A recombinant *Bacillus subtilis* vaccine to induce CD8+ T cell response

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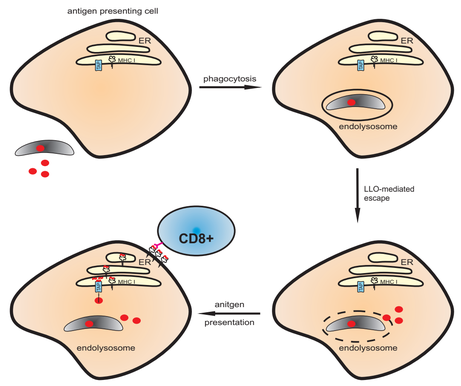
BACKGROUND

Cytotoxic CD8+ T cells (CTLs) play an important role in eradication of many intracellular pathogens, as well as in destruction of cancer cells. Interaction of the T cell receptor of the lymphocytes (TCR) with major histocompatibility complex class I molecules (MHC class I) located at the surface of the antigen presenting cells (APCs), such as dendritic cells (DCs) or macrophages, provide specificity of the interaction between those two types of cells. After recognizing the MHC I – peptide complexes of infected cells and receiving costimulation from the CD4+ T lymphocytes, CTLs become activated, what causes the death of the target cells by apoptosis and release of membrane perforating granzymes and perforins. More importantly, a population of memory T cells is generated, thereby leading to resistance to a pathogen, whose antigen was presented. Different strategies can be used to deliver antigens to the cytosol of APCs, among which that based on bacterial carriers constitutes probably the most studied and the most promising one. Both attenuated and commensal microorganisms are used in the next generation vaccine design. However, due to the potential risk of conversion to a virulent strain and infection development, pathogenic vectors are less likely to use. A major drawback of the systems based on non-pathogenic bacteria is the lack of invasiveness, so the antigen delivery could be less effective than in case of pathogenic bacteria. To overcome this problem, virulence determinants of intracellular pathogens could be expressed in non-virulent species. Among proteins, whose activities promote bacterial invasion and escape from the vacuole to the cytoplasm of the host cell, there are numerous virulence determinants such as listeriolysin O (LLO) from Listeria monocytogenes, a model intracellular pathogen. Interestingly, LLO is simultaneously a major virulence factor and a major immunogen, what makes it an ideal adjuvant candidate [1].



OBJECTIVES

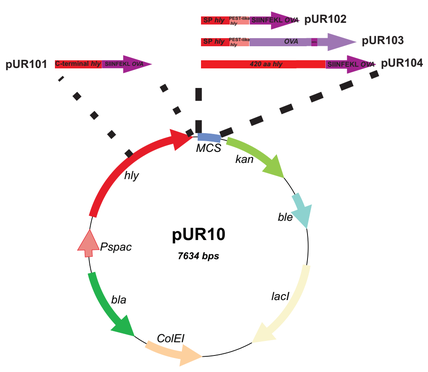
The aim of this project was construction of a series of bacterial delivery vectors based on the non-pathogenic, model Gram-positive bacterium *Bacillus subtilis*, as well as evaluation of their potency to deliver different variants of the model antigen, chicken egg ovalbumin (OVA), to the cytosol of dendritic cells of JAWS II line. Along with examining the presentation of the OVA antigens in the MHC I complexes, ability to induce the response of cytotoxic T cell lymphocytes was evaluated.

MATERIALS AND METHODS

B. subtilis strains producing recombinant fusion antigens were obtained by standard methods of molecular cloning. All constructed delivery vectors expressed listerial LLO toxin enabling escape from eukaryotic vacuole and consequently facilitating heterologous production of immunogenic proteins in the cytosol of APCs [2]. Ability to invade eukaryotic cells, as well as cytotoxicity and hemolytic activity were examined. Presentation of the OVA epitope in the context of the MHC I molecules was evaluated by flow cytometry after staining the *Bacillus*-infected DCs with 25-D1.16 antibody recognizing H-2Kb-OVA257-264 complexes. The ability of B. subtilis to elicit T cell activation and proliferative response of CFSE-labelled cytotoxic CD8+ T cells OT-I isolated from transgenic murine lymph nodes was determined by flow cytometry [3].

