ВЛИЯНИЕ CFP-10/ESAT-6 СЕКРЕТОРНЫХ ПРОТЕИНОВ НА ДОЛГОВРЕМЕННУЮ НЕСПЕЦИФИЧЕСКУЮ ИММУНОЛОГИЧЕСКУЮ ПАМЯТЬ В МАКРОФАГАХ МЫШЕЙ

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Резюме. Клетки врожденного иммунитета (моноциты/макрофаги, NK) могут также развивать иммунную память, что означает, что эти клетки обучаются после их первой встречи с патогенами, так что они проявляют неспецифический иммунологический ответ на этот же или другой патоген. Бацилла Кальметта–Герена (БЦЖ) индуцирует во врожденных иммунных клетках неспецифическую врожденную память (тренированный иммунитет). Исследовали неспецифическую врожденную память в макрофагах мышей BALB/c в ответ на микобактерии, имеющие или не имеющие в геноме RD1 область. Мышей иммунизировали вакциной БЦЖ, на 7-й день выделяли перитонеальные макрофаги и стимулировали их бактериальным липополисахаридом, CFP-10 или ESAT-6. Кроме этого, мышей иммунизировали вакциной *Mycobacterium tuberculosis* уро-BCG ($RD1^-$) и штаммом *Mycobacterium tuberculosis* H37Rv ($RD1^+$) подкожно или внутривенно, на 4-й день выделяли перитонеальные макрофаги и стимулировали липополисахаридом. Альвеолярные макрофаги получали из эксплантатов легких мышей инфицированных *Mycobacterium tuberculosis* штамма H37Rv мышей, наращивали до конфлуэнтности 70-80% и далее стимулированы их липополисахаридом. В кондиционированной среде макрофагов исследовали уровень лактата, цитокинов и глюкозы. Показано, что перитонеальные макрофаги от мышей, праймированных вакциной БЦЖ, в ответ на CFP-6 и ESAT-10 увеличили уровень продукции IL-1β, TNFα и лактата ($p < 0,05$). Необходимо отметить тот факт, что липополисахарид также увеличивал продукцию IL-1β, TNFα и потребление глюкозы праймированными вакциной БЦЖ перитонеальными макрофагами ($p < 0,05$). Показано, что перитонеальные макрофаги, праймированные Уро-БЦЖ, увеличивали спонтанную продукцию IL-1β и снижали спонтанную продукцию TNFα ($p < 0,05$). В случае праймирования макрофагов подкожным или внутривенным способом введения *Mycobacterium tuberculosis* штамм H37Rv по-разному влияли на продукцию цитокинов – снижали продукцию IL-1β и увеличивали TNFα и IL-10. В ответ на липополисахарид перитонеальные макрофаги увеличивали продукцию IL-1β, TNFα, IL-10 и потребление глюкозы ($p < 0,05$). Способ праймирования макрофагов *Mycobacterium tuberculosis* штамм H37Rv также вел к разнонаправленному

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уровню продукции цитокинов. Было показано, что альвеолярные макрофаги сохраняли тренированный иммунитет, так, они продуцировали повышенные уровни IL-1β, TNFα, IL-10 ($p < 0,05$). Таким образом, макрофаги мышей сформировали фенотип тренированного иммунитета в ответ на различные типы микобактерий, который сохраняется длительное время после первичного контакта с патогеном, в частности в альвеолярных макрофагах.

Ключевые слова: вакцина БЦЖ, штаммы Mycobacterium tuberculosis, цитокины, лактат, глюкоза, оксид азота

