macrophages exhibit potent anticancer activity. The effect of some bacteria on macrophages in vitro has been studied in considerable detail. There are data on the local effects of microbiota on the intestinal macrophages. However, the data on the effect of microbiota on tumor-associated macrophages of distant tumors is insufficient. So, the aim of this study was to evaluate the influence of *Bifidobacterium animalis* subsp. *lactis* BB-12 and *Lactobacillus rhamnosus* GG supplementation on the polarization of tumor-associated macrophages in mice bearing solid Ehrlich carcinoma.

Materials and Methods

Mice. The study was carried out on female Balb/c mice of 2.0—2.5 months old, weighing 19—22 g, which were bred at the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (IEPOR) of the National Academy of Sciences of Ukraine. The animals were kept in standard vivarium conditions with natural lighting and provided with food and water ad libitum. The mice were handled and kept in accordance with the standard international rules of biological ethics and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [8].

Bacterial strains. Lyophilized *B. animalis* subsp. *lactis* BB-12 (Lek Pharmaceuticals, Ljubljana, Slovenia) or *L. rhamnosus* GG (Probiotical S.p.A., Switzerland) cells were used as probiotic preparations. The probiotics were administered via gavage at a dose of 7×10^5 CFU/mouse per administration, daily, for 28 days.

Tumor strain. Ehrlich adenocarcinoma cells were obtained from the IEPOR Bank of Cell Lines from Human and Animal Tissues. To induce tumor growth, 5×10^5 Ehrlich carcinoma cells were injected intramuscularly into the right hind leg of experimental animals.

Experimental design. Mice (n = 27; 3 mice per group per one time point) were randomly divided into three groups defined hereinafter as Bifido, Lacto, and tumor control. Starting on day 2 after tumor transplantation, the groups were administered with BB-12, LGG, or 0.9% NaCl, respectively, in the dosage described above. On days 14, 21, and 28 of tumor growth, the mice were sacrificed, the tumor nodules were excised, and tumor-associated macrophages (TAMs) were isolated and subjected to further investigation.

Isolation of TAMs was performed as previously described [9]. The tumor nodules were aseptically

removed, shredded with scissors, and homogenized using a Potter's homogenizer in 199 medium supplemented with 10% bovine serum. Following two consecutive washes with 199 medium at 550 g for 10 min, the cells were resuspended in 199 medium and counted. Then, 1×10^6 cells were placed in 96-well flat-bottomed plates and cultured for 2 h at 37 °C, 5% CO_2, and 100% humidity. The non-adherent cells were removed, and the adherent cells were washed twice with 0.9% NaCl solution before further investigation.

Cytotoxic activity of TAMs (CTA) was determined by an MTT assay [10]. Ehrlich carcinoma cells were used as targets. In brief, 2×10^4 target cells in RPMI-1640 medium supplemented with 10% fetal bovine serum and antibiotics (all reagents from Sigma, USA) were placed in flat-bottomed 96-well plates containing adhered macrophages ($4 \times \times 10^5$ cells/well) and incubated for 18 h in an atmosphere of 100% humidity, 5% CO_2 at 37 °C. Control wells contained either target cells or adherent macrophages. After that, 0.01 mL of MTT solution/well (5 mg/mL, Sigma, USA) was added, and incubation continued for 2 h. Then the plates were centrifuged (550 g for 15 min) and washed twice with 0.9% NaCl solution. Finally, 0.12 mL of KOH (2 mol/L) and 0.14 mL of DMSO (50% solution) were added to each well. The optical density was measured at $\lambda = 545$ nm vs $\lambda = 630$ nm with a micro-ELISA reader (StatFax-2100, USA). Each sample was analyzed in triplicate.