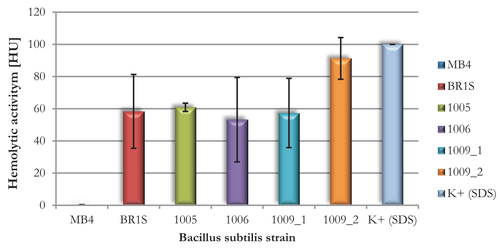
RESULTS

Construction of the LLO-OVA producing *B. subtilis* strains



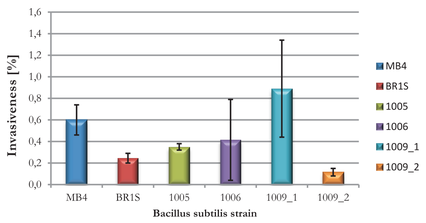
Vectors pUR101 - pUR104 were achieved respectively by cloning: **a)** OVA epitope (SIINFEKL) sequence fused to C-terminal sequence of LLO, **b)** fusion sequence LLO-OVA (N-terminal sequence of LLO coding ribosome binding site (RBS), signal peptide (SP) and PEST-like sequence fused to the sequence of SIINFEKL), **c)** fusion sequence LLO-OVA (N-terminal sequence of LLO coding RBS, SP and PEST-like sequence fused to the complete sequence of OVA), **d)** partial LLO sequence coding first 420 aa fused to SIINFEKL sequence). Truncated form of LLO lacking 84 C-terminal amino acids (domain 4 of the protein) was used as it retains its activity and is essential for IFN-γ-inducing activity, what plays an important role especially in inducing protective immunity.

Hemolytic activity of the LLO-OVA producing *B. subtilis* strains



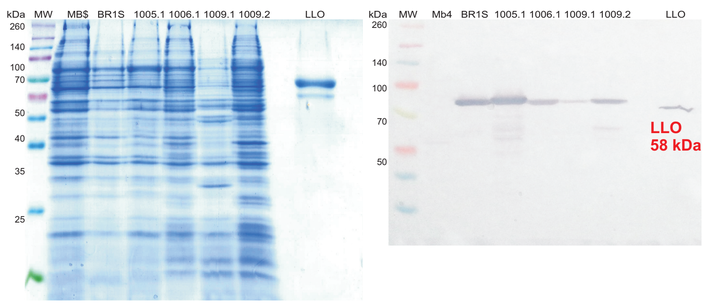
Hemolytic activity was assayed on sheep erythrocytes (SRBC). The erythrocytes were washed three times with PBS, and suspended to a final concentration of 10% in PBS (pH 7,4). The bacterial culture supernatant was diluted 50-fold in 1% erythrocyte suspension. The prepared solution was incubated for 30 minutes at 37 °C, and then centrifuged for 3 minutes at 1200 RPM. The released hemoglobin was measured spectrophotometrically at 410 nm. Hemolytic units (HU) were calculated after setting 0 HU as the activity of negative control, and 100 HU as total hemolysis (sample lysed with 0,01 % SDS).

Invasiveness of the LLO-OVA producing *B. subtilis* strains



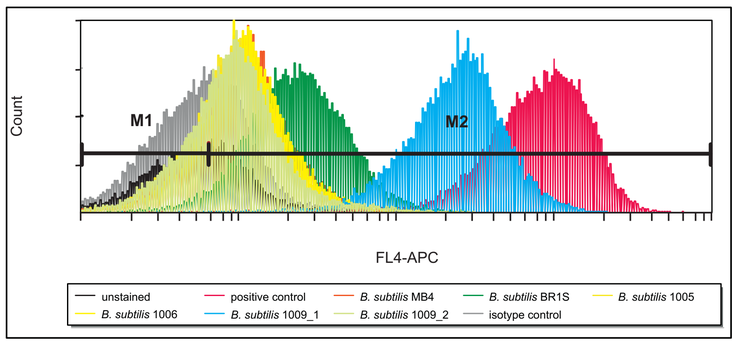
For infection, bacterial inoculum was added to wells of 24-well plate with JAWS II DCs at multiplicity of infection (MOI) of 10. The plates were incubated at 37 °C with 5% CO2 for 30 minutes. To remove extracellular bacteria, each well was washed 3 times with PBS pH 7,4 and incubated with RPMI-1640 medium containing gentamycin (100 μg/ml) for 1 hour. Cells were washed again with PBS, lysed with 1 % Triton X-100 and plated.

Listeriolysin secretion by *Bacillus subtilis* strains



Supernatant proteins were isolated from the culture supernatants of IPTG-inducted strains by TCA / ethanol precipitation, and subsequently subjected to SDS-PAGE. For Western-blotting, gels were blotted onto PVDF membrane, which, after blocking, was incubated with primary rabbit anti-LLO antibody and secondary goat anti-rabbit antibody conjugated with alkaline phosphatase.

