ИЗМЕНЕНИЯ СОСТАВА МИКРОБИОТЫ КИШЕЧНИКА, АССОЦИИРОВАННЫЕ С ДЕФИЦИТОМ IL-6

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Резюме. Интерлейкин-6 (IL-6) – цитокин широкого спектра действия, который участвует в иммунной, нервной и эндокринной регуляции многих биологических процессов. IL-6 выполняет как гомеостатические, так и патогенные функции, в том числе он является одним из ключевых участников цитокинового шторма при COVID-19, а также контролирует выработку белков острой фазы при воспалении. IL-6 вовлечен в поддержание кишечного гомеостаза и играет ключевую роль как в индукции воспаления, так и в восстановлении кишечника после повреждения. В свою очередь, комменсальная микробиота – населяющие кишечник организма-хозяина эукариоты, прокариоты и вирусы – представляет собой один из ключевых факторов, модулирующих иммунный ответ в кишечнике. Так, преобладание определенных групп организмов связывают с развитием воспаления кишечника, а пробиотики и антибиотики успешно применяются как поддерживающая терапия при воспалительных заболеваниях кишечника. IL-6 необходим для поддержания барьерной функции кишечника, поскольку передача сигнала от данного цитокина модулирует пролиферацию клеток кишечника, что необходимо для их своевременного обновления как в гомеостазе, так и при воспалении. Установлено, что генетическая инактивация *IL6* способствует развитию кишечного воспаления, при этом вклад IL-6 в регуляцию состава микробиоты остается неясным. Для изучения этого вопроса был проведен анализ образцов стула наивных мышей дикого типа и мышей, дефицитных по *IL6* (IL-6 KO), полученных на генетической основе C57Bl/6. Установлено, что у нокаутных мышей на фоне дефицита IL-6 наблюдаются значительные изменения в представленности отдельных таксономических групп, которые, предположительно, и обеспечивают чувствительность IL-6 KO к развитию колита. Было обнаружено, что у IL-6 KO мышей по сравнению с мышами дикого типа наиболее существенно снижается относительное содержание *Firmicutes* и *Clostridiales* и повышается – *Bacteroides*. Полученные нами данные о снижении представленности *Firmicutes* у мышей с дефицитом IL-6, а также о снижении представленности *Lactobacillaceae* и других крупных таксонов говорят о том, что композиция микробиоты IL-6 KO мышей отчасти похожа на композицию микробиоты, характерную для хронического воспаления кишечника. Настоящая работа представляет основу для дальнейших исследований вклада IL-6-опосредованных изменений микробиоты в поддержание гомеостаза кишечника и развитие воспаления.

Ключевые слова: IL-6, микробиота, воспаление кишечника, маркеры воспаления, мышиные модели, кохаузинг

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CHANGES IN THE COMPOSITION OF THE INTESTINAL MICROBIOTA ASSOCIATED WITH IL-6 DEFICIENCY

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Abstract. Interleukin-6 (IL-6) is a broad-spectrum cytokine involved in the immune, nervous, and endocrine regulation of many biological processes. IL-6 performs both homeostatic and pathogenic functions. It is one of the key factors in the cytokine storm in COVID-19, and it also controls the production of acute phase proteins during inflammation. IL-6 is involved in the maintenance of intestinal homeostasis and is required for both the induction of inflammation and the repair of the injured intestinal tissue. In turn, the commensal microbiota, represented by eukaryotes, prokaryotes, and viruses, is one of the key factors modulating the immune response in the gut. The predominance of certain groups of commensal microorganisms is associated with the development of intestinal inflammation, while probiotics and antibiotics are successfully used to control inflammatory bowel disease. IL-6 is also necessary to maintain the barrier function of the intestine by modulating the proliferation of intestinal cells, which is necessary for their timely renewal both in homeostasis and inflammation. It has been established that the genetic inactivation of *IL6* contributes to the development of intestinal inflammation, while the involvement of IL-6 in the control of the gut microbiota composition remains unclear. To investigate this issue, we analyzed stool samples from wild-type naive mice and mice deficient in *IL6* (IL-6 KO) generated on the C57Bl/6 genetic background. It has been determined that IL-6 KO shows significant changes in some taxonomic groups of commensals, which may explain the sensitivity of IL-6 KO to the development of colitis. Interestingly, the relative contents of *Firmicutes* and *Clostridiales* are significantly reduced, whereas *Bacteroides* are increased in IL-6 KO as compared with wild-type mice. Our data on the reduction of *Firmicutes, Lactobacillaceae*, and other large taxa in IL-6 deficient mice suggest that the microbiota composition of IL-6 KO mice is somewhat similar to that of mice with chronic intestinal inflammation. Our study serves as a perspective for further research on the contribution of IL-6-mediated changes in the microbiota composition to the maintenance of intestinal homeostasis and the development of chronic gut inflammation.

