OncoImmunology 2:1, e22944; January 2013; © 2013 Landes Bioscience

Intravaginal live attenuated *Salmonella* increase local antitumor vaccine-specific CD8+ T cells

Loane Decrausaz, Christelle Pythoud, Sonia Domingos-Pereira, Laurent Derré, Patrice Jichlinski and Denise Nardelli-Haefliger*

Department of Urology; Centre Hospitalier Universitaire Vaudois and University of Lausanne; Lausanne, Switzerland

Keywords: bacterial immunostimulant, CD8+ T cells, genital mucosa, human papillomavirus, vaccination

We have recently reported that the intravaginal instillation of synthetic Toll-like receptor 3 (TLR3) or TLR9 agonists after a subcutaneous vaccination against human papillomavirus E7 highly increases (~5-fold) the number of vaccine-specific CD8+ T cells in the genital mucosa of mice, without affecting E7-specific systemic responses. Here, we show that the instillation of live attenuated *Salmonella enterica* serovar Typhimurium similarly, though more efficiently (~15-fold), increases both E7-specific and total CD8+ T cells in the genital mucosa. Cancer immunotherapeutic strategies combining vaccination with local immunostimulation with live bacteria deserve further investigations.

Introduction

Therapeutic human papillomavirus (HPV) vaccines targeting the E6 and/or E7 HPV oncogenes were mainly designed to induce specific cytotoxic T lymphocyte (CTL) responses against cervical cancer, the second leading cause of cancer deaths in women worldwide.[1] Despite impressive results in animal models, their application in humans has shown modest clinical effectiveness (reviewed in ref. 2). We have recently reported that the combination of antigen-specific vaccination followed by the induction of local inflammation by intravaginal (IVAG) instillation of Toll-like receptor (TLR) agonists (i.e., CpG oligonucleotides, CpG, a TLR9 agonist or poly(I:C), PIC, a TLR3 agonist) is able to promote the accumulation of both total and antigen-specific CTLs in the genital mucosa (GM) of mice.[3] Most interestingly, repeated IVAG CpG instillations after E7-targeting vaccination eventually led to the regression of large genital HPV-induced tumors. GM-localized CD8+ T cells recruited in response to CpG preferentially expressed CCR5 and CXCR3 chemokine receptors and E-selectin ligands, suggesting that they accumulated in the GM through the interaction with CpG-induced CCL5, CCL3, CCL4, CXCL9, CXCL10, CXCL11 and/or E-selectin.[3] Other TLR ligands are known to modify the expression of selectins, integrins, chemokines and chemokine receptors, which may affect T-cell migration to effector sites.[4] Among these, live bacteria, such as *Salmonella,* can induce pro-inflammatory responses via bacterial components, including lipopolysaccharide (LPS, a TLR4 agonist),[5] flagellin (a TLR5 agonist)[6] and/or bacterial DNA (TLR9 agonist), not only when delivered orally (their normal route of infection), but also when administered in vagina.[7] In addition, attenuated *Salmonella* can be easily engineered to deliver heterologous antigens[8,9] and is used as a vaccine (strain Ty21a,[10] Vivotif®) against typhoid fever by the oral route since decades, with an excellent safety record.[11] Here, we have investigated whether attenuated *Salmonella enterica* serovar Typhimurium vaccine strains would act as an IVAG immunostimulant after E7 vaccination in mice.

Result and Discussion

Intravaginal instillation of live attenuated *Salmonella enterica* serovar Typhimurium after a subcutaneous (s.c.) E7 vaccination increased E7-specific effector CD8+ T cells in the cervix-vagina (CV). All C57Bl/6 mice were first synchronized in a diestrus-like status to avoid possible variations in the IVAG immunostimulatory activity along the estrous cycle. Groups of mice were s.c. immunized with a long synthetic E7 peptide together with adjuvants[12] and 5 d later PBS (as control), CpG or ~5×10^8 CFU of PhoPc attenuated *Salmonella* expressing an irrelevant antigen (PhoPckanL1S)[13] were administered in vagina. Mice were sacrificed at day 9, and cells recovered from CV were analyzed (**Fig. 1**) by ex-vivo interferon γ (IFNγ) ELISPOT assays using the H-2Db restricted $E7_{49-57}$ CTL peptide.[12] As previously shown,[3] IVAG CpG significantly increased (by ~5-fold) E7-specific effector CD8+ T cells in the CV (means ± SEM, E7-specific IFNγ-secreting cells/10^5 CV cells of 71 ± 8 as compared with 14 ± 5 after intravaginal PBS, $p < 0.0001$ by one-way ANOVA and Tukey's post-test). More interestingly, intravaginal PhoPc was even more efficient, leading to a ~15-fold increased number of E7-specific IFNγ-secreting cells/10^5 CV cells (196 ± 46, $p < 0.0001$ and $p < 0.05$, as compared with intravaginal PBS and intravaginal CpG, respectively). The PhoPc strain has a mutation in the two-component regulatory system *phoP*/*phoQ*, which controls more than 40 different