EFFECT OF CFP-10/ESAT-6 SECRETORY PROTEINS ON LONG-TERM NON-SPECIFIC IMMUNOLOGICAL MEMORY IN MOUSE MACROPHAGES

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Abstract. Innate immune cells (monocytes/macrophages, NK) can also develop immune memory, which means that these cells are trained after their first encounter with pathogens so that they exhibit a nonspecific immunological response to the same or another pathogen. Bacilli Calmette–Gu rin (BCG) induces nonspecific innate memory (trained immunity) in innate immune cells. We examined nonspecific innate memory in macrophages of BALB/c mice in response to mycobacteria with or without the RD1 region in the genome. Mice were immunized with BCG vaccine, and peritoneal macrophages were isolated on day 7, and then stimulated with bacterial lipopolysaccharide, CFP-10, or ESAT-6. In addition, mice were immunized with *Mycobacterium tuberculosis* uro-BCG vaccine ($RD1^-$) and *Mycobacterium tuberculosis* strain H37Rv ($RD1^+$) subcutaneously or intravenously; peritoneal macrophages were isolated and stimulated with lipopolysaccharide on day 4. Alveolar macrophages were obtained from lung explants of mice infected with *Mycobacterium tuberculosis* strain H37Rv mice, were expanded to confluence 70-80% and further stimulated with lipopolysaccharide. Lactate, cytokines, and glucose levels were examined in conditioned macrophage medium. Peritoneal macrophages from mice primed with BCG vaccine were shown to increase IL-1β, TNFα, and lactate production in response to CFP-6 and ESAT-10 ($p < 0.05$). Of note is the fact that lipopolysaccharide also increased production of IL-1β, TNFα, and also increased glucose uptake by peritoneal macrophages primed with BCG vaccine ($p < 0.05$). Peritoneal macrophages primed with Uro-BCG were shown to increase spontaneous production of IL-1β and decrease spontaneous production of TNFα ($p < 0.05$). When macrophages were primed by subcutaneous or intravenous administration of *Mycobacterium tuberculosis* strain H37Rv differentially affected cytokine production, by decreasing IL-1β production and increasing TNFα and IL-10, was observed. In response to lipopolysaccharide, peritoneal macrophages increased IL-1β, TNFα, IL-10 production and glucose consumption ($p < 0.05$). The mode of priming of macrophages with *Mycobacterium tuberculosis* strain H37Rv also led to multidirectional levels of cytokine production. Alveolar macrophages were shown to retain trained immunity, as they produced elevated levels of IL-1β, TNFα, and IL-10 ($p < 0.05$). Thus, mouse macrophages formed a trained immunity phenotype in response to different types of mycobacteria, which persists for a long time after primary contact with the pathogen, particularly in alveolar macrophages.

Keywords: BCG vaccine, strain of Mycobacterium tuberculosis, cytokines, lactate, glucose, NO

Introduction

Macrophages (Mf) are the main host cells of *Mycobacterium tuberculosis* (MBT), which could suppress the functional activity of these cells [2]. The BCG vaccine strain is able, through the induction of epigenetic reprogramming and metabolic changes in innate immune cells, to elicit trained immunity (TI). TI capable developed highest response to secondary exposure to homologous or heterologous pathogens, and protect from tuberculosis [14]. MBT virulence is associated with the presence of the RD1 region in the genome encoding CFP-10 and ESAT-6 [1, 3, 6, 7, 11]. It was obtained increased production of IFNγ, TNFα and IL-10 by lymphocytes in response to CFP-10/ESAT-6 stimulus [1, 15].

The aim of the study was to evaluate the severity of the induction of trained immunity taking into account the presence or absence of the RD1 region in the

mycobacterial genome in the resident macrophages of BALB/c mice.

Materials and methods

The study was performed in compliance with Directive 2010/63/EU of the European Parliament and Council on the protection of animals used for scientific purposes. Experiment (1) BALB/c mice were immunized with hot-inactivated BCG vaccine (0.1 mg intraperitoneally, Microgen, RF), and then peritoneal macrophages (pMf) were isolated on day 7. 10^6 pMf/well in RPMI-1640 medium (Paneco, Russia) supplemented with 2 mM L-glutamine (Sigma, USA), 5 mM HEPES-buffer (Sigma, USA), 10% FCS (Gibco, USA) and LPS (0 and 0.5 μg/mL lipopolysaccharide, Sigma, USA), CFP-10 and ESAT-6 (0 and 10 μg/mL, Oxford Immunotech, UK) were incubated in 24-well plates (TPP, Switzerland) for 24 hours in a CO_2 incubator (Sanyo, Japan).

