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Activated Charcoal Enhanced the Antigen-expression and Dendritic Cell Maturation of the Vaccine Using *Listeria*-platform

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ABSTRACT

Background: *Listeria monocytogenes* (LM) has been used as a vaccine vector based upon its ability to induce a strong cell-mediated immune response. LM inactivated with γ -irradiation retains immunogenic properties and is an attractive platform for clinical use since it would have improved safety concerns compared to live vectors. Activated charcoal has been shown to enhance expression of LM proteins such as PrfA.

Aim: To investigate the effect of various growth conditions supplemented with activated charcoal on recombinant antigen expression.

Methods: We prepared γ -irradiated ovalbumin-expressing LM (LM-OVA) after growth under various culture conditions. We cultured LM-OVA at various temperatures including 25°C, 37°C and 37°C with activated charcoal and compared OVA expression by western blot analysis, dendritic cells maturation and OVA-specific T cells.

Results: The OVA expression was highest in γ -irradiated LM-OVA grown with activated charcoal at 37°C. Compared to other growth conditions, γ -irradiated LM-OVA grown with activated charcoal at 37°C induce better DC maturation as well as production of the highest number of antigen-specific IFN γ -secreting T cells.

Conclusion: The further study should be demonstrated the potential to alter growth conditions to enhance OVA expression resulting for vaccine vectors, thereby improving their safety and efficacy.

INTRODUCTION

Live attenuation and inactivation of bacterial pathogens have been used as vaccination strategies since the beginning of the development of vaccines.⁽¹⁾ Inactivation of vaccines, for example by application of heat, is a classic tool that results in safe vaccines with relatively weak immunogenicity. In contrast, live attenuated vaccines raise safety concerns but induce long-term immunity. Live, attenuated strains of many bacteria that synthesize and secrete foreign antigens are being developed as vaccines for a number of infectious diseases and cancers.⁽²⁾

The most generally used bacterial vectors include microbes such as *Listeria monocytogenes* (LM)⁽³⁾, *Escherichia coli*,⁽⁴⁾ and *Salmonella*.⁽⁵⁾ Bacteria-based vaccines have advantages compared to other antigen delivery strategies, including low cost of production, absence of animal products, genetic stability, and safety.⁽⁶⁾ Bacterial vaccination induces innate immunity and stimulates an adaptive immune response, as seen in LM-based vaccines delivering a tumor-associated antigen (TAA) that resulted in efficacious anti-tumor effects.⁽⁷⁻⁹⁾ LM, a facultative anaerobe, intracellular bacterium, is the causative agent of listeriosis. It is one of the most virulent food-borne pathogens, with 20 to 30 percent of clinical infections resulting in death.⁽¹⁰⁾ A number of biological properties make LM a promising platform for the development of vaccines, particularly those used against infectious diseases or cancers. An infection with LM is accompanied by a strong innate immune reaction that leads to pronounced specific cellular immune responses, including both CD8⁺ and CD4⁺ T-cell activation of a Th₁ phenotype.⁽¹¹⁾ Systemic infection of mice with LM has been an important model for understanding listeriosis and host responses to other intracellular pathogens. Infection of both phagocytic and non-phagocytic cells depends on bacterial expression of listeriolysin O (LLO)⁽¹²⁾, actin nucleator A (ActA)⁽¹³⁾ and pluri-potential transcription factor (PrfA).⁽¹⁴⁾ LLO mediates escape of LM from phagosomes into the cytosol, resulting in MHC class I presentation of LM antigens to CD8⁺ T cells. In the cytoplasm, ActA protein is switched on, allowing polymerization of host cell actin that propels bacterial spread from cell to cell.

A previous study reported induction of virulence factor expression by activated charcoal in LM.⁽¹⁵⁾ PrfA is proposed as a major regulator of LM virulent gene expression, especially for presentation of a putative PrfA box group I. Brain heart infusion broth supplemented with activated charcoal (BHIC) has been reported to optimize virulent gene expression.⁽¹⁶⁾ However, the exact mechanism of induction by activated charcoal is still unknown.