*Correspondence to: Denise Nardelli-Haefliger; Email: dnardell@hospvd.ch
Submitted: 09/26/12; Revised: 11/16/12; Accepted: 11/17/12
http://dx.doi.org/10.4161/onci.22944
Citation: Decrausaz L, Pythoud C, Domingos-Pereira S, Derré L, Jichlinski P, Nardelli-Haefliger D. Intravaginal live attenuated *Salmonella* increase local antitumor vaccine-specific CD8+ T cells. OncoImmunology 2013; 2:e22944

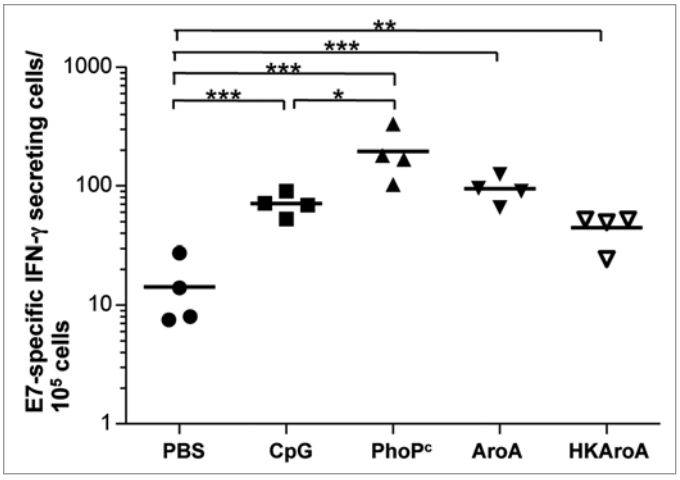

Figure 1. IVAG PhoP^c or AroA bacteria administered after E7 immunization increased E7-specific effector CD8$^+$ T cells in the cervix-vagina. Groups of mice were *s.c.* immunized with 50 μg $E7_{1–98}$ + 10 μg CpG + 0.4 μg *Escherichia coli* heat labile toxin (E7 vaccine)[12] and 5 days later instilled in vagina with PBS, 100 μg CpG, ~5x 10^8 CFU of PhoP^c, AroA or heat-killed (HK) AroA bacteria. Mice were sacrificed three days later and cells recovered from the cervix-vagina were analyzed by ex vivo IFNγ ELISPOT as previously described.[12] The numbers of $E7_{49–57}$ specific IFNγ-secreting cells/10^5 cells are indicated. Horizontal bars represent mean responses. Significant differences are indicated by *p < 0.05, **p < 0.01 and ***p < 0.001 following one-way ANOVA and Tukey's post test (GraphPad Prism 5).

genes required for intracellular survival and resistance to innate immune defense mechanisms.[14,15] We thus further tested a differently attenuated auxotrophic mutant *Salmonella* strain, AroA (AroAkanL1S),[13] which depends on *p*-aminobenzoic acid and 2,3-dihydroxybenzoate for the synthesis of aromatic amino acids and growth.[16] IVAG AroA after E7-vaccination also significantly increased E7-specific IFNγ-secreting cells/10^5 CV cells (95 ± 12, p < 0.0001 as compared with intravaginal PBS, p = non significant as compared with PhoP^c). Because these bacteria can persist in the CV after intravaginal infection,[7] we tested whether viability was influencing their intravaginal immunostimulatory ability. Indeed intravaginal heat-killed (HK) AroA bacteria were still able to significantly increase E7-specific IFNγ-secreting cells/10^5 CV cells (45 ± 7, p < 0.01 as compared with intravaginal PBS), though less efficiently than live AroA cells (p < 0.05 by Student's t-tests).

