Bacterial ghosts as adjuvants in syngeneic tumour cell lysate-based anticancer vaccination in a murine lung carcinoma model

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Abstract. Instead of relying on external anticancer factors for treatment, immunotherapy utilizes the host's own immune system and directs it against given tumour antigens. This study demonstrated that it is possible to overcome the documented immunosuppressive properties of tumour cell lysate by supplementing it with appropriate adjuvant. Lewis lung carcinoma (LLC)-challenged C57BL/6 mice were treated with LLC cryo-lysate mixed with either bacterial ghosts (BGs) generated from *E. coli* Nissle 1917 or *B. subtilis* 70 kDa protein as adjuvants. Median and overall survival, the size of metastatic foci in lung tissue and levels of circulating CD8a$^+$ T cells were evaluated and compared to the untreated control mice or mice treated with LLC lysate alone. After primary tumour removal, a course of three subcutaneous vaccinations with LLC lysate supplemented with BGs led to a significant increase in overall survival (80% after 84 days of follow-up vs. 40% in untreated control mice), a significant increase in circulating CD8a$^+$ T cells (16.57 vs. 12.6% in untreated control mice) and a significant decrease in metastasis foci area and incidence. LLC lysate supplemented with *B. subtilis* protein also improved the inspected parameters in the treated mice, when compared against the untreated control mice, but not to a significant degree. Therefore, whole cell lysate supplemented with BGs emerges as an immunostimulatory construct with potential clinical applications in cancer treatment.

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Introduction

The immune system plays a critical role in the prevention of tumour development. The sophisticated bidirectional interaction between cancer cells and the immune system is defined as cancer immunoediting (1). Unfortunately, during this dynamic process, malignant cells may acquire various molecular and cellular mechanisms enabling them to escape immune-mediated control and manifest as clinically apparent cancer. Various immune evasion mechanisms can be grouped into immunoselection (antigen loss on cancer cell surface) and immunosubversion (tumour-driven active creation of local and systemic immunosuppressive milieu) (2). Due to impaired and disbalanced antitumour immunity in cancer patients (3,4), immune response-modulating strategies, known as tumour immunotherapy, have been introduced into clinical practice. Among the various types of cancer immunotherapy (5), therapeutic cancer vaccination is one of the most promising approaches, especially when combined with other immunotherapeutic strategies as well as cancer chemotherapy, radiation therapy and targeted therapies (6).

Therapeutic cancer vaccination aims at inducing and/or augmenting cytotoxic cellular immune responses that are able to quantitatively and qualitatively overwhelm the cancer-driven immunosuppressive arm of antitumour immune response (7). Therapeutic vaccines exploit mostly dendritic cells (DCs) that are the main initiators and orchestrators of adaptive immune responses (8). DCs can be targeted either *in situ* or generated *ex vivo* and reinjected back into the same patient to achieve their therapeutic effect. Selection of proper tumour-associated antigens (TAAs) and induction of optimal DC maturation are the critical steps in therapeutic cancer vaccination (9). Based on antigen selection strategy, therapeutic vaccines can be mono- or oligovalent (using one or several defined TAAs) and polyvalent (a variety of undefined TAAs is used). In theory, polyvalent vaccines should be superior to mono- or oligovalent vaccines, since the former mobilize the immune system to target more than just one or few TAA(s), which could be

potentially lost in individual cases (10). Moreover, tumours are known to be heterogeneous (11); therefore targeting one antigen may target only a portion of the whole tumour cell spectrum.

Tumour cell lysate (TCL) contains a mixture of proteins resulting from induced lysis of tumour cells, which ensures a broad spectrum of target antigens (12). Furthermore, the TCL approach to vaccination does not require *a priori* knowledge of relevant TAAs and targets also potentially unknown TAAs (13). The ease of manufacturing and storage, lack of limitations dictated by host-specificity and no obligation to know specific TAAs make TCL an appealing vaccine candidate. On the other hand, TCL contains not only immunogenic TAAs, but also various immunosuppressants naturally occurring in cancer cells, such as hyaluronan, known for inducing tolerogenic rather than immunogenic maturation of DCs and macrophages (14). Agents, such as Fas ligand (15) or transforming growth factor-β (16), inducing the apoptosis of immune cells are also thought to be present in TCL (17). However, the negative effects of immunosuppressive components present within TCL may potentially be compensated by properly selected immunostimulatory adjuvants that are otherwise used for the induction of DC maturation (9).