NO production was measured by the standard Griess reaction [11]. In brief, cell suspensions ($2 \times \times 10^6$ cells/well) were placed in 96-well flat-bottom tissue culture plates containing 200 μL of medium and cultured for 24 h. At the end of the incubation period, supernatants were collected, and NO production was quantified by the accumulation of nitrite (as a stable metabolite of NO) by the Griess reaction. An aliquot of culture supernatant (100 μL) was mixed with an equal volume of the Griess reagent (Acros Organics, Belgium) and incubated for 1 h at room temperature in the dark. The reaction products were colorimetrically quantified at $\lambda = 550$ nm. The conversion of absorbance to micromolar concentrations of NO was deduced from a standard curve using a known concentration of $NaNO_2$ prepared in the same medium and treated with Griess reagent under the same conditions. Amount of NO was expressed in μM NO_2^- per 10^6 cells.

Arginase activity was determined by measuring the rate of urea formation [11, 12]. Macrophages were lysed by double freezing and melting. Then 50 μL of 50 mM Tris-HCl (pH 7.4) and 10 μL of 50 mM $MnCl_2$ were added to each sample. The samples were then heated at 56 °C for 10 min, and 100 μL of 0.5 M L-arginine (pH 9.7) was added and further incubated for 30 min at 37 °C. The reaction was stopped with 800 μL of acid mixture (1:3:7, 96% H_2SO_4 : 85% H_3PO_4 : H_2O). Then 40 μL of α-isonitrosopropriophenone (Sigma—Aldrich, USA) was added to the solution, heated for 30 min at 95 °C, and incubated for 30 min at 4 °C. Urea concentration was measured spectrophotometrically at $\lambda = 550$ nm. The values of optical density were converted to micrograms based on the calibration curve of standard urea solutions. The data were presented in units per 10^6 cells. One unit of arginase activity corresponds to the amount of enzyme that hydrolyzes 1 μmol of arginine per 1 min.

Reactive oxygen species production. In brief, 0.5×10^6 TAMs in 0.5 mL of 15 μM of a 2′,7′-dichlorofluorescein diacetate (Sigma—Aldrich, USA) solution were placed in microtubes and incubated for 40 min at 37 °C. After staining, the cells were washed in 1.5 mL of phosphate-buffer saline, centrifuged at 400 g for 5 min, resuspended in 0.5 mL of phosphate-buffered saline, and analyzed immediately on a flow cytometer (DxFlex, Beckman Coulter, USA) using the FITC channel (525/40 nm BP light filter). The mean fluorescence value (mean FITC-A) was calculated from the fluorescence distribution histograms. The fluorescence of cells not treated with 2′,7′-dichlorofluorescein diacetate was measured and subtracted from the average FITC-A fluorescence intensity. At least 10,000 events were analyzed in each sample. The obtained data were analyzed using the CytExpert for DxFlex program [13].

Statistical processing of the results was performed using standard methods of variational statistics with GraphPad Prism 8.0.1 (Graphpad Software Inc., USA). The significance of the differences between the groups was assessed using Student's *t*-test. The difference was considered significant at $p < 0.05$.

Results and Discussion

We have studied TAMs isolated from Ehrlich carcinoma of mice treated with BB-12 or LGG adminis-

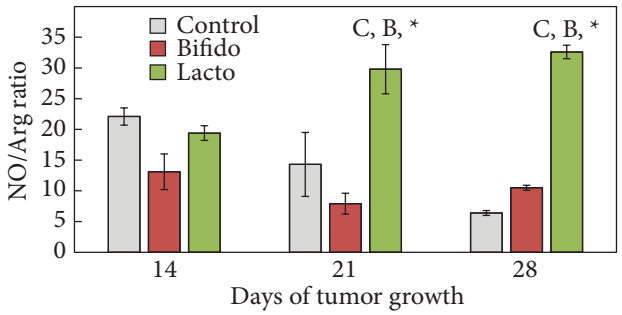

Fig. 1. The NO/Arg ratio in TAMs of mice administered with BB-12 or LGG after transplantation with Ehrlich carcinoma cells. $^{C}p \le 0.05$ compared to the control group; $^{B}p \le 0.05$ compared to the Bifido group; $^{*}p \le 0.05$ compared to the data on day 14 in the group