Keywords: IL-6, microbiota, intestinal inflammation, inflammatory markers, mouse models, cohousing K

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Introduction

The commensal microbiota is comprised of eukaryotes and prokaryotes inhabiting the body of a host organism, including fungi and protozoa, archaea, bacteria, as well as viruses and bacteriophages. The maternal microbiota plays a key role in shaping the commensal microbial community of the child. With age, the composition of the microbial community undergoes significant changes and is regulated by many internal and external factors. Microbes inhabit both external and internal surfaces of the human body, including the gastrointestinal, respiratory and urogenital tracts, skin, oral mucosa and conjunctiva, but the main contribution to the total microbial biomass is made by the microbiota of the large intestine. Qualitative and quantitative composition varies significantly between different individuals. However, it is assumed that the human microbiota is represented by the core bacteria, in particular, belonging to the genera *Faecalibacterium, Ruminococcus, Eubacterium* and *Dorea* (*Firmicutes*), *Bacteroides* and *Alistipes* (*Bacteroidetes*) and *Bifidobacterium* (*Actinobacteria*), found in most people and constituting a significant proportion of the total number of microbial cells in the intestine [14].

In the process of co-evolution of mammals and their microbial symbionts, stable mutualistic relationships were formed between them. As a result, the microbiota began to participate in many physiological processes of the host organism. In particular, it is necessary for the development and normal functioning of the immune system [12]. Disturbances in the immune system can trigger inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease. Disruption in the control of microbiota by the host organism is one of the prerequisites for the development of inflammatory bowel disease [8]. For example, genomic mutations associated with an increased risk

of intestinal inflammation encode genes involved in recognizing and destroying microorganisms, as well as in maintaining the normal functioning of the intestinal barrier [11].

Mouse models significantly contributed to our understanding of the physiological and inflammatory functions of intestinal commensals. It was demonstrated that the microbiota is necessary for the development of intestinal inflammation, and the use of antibiotics facilitates the course of the disease [13]. In addition, transfer of fecal microbiota from mice with colitis results in intestinal inflammation in healthy recipient mice [6]. Thus, the study of the microbiota associated with intestinal inflammation is of particular interest. However, in addition to studies of changes in the microbiota that occur during intestinal inflammation, studies aimed at searching for prognostic markers of inflammation are also relevant.

One of the key factors that maintains the barrier function of the gut is IL-6. IL-6 deficiency is associated with increased susceptibility to inflammation in a mouse model of DSS-induced colitis [7], and pharmacological blockade of IL-6 signaling in humans is associated with an increased risk of intestinal perforations [10]. This effect is attributed to the ability of IL-6 to stimulate the proliferation of epithelial cells and to prevent their apoptosis. Despite the interest in the molecular mechanisms mediating the protective functions of IL-6 in the context of intestinal inflammation, the effect of this cytokine on modulating the composition of the intestinal microbiota has not yet been addressed. In addition to identification of taxonomic groups composing gut microbiota, the search for predictive markers of intestinal inflammation, is also of particular interest for fundamental science and medicine. The present work focuses on the identification of bacterial groups, which may serve as indicators of colitis development by studying IL-6-deficient mice that are highly sensitive to intestinal inflammation.

Materials and methods

Mice

The work was carried out on C57Bl/6 wild-type and *IL-6* knockout (IL-6 KO) mice, generated on C57Bl/6 genetic background. Mice at the age of 6-8 weeks were obtained from the SPF animal facility of the Institute of Cytology and Genetics of the Siberian Branch of the Russian Academy of Sciences. They were housed in individually ventilated Tecniplast EM500 cages with hygienic dust-free bedding at a constant temperature of 21±3 °C, humidity 40±10%, and 12-hour day/night cycles with food and water ad libitum at the SPF animal facility of EIMB RAS

Collection of stool samples and DNA isolation

Stool samples were collected in 2 mL tubes, DNA was isolated on the same day using the Qiagen QIAamp Fast DNA Stool Mini Kit according to the manufacturer's protocol. Briefly, 1 mL of InhibitEX buffer was added to the tube and the contents were triturated with a plastic swab. Next, the sample was incubated for 10 min at 70 °C and 5 min at 95 °C. It was vigorously mixed on a vortex for 15 seconds and centrifuged at 4 °C 14000 g for 1 minute. 600 μL of the supernatant was transferred to a new tube containing proteinase K, after which 600 μL of AL buffer was added, incubated for 10 min at 70 °C, and 600 μL of 96% ethanol was added. After that, 600 μL of the sample was transferred to a DNA extraction column and centrifuged, the liquid was removed from the liquid collection tube and the application was repeated until the sample was completely loaded on the column. Then the column was washed with buffers AW1 and AW2 with centrifugation for 3 min. After that, the liquid collection tube was replaced with a clean tube, 50 μL of ATE buffer was applied to the column, centrifuged for 1 min to elute the DNA. The DNA concentration was measured on an Implen NanoPhotometer N50 spectrophotometer, the samples were stored at -80 °C.