Adjuvants used in therapeutic cancer vaccination include Toll-like receptor (TLR) agonists [e.g. imiquimod, resiquimod and lypopolysaccharide (LPS)], cytokines (e.g. granulocyte-macrophage colony-stimulating factor, interferons and interleukins), prostaglandin E_2 and their combinations (18). It was demonstrated that proper combinations of various TLR agonists are needed for adequate induction of DC maturation (19). Hence the use of natural sources of multiple adjuvants may optimize therapeutic cancer vaccination strategies. Indeed, it was demonstrated that parts of, or whole, inactivated, pathogens have a positive impact on the effectiveness of the TCL-derived vaccines. Experiments on animals and human trials were performed with viral particles *per se* (20), but also as an antigen delivery system, i.e. virosomes. Virosomes are spherical nanoparticles consisting of a non-viral component: a phospholipid bilayer, with an embedded viral component on the surface: influenza virus hemagglutinin and neuraminidase are the most popular choice. The viral components enable virosomes to fuse with antigen presenting cells (APCs) and release their contents directly into the APC cytoplasm, triggering immune response against the antigen in question (21). Virosomes also possess immunostimulatory properties on their own (22), and virosomal vaccines are capable of eliciting both T_h and CTL responses (23). Their efficacy has been demonstrated in many animal models (24) and in clinical trials, e.g. where virosomal vaccine containing Her2/neu peptides induced specific antibody production and a decrease in circulating regulatory T-lymphocytes in metastatic breast cancer patients (25).

A possible downside to virosomes is the fact that, due to the presence of only selected viral proteins, they only have a fraction of viral full immunostimulatory potential. The rather limited capacity of a single virosome is also considered a drawback. In this regard, bacterial ghosts (BGs), empty and intact non-living bacterial cell envelopes, comprise a platform that can serve both as a source of multiple adjuvants and a system for antigen delivery to DCs. BGs are generated by controlled expression of bacteriophage-cloned protein E-inducing lysis of Gram-negative bacteria (26,27). The product of E protein-specific lysis is the intact shell of the bacteria with a conserved surface and periplasmic molecules that serve as danger signals and immune potentiators (26,28,29) and an empty inside, which can be loaded with the desired antigens. Such a construct is ready to use as a compound delivery system (30), an anti-bacterial vaccine on its own (31,32) or a system for the induction of DC maturation and loading them with antigens, such as TAAs, in a single-step process (33). BGs represent a novel approach towards vaccination, immunomodulation and drug delivery boasting various product advantages. They are stable at room temperature (RT), non-living and carry almost no residual DNA. Having the external immunological properties of living bacteria, BGs act as a natural adjuvant (34).

Bacillus subtilis (*B. subtilis)* is known to have anticancer properties, both on its own, as well as its metabolites. *B. subtilis* protein metabolites retrieved from culture medium filtrate have been described as lectins (35) and have been investigated as potential anticancer agents due to their cytotoxic properties (36). Their application as adjuvant has been first tested in a murine sarcoma model, in combination with probiotics (36,37), interferon-γ (38) or on its own (39), where tumour growth inhibition, survival benefit, as well as stimulation of macrophages and spleen mononuclear cells were reported. Lewis lung carcinoma (LLC) model in C57BL/6 mice was also used with promising results, regarding tumour inhibition index (40,41), activity of antitumor effector cells (40-42) and mouse survival (41,42). *B. subtilis* is also known to have immunostimulatory properties on DCs; both on its own (43), as well as its metabolites (44).

In the present study, we investigated the immunostimulatory potential of *E. coli* Nissle 1917 BGs (as a source of multiple immune potentiators) and *B. subtilis* B-7025 70 kDa (*B.s.* B-7025) protein isolates as candidate adjuvants for TCL-based therapeutic cancer vaccine in a murine lung cancer model.

Materials and methods

Mice and cell lines. Eight- to 12-week-old female C57BL/6 mice were obtained from the State Research Institute Centre for Innovative Medicine (Vilnius, Lithuania). Mice were housed in plastic cages (10 mice/cage) under normal daylight conditions with water and food *ad libitum*. All animal procedures were carried out in accordance with the Directive of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes along with approval of the Lithuania State Food and Veterinary Service.

Murine metastatic Lewis lung carcinoma LLC1 (LLC) cell line was a kind gift from R.E. Kavestky Institute of Experimental Pathology, Oncology and Radiobiology (Kyiv, Ukraine). Cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) containing 2 mM L-glutamine, 10% foetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (both from Gibco, Paisley, UK) in humidified atmosphere containing 5% CO_2 at 37˚C. LLC cells were used for tumour implantation and for preparation of autologous tumour lysate.