LM based-vaccination strategies focusing on synthesis and secretion of foreign antigens are being developed as vaccines for a number of infectious diseases and cancers.⁽¹⁷⁻¹⁸⁾ γ -irradiated LM has been proposed as a safe yet immunogenic alternative to live attenuated vectors.⁽³⁾ We hypothesized that growth conditions prior to γ -irradiation of LM-OVA would influence expression of recombinant expressed antigens and immunogenicity.

MATERIALS AND METHODS

Bacteria, growth conditions and animals

Listeria monocytogenes wild type (LM-WT) strain 10403s and ovalbumin-expressing LM (LM-OVA)⁽¹⁹⁾ were kindly provided from Raz E. (Rheumatology Section, University of California San Diego 10403s was originally from Dan Portnoy at UC Berkeley, and LM-OVA was originally from H. Shao at U Penn). The LM were cultured from frozen stock in BHI and plated on BHI agar to determine colony-forming units. The LM was classified and cultured into three groups, 25°C, 37°C, and 37°C supplement with 0.02% activated charcoal (CECA S, Hauts de Seine, France).⁽¹⁵⁾ The cultured growth conditions were shaken at 37°C for 8 hr and after that adjusted by using 1:10 dilution into fresh broth up to OD600 equal to 1 as modified from Ripio *et al.*⁽²⁰⁾ For irradiation, the cultured LM were washed and re-suspended with PBS (pH 7.2) at 10¹⁰ bacteria/ml and exposed to 600 krad of the γ -irradiation over 380 min with a 137 Cesium source.⁽³⁾ C57BL/6 mice (6 mice per group and duplicated experiments) were obtained from University of California San Diego (UCSD) animal facilities (bred in-house).

Western blot analysis

Protein samples (20 μ l) were separated on 10% SDS–polyacrylamide gels and were

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analyzed by western blotting. Fractionated proteins were then transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences, Germany) using transfer buffer (48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% [vol/vol] ethanol) and a semi-dry electro-transfer unit. The membranes were blocked with 5% low-fat milk, washed, and incubated with polyclonal rabbit antibodies directed against OVA. Peroxidase-conjugated goat-anti-rabbit immunoglobulin G (Dianova, Germany) was used as the second antibody. Bands were visualized by incubation of each membrane with western blot detection reagents (Amersham Biosciences) and exposure to Kodak XAR film.

Bone-marrow-derived dendritic cells

Mouse bone-marrow-derived DCs were cultured as previously described. In brief, bone marrow from femurs and tibia of C57BL/6 mice was plated on day 0 at 5×10^5 cells/ml in DC medium, which consisted of RPMI supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml recombinant murine GM-CSF. On day 3, an equal volume of DC medium was added. The non-adherent cells were harvested on day 6. Flow cytometry, after staining with antibody against the following cell-surface marker was conducted to characterize the cultured cells after stimulation of CD40.⁽³⁾

Intracellular cytokine staining and flow cytometry

Naïve and vaccinated mice were intraperitoneally challenged with 10^5 live LM 2 weeks after the last vaccination. Three days after challenge, splenocytes were harvested and inoculated for 6 hr in the absence or presence of whole irradiated LM-OVA peptide (SIINFEKL, PeptidoGenic Research).⁽²¹⁾ GolgiStop was added during the last 4 hr of incubation. Cells were stained for CD4, CD8 and IFN- γ according to the manufacturer's instructions (BD PharMingen). Flow cytometry, was acquired on FACSCalibur flow cytometer with cellQuest Software after staining of the cells with the indicated antibodies in PBS containing 0.5% BSA and 0.05% sodium azide according to standard protocols. Data were analyzed with FlowJo software, respectively.⁽³⁾

Statistical analysis

Mann-Whitney's U-test was used to determine the statistical significance of the values obtained and $P < 0.05$ was considered statistically significant.

RESULTS

Effect of LM-OVA growth conditions on OVA expression by western blot analysis

OVA expression after γ -irradiation of LM-OVA grown in various growth conditions is shown in Figure 1. Compared to other conditions, there was similar pattern of various LM-OVA expression. Slightly increase was found in OVA expression when LM-OVA were grown at 37°C supplemented with activated charcoal.