Bacterial expression of E7 modestly improved the recruitment of E7-specific CD8$^+$ T cells in the cervix-vagina upon IVAG instillation. We wondered whether the expression of E7 by IVAG bacteria may further increase the recruitment of E7-specific CD8$^+$ T cells in the CV by locally boosting vaccine-specific immune responses. For this purpose, we engineered a PhoP^c strain that carried a plasmid (pFSnsd-kan3-mtHsp70HPV16$E7_{Δ21–26}E6_{Δ118–122}$, **Fig. 2A**) expressing, under the prokaryotic *trc* promoter, non-oncogenic forms of E7 ($E7_{Δ21–26}$)[17] and E6 ($E6_{Δ118–122}$)[18] fused to the heat-shock protein (Hsp)70 of *Mycobacterium tuberculosis* (mt) (see lanes HspE7E6 in **Fig. 2B**).[19,20] However, the IVAG administration of the E7-expressing PhoP^c (Phop^{c}E7) bacteria following E7 vaccination was only slightly more efficient than PhoP^c bacteria at increasing the number of E7-specific IFNγ-secreting cells in the CV (191 ± 42 and 167 ± 29 at day 9, respectively, p = non significant, **Fig. 2C**). Even when examined at a later time point (day 15) to accommodate possible local antigen-presentation and specific T-cell proliferation variations, no significant difference between the two intravaginal recombinant *Salmonella* strains could be observed (39 ± 11 and 32 ± 5, respectively, **Fig. 2C**). A subset of CV cells from day 9 (n = 4) were also examined by flow cytometry upon anti-CD8 and tetramer staining (TetE7, based on the H-2Db restricted $E7_{49–57}$ CTL peptide, see **Table 1**). A slightly higher number of TetE7$^+$CD8$^+$ cells was again observed upon IVAG PhoP^{c}E7, as compared with IVAG PhoP^c (p = non significant), but, more interestingly, the percentage of TetE7 CD8$^+$ T cells among total CD8$^+$ T cells appeared significantly higher than after the instillation of IVAG PhoP^c bacteria (p < 0.001) or IVAG PBS (p < 0.05). The fact that E7-specific CD8$^+$ T cells were enriched in the CD8$^+$ T-cell population of the GM when IVAG *Salmonella* expressed E7 suggests that indeed some local boosting had occurred. This is in agreement with previous reports on the ability of recombinant PhoP^c cells to induce antigen-specific antibodies and cell-mediated immune responses after IVAG immunization,[7,21] although the modest effect observed in our case suggest that the expression of E7 was too low or not enough immunogenic in our recombinant PhoP^c strain after a single IVAG instillation. Indeed, PhoP^{c}E7 bacteria were also unable to prevent tumor implantation when used as a vaccine in a prophylactic setting (data not shown). Given the accumulating success of *Salmonella* as gene delivery system,[22] new constructs using an eukaryotic promoter for E7 expression would be worth testing. Interestingly, and despite of the fact that PhoP^c bacteria are surviving for three weeks in the CV at 10^4 CFU,[7] the E7-specific response was greatly decreased at day 15, as compared with day 9 (p < 0.01), though it was still significantly higher than after the IVAG administration of PBS (p < 0.05). This may suggest that an IVAG dose of 10^4 CFU is not sufficient to maintain a high number of E7-specific CD8$^+$ T cells in the CV or, on the contrary, that the chronic presence of bacteria is counterproductive. In the case of CpG, three consecutive IVAG doses administered at days 6, 9 and 12 were able to sustain a high E7-specific immune response until day 15, while successive PIC instillation were ineffective.[3] The analysis of peripheral blood mononuclear

Table 1. TetE7 CD8$^+$ and total CD8$^+$ T cells in the genital mucosa (n = 4) after IVAG instillation of Phop^c bacteria expressing or not E7

IVAG immunostimulant	TetE7$^+$CD8$^{+†}$	Total CD8$^+$ T cells†	% TetE7$^+$CD8$^+$/total CD8$^{+†}$
PBS	0.12 ± 0.04	0.66 ± 0.19	17.4 ± 0.4
Phop^c	1.83 ± 0.43***	10.94 ± 3.06***	16.9 ± 0.9
Phop^{c}E7	2.28 ± 0.31***	9.57 ± 0.92***	22.6 ± 0.8*,¶¶

†Mean percentages ± SEM; *p< 0.05 as compared to IVAG PBS; *** p< 0.001as compared to IVAG PBS; ¶¶p< 0.001 as compared to IVAG PhoP^c.