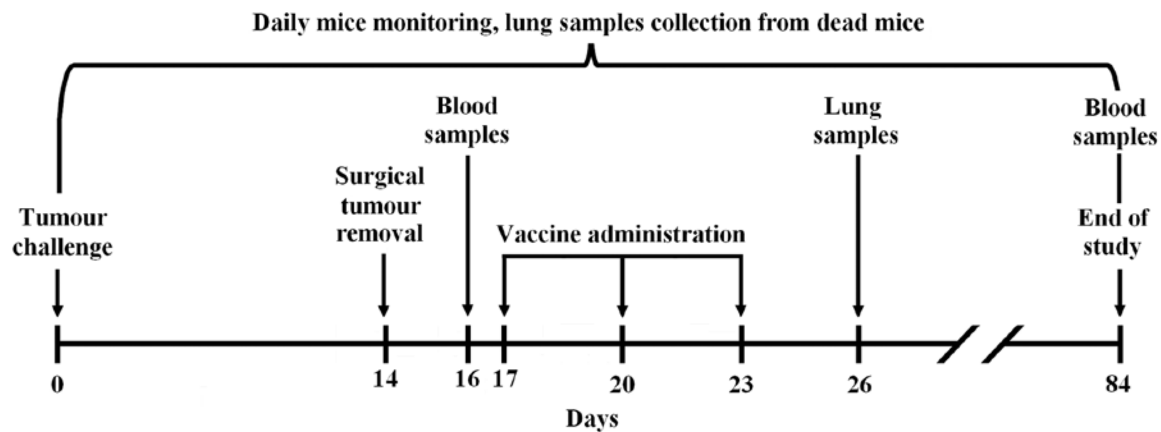

Figure 1. Experimental anticancer vaccination and sampling scheme for C57BL/6 mice challenged with Lewis lung carcinoma (LLC). Experimental design depicted from the time of tumour challenge (day 0) until the end of the survival observation (day 84).

BG preparation. Probiotic Gram-negative strain *E. coli* Nissle 1917 was used for the generation of BGs. BGs were produced by the controlled expression of the phage-derived lysis protein E, as we have described previously (45). Due to the safety reasons, the BG preparation was treated with β-propiolactone (Ferak, Berlin, Germany) to fully inactivate all residual non-lysed viable bacterial cells and DNA present in BG suspension, followed by extensive washing via tangential flow filtration method (45). Aliquots of washed BGs were frozen at -80˚C and lyophilized. Dry-powdered product was stored at RT until further use.

B. subtilis B-7025 protein preparation. The *B. subtilis* B-7025 protein with molecular mass of 70 kDa was retrieved from culture filtrate on day 10 by protein precipitation with ammonium sulfate (Alpharus NVP, Ukraine), followed by chromatographic separation on a Sephacryl column S-200 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), as described elsewhere (46,47). Acquired protein mix is not structural bacterial protein, but rather lectin metabolite of *B. subtilis* B-7025 (35,37,46,47). The protein concentration was determined by the Lowry method (48). The protein metabolite purity was verified by sodium dodecylsulphate polyacrylamide gel electrophoresis. The protein concentrate was sterilised using 0.20-μm filters (Sigma, St. Louis, MO, USA) and stored at -20˚C.

Preparation of autologous tumour lysate. To prepare autologous tumour lysate, LLC cells were collected via trypsin digestion (Gibco), washed with phosphate-buffered saline (PBS) (Lonza) 3 times and resuspended in 1 ml of PBS. Cells were treated with 6 freeze-thaw lysing cycles using liquid nitrogen and a 37˚C water bath in an alternating manner. Cells were centrifuged at 4,000 x g for 10 min, and the supernatant was collected and passed through a 0.2-μm syringe filter (Corning, Inc., Corning, NY, USA). Protein concentration was determined by the the Lowry method (48). Protein concentration in TCL was 150 μg/100 μl of PBS/dose.

Formulations of therapeutic vaccines. The first vaccine was generated by mixing 150 μg of LLC lysate with 0.1 mg ($2x10^8$ particles) of BGs in 100 μl of PBS per dose ('LLC+BGs' vaccine). The second vaccine was generated by mixing 150 μg of LLC lysate with 150 μg of *B. subtilis* B-7025 70 kDa protein mix in 100 μl of PBS per dose ('LLC+*B.s*.B-7025' vaccine). The third vaccine consisted of LLC lysate alone, i.e. without adjuvant. Injection of PBS alone served as a negative control (control group).