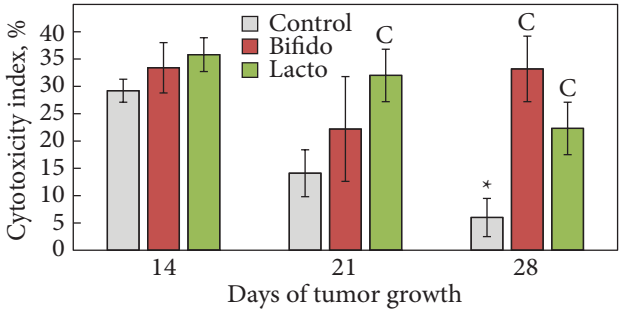

Fig. 2. The cytotoxic activity of TAMs isolated from mice administered with BB-12 or LGG after transplantation with Ehrlich carcinoma cells. $^{C}p \le 0.05$ compared to the control group; $^{*}p \le 0.05$ compared to the data on day 14 in the group

tered through gavage. The study design is described in detail in the Materials and Methods section.

First, the NO production and arginase activity in TAMs were studied, since differently polarized macrophages utilize different ways to metabolize arginine, and these ways are concurrent and competitive [14]. The ratio of NO level to arginase activity more clearly depicts which arginine metabolism pathway prevails. As illustrated in Fig. 1, during tumor growth in the control group, the NO/Arg ratio declined due to a slight decrease in NO production alongside an increase in arginase activity (see Table 1), indicating that TAMs in the control mice had acquired M2 polarization. Predominant M2 polarization is characteristic of TAMs isolated from tumors of different localizations in humans and rodents and is often associated with poor prognosis [15].

Table 1. **The level of NO production and arginase activity in TAMs after administration of BB-12 or LGG to mice with transplanted Ehrlich carcinoma**

Group of animals	Days of tumor growth		
	14	21	28
NO production			
Control	24.9 ± 2.1	24.4 ± 2.8	16.6 ± 2.8*
Bifido	25.1 ± 2.5	19.1 ± 4.1	26.3 ± 1.0 ^{C}
Lacto	30.0 ± 1.9	22.6 ± 4.7	31.0 ± 1.1 $^{C, B}$
Arginase activity			
Control	1.13 ± 0.06	1.80 ± 0.40	2.18 ± 0.64
Bifido	2.34 ± 0.55	2.40 ± 0.30	2.50 ± 0.07
Lacto	1.55 ± 0.18	0.80 ± 0.02$^{C, B}$	0.95 ± 0.07^{B}

Notes: $^{C}p \le 0.05$ compared to the control group; $^{B}p \le 0.05$ compared to the Bifido group; $^{*}p \le 0.05$ compared to the data on day 14 in the group.

On the contrary, in mice receiving LGG, the NO/Arg ratio increased during the experiment, clearly indicating an M1 polarization state in TAMs. In this group of mice, the NO level (which belongs to the cytotoxic machinery of macrophages) remained high throughout the experiment, exceeding that of the control and Bifido groups on day 28 of tumor growth. Conversely, arginase activity was lower than in the control and Bifido groups (see Table 1).

The NO/Arg ratio in mice receiving BB-12 indicated a state of polarization closely resembling the M2 type, characterized by high arginase activity. However, the level of NO production remained stable throughout the experiment.

The loss of cytotoxic activity by M2 macrophages is another feature that allows us to distinguish between M1- and M2-polarized macrophages [16]. As illustrated in Fig. 2, TAMs in control tumor-bearing mice gradually lost the ability to kill Ehrlich carcinoma cells in vitro. In this group, cytotoxic activity decreased from 29.2% on day 14 to 6.0% on day 28 of tumor growth ($p \le 0.05$ compared to both treated groups and the data on day 14 in the group), which evidenced M2 polarization of TAMs. In contrast, the cytotoxic activity of TAMs in the treated groups did not change significantly throughout the experiment and remained higher than in the control group on day 28 of tumor growth. There were no significant differences between the Bifido and Lacto groups.

