МОДУЛЯЦИЯ *LACTOBACILLUS JOHNSONII* ДЕНДРИТНЫХ КЛЕТОК КОСТНО-МОЗГОВОГО ПРОИСХОЖДЕНИЯ У МЫШЕЙ, НЕСУЩИХ НУЛЕВУЮ МУТАЦИЮ В ГЕНЕ MUC2

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Резюме. В кишечнике обитает более триллиона бактерий, которые нарабатывают до 60% метаболитов хозяина. Поэтому кишечный микробиом играет важную роль в регуляции иммунного ответа хозяина. В настоящее время получено много данных не только о влиянии пробиотических штаммов бактерий на развитие патологий, связанных с дисбиозами и нарушениями метаболического обмена, но и о важной роли бактерий в лечении воспаления, онкологии и нейродегенеративных нарушений. Изучение влияния пробиотических штаммов на лечение различных патологий проводят на экспериментальных животных с нарушением работы генов, приводящих к данной патологии. Для понимания механизма прямого действия пробиотиков используют клетки здоровых мышей или перевиваемые культуры клеток в *in vitro* исследованиях. Однако проводится довольно мало исследований эффекта пробиотических штаммов на клетки, полученные от животных с патологией. В данной работе мы исследовали фенотип дендритных клеток (ДК) мышей Muc2$^{-/-}$ с признаками хронического воспаления кишечника и оценивали какой эффект *L. johnsonii* оказывает на функциональную активность ДК. Известно, что ключевыми признаками всех экспериментальных моделей воспалительных заболеваний кишечника (ВЗК) являются истончение защитного муцинового слоя в кишечнике и изменение кишечной микрофлоры. В нашей работе мы сравнили эффективность созревания и активации ДК, полученных из костного мозга мышей с мутацией в гене Muc2, и ДК, полученных от здоровых мышей линии C57BL/6 свободных от специфических видовых патогенов. А также оценили экспрессию костимуляторных молекул, пролиферативный индекс и возможность активации Т-регуляторного ответа ДК, которые которые были стимулированы пробиотическими бактериями *L. johnsonii*.

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Методом проточной цитометрии оценивали экспрессию клеточных маркеров дендритных и Т-клеток с помощью антител к вне- и внутриклеточным белкам. Пролиферативную активность спленоцитов оценивали с помощью WST теста.

В работе было показано, что дендритные клетки, полученные из костного мозга мышей с нулевой мутацией гена Muc2 имели незрелый фенотип по основным маркерам ДК. Дендритные клетки Muc2$^{-/-}$ мышей не могли эффективно стимулировать пролиферацию аллогенных и сингенных Т-клеток. Пробиотический штамм *L. johnsonii* был способен не только стимулировать созревание дендритных клеток, полученных от Muc2$^{-/-}$мышей, но и повышать экспрессию FoxP3 на CD25^{+} Т-клетках, которые ко-культивировали с дендритными клетками.

Таким образом, мы полагаем, что данный пробиотический штамм бактерий может снижать признаки воспаления и уменьшать проявление патологических нарушений у мышей с признаками развития ВЗК.

Ключевые слова: дендритные клетки, Lactobacillus, Т-регуляторные клетки, Muc2, воспаление, кишечник

LACTOBACILLUS JOHNSONII MODULATION OF BONE MARROW-DERIVED DENDRITIC CELLS GENERATED FROM MICE WITH NULL MUTATION OF THE MUC2 GENE

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Abstract. The gut is inhabited by a trillion bacteria that produce up to 60% of the host's metabolites. The gut microbiome plays an important role in regulating host immune function. A lot of research concerned the effect of probiotic on the pathologies associated not only with dysbiosis and metabolic disorders, but there is breakthrough in the treatment of inflammation, oncology and neurodegenerative disorders. Animals with mutation of the genes leading to pathology used to assay probiotic effect. To understand direct action of probiotics, cells derived from control mice or cell culture of tumor genesis *in vitro* studies are used. However, there is little research of the probiotic effect on cells derived from mice with pathology. In this study, we assessed the phenotypes of dendric cells derived from Muc2$^{-/-}$ mice with chronic inflammation and assessed the effect of *L. johnsonii* on the dendric cells. It is known that the key features of IBD models are thinning of mucin layer and changes in the intestinal microbiome. We compared the efficiency of maturation and activation of dendric cells derived from the bone marrow of Muc2$^{-/-}$ mice and dendric cells obtained from healthy C57BL/6 mice free from specific species pathogens. We evaluated the expression of co-stimulatory molecules, the proliferative index, and the ability to trigger the T regulatory response of dendric cells, which were stimulated with the probiotic *L. johnsonii*.