Real-time quantitative PCR (qRT-PCR)

To analyze the composition of the intestinal microbiota, DNA samples were diluted with mQ to a concentration of 10 ng/μL. The reaction mix for one sample consisted of 4 μL qPCR premix (Evrogen qPCRmix-HS SYBR+LowROX), 1 μL each of forward and reverse 10 μM primers (Evrogen) (Table 1), 12 μL mQ and 2 μL DNA. Each reaction was run in duplicate in 96-well Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate, qRT-PCR was performed on a QuantStudio 6 Pro amplifier.

In stool samples, the representation of bacterial taxa was assessed (Table 1). The reference group was Eubacteria (Barman et al., 2008). The amplification mode was the same for all primers: 95 °C 5 min, 40X (95 °C 15 sec, 60 °C 30 sec, 72 °C 30 sec).

Analysis of the relative abundance (A) of the bacterial taxon (x) in the intestinal microbiota was performed as follows:

$A(x) = 2^{Ct(Eubacteria) - Ct(x)}$, where

Ct is the amplification cycle averaged over two repetitions, at which the content of the product in the reaction mixture reaches the threshold value (Kruglov et al., 2013). Statistical processing of the results was carried out using the GraphPad Prism 8 software. The compliance of the sample with a normal distribution was checked using the Shapiro–Wilk test. If the data were normally distributed, statistical significance was assessed using a t-test. A p-value less than 0.05 was considered statistically significant.

Results and discussion

To determine the role of IL-6 in maintaining the normal composition of the gut microbiota, DNA was isolated from stool samples and the content of bacterial taxa in IL-6 KO naive and wild-type

TABLE 1. PRIMER SEQUENCES FOR DETERMINING THE COMPOSITION OF THE INTESTINAL MICROBIOTA WITH qRT-PCR

Target	Primer type	Sequence
Actinobacteria	F	TGTAGCGGTGGAATGCGC
	R	AATTAAGCCACATGCTCCGCT
Bacteroides	F	GGTTCTGAGAGGAAGGTCCC
	R	GCTGCCTCCCGTAGGAGT
Clostridiales	F	ACTCCTACGGGAGGCAGC
	R	GCTTCTTAGTCAGGTACCGTCAT
Enterobacteriaceae	F	GTGCCAGCAGCCGCGGTAA
	R	GCCTCAAGGGCACAACCTCCAAG
Epsilonproteobacteria	F	AGGCTTGACATTGATAGAATC
	R	CTTACGAAGGCAGTCTCCTTA
Eubacteria	F	ACTCCTACGGGAGGCAGCAGT
	R	ATTACCGCGGCTGCTGGC
Firmicutes	F	GGAGYATGTGGTTTAATTCGAAGCA
	R	AGCTGACGACAACCATGCAC
Lactobacillaceae	F	AGCAGTAGGGAATCTTCCA
	R	CACCGCTACACATGGAG

mice was studied (Figure 1). For analysis, we selected groups of bacteria with relative content changes during intestinal inflammation, namely the kingdom *Firmicutes* [15], its family *Lactobacillaceae* [9], and the order *Clostridiales* [1]; *Bacteroides* – a large genus of the kingdom *Bacteroidetes* [3]; kingdoms of *Actinobacteria*; *Epsilonproteobacteria* and *Betaproteobacteria* – classes of the kingdom *Proteobacteria* [15] – and *Enterobacteriaceae* – families of the class *Deltaproteobacteria* of the same kingdom [9].

We found that the relative content of *Firmicutes* and *Clostridiales* was significantly reduced while the content of *Bacteroides* was increased in IL-6 KO as compared with wild-type mice (Figure 1A, B, C). A less pronounced decrease was observed in IL-6