Tumour challenge and therapeutic vaccination. Mice received a subcutaneous (s.c.) injection of $3x10^5$ LLC cells in the left hind-foot on day 0. On day 14, primary tumours were surgically removed by amputating the foot. Mice with the primary tumour removed were subsequently treated with either LLC+BGs vaccine (n=13), or LLC+*B.s*.B-7025 vaccine (n=13), or LLC lysate alone (n=11). Tumour-challenged, but untreated mice (n=13) served as a control group. Mice in the treated groups were vaccinated according to the same scheme; each vaccine was injected s.c. into the nape of the neck on days 17, 20 and 23. See vaccination scheme in Fig. 1. Mice were observed until day 84.

Sampling. In order to assess the anticancer effectiveness of the vaccines, mouse lung and blood samples were obtained (Fig. 1). Lungs were analysed for metastasis since they are the preferred metastatic location for the LLC cell line (49). Blood samples were collected from the hip vein before vaccination onset (day 16) and from the surviving mice of each experimental group at the end of the experiment (day 84). Three days after the completion of the therapeutic vaccination course (day 26), 3 mice from each group were sacrificed to determine the presence of micrometastases. Mice found dead during the follow-up period underwent histological analysis as well. The survival of mice was observed daily throughout the experiment, with the exception of mice sacrificed on day 26, which were not taken into account.

Analysis of metastasis. In each case, lung tissue was fixed in 10% neutral buffered formalin, dehydrated in alcohol baths (70% for 12 h/90% for 12 h/100% for 24 h) and embedded in paraffin. The formed paraffin blocks were cut with a microtome into slices 3-μm thick. The sections were deparaffinised, rehydrated and stained with hematoxylin and eosin morphological stain. Each section was examined with a light

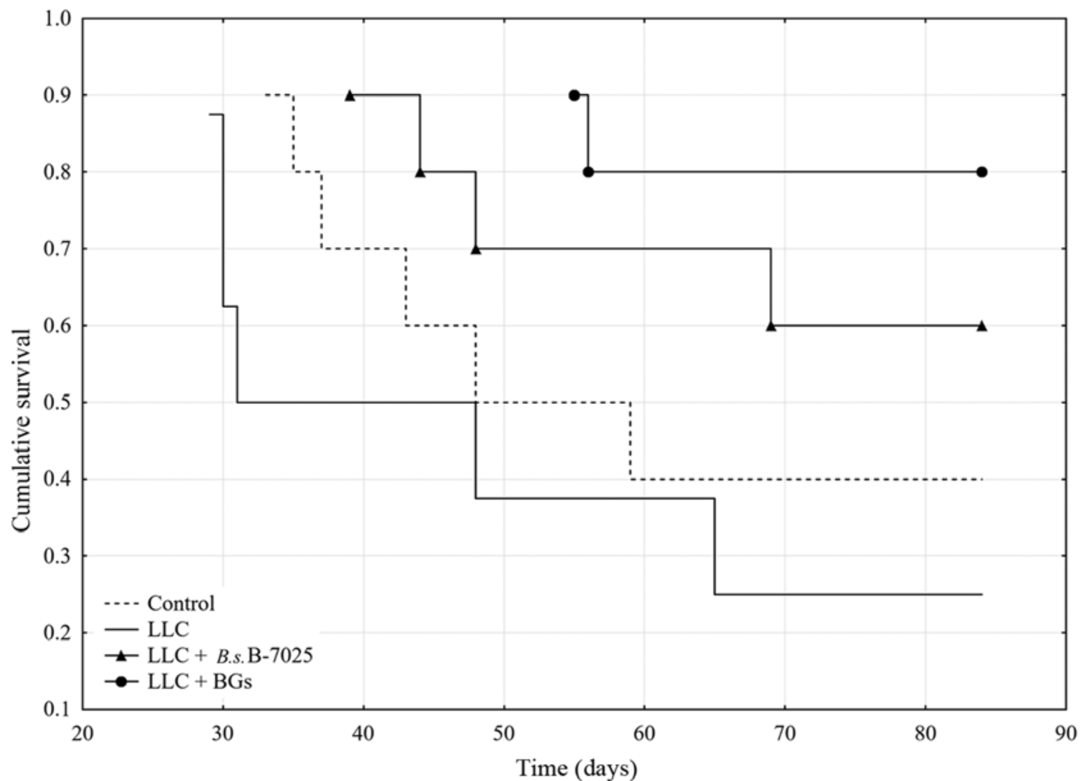

Figure 2. Kaplan-Meier survival curves for C57BL/6 mice undergoing anticancer autologous lysate-based therapeutic vaccination. Control, Lewis lung carcinoma (LLC), LLC+*B.s.*B-7025 and LLC+BGs groups are plotted from observation day 25 to 84.