Markers of dendritic and T cells were assessed by flow cytometry using antibodies to extra- and intracellular proteins. The proliferative activity of splenocytes was assessed using the WST test.

It was shown that dendric cells derived from the Muc2$^{-/-}$ had an immature phenotype. Dendric cells of Muc2$^{-/-}$ mice could not effectively stimulate the proliferation of allogeneic and syngeneic T cells. *L. johnsonii* was able not only to stimulate the maturation of dendric cells derived from Muc2$^{-/-}$ mice, but also to increase the expression of FoxP3 on CD25^{+} T cell that were co-cultured with DCs.

Thus, we believe that this probiotic bacterium can reduce signs of inflammation and reduce pathological processes in animals of an experimental model of IBD *in vivo*.

Keywords: dendritic cells, Lactobacillus, T regulator cells, Muc2 gene, inflammation, intestine

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Introduction

It is widely accepted that the gut microbiota plays an important role in multiple functions of the host. The human gut is inhabited by a trillion of bacteria, represented by 10^4 various species. The number of intestinal bacteria genes by 150 times exceeds the one of human genes, and its metabolites account for 60% of all blood metabolites in human body [3]. The microbiome actively participates in regulation of the host homeostasis, has a systemic effect on the populations of cells of innate and adaptive immunity, and provides the supply of certain essential nutritional components both to the host, as well as to its symbionts in the microbial community. In a healthy state, these relationships are well balanced, but disruption of this balance can contribute to various conditions, including inflammatory bowel disease (IBD) and atopy [7].

An association between the gut microbiota misfunctioning and the risk or presence of specific human diseases has been published recently [1]. In order to restore the balance, food supplements containing some beneficial bacteria species, such as of *Lactobacillus* and *Bifidobacterium* genera, are widely used as probiotics. For example, *Lactobacillus johnsonii* is one of the lactobacilli species, which is used as a probiotic drug. It alleviates the symptoms of various diseases, such as type I diabetes [8]. However, the mechanisms by which these individual probiotics modulate host response and immunity are diverse and often strain-specific. It has previously been shown that some lactic acid bacteria can have a direct effect on DCs, monocytes and tissue macrophages and, to a lesser extent, on B cells [6].

Currently, studies of the effect of probiotic drugs on the immune system are carried out on healthy mice with SPF status, but probiotics are most widely used to correct the composition of the microbiota in IBD patients. It is known that key signatures of all IBD models are the thinning of the protective mucin layer in the intestine and changes in the intestinal microflora [2]. One of the IBD model are Muc2-/- mice carrying a mutation in the Muc2 gene, which leads to disruption of the protective mucin layer in the small and large intestines. In our work, we compared the efficiency of maturation and activation of DCs obtained from the bone marrow of mice with a mutation in the Muc2 gene and DCs obtained from healthy C57BL/6 mice with SPF status. We also evaluated the expression of co-stimulatory molecules, the proliferative index, and the ability to trigger the T regulatory response of DCs, which were stimulated with the probiotic *L. johnsonii*.