Experiment (2) BALB/c mice were immunized with MBT uro-BCG medac strain RIVM ($RD1^-$) or H37Rv ($RD1^+$) subcutaneously (s.c.) or intravenously (i.v.). On day 4, 10^6 pMf/well in RPMI-1640 medium supplemented with 2 mM L-glutamine, 5 mM HEPES buffer, 10% FCS, and LPS (0 and 0.5 μg/mL) were incubated in 24-well plates for 24 hours in a CO_2 incubator (Sanyo, Japan).

Experiment (3) Alveolar macrophages (aMf) were isolated from BALB/c mice infected with MBT strain H37Rv by migration of cells from the explants, and expanded to achieve 70-80% of confluence. Then 10^6 aMf/well in RPMI-1640 medium supplemented with 2 mM L-glutamine, 5 mM HEPES buffer, 10% FCS, and LPS (0 and 0.5 μg/mL) were incubated in 24-well plates for 24 hours in a CO_2 incubator (Sanyo, Japan). Lactate, NO, cytokines, and glucose levels were determined in conditioned Mf media. The level of stable nitric oxide (NO)-nitrite (NO2-) metabolites was assessed using the Griess reagent. Lactate using the Lactate-Novo kit, IL-1β, IL-10, and TNFα (Vector-BEST, Russia) were assessed according to the manufacturer's instructions, and glucose was assessed using the Glucose-Novo kit (Vector-BEST, Russia).

The data was statistically processed using Statistica 10.0 for Windows. The normality of the distribution of the data obtained was assessed using the Shapiro–Wilk's w-criterion. Data in the table 1 were presented as mean and standard deviation (M±SD); statistical significance of intergroup differences was assessed by ANOVA with Bonferroni post hoc test and was accepted at $p < 0.05$.

Results and discussion

As shown in the Table 1, CFP-10 and ESAT-6 increased the production of IL-1β, TNFα, and lactate by BCG-primed pMf ($p < 0.05$). In response to the second stimulus, pMf increased production of IL-1β, TNFα, NO, and lactate compared to BCG-primed cells alone ($p < 0.05$). The combination of CPF-10, ESAT-6, CFP-10/ESAT-6 with LPS increased pMf IL-1β production and decreased TNFα production ($p < 0.05$). The non-virulent strain of MBT increased IL-1β, TNFα, and IL-10 production ($p < 0.05$). At the same time, immunization s.c. with a virulent strain of MBT increased IL-1β, IL-10 production and decreased TNFα production, while i.v. MBT administration resulted in increased TNFα production and decreased IL-1β, IL-10 production compared to basal levels ($p < 0.05$).

LPS stimulation of pMf primed *in vivo* in the group of mice immunized with the non-virulent MBT strain increased IL-1β and TNFα production compared to the same indices in intact animals ($p < 0.05$). pMf from the mice primed with the virulent strain of MBT (s.c. injection) responded to the LPS stimulus with an increase in TNFα and IL-1β production, and a decrease in TNFα and IL-10 production ($p < 0.05$) when immunized by the i.v. Comparing the effects of the vaccine administration route on the induction of trained immunity in the pMf of mice without a second stimulus, we noted the fact that the production of IL-1β and TNFα was higher with i.v. immunization with the non-virulent MBT strain ($p < 0.05$).

In the group of mice immunized with the virulent strain of MBT, multidirectional production of IL-1β and TNFα was shown. The second stimulus in the case of pMf from mice immunized with the i.v. contributed to the increase in the levels of all cytokines, while the maximum increase in the production of only TNFα ($p < 0.05$) was observed when s.c. immunization with the virulent strain of MBT was used.

Intravenous immunization with the virulent strain promoted an increase in the production of pMf IL-1β and a decrease in the production of TNFα and IL-10 in response to LPS, while b/c immunization showed an increase in the production of only TNFα ($p < 0.05$). It seemed legitimate to evaluate the effect of mycobacteria on the expression of trained immunity *in vivo* in mice. Thus, aMf in response to LPS stimulus responded with increased production of IL-1β, TNFα, IL-10, NO ($p < 0.05$).