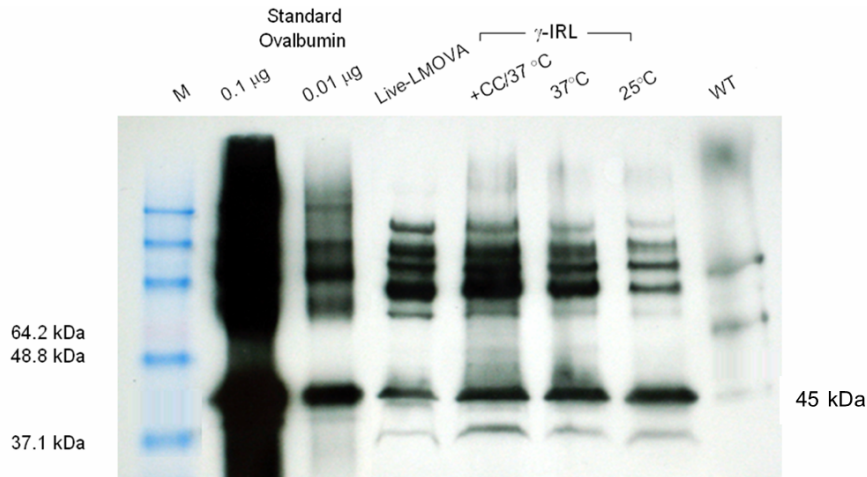


Figure 1. OVA expression is affected by *LM*-OVA growth conditions. The OVA (45 kD) expression was assessed followed by γ -irradiated *LM*-OVA grown under the indicated conditions at 25°C, 37°C, 37°C supplemented with activated charcoal compared to live-*LM*-OVA and *LM*-wild type. Standard ovalbumin of 0.1 μ g and 0.01 μ g were used as positive control OVA expression. Slightly increase *LM*-OVA expression of at 37°C supplemented with activated charcoal than other condition was shown. [Remark: M; marker, CC; activated charcoal, WT; wild type, γ -IRR; γ -irradiated *Listeria*, *LM*-OVA; *Listeria monocytogenes*-ovalbumin].

Effect of *LM*-OVA growth conditions on dendritic cells

Induction of and adaptive immune response requires DC maturation and antigen presentation as shown in Figure 2. We assessed the ability of the various γ -irradiated *LM*-OVA preparations to induce DC maturation. *LM*-OVA grown at 37°C with activated charcoal showed the greatest ability to upregulate the maturation marker CD40 on DC. The result showed increase of specific induction, continuously.

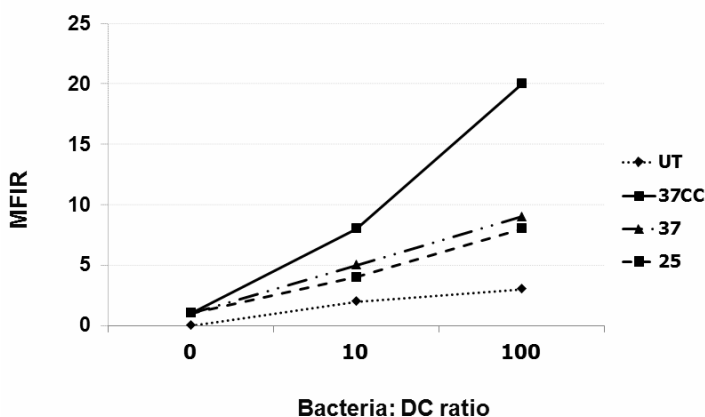


Figure 2.