Materials and methods

Generation of bone-marrow-derived dendritic cells

Bone marrow-derived DCs were generated from bone marrow cells isolated from the femur and tibia of female 7-8-week-old C57BL/6 and Muc2-/- mice by flushing out the cells with PBS using a sterile syringe. The harvested cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 50 μg/mL gentamicin, 100 μg/mL penicillin-streptomycin (Gibco, China) (complete culture medium). Then, the cells were pooled and plated on 25 cm^2 flasks at a density of 1×10^6 cell/mL. Subsequently, growth factors GM-CSF (BioLegend, USA) and IL-4 (BioLegend, USA) were added into the medium to a final concentration of 20 ng/mL. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was replaced on day 3 with fresh growth factors. On day 7, the cells were gently collected by cell scraper from the plate. The cells were plated into 24-well plates for priming by *L. johnsonii* in proportion 1:10 (MOI 0.1) for 2 days in duplicates.

Flow cytometry analysis of DCs phenotype

The cells were collected after incubation, washed with PBS, 1×10^6 cells were taken into the analysis. Dead cells were stained with Zombie Violet dye (BioLegend, USA) diluted in PBS (1:400). Then, cells were labeled with monoclonal antibodies conjugated with fluorochromes to surface antigens (BioLegend, USA): anti-CD11c-APC, anti-CD80-FITC, anti-CD86-APC/Cy7, anti-CD83-PE/Cy7, anti-I-Ab-PerCP/Cy5.5. Flow cytometry was performed using FACS CantoII flow cytometer (Becton Dickinson, USA), and the data were analyzed with the BD FACSDiva 6.0 software.

Mixed lymphocyte reaction

The effect of DCs, primed by *L. johnsonii*, on the proliferation of syngeneic and allogeneic splenocytes was assessed by WST-1 method. We obtained splenocyte suspensions from the spleens of C57BL/6, $Muc2^{-/-}$ and BALB/c mice. The immature DCs were collected on day 7 and plated on 96-well plate in a concentration 2×10^5 cells/mL in RPMI-1640 complete culture medium, supplemented IL-4 (20 ng/mL), GM-CSF (20 ng/mL). Then, bacteria were added at MOI 0.1, and DCs were incubated for 2 days at 37 °C in a humidified atmosphere containing 5% CO_2.

After culturing, DCs were treated with 50 ng/mL mitomycin C (Roche Diagnostics GmbH, Germany) for 1h. Then, DCs were washed with RPMI-1640 complete culture medium. 2×10^5 splenocytes in RPMI-1640 complete culture medium were added to DCs. The ratio of DCs to splenocytes was 1:10. Splenocytes without DCs were used as a control. To measure the proliferation of syngeneic cells we used splenocytes from C57BL/6 and Muc2$^{-/-}$ respectively, to measure the proliferation of allogeneic cells – splenocytes from BALB/c for C57BL/6 and Muc2$^{-/-}$ DCs. Cells co-cultured in 96-well plate at 37 °C for 72 h in a humidified atmosphere containing of 5% CO_2. Next, WST-1 solution (Roche Diagnostics GmbH, Germany) was added to each well for an additional 6 h incubation. The optical density was measured at 450 nm with reference at 620 nm by reader Infinite® F50 (Tecan, Switzerland).

***In vitro* generation of Treg from naive CD4$^+$T cells**

Naive CD4$^+$CD44$^-$T cells were isolated from the spleens of C57BL/6 and Muc2$^{-/-}$ mice using a naive CD4$^+$T cells isolation kit (Miltenyi Biotec Inc., Germany). The immature DCs (2×10^4 cells/well) cultured with or without *L. johnsonii* at MOI 0.1 in RPMI-1640 complete culture medium, supplemented IL-4 (20 ng/mL), GM-CSF (20 ng/mL) for 2 days at 37 °C containing of 5% CO_2. Then, culture medium was gently discarded from the wells, and syngeneic purified naive CD4$^+$T cells (2×10^6 cells/mL) added to DCs for 72 h in a culture medium containing 100 ME/mL of recombinant human IL-2. Brefeldin A (5 μg/mL; BioLegend, USA) was added to the wells 20 hours before cell harvested to evaluate IL-10$^+$Tregs.