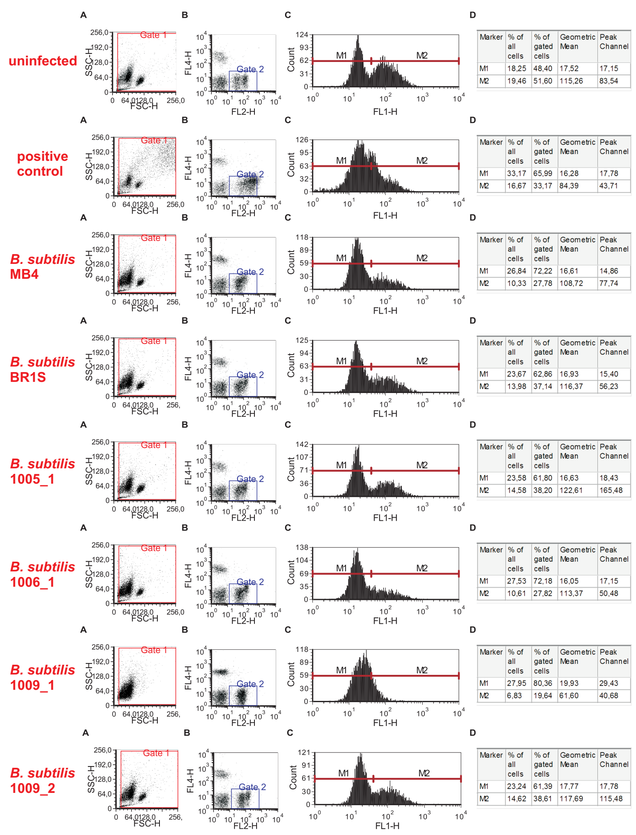
OVA epitope presentation by JAWS II dendritic cells (DCs) infeced with different strains producing LLO-OVA fusion proteins



Sample	Gate	# of Events	% of gated cells	Median	Geometric Mean	CV	Peak Value	Peak Channel
unstained	M1	6186	58,4	3,7	3,3	42,5	286,0	1,0
	M2	4403	41,6	9,7	11,0	83,1	228,0	6,7
isotype control	M1	11745	56,1	3,8	3,4	41,9	486,0	1,0
	M2	9192	43,9	10,0	11,3	422,5	443,0	6,7
positive control	M1	78	0,4	3,2	3,0	44,2	5,0	2,7
	M2	20958	99,6	835,4	768,0	67,1	447,0	1000,0
non-infected	M1	9337	44,3	3,9	3,6	39,8	414,0	6,0
	M2	11724	55,7	10,8	11,7	94,7	502,0	9,0
***B. subtilis* MB4**	M1	4782	22,0	4,5	4,0	34,5	313,0	6,5
	M2	16969	78,0	12,4	13,6	114,1	581,0	11,6
***B. subtilis* BR1S**	M1	887	4,1	4,9	4,3	31,8	68,0	6,0
	M2	20692	95,9	24,6	25,1	91,9	465,0	22,9
***B. subtilis* 1005**	M1	5759	26,8	4,5	4,0	34,4	384,0	6,5
	M2	15749	73,2	12,0	13,3	127,4	566,0	9,7
***B. subtilis* 1006**	M1	4756	21,9	4,5	4,1	33,8	316,0	6,5
	M2	16940	78,1	12,4	13,8	113,9	598,0	9,3
***B. subtilis* 1009_1**	M1	70	0,3	3,3	3,0	50,6	7,0	1,0
	M2	21547	99,7	245,8	231,8	74,1	567,0	245,8
***B. subtilis* 1009_2**	M1	6328	29,5	4,5	4,0	35,3	397,0	6,5
	M2	15126	70,5	11,6	13,2	202,3	566,0	9,7

Presentation of immunogenic peptide was evaluated by flow cytometry after staining DCs infected by *B. subtilis* with BD Biosciences 25D-1.16 antibody capable of recognizing oktapeptide SIINFEKL in a complex of MHC class I molecules ($H2\text{-}K^b$). DCs pulsed with a wild-type strain *B. subtilis* MB4 (non-haemolytic) and BR1S (haemolytic) strains were included as negative controls, while DCs pulsed with 10 ng/ml SIINFEKL peptide were used as a positive control of peptide presentation. Data were aquired by FACSCalibur cytometer and processed using FCS Express 4 Flow programme (De Novo Software).

Cytotoxic T cells activation by JAWS II dendritic cells infected with LLO-OVA *B. subtilis* strains



Lymphocytes isolated from lymph nodes of OT-I mice were stained with CFSE and incubated with *B. subtilis* LLO-OVA infected JAWS II dendritic cells. After 3 days T-cells were collected and subsequently stained with anti-CD4 and anti-CD8 monoclonal antibodies. T-cell proliferation was evaluated by measuring the dilution of CFSE labelling in respective CD8+ population using flow cytometry.

CONCLUSIONS

Tests conducted during the described study revealed that *B. subtilis* strain 1009_1 producing whole-length OVA antigen fused to N-terminal sequence of LLO is most capable to induce the presentation of the OVA antigen on the surface of APCs and to activate response of cytotoxic T cells. Comparison of strains producing different variants of LLO-OVA antigens indicated that 1009_1 strain is the most promising candidate for an effective antigen-delivery vector.

References

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[2] Bielecki J., Youngman P., Connelly P., Portnoy D. A. 1990. Bacillus subtilis expressing a haemolysin gene from Listeria monocytogenes can grow in mammalian cells. Nature 345: 175–176
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