Tuberculosis is considered to be a global emergency worldwide [8]. The BCG vaccine is primarily a tuberculosis vaccine that also has a protective effect against leprosy, Buruli ulcer, and other non-tuberculosis mycobacterioses as a consequence of epigenetic and metabolic reprogramming of innate immunity cells [2, 14]. We observed an increase in pro-inflammatory cytokine production by BCG vaccine-primed pMf in response to CFP-10, ESAT-6, but no such pattern was found in response to the second stimulus (LPS). It is known that cytokines are involved in the pathogenesis of tuberculosis: their levels change with the stage of the pathological process,

TABLE 1. CYTOKINE PRODUCTION BY MACROPHAGES OF BALB/c MICE DURING INDUCTION OF TRAINED IMMUNITY *IN VITRO* AND *IN VIVO* (M±SD)

Parameter	IL-1β, pg/mL	TNFα, pg/mL	IL-10, pg/mL	NO, μM/mL	Lactate, mM	Glucose consumption, mM
Experiment 1						
Without second stimulus						
Basal	72.3±3.1	85.0±7.1	71.7±1.9	4.76±0.62	2.590±0.001	6.27±0.22
CFP-10	106.0±3.5*	106.6±1.2*	61.5±8.3	4.28±0.48	3.07±0.11*	6.04±0.09
ESAT-6	111.2±6.3*	193.3±4.4*	71.9±5.3	4.37±0.90	3.04±0.13*	6.15±0.21
CFF-10/ESAT-6	61.0±0.6*	174.3±3.2*	80.4±6.1	5.12±0.52	2.94±0.13*	5.77±0.21
In response to LPS						
LPS	80.3±1.9*	132.1±1.9*	69.1±3.8	6.83±0.13*	2.80±0.02	6.12±0.22
CFP-10	83.4±3.9	145.3±5.4#	68.0±1.9	4.08±0.21#	2.90±0.24	5.93±0.19
ESAT-6	141.4±1.5#	120.1±5.0#	67.3±3.5	4.56±0.50#	3.22±.2.00#	5.71±0.22
CFP-10/ESAT-6	107.3±1.8#	130.3±1.8	65.7±2.1	4.07±0.06#	2.89±0.03#	4.33±0.21#
Experiment 2						
Without second stimulus						
Untreated	61.8±3.1	123.4±0.8	61.5±0.4	2.83±0.05	2.29±0.11	3.33±0.14
Uro-BCG, s.c.	77.6±0.3* •	95.1±1.8* •	127.9±9.6* •	2.80±0.02	2.96±0.97	2.95±0.26
Uro-BCG, i.v.	84.9±0.6*	114.3±0.4*	76.1±3.6*	2.85±0.08	3.04±0.73	2.01±0.28*
H37Rv, s.c.	92.0±0.7* •	61.7±1.5* •	66.0±0.8* •	2.80±0.03	2.76±0.24	1.65±0.40*
H37Rv, i.v.	54.9±0.9*	153.5±3.9*	57.4±1.3*	2.87±0.03	2.44±0.15	1.75±0.04*
In response to LPS						
Untreated	58.5±1.4	74.4±1.9	73.8±4.6	6.90±0.12	2.58±0.03	3.84±0.12
Uro-BCG, s.c.	74.6±0.7# •	218.3±4.2# •	67.6±2.9	6.05±0.07#	2.88±0.31	3.43±0.13#
Uro-BCG, i.v.	139.1±1.7#	153.3±2.5#	131.7±0.4#	6.08±0.12#	2.92±0.13#	2.41±0.09#
H37Rv, s.c.	62.6±7.4	275.7±1.9# •	57.5±2.6#	5.41±0.06# •	3.00±0.21#	2.24±0.29#
H37Rv, i.v.	73.7±0.7#	48.8±0.5#	60.9±2.6#	4.91±0.10#	2.71±0.10	2.06±0.11#
Experiment 3						
Basal	88.75±0.66	74.94±3.58	56.16±2.02	6.97±0.83	5.54±0.15	1.23±0.35
LPS	116.35±2.09*	105.26±2.05*	73.36±4.13*	9.71±1.81*	5.58±0.08	1.32±0.32

Note. $p < 0.05$ *, with basal; #, with LPS; •, with i.v. administration.

polychemotherapy and can serve as an indicator of the effectiveness of treatment [9].

