Kobe J. Med. Sci., Vol. 58, No. 3, pp. E63-E71, 2012

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

SURASAK WANRAM[1, 2 *], DONALD GUINEY[3], PAWANA PANOMKET[1], and PATCHAREE JEARANAIKOON[2]

[1]*Department of Pathology, College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani, Thailand*

[2]*Centre for Research and Development of Medical Diagnostic Laboratories (CMDL), Faculty of Associated Medical Sciences, Khon Kaen University, Thailand,*

[3]*Department of Medicine, Faculty of Medicine, University of California San Diego, USA*

Received 11 May 2012 / Accepted 13 June 2012

Key Words: *Listeria monocytogenes* (*LM*), γ-irradiated *LM*-ovalbumin (OVA), Activated charcoal supplement, *LM*-based vaccines

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 IFNγ-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)

Phone: +66-45-353-900 ext. 5843 Fax: +66-45-353-901 E-mail: Mdsurawa@ubu.ac.th

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 Th_1 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 10^{10} 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 µl) were separated on 10% SDS–polyacrylamide gels and were

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 $5x10^5$ 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 μg/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 signification.

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.

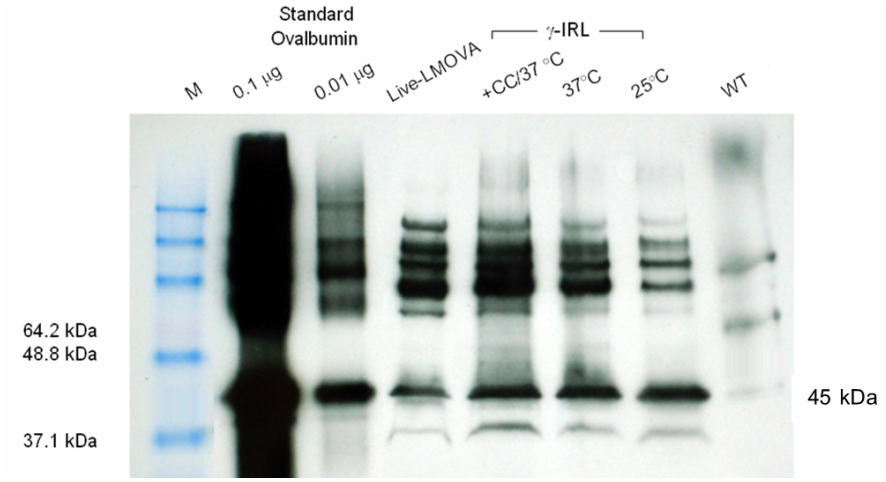

Figure 1. OVA expression is affected by *LM*-OVA growth conditions. The OVA (45 kD) expression was assessed followed by γ-irradiated *LM*-OVA grown under the indicated conditions at 25°C, 37°C, 37°C supplemented with activated charcoal compared to live-*LM*-OVA and *LM*-wild type. Standard ovalbumin of 0.1 µg and 0.01 µg 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-IRL; 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.

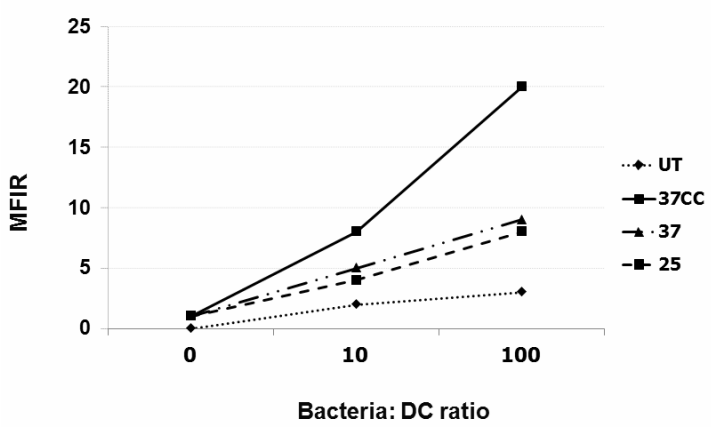

Figure 2. DCs maturation by γ-irradiated *LM*-OVA. Expression of the DCs maturation marker CD40 after incubation of DC with *LM*-OVA that had been irradiated after exposure to the indicated growth conditions (25°C, 37°C and 37°C supplemented with activated charcoal). C57BL/6 bone-marrow-derived DCs were 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.