Flow cytometry analysis of Treg

After co-culturing, cells were collected, washed twice with PBS, then cells were stained with monoclonal antibodies against mouse surface (BioLegend, USA) CD3-FITC, CD4-APC, CD25-PerCP and intracellular markers (BioLegend, USA) FoxP3-PE, IL-10-APC/Cy7. For intracellular staining, cells were fixed and permeabilized using True-Nuclear™ Transcription Factor Buffer Set (BioLegend, USA). Subsequent analyses were performed by FACS CantoII (Becton Dickinson, USA).

Statistical distribution had not normally distributed data; the analysis of the data was performed by non-parametric two-way PERMANOVA test with post-hoc Bonferroni test. All data are presented as mean ± standard diversity.

Results and discussion

To analyze the phenotype of bone-marrow DCs from mice with intestinal inflammation and investigate the effect of co-culture DCs with probiotic bacteria we measured expression of co-stimulated molecules of DCs by flow cytometry. Percentage of CD80$^+$DCs generated from born-marrow of Muc2$^{-/-}$ mice were significantly lower compared to C57BL/6 mice DCs (Two-way PERMANOVA test $F(1.17) = 39.33$ $p < 0.0001$) (Figure 1A). At the same time, DCs priming by *L. johnsonii* and interaction of two factors (genotypes and bacteria primering) did not affect the percentage of CD80$^+$DC (Two-way PERMANOVA test $F(1.17) = 3.59$ ND and $F(1.17) = 0.99$ ND respectively). Culturing DC with *L. johnsonii* significant increased percentage of CD83$^+$DCs (Figure 1B) (Two-way PERMANOVA test $F(1.17) = 8.60$ $p = 0.01$). However, percentage of CD83$^+$DC did not depend on mice genotype and interaction two factors (Two-way PERMANOVA test $F(1.17) = 0.53$ ND and $F(1.17) = 1.35$ ND respectively).

As for another DC co-stimulatory molecule, CD86$^+$, it depended both on mice genotype and DCs priming by *L. johnsonii* (Two-way PERMANOVA test $F(1.17) = 22.55$ $p < 0.001$ and $F(1.17) = 26.84$ $p < 0.001$). Thus, higher level of CD86$^+$DCs were observed in cell obtained from C57BL/6 mice than from Muc2$^{-/-}$ mice and it was independent on culturing with or without *L. johnsonii*. DCs priming by bacteria led to enhanced CD86 expression (Figure 1C). Percentage of MHC I-A^b DCs depended on presence of *L. johnsonii* in cell culture and was similar between Muc2$^{-/-}$ and C57BL/6 mice as well as interaction of two factors (Two-way PERMANOVA test $F(1.17) = 11.62$ $p < 0.01$ and $F(1.17) = 0.36$ ND and $F(1.17) = 0.05$ ND respectively). Thus, addition of bacteria increased percentage of MHC I-A^b DCs of both genotypes of mice. After priming by *L. johnsonii* percentage of CD86$^+$DC became higher in C57BL/6 mice compared to Muc2$^{-/-}$ mice (Figure 1D).

To investigate the functional capacity of DCs treated with *L. johnosonii* to stimulate allogeneic and syngeneic T cell proliferation we used WST assay. Proliferation index depended on mice genotype but not on treatment of probiotic and interaction of two factors (Two-way PERMANOVA test $F(1.23) = 35.08$ $p < 0.0001$ and $F(1.23) = 0.46$ ND and $F(1.23) = 0.41$ ND respectively). Thus, T cell proliferation activity of DCs generated from C57BL/6 mice was higher than DCs from Muc2$^{-/-}$ mice and it was independent of priming by bacteria. However, only proliferation index of DCs from C57BL/6 mice was higher than proliferation activity of intact BALB/c splenocytes (Figure 2A). There was no effect of DCs from Muc2$^{-/-}$ mice to stimulate allogenic T cell proliferation.