microscope in order to identify the tumour-infiltrated areas. Images were captured with an automated Leica DM50000 B microscope equipped with a Leica DFC420 C digital camera (Leica, Wetzlar, Germany). Images were processed using the 'Sharpen' option in the 'ImageJ' image analysis program (50). The area of all tumour nodules that were found in lung tissue was estimated by 'Freehand selection tool' in 'ImageJ', and measurements were expressed in mm^2.

Flow cytometry. Blood samples were collected and analysed by flow cytometry for CD8a surface expression at two different time-points (days 26 and 84). One hundred microliters of whole blood was stained with PE-Cy7-conjugated rat anti-mouse CD8a antibody (cat 552877), according to the manufacturer's instructions and lysed with FACS lysing solution (both from BD Pharmingen, San Diego, CA, USA). Samples were acquired with LSR II flow cytometer (BD Pharmingen), using 488 nm excitation laser and 780/60 band pass filter. At least $1x10^5$ cells were analysed with FACSDiva software (BD Pharmingen). Singlets and alive cells were identified based on forward (FSC) and side (SSC) scatter profile.

Statistical analysis. All statistical analyses were performed using Statistica 10 software (StatSoft, Tulsa, OK, USA). Survival was evaluated by Kaplan-Meier method and differences in survival distributions were assessed using ANOVA. Weighted ANOVA variant was used to take into account the differences in the mouse count in the groups. Post-hoc analysis was performed using the Dunnett's test. Differences between tumour areas in lung histochemistry slides were assessed using the Mann-Whitney U-test. Flow cytometry data were analysed with the unpaired, two-tailed Student's t-test. Differences were considered statistically significant for P-values <0.05.

Results

Survival analysis. The differences in the survival between the groups were analysed using several statistical models. Kaplan-Meier survival curves are displayed in Fig. 2. By the end of the observation period (day 84) the survival rate in the LLC+BGs group, LLC+*B.s.*B-7025 group, LLC group and control group was 80, 60, 25 and 40%, respectively.

ANOVA survival analysis [F(2,25)=5.0116, P=0.01478] indicated statistically significant differences in survival times between the groups (Table I). Post-hoc analysis showed that mice in the LLC+BGs group, but neither in the LLC+*B.s.*B-7025 group nor the LLC group, survived significantly longer compared with the untreated control. This indicated that treatment with LLC+*B.s.*B-7025 and non-adjuvanted LLC lysate had no therapeutic effect in this experimental setting. There was no significant survival difference between mice treated with LLC+BGs and LLC+*B.s.*B-7025 vaccines, but the group treated with LLC lysate alone had statistically lower survival than the groups treated with LLC+BGs or LLC+*B.s.*B-7025 vaccines (Table II).

Analysis of metastasis. Three days after the last vaccination, no metastatic foci were found in the sacrificed mice in any of the groups. On the other hand, morphological analysis of the mice that died during the follow-up period revealed

Table I. Survival of the C57BL/6 mice after removal of LLC tumour and subsequent therapeutic vaccination with various formulations of syngeneic LLC lysate.

Group	Days (median)	Days (mean)	Days (Std.Err.)	Days (-95.00%)	Days (+95.00%)	n (total=28)
Control	53.5	59	7	43	75	10
LLC	39.5	50	9	30	70	8
LLC+*B.s.*B-7025	84.0	70	6	57	84	10
LLC+BGs	84.0	78	4	70	87	10

LLC, Lewis lung carcinoma; BGs, bacterial ghosts; *B.s.*B-7025, *B. subtilis* B-7025 70 kDa.

Table II. ANOVA post-hoc analysis of survival (Dunnett's method).