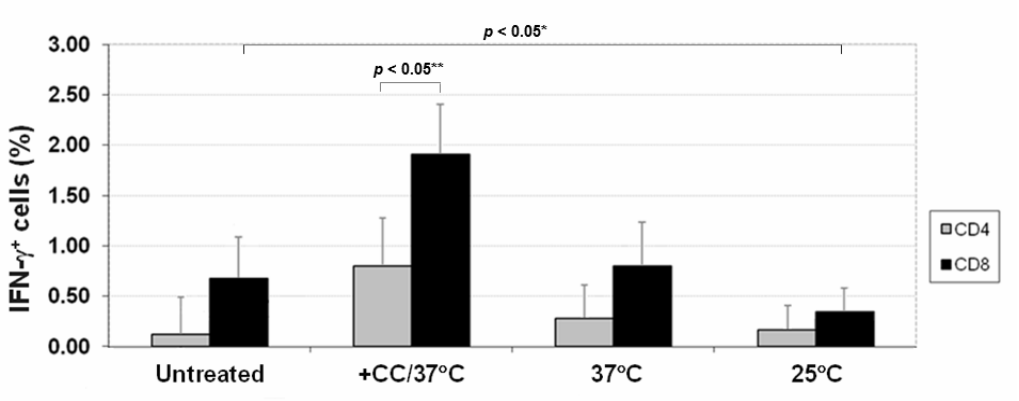

Figure 3. T cells induction by γ-irradiated *LM*-OVA. The γ-irradiated *LM*-OVA after growth under indicated conditions (25°C, 37°C and 37°C supplemented with activated charcoal) or live *LM*-OVA growth at 37°C were used to immunize mice (n = 6 mice/ group). OVA-specific IFN-γ^+ CD4 and CD8 T cell frequencies (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 TNFα blockers. They can cause serious and potentially fatal outcomes in patients treated with TNFα 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.

ACKNOWLEDGEMENTS

This study is supported by a Commission of Higher Education (CHE) grant associated with Ubon Ratchathani University (UBU) and Khon Kaen University (KKU). The Centre for Research and Development of Medical Diagnostic Laboratories (CMDL) is the site of collaboration between these institutions. The authors would like to thank those who helped and encouraged this work; Temduang Limpaiboon and Chanvit Leelayuwat (CMDL, KKU, Thailand), Eyal Raz (Department of Rheumatology, UCSD, USA) and Sandip Datta (NIAID, NIH, USA). Thanks also to Bob Tremayne (UBU) for English assistance.

REFERENCES

1. **Plotkin SA**. 2005. "Vaccines: past, present and future." Nat Med **11** (4 Suppl): S5-11.

2. **Shahabi V, Maciag PC, Rivera S and Wallecha A.** 2010. "Live, attenuated strains of Listeria and Salmonella as vaccine vectors in cancer treatment." Bioeng Bugs **1** (4): 235-243.

3. **Datta SK, Okamoto S, Hayashi T, Shin SS, Mihajlov I, Fermin A, Guiney DG, Fierer J and Raz E.** 2006. "Vaccination with irradiated Listeria induces protective T cell immunity." Immunity **25** (1): 143-152.

4. **Jeong S, Marks BP, Ryser ET and Moosekian SR.** 2010. "Inactivation of Escherichia coli O157:H7 on lettuce, using low-energy X-ray irradiation." J Food Prot **73** (3): 547-551.

5. **Begum RH, Rahman H and Ahmed G.** 2011. "Development and evaluation of gamma irradiated toxoid vaccine of Salmonella enterica var Typhimurium." Vet Microbiol **153** (1-2): 191-197.

6. **Surendran N, Hiltbold EM, Heid B, Sriranganathan N, Boyle SM, Zimmerman KL and Witonsky SG.** 2010. "Heat-killed and gamma-irradiated Brucella strain RB51 stimulates enhanced dendritic cell activation, but not function compared with the virulent smooth strain 2308." FEMS Immunol Med Microbiol **60** (2): 147-155.

7. **Baban CK, Cronin M, O'Hanlon D, O'Sullivan GC and Tangney M**. 2010. "Bacteria as vectors for gene therapy of cancer." Bioeng Bugs **1**(6): 385-394.

8. **Tangney M**. 2010. "Gene therapy for cancer: dairy bacteria as delivery vectors." Discov Med **10** (52): 195-200.

9. **Tangney M and Gahan CG.** 2010. "Listeria monocytogenes as a vector for anti-cancer therapies." Curr Gene Ther **10** (1): 46-55.

10. **Ramaswamy V, Cresence VM, Rejitha JS, Lekshmi MU, Dharsana KS, Prasad SP and Vijila HM.** 2007. "Listeria--review of epidemiology and pathogenesis." J Microbiol Immunol Infect **40** (1): 4-13.

11. **Zenewicz LA and Shen H.** 2007. "Innate and adaptive immune responses to Listeria monocytogenes: a short overview." Microbes Infect **9** (10): 1208-1215.

12. **Gaillard JL, Berche P, Mounier J, Richard S and Sansonetti P.** 1987. "In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2." Infect Immun **55** (11): 2822-2829.

13. **Tilney LG and Portnoy DA.** 1989. "Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, Listeria monocytogenes." J Cell Biol **109** (4 Pt 1): 1597-1608.

14. **Portnoy DA, Chakraborty T, Goebel W and Cossart P**. 1992. "Molecular determinants of Listeria monocytogenes pathogenesis." Infect Immun **60** (4): 1263-1267.

15. **Ermolaeva S, Belyi Y and Tartakovskii I**. 1999. "Characteristics of induction of virulence factor expression by activated charcoal in Listeria monocytogenes." FEMS Microbiol Lett **174** (1): 137-141.