Macrophages can perform cytotoxic activity via different mechanisms, and the production of ROS is one of them [15]. In our study, ROS production exhibited a very similar pattern in all groups (Fig. 3) with an increase in the production reach-

ing its peak on day 21 of tumor growth, followed by a sharp decrease on day 28. TAMs in the Lacto group demonstrated the highest rates of ROS production compared to both the control and Bifido groups at the first two observation points ($p \le 0.05$ for both groups on days 14 and 21 of tumor growth). In the Bifido group, ROS production did not differ from the control group except on day 14 of tumor growth.

Two probiotic bacteria under study elicited clearly different polarization states of TAMs. In the group supplemented with LGG, TAMs demonstrated classical M1 polarization with low arginase activity and high production of NO, ROS, and high cytotoxic activity. These results are consistent with literature data, demonstrating that, although LGG can stimulate both Th1 and Th2 cytokines, the IFN-γ/IL-4 ratio indicates a pro-Th1 bias in the effects of LGG observed in vivo in various immunocompetent organs [17] and in different macrophage models in vitro [18, 19].

On the contrary, TAMs from mice treated with BB-12 exhibited M2 polarization, albeit distinct from the typical M2 polarization observed in TAMs from the control group. The main difference lies in the Bifido group TAMs' ability to eliminate Ehrlich carcinoma cells in vitro. Since TAMs' cytotoxic activity in this group did not correlate with NO or ROS production (unpublished results), it may be mediated via TNF-α production. Although the TNF-α level was not analyzed in this experiment, the ability of BB-12 to induce its production has been well demonstrated by other researchers. For instance, the faecal precipitates obtained during the consumption of BB-12-enriched yoghurt induced the high TNF-α response in J774.1 cells without affecting IL-1α and IL-10 production [20]. Similar results were obtained in another study, where in vitro stimulation of dendritic cells derived from human monocytes with BB-12 resulted in the induction of IL-12 and TNF-α to a high degree and IL-10 to a low degree. In the same settings, peripheral blood mononuclear cells responded with high levels of IL-10, IFN-γ, and TNF-α [21]. BB-12 was found to be one of the most potent inducers of TNFα and IFN-γ production compared to the other 18 tested bacteria [22]. Moreover, BB-12 was found to induce the production of both pro- and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-10, IL-12, and IFN-γ) by monocyte-derived

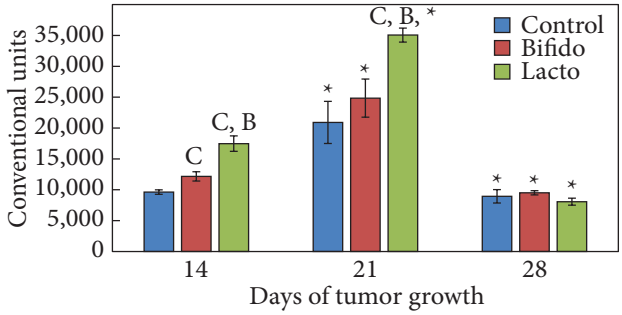

Fig. 3. The production of ROS by TAMs isolated from mice administered with BB-12 or LGG after transplantation with Ehrlich carcinoma cells. ^{C} $p \le 0.05$ compared to the control group; ^{B} $p \le 0.05$ compared to the Bifido group; * $p \le 0.05$ compared to the previous observation point in the group

dendritic cells [5]. In this study, LGG emerged as a poor stimulator of monocyte-derived dendritic cells, failing to increase cytokine production compared to unstimulated cells. This corresponds to our results obtained on intact mice administered with LGG [23], pointing to the dependence of probiotic's effects on the state of the host's immune system before stimulation. Thus, it appears that BB-12 is capable of eliciting TNF-α production, at least in vitro. Since a high arginase activity and TNF-α production are characteristic of M2b polarized macrophages [15], this allows us to speculate that supplementation with BB-12 leads to TAMs polarization to the M2b subtype in this group of mice.