Our data point to the fact that we have partially succeeded in reproducing the phenomenon of trained immunity in pMf in response to stimulation with BCG vaccine. Thus, BCG vaccine-primed pMf in response to the second stimulus (LPS) increased cytokine and nitric oxide production, but did not increase lactate production or glucose consumption. Our findings on the effect of mycobacterial proteins on the secretory potential of primed BCG vaccine pMf are interesting. On Mf derivatives of THP-1 (cultured with phorbol-12-myristate-13-acetate), ESAT-6, CFP-10 and ESAT-6/CFP-10 were shown to significantly reduce NO and reactive oxygen species (ROS) production [12]. The RAW264.7 cell line, in response to ESAT-6, CFP-10 and ESAT-6/CFP-10 stimulation, reduced spontaneous and LPS-stimulated reactive oxygen species (ROS) production [5]. In the murine monocyte line ANA-1, CFP-10/ESAT-6 was shown to stimulate NO and IL-12 production in response to IFNγ stimulation, which is abolished when the cells are treated with AG490, a selective inhibitor of the JAK/STAT signaling pathway [7].

At the same time, recombinant CFP-10/ESAT-6 promotes dose-dependent increase of TNFα production by human monocytes and THP-1 cells, enhances CD80 and CD40 expression, increased IFNγ-induced TNFα production and HLA-DR expression [4]. Our data indicates stimulation of IL-1β, TNFα production by pMf after priming them with BCG vaccine, but we did not observe changes in

cellular production of NO. The differences detected with the literature data may be a consequence of the use of pMf in our work rather than monocytic cell lines and peripheral blood monocytes or macrophages derived from human or animal bone marrow. MBT secretory proteins encoded by the RD-1 region play an essential role in mycobacterial virulence, as they can inhibit ROS production [5].

Mycobacterial virulence also depends on the ability of mycobacteria to inhibit the IL-1β-dependent pro-inflammatory response, the suppression of apoptosis, delayed recruitment and activation of adaptive immunity cells [2]. On this basis, we investigated the effect of the method of mycobacterial administration and their virulence on the expression of trained immunity in Mf. Thus, vaccination with uro-BCV enhances the production of IL-1β by primed pMf. The pMf from mice vaccinated with MBT strain H37rv by s.c. administration produced higher levels of IL-1β, while in mice vaccinated by i.v. administration – TNFα. The pMf primed with non-virulent or virulent strains of MBT increased production of IL-1β, TNFα, and NO in response to LPS.

Consequently, MBT virulence and mode of immunization are not essential in the induction of trained immunity in the Mf. The vaccine strain of Mycobacterium BCG differs from virulent strains of MBT in the pattern of dissemination from aMf to other myeloid cells, mainly neutrophils and recruiting macrophages, which plays an essential role in MBT dissemination [10]. In addition, the aerosol route of BCG administration has been shown to develop resistance to *Streptococcus pneumonia* infection in mice. NRF2 (immune response regulator) aMf from knockout mice has been shown to initiate a significant pro-inflammatory response as early as day 10 of infection with MBT [13]. On this basis, we obtained aMf from mice with tuberculosis process induced by i.v. injection of MBT strain H37Rv. In response to LPS, aMf increased the production of both IL-1β, TNFα, IL-10, and NO. Consequently, taking into account the time required to obtain a primary culture of aMf (2-3 weeks), we can judge about the persistence of priming of these cells to MBT.

Conclusion

Thus, MBT vaccination irrespective of virulence and method of administration induces trained immunity in Mf. Moreover, mycobacterial secretory proteins CFP-10, ESAT-6 may act as substrates capable of influencing trained immunity. Judging by the increased production of aMf cytokines from MBT-infected mice, trained immunity in cells *in vitro* is preserved for a long-time.

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