16. **Milohanic E, Glaser P, Coppée JY, Frangeul L, Vega Y, Vázquez-Boland JA, Kunst F, Cossart P and Buchrieser C**. 2003. "Transcriptome analysis of Listeria monocytogenes identifies three groups of genes differently regulated by PrfA." Mol Microbiol **47** (6): 1613-1625.

17. **Kim SH, Castro F, Paterson Y and Gravekamp C.** 2009. "High efficacy of a Listeria-based vaccine against metastatic breast cancer reveals a dual mode of action." Cancer Res **69** (14): 5860-5866.

18. **Singh R and Wallecha A**. 2011. "Cancer immunotherapy using recombinant Listeria monocytogenes: transition from bench to clinic." Hum Vaccin **7** (5): 497-505.

19. **Pope C, Kim SK, Marzo A, Masopust D, Williams K, Jiang J, Shen H and Lefrançois L.** 2001. "Organ-specific regulation of the CD8 T cell response to Listeria monocytogenes infection." J Immunol **166** (5): 3402-3409.

20. **Ripio MT, Domínguez-Bernal G, Suárez M, Brehm K, Berche P and Vázquez-Boland JA**. 1996. "Transcriptional activation of virulence genes in wild-type strains of Listeria monocytogenes in response to a change in the extracellular medium composition." Res Microbiol **147** (5): 371-384.

21. **Karttunen J, Sanderson S and Shastri N.** 1992. "Detection of rare antigen-presenting cells by the lacZ T-cell activation assay suggests an expression cloning strategy for T-cell antigens." Proc Natl Acad Sci U S A **89** (13): 6020-6024.

22. **Dramsi S, Lebrun M and Cossart P.** 1996. "Molecular and genetic determinants involved in invasion of mammalian cells by Listeria monocytogenes." Curr Top Microbiol Immunol **209**: 61-77.

23. **Hoffman PS, Pine L and Bell S.** 1983. "Production of superoxide and hydrogen peroxide in medium used to culture Legionella pneumophila: catalytic decomposition by charcoal." Appl Environ Microbiol **45** (3): 784-791.

24. **Matsuhashi M, Endoh K, Pankrushina AN, Watanabe H, Yamamura H, Komiyama M, Endo S, Tobi M, Ohshima H, Mano Y, Hyodo M, Kaneko T and Otani S.** 1997. "Growth-promoting effect of carbon material upon bacterial cells propagating through a distance." J Gen Appl Microbiol **43** (4): 225-230.

25. **Edelstein PH and Edelstein MA.** 2010. "Comparison of the plating efficiencies and shelf lives of three different commercial buffered charcoal yeast extract media supplemented with alpha-ketoglutaric acid." J Clin Microbiol **48** (5): 1882-1883.

26. **Tubach F, Ravaud P, Salmon-Céron D, Petitpain N, Brocq O, Grados F, Guillaume JC, Leport J, Roudaut A, Solau-Gervais E, Lemann M, Mariette X and Lortholary O; Recherce Axée sur la Tolérance des Biothérapies Group**. 2006. "Emergence of Legionella pneumophila pneumonia in patients receiving tumor necrosis factor-alpha antagonists." Clin Infect Dis **43**(10): e95-100.

27. **Ramos JM, García-Sepulcre MF, Masiá M, Brotons A, Grau MC and Gutiérrez F.** 2010. "Listeria monocytogenes infection in patients with inflammatory bowel diseases receiving anti-tumor necrosis factor therapy." Rev Esp Enferm Dig **102** (10): 614-616.

28. **Brzoza KL, Rockel AB and Hiltbold EM.** 2004. "Cytoplasmic entry of Listeria monocytogenes enhances dendritic cell maturation and T cell differentiation and function." J Immunol **173** (4): 2641-2651.

29. **Khamisabadi M, Arab S, Motamedi M, Khansari N, Moazzeni SM, Gheflati Z and Hadjati J.** 2008. "Listeria monocytogenes activated dendritic cell based vaccine for prevention of experimental tumor in mice." Iran J Immunol **5**(1): 36-44.

30. **Reinicke AT, Omilusik KD, Basha G and Jefferies WA**. 2009. "Dendritic cell cross-priming is essential for immune responses to Listeria monocytogenes." PLoS One **4**(10): e7210.

31. **Datta SK, Redecke V, Prilliman KR, Takabayashi K, Corr M, Tallant T, DiDonato J, Dziarski R, Akira S, Schoenberger SP and Raz E.** 2003. "A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells." J Immunol **170**: 4102-4110.

32. **Nagata T and Koide Y.** 2010. "Induction of Specific CD8 T Cells against Intracellular Bacteria by CD8 T-Cell-Oriented Immunization Approaches." J Biomed Biotechnol: **764542**.

33. **Xu M and Dai MS.** 2010. "Recombinant E. coli LLO/OVA induces murine BMDCs maturation via TLR4 and NOD1 receptor and promotes specific cytotoxic T cell immunity." Biomed Environ Sci **23**(5): 350-356.