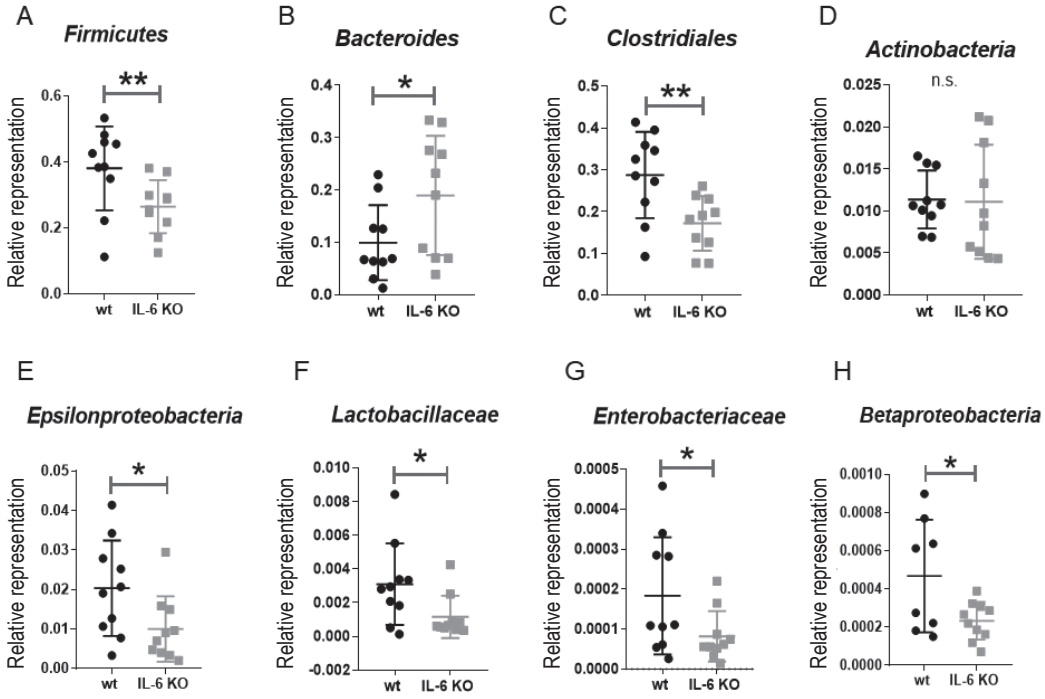

Figure 1. IL-6 deficiency is associated with altered gut microbiota composition in naive mice compared to wild-type control mice

Note. Relative representation of *Firmicutes* (A), *Bacteroides* (B), *Clostridiales* (C), *Actinobacteria* (D), *Epsilonproteobacteria* (E), *Lactobacillaceae* (F), *Enterobacteriaceae* (G) и *Betaproteobacteria* (H) in stool samples of naïve IL-6 KO mice and wild-type mice. *, $p < 0.05$; **, $p < 0.01$. Data are presented as mean and standard deviation.

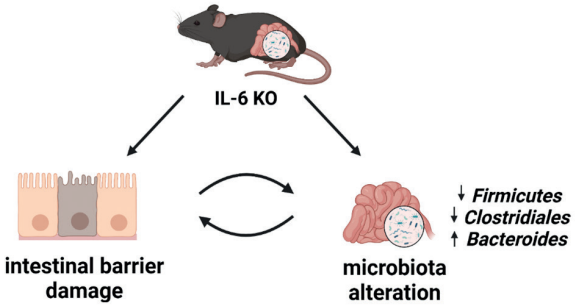

Figure 2. Altered microbiota of IL-6 KO mice may impact predisposition to intestinal inflammation

KO mice in relation to the *Epsilonproteobacteria*, *Lactobacillaceae*, *Betaproteobacteria*, and *Enterobacteriaceae* (Figure 1E, F, G, H). There were no differences in the relative content of Actinobacteria in mice of different genotypes (Figure 1D).

Inflammatory bowel disease is associated with a disturbance in the composition of the intestinal microbiota, which results both in a decrease in its overall diversity and in a change in the representation of individual taxa of microorganisms [4]. In particular, the gut microbiota of patients with chronic inflammation is enriched in the *Proteobacteria* and *Actinobacteria* kingdoms, while the relative content of the *Firmicutes* kingdom is, on the contrary, reduced [5]. Among the *Clostridiales*, both pathogenic and protective microorganisms are found; at the same time, the decrease in *Lactobacillaceae* in most studies is associated with the development of inflammation [2]. Despite an increase in protective *Bacteroides* [3], a significant decrease in *Firmicutes* in IL-6 deficient mice, as well as a decrease in *Lactobacillaceae* and other large taxa, suggests that the microbiota composition of IL-6 KO mice is somewhat similar to the microbiota composition characteristic of chronic intestinal inflammation.

Conclusion

Our results indicate that IL-6 is involved in the control of the intestinal microbiota, while IL-6 deficiency is associated with changes in its composition. How exactly this shift in the composition of the gut microbiota affects the functionality of the immune system remains unclear and needs to be further addressed. At the same time, our data show that the microbiota of IL-6 KO differs from that of wild-type mice, supporting our hypothesis that mice predisposed to gut inflammation may have an altered microbiota (Figure 2). We plan to identify specific representatives of the microbiota, the manipulation of which may have a therapeutic effect in the context of intestinal inflammation and colorectal cancer. Determination of taxa, the change in the representation of which can serve as a marker of future inflammation, is of particular interest and requires further research.

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