The most prominent characteristic of the M2b polarization is the production of both immunosuppressive (IL-10) and immunostimulating (TNF-α, IL-1β, IL-6, CXCL1, CXCL2, and CXCL3) cytokines [15]. Furthermore, unlike M2a macrophages, M2b macrophages rely more on anaerobic glycolysis than on the TCA cycle and OXPHOS for ATP production [15], thus resembling the metabolic pattern of M1 polarized cells. At first glance, M2b macrophages appear to be less pro-tumorigenic than the other types of M2 macrophages. Their functional state looks like intermediate between the anticancer M1 type and the pro-cancerous M2 type. From this point of view, M2b polarization induced by BB-12 can be treated in at least two different ways. On the one hand, BB-12 supplementation appears to protect TAMs from early M2 polarization, enabling them to maintain their cytotoxic and anticancer activity for a longer period. On the other hand, the substances they produce may be involved in cancer progression. At least, TNF-α can

possess both immunostimulating and cytotoxic as well as immunosuppressive and proangiogenic activities [24]. Moreover, a "primary marker" of M2b macrophages is considered IL-10 [24], a potent anti-inflammatory and immunosuppressive cytokine. Therefore, the effects of BB-12 on TAMs require thorough elucidation.

Supposedly, the difference in TAM polarization observed in our experiment is due to the disparity in metabolic signals produced by BB-12 and LGG. It appears that the products of tryptophan metabolism and short-chain fatty acids (SCFAs) are the main signals produced by these probiotics that could influence the activity of TAMs [25]. The mechanisms of interaction of tryptophan metabolites with macrophages have been studied using different experimental models [26—28], as well as pro-inflammatory and anti-inflammatory influence of metabolites and components of bifidobacteria and lactobacilli [29—32]. However, it remains unclear yet, which probiotic components play a leading role in TAM polarization.

Thus, the obtained results indicated that consumption of two probiotics, BB-12 and LGG, affected polarization of TAMs in distantly located experimental tumors. In general, these data are coherent with the effects of these bacteria studied in different macrophage models described so far. The mechanisms of such effects revealed in our experiment require further investigation.

Acknowledgment

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ВПЛИВ *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* BB-12
ТА *LACTOBACILLUS RHAMNOSUS* GG НА ПОЛЯРИЗАЦІЮ
ПУХЛИНОАСОЦІЙОВАНИХ МАКРОФАГІВ

Мікробіота має значний вплив на імунну систему організму господаря. Однак вплив мікробіому суттєво залежить від виду та штаму мікроорганізму та контексту застосування. **Метою** дослідження є оцінка впливу *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) та *Lactobacillus rhamnosus* GG (LGG) на поляризацію пухлиноасоційованих макрофагів у мишей з солідною карциномою Ерліха. **Матеріали та методи.** Самкам мишей Balb/c з перещепленою солідною карциномою Ерліха вводили через зонд BB-12, LGG або 0,9% NaCl. На 14, 21 і 28 день росту з пухлинної тканини виділяли макрофаги та досліджували їхній функціональний стан. Утворення оксиду азоту (NO) вимірювали за допомогою стандартної реакції Грісса. Активність аргінази визначали на основі вимірювань кількості сечовини. Вироблення активних форм кисню (ROS) перевіряли за допомогою проточної цитометрії. Цитотоксичну активність оцінювали за допомогою MTT-тесту. **Результати.** Застосування різних бактерій викликало різні стани поляризації пухлиноасоційованих макрофагів. У групі мишей, що отримували LGG, макрофаги були поляризовані за M1 типом, що характеризувався низькою аргіназною активністю, але високим виробленням NO і ROS, а також цитотоксичною активністю щодо клітин карциноми Ерліха. Пухлиноасоційовані макрофаги мишей, які отримували BB-12, демонстрували поляризацію M2 (ймовірно, M2b), що характеризувалася високою аргіназною активністю поряд із збереженою цитотоксичною активністю щодо клітин карциноми Ерліха in vitro. **Висновок.** Пероральне застосування пробіотиків впливає на поляризацію пухлиноасоційованих макрофагів. Імовірно, що BB-12 і LGG мають різний терапевтичний потенціал.

Ключові слова: *Bifidobacterium animalis* subsp. *lactis* BB-12; *Lactobacillus rhamnosus* GG, карцинома Ерліха, пухлиноасоційовані макрофаги, поляризація