DCs maturation by γ -irradiated *LM*-OVA. Expression of the DCs maturation marker CD40 after incubation of DC with *LM*-OVA that had been irradiated after exposure to the indicated growth conditions (25°C, 37°C and 37°C supplemented with activated charcoal). C57BL/6 bone-marrow-derived DCs were cultured overnight with γ -irradiated *LM*-OVA

(25°C, 37°C and 37°C supplemented with activated charcoal) or live *LM*. CD40 induction was determined by flow cytometry and expressed as mean fluorescence intensity ratio (MFIR = fluorescence of treated cells/ fluorescence of untreated cells)

OVA-specific T cells showed the most susceptible by activated charcoal condition

Findings showed the most quantity of CD8⁺ T-cells regarding the percentage of IFN- γ positive cells (see Figure 3). The irradiated LM-OVA with activated charcoal condition showed the most susceptibility to infect regarding CD8⁺ T-cells presentation. The highest observation was represented at 37°C supplemented with activated charcoal growth situation as well as untreated--non γ -irradiated LM-OVA. The induction of γ -irradiated LM-OVA caused by its activation was also shown to be the highest percentage in CD8⁺ rather than CD4⁺ T-cells respectively.

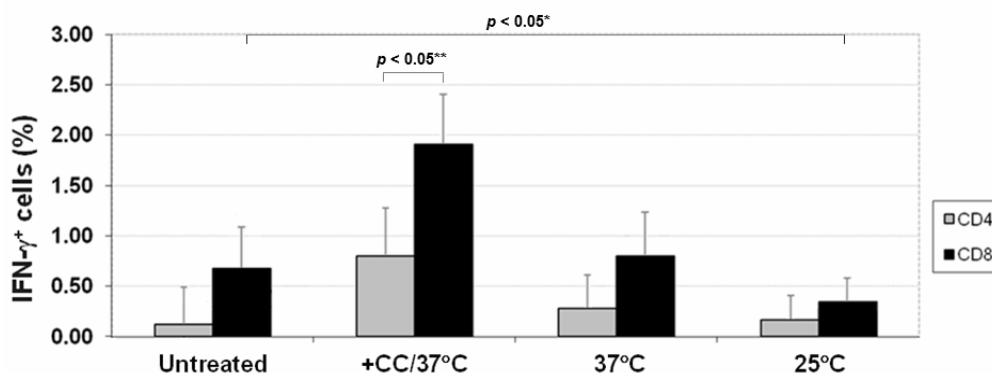


Figure 3. T cells induction by γ -irradiated LM-OVA. The γ -irradiated LM-OVA after growth under indicated conditions (25°C, 37°C and 37°C supplemented with activated charcoal) or live LM-OVA growth at 37°C were used to immunize mice (n = 6 mice/ group). OVA-specific IFN- γ ⁺ CD4 and CD8 T cell frequencies (as a percentage of total cells) was determined by flow cytometry. The most induction of DC maturation between CD4 and CD8 T cell was significant different ($p < 0.05$; Mann Whitney U test) at 37°C supplemented with activated charcoal as well as untreated (non γ -irradiated LM-OVA; live LM-OVA). [Remark: CC; activated charcoal, WT; wild type, γ -IRL; γ -irradiated *Listeria*, LM-OVA; *Listeria monocytogenes*-ovalbumin].

DISCUSSION

Virulent genes of LM-WT are essentially controlled by the major transcriptional regulator PrfA because the PrfA activates genes of virulent gene clusters of LM, including *prfA*, *plcA*, *hly*, *mpl*, *actA* and *plcB*, as well as the expression of *inlA* and *inlB* which have invasion protein properties.⁽²²⁾ The suitable synthesis condition is activated at 37°C and cultured in the presence of activated charcoal.^(15-16, 20) It has been reported that activated charcoal can promote growth due to decomposition of active oxygen⁽²³⁾ and carbon source⁽²⁴⁾. This is similar to this study's hypothesis that the supplemented with activated charcoal can induce essential protein expression of LM-OVA. The study's findings showed the percentages growth observed in three different matrices for 25°C, 37°C, and 37°C supplemented with activated charcoal compared to wild type live LM-OVA. The populations of LM-OVA were slightly different in various expressions of LM-OVA compared to LM-WT. The highest expression of LM-OVA was found at 37°C supplemented with activated charcoal by western blot analysis. Although, enhancement of virulent gene expression by LM mutant as well as wild type have been previously studied, and especially for a presence of activated charcoal are well known⁽¹⁵⁾, but our study showed effect of altered LM-OVA expression after using γ -irradiation technique. It should be investigated for the further

enhancing vaccination and prolong immunity.