Group	P-value			
Control	-	0.3486	0.9834	0.9989
LLC	0.9621	-	0.9989	1.0000
LLC+*B.s.*B-7025	0.2296	**0.0485**	-	0.9559
LLC+BGs	**0.0475**	**0.0070**	0.3750	-

Statistically significant differences are +marked in bold. LLC, Lewis lung carcinoma; BGs, bacterial ghosts; *B.s.*B-7025, *B. subtilis* B-7025 70 kDa.

development of pulmonary metastatic foci in 100% of the analysed control and LLC samples. In contrast, only 33% of the analysed lung samples of both the LLC+*B.s.*B-7025 and LLC+BGs groups displayed signs of metastasis. Fig. 3 shows representative lung slices presenting various spreads of metastasis in the investigated groups. The mean size of the metastatic tumours in the LLC group was 0.452±0.3 mm^2, whereas in the LLC+*B.s.*B-7025 group it was 0.016±0.013 mm^2 and in the LLC+BGs group only 0.006±0.0001 mm^2. Mann-Whitney U-test confirmed that the LLC+BGs metastasis size was significantly smaller then that of the LLC group (P=0.019), unlike the LLC+*B.s.*B-7025 group (P=0.514).

Flow cytometry. Whole blood samples were analysed for CD8a surface antigen expression at two different time-points (before the vaccination and at the end of the study). Representative graphs are shown in Fig. 4. Summarised results are presented in Fig. 5. The number of cells expressing CD8a remained at the same level before and after vaccination in both the control and LLC+*B.s.*B-7025 groups. On the other hand, compared to the measurement taken before the vaccinations, the mice treated with non-adjuvanted LLC lysate had a significantly lower CD8a count in the blood at the end of the study (P<0.001), whereas the mice in the LLC+BGs group had a significantly higher $CD8a^+$ cell count at the end of the study (P=0.019). Notably, at the end of the study, CD8a expression in the LLC+BGs group was significantly higher than that noted in the control (p=0.047), LLC+*B.s.*B-7025 (P=0.026) and LLC (P=0.002) groups. Mice treated with LLC+*B.s.*B-7025 vaccine had a higher $CD8a^+$ cell count than that noted in the mice treated with the LLC lysate alone (P<0.001), but not statistically different from the control group (P=0.782) (Fig. 5).

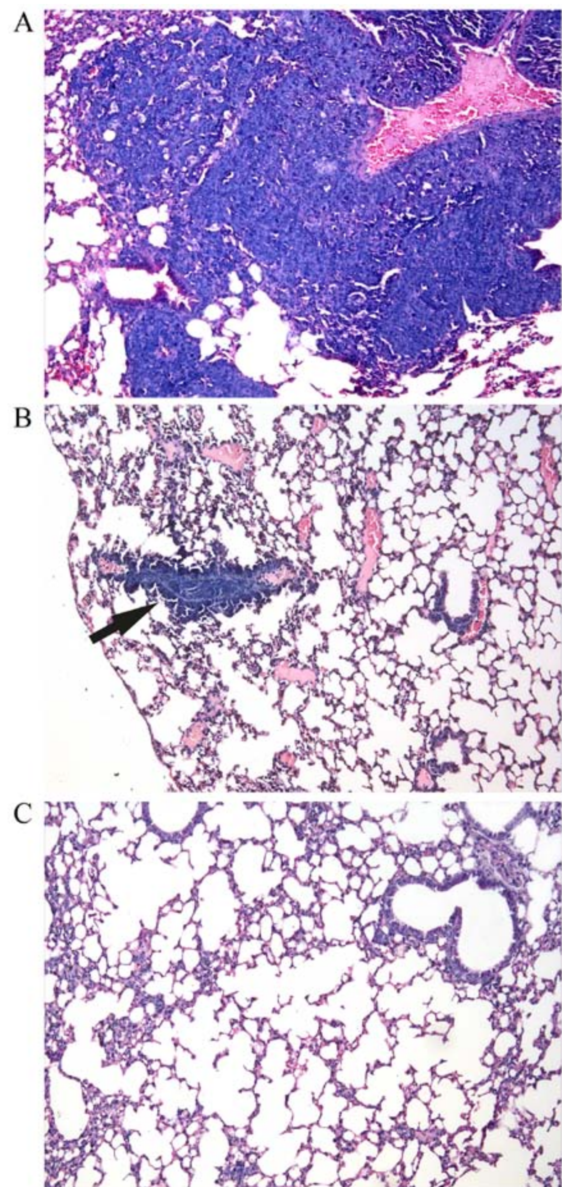

Figure 3. Histochemical staining of lung samples from the dead C57BL/6 mice. Staining, hematoxylin and eosin. Magnification, x100. (A) Metastatic focus fills the entire field of view. (B) Metastatic focus is indicated by arrows. (C) Clean lung slice with no sign of metastasis.