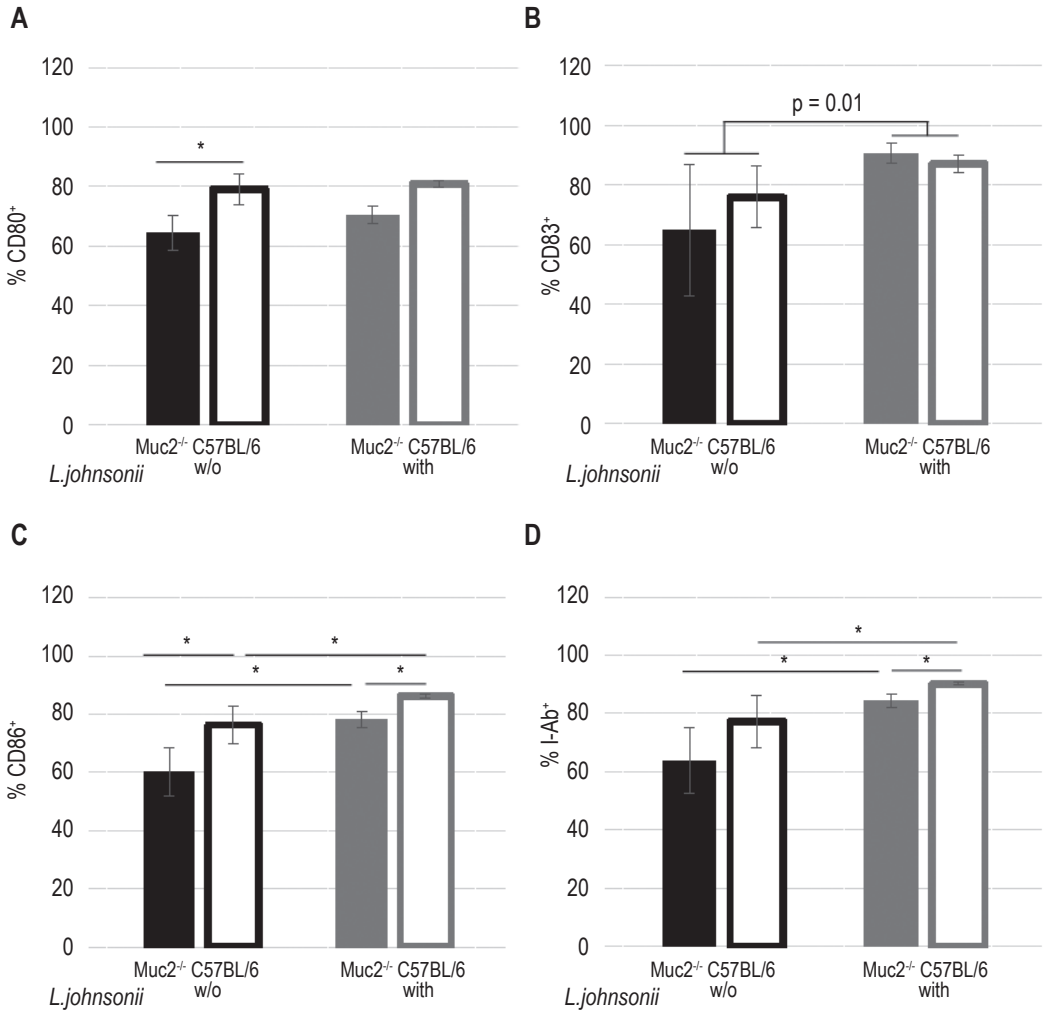

Figure 1. Bone-marrow derived dendric cells from C57BL/6 and Muc2$^{-/-}$ cultured with and without *L. johnsonii*

Note. (A) Percentage of CD80^{+}DCs gated on CD11b^{+} cells. (B) Percentage of CD83^{+}DCs gated on CD11b^{+} cells. (C) Percentage of CD86^{+}DCs gated on CD11b^{+} cells. (D) Percentage of MHC I-Ab^{+} DCs gated on CD11b^{+} cells. *, $p < 0.05$ Post-hoc Bonferroni test; $p < 0.01$ Effect primed with *L. johnsonii* Two-way PERMANOVA test.