The study suggests that the activated charcoal at 37°C can induce *LM*-OVA expression. Although, it remains unclear how activated charcoal is essential for *LM*-OVA growth, it is reasonable that activated charcoal promotes the growth of some bacteria on nutritional media due to removal of toxic compound.⁽¹⁵⁾ A 2010 study of necessary activated charcoal reported that charcoal yeast extract media was proposed as a crucial factor for growth of *Legionella pneumophila*.⁽²⁵⁾ Both *Legionella* and *Listeria* have been added to the Boxed Warning for the entire class of TNF α blockers. They can cause serious and potentially fatal outcomes in patients treated with TNF α blockers.⁽²⁶⁻²⁷⁾ Therefore, it appears that the supplements of activated charcoal at 37°C of *LM*-OVA should be the suitable growth condition as induced protein expression procedure resulting for further immune-therapeutic rationale, especially used with the γ -irradiation.

Our study examined the efficacy of γ -irradiated induction as DC are the most powerful antigen presenting cells that initiate the primary immune response.⁽²⁸⁻³⁰⁾ The representation of *ex vivo* with γ -irradiated *LM*-OVA to DC promoted cellular immunity at 37°C supplemented with activated charcoal growth condition doing well. These results indicated that induction of γ -irradiated *LM*-OVA at 37°C supplemented with activated charcoal induced DC maturation more successfully than *LM*-WT. Our result did not show increase of specific ratio of DCs with antigen presentation, continuously. We showed only initial induction of antigen presentation of DCs with recombinant *LM*-OVA expression after γ -irradiation. Our results referred that specific immunity by MHC class II induction is required. In the fact that activation of immunity is kinetic bacterial infections both innate and adaptive response, therefor more investigation such as prolong antigen presentation, duration, antigen persistence and induction of rapid recall effector functions should be further elucidated.

Previous studied found that irradiated *LM* can induce CD4⁺ and CD8⁺ T cell response.⁽³¹⁾ It has been proposed that dependent on both CD4⁺ and CD8⁺ T cells can induce immunity. Similar to our study that using platform of γ -irradiated *LM*-OVA with the optimal growth condition showed immunity induction, resulting as a further therapeutic vaccine method was supported. This study introduced more information of the basic tool of different growth conditions followed by the γ -irradiated *LM*-OVA. The hypothesis of this study was that appropriate *LM*-OVA expression in interested growths condition with their γ -irradiation should induce more immunity. The *in vivo* mouse vaccinated cellular immunity via CD8⁺ followed by CD4⁺ responsive cells at 37°C supplemented with activated charcoal was the strongest IFN- γ induction. The findings similar to induction of specific CD8⁺ T-cells against *LM*-OVA can be the cellular arm of adaptive immunity are important⁽³²⁻³³⁾ especially as this study showed an interested protocol of supplemented with activated charcoal after the γ -irradiated *LM*-OVA technique. So, this investigation gave the important tool for enhancement of immunogenicity expression and strongly supported by the vaccination with irradiated *LM* induced protective T cell immunity, previously.⁽³⁾ The future investigation on γ -irradiated *LM*-OVA induce protective immunity of platform of activated charcoal should be explored. Moreover, comparison of various interested protein expression as well as different bacterial vectors should be required.

The γ -irradiated *LM*-OVA was recognized as an appropriate rationale for use as therapeutic vaccination bacterial induced immunity.⁽³⁾ The optimal condition of 37°C supplemented with activated charcoal may be necessary and an important growth condition to induce interested protein expression. Additional experiments are required to define what and how the exact mechanisms of activated charcoal are. It may be used as a supported

platform for important technique to improve interested protein expression. However, future study needs to focus on further specific targets of essential regulatory pathways, prolong immunity induction as well as protection study should be investigated. Finally, suitable strategies of enhancing prophylactic and therapeutic LM-based vaccines as well as cancer immunology association should be further elucidated.

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