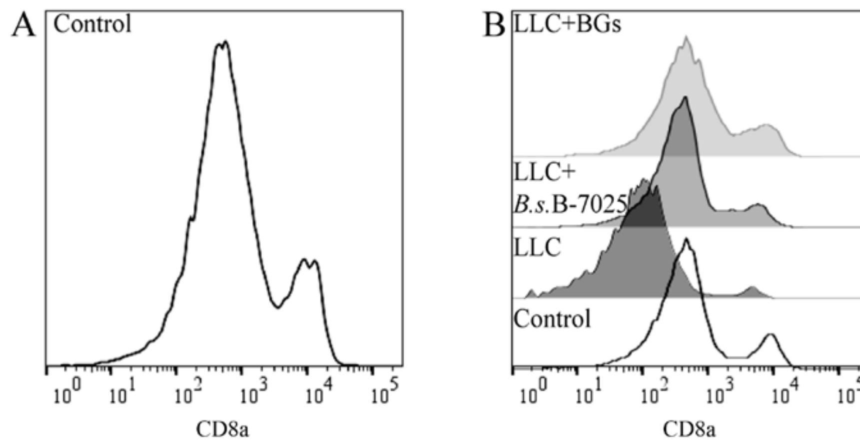

Figure 4. Half-offset histograms of the CD8a signal registered on a flow cytometer before vaccination (A) and at the end of the study (B). Control (white) and the three therapeutic groups are displayed (shades of grey).

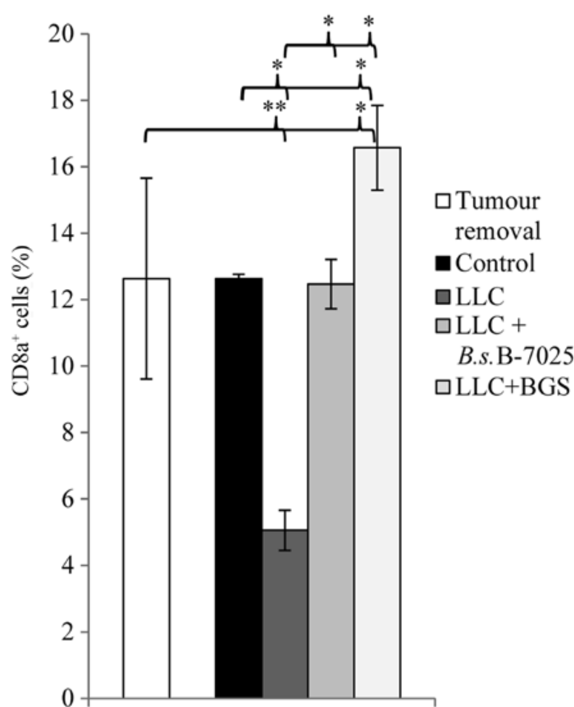

Figure 5. Data represent the mean ± SD of three independent measurements performed using murine lymphocytes obtained before the vaccination treatment (white) and at the end of the study in the various treatment groups (shades of grey). See Fig. 1 for sampling details. *P<0.05 and **P<0.01.

Discussion

In the present study, C57BL/6 mice, inoculated with metastasizing LLC cells, received adjuvant treatment with therapeutic vaccines following surgical removal of primary tumour. The proposed approach is clinically relevant, due to the fact that management of micrometastatic or unresectable metastatic disease in patients after primary tumour resection is still a challenge in modern oncology (51,52). Primary tumour removal was introduced to focus more on metastasis inhibition rather than on tumour growth inhibition. Lungs were investigated for metastatic foci due to the known affinity of LLC cells, which spread preferentially to lung tissue (49). Syngeneic LLC lysate was used as a vaccine (source of tumour antigens for immunization). To counter the potential immunosuppressive characteristics of the tumour lysate (17) and augment the evolving antitumour immune responses, two distinct bacterial-based adjuvants were used with the therapeutic vaccination. These adjuvants included either *B. subtilis* B-7025 70 kDa protein isolates or bacterial ghost generated from probiotic *E. coli* Nissle 1917. Whole bacterial-based adjuvant systems gained attention in oncology with the introduction of bacillus Calmette-Guérin immunotherapy for the treatment of non-muscle invasive bladder cancer (53). Currently this type of cancer immunotherapy was further improved by manipulating bacteria and loading them with tumour antigens in order to ensure tumour antigen specificity of the elicited immune responses. Such innovative approaches include live, attenuated *Listeria monocytogenes* bacteria consisting of gene deletions to diminish their pathogenicity and engineered to express tumour antigens (54). Recent data have shown that immunotherapy with mesothelin-expressing, live, attenuated *L. monocytogenes* CRS-207 plus chemotherapy demonstrate encouraging clinical activity in patients with malignant pleural mesothelioma (55). Our investigated BG platform also emerges as a very promising immunotherapeutic tool of this kind. It may even be safer, since it exploits completely non-living and intact bacterial envelopes rather than attenuated or killed, but metabolically active, bacteria.