On the contrary, syngeneic proliferation index was dependent on probiotic treatment and interaction of two factors but not on mice genotype (Two-way PERMANOVA test $F(1.23) = 6.49$ $p < 0.05$ and $F(1.23) = 33.88$ $p < 0.001$ and $F(1.23) = 0.90$ ND respectively). Immature DC from C57BL/6 mice demonstrate more proliferation activity than DC of Muc2$^{-/-}$ mice but treatment with probiotic reversed it and significantly decreased the index. Cell proliferation stimulated by mature and immature DCs was compared to splenocytes proliferation in steady state without addition of DCs (Figure 2B).

To understand whether DCs are capable of stimulating T regulatory response, we co-cultured splenocytes with DCs and assessed the percentage of CD25^{+}FoxP3^{+} and IL-10^{+} cells. High impact of culturing DCs with *L. johnsonii* was detected on percentage of CD25^{+}FoxP3^{+} cells only (Two-way PERMANOVA test $F(1.23) = 10.99$ $p < 0.001$) Mice genotype and interaction of two factors did not affect the percentage of CD25^{+}FoxP3^{+} cells (Two-way PERMANOVA test $F(1.23) = 0.60$ ND and $F(1.23) = 0.24$ ND respectively). Splenocytes became tolerogenic (enhanced expression CD25 and FoxP3) only during co-culture with mature DCs of Muc2$^{-/-}$ mice that were stimulated with *L. johnsonii*. Immature and mature DC of C57BL/6 did not stimulate activation of T regulatory cells (Figure 2C). At the same time, there were no effects of any factors to the percentage of IL-10^{+}T cell (Two-way PERMANOVA test $F(1.23) = 2.98$ ND and $F(1.23) = 2.38$ ND and $F(1.23) = 0.66$ ND respectively) (Figure 2D).

Thus, priming by *L. johnsonii* had no impact on DCs ability to induce proliferation of allogeneic splenocytes. However, in Muc2$^{-/-}$ mice functional capacity of DCs was reduced compared to DCs of