The study results showed that treatment of tumour-bearing mice with LLC+BGs or LLC+*B.s.*B-7025 prevented the formation of lung metastasis in 67% of the mice, while no protective effect was detected in the mice treated with LLC lysate-alone and in mice without treatment (control group) (Fig. 3). Although the median overall survival of the mice treated either with LLC+BGs and LLC+*B.s.*B-7025 was the same (84 days) and increased compared with the controls (53.5 days), only the treatment with LLC+BGs led to a statistically significant median overall survival (Fig. 2). Moreover, the survival rate of the mice from the LLC+BGs treatment group at day 84 was significantly increased when compared with the survival rate of mice from the LLC+*B.s.*B-7025 treatment group (80 vs. 60%). Mice treated with non-adjuvant LLC lysate had a significantly shorter survival than those treated with LLC+BGs or LLC+*B.s.*B-7025

Cytotoxic lymphocytes are thought to be the key factor in successful anticancer immune response (56). Smaller metastatic foci found in lung samples (Fig. 3) can explain why the LLC+BGs vaccine achieved a better survival rate than LLC+*B.s.*B-7025. Smaller metastatic foci found in lung samples of the mice treated with the LLC+BGs vaccine (compared to lung samples of mice from other treatment groups) along with improved median overall survival of mice might be associated with the detected elevated numbers of circulating CD8a$^+$ T cells at the end of the study. Subcutaneous administration of vaccine made of LLC+BGs elicited a significantly higher number of circulating CD8a$^+$ T cells in the tumour-bearing mice compared to the numbers of total circulating CD8a$^+$ T cells detected in blood samples obtained from the non-treated mice as well as from mice treated with the vaccine made of LLC+*B.s.*B-7025 and LLC alone. Moreover, only mice treated with LLC+BGs vaccine, compared to other treatment groups, had at the end of the study a significantly higher number of circulating CD8a$^+$ T cells than before the vaccination onset (Fig. 5). Noteworthy, the standard deviation of CD8a$^+$ cells in the LLC+BGs group was three times smaller when measured in the surviving vs. the dead mice at the end of the study (data not shown). This fact implies that the surviving mice can be characterised by a successful CTL mobilisation. This is in line with already published data, where authors also detected higher CD8a levels only in responder mice, treated with a TCL-based vaccine (57).

Notably, the level of blood CD8a$^+$ T cells was significantly decreased in the mice treated with the non-adjuvant-modified LLC lysate compared with the controls and mice treated with adjuvanted LLC lysates (Fig. 5). In addition, mice treated with non-adjuvanted LLC lysate showed the lowest survival rate of only 25% compared with 80, 60 and 40% in the LLC+BGs, LLC+*B.s.*B-7025 and control groups, respectively. We assume that various immunosuppressants present in the tumour lysate were responsible for the decrease in CD8a$^+$ T cell population as a result of predominant cancer-associated immunosuppressive environment. However, coupling of LCC lysate vaccine preparations with strong immunostimulators, especially with BGs, can modulate the character of LCC lysate from immunosuppressive to immunostimulatory by improving both the recognition and presentation of tumour-associated neo-antigens by professional antigen-presenting cells capable then of activating and (re)stimulating effector immune competent cells. Indeed it has been previously shown that the generation of tolerogenic DCs was induced by culturing immature DCs from healthy donors with the plasma of pancreatic cancer patients (58) or supernatants of various tumour cell lines (14,59).

In the light of this preliminary study, BGs emerge as a novel adjuvant and antigen delivery platform for TCL-based therapeutic cancer vaccination. This straightforward and potentially clinically effective immunotherapeutical approach requires more extensive pre-clinical investigation and warrants consideration outside the animal models.

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