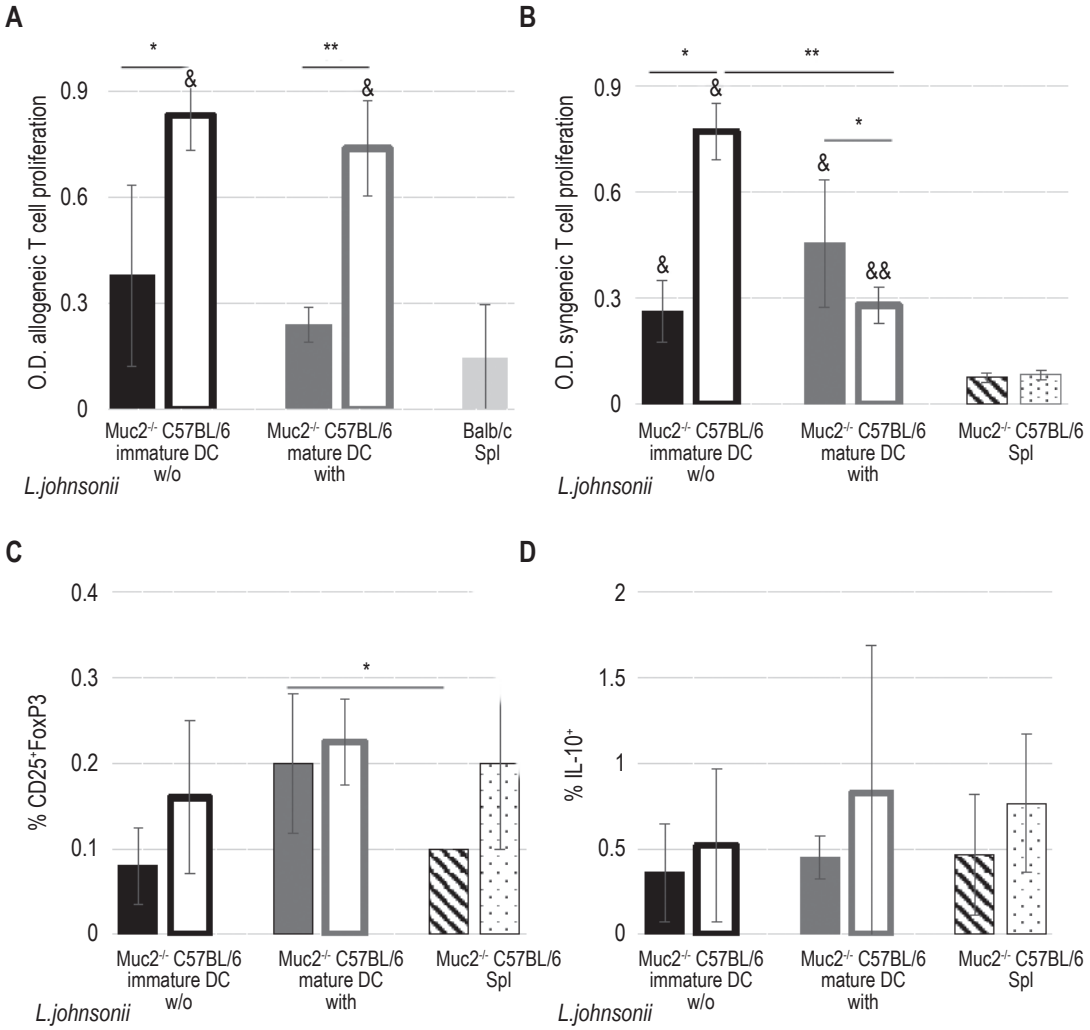

Figure 2. Co-cultivation of splenocytes and born-marrow derived DCs from C57BL/6 and Muc2$^{-/-}$ mice cultured with and without *L. johnsonii*

Note. (A) Proliferation index of allogeneic T cells of BALB/c mice co-cultured with DCs. (B) Proliferation index of syngeneic T cell co-cultured with DCs. (C) Percentage of CD25^{+}FoxP3^{+}T cells co-cultured with DCs. (D) Percentage of IL-10^{+}T cells co-cultured with DCs. *, $p < 0.05$; **, $p < 0.01$ Post-hoc Bonferroni test; $^{\&}$, $^{\&\&}$, Mann–Whitney test: difference from mono cultivation of splenocytes respectively.

C57Bl/6 mice. This effect is consistent with immature phenotype of DCs from Muc2$^{-/-}$ mice, which have lower expression of co-stimulatory molecules CD80, CD86 and CD83 compared to DCs from C57BL/6 mice. Moreover, DC from Muc2$^{-/-}$ culture with *L. johnsonii* stimulated tolerogenic cells that could be associated with immature phenotype of DC.

We have shown that Muc2$^{-/-}$ mice that model IBD generate an immature DC phenotype compared to control animals. This effect was shown for the first time. Previous studies have analyzed functions of DCs from animals without inflammation [4]. The addition of probiotic bacteria to DC culture stimulated maturation, but the genotype of mice from which cells were generated had no impact on maturation. The immature phenotype of DCs from Muc2$^{-/-}$ mice shows a low potential for T cell proliferation, both allogeneic and syngeneic. Whereas C57BL/6 mature DCs primed with the probiotic stimulated proliferation of foreign T cells and reduced the proliferative index of their own splenocytes. It has been shown that IL-10 is able to reduce proliferative activity, but in our experiment, the percentage of IL-10^{+} cells was not changed [10]. At the same time, the immature phenotype DCs from Muc2$^{-/-}$ mice in combination with the probiotic showed a significant increase in proliferation of tolerogenic cells. Previously, it was shown that *L. johnsonii* causes an increase in TGF and IL-10 production of $CaCo_2$ cells [5]. However, effect on DCs generated from mice with chronic inflammation was shown for the first time. In the literature is discussed that immature

DCs are able to stimulate tolerogenic response more effectively [9].

Conclusion

We have demonstrated impaired growth of DCs from the bone marrow of mice with a null mutation of the Muc2 gene, which did not stimulate the proliferation of allogeneic and syngeneic T cells. *L. johnsonii* was able not only to stimulate DC maturation, but also to increase FoxP3 expression on $CD25^+$ cells when cultured with DC. It can be assumed that the probiotic will have a beneficial effect on inflammation and reduce pathological processes in animals *in vivo*.

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