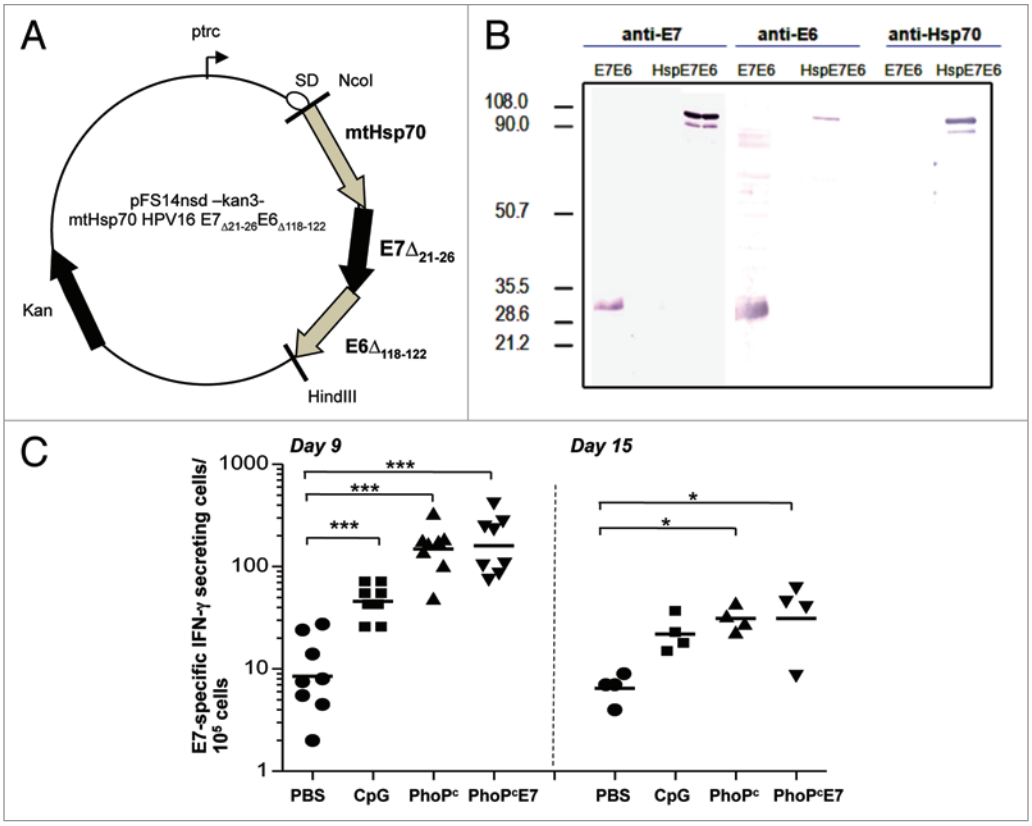

Figure 2. Bacterial expression of E7 modestly influenced the fold-increase of E7-specific CD8+ T cells in the cervix-vagina upon intravaginal instillation. (**A**) The plasmid pFSnsd-kan3-mtHsp70HPV16E7$_{\Delta 21-26}$E6$_{\Delta 118-122}$ contains within the *Nco*I and *Hind*III restrictions sites, instead of L1S,[13] a sequence encompassing the full lenght *Mycobacterium tuberculosis* (mt) heat-shock protein 70 (Hsp70) open reading frame (ORF) fused to the E7 ORF deleted from residues 21 to 26 and the E6 ORF deleted from residues 118–122. (**B**) Expression of the fusion protein (~100 kDa) in PhoPc bacterial lysates (lane HspE7E6) is detected with polyclonal anti-E7 and anti-E6 antibodies (kindly provided by Dr. John Schiller, NCI, Bethesda, USA) as well as with a monoclonal anti-mtHsp70 antibody (HyTest Ltd, Turku, Finland). For comparison, E7 and E6 only are detected in bacterial lysates from PhoPc cells expressing an E7-E6 fusion without Hsp70 (~32 kDa). (**C**) Mice *s.c.* immunized with the adjuvanted E7 vaccine and receiving 5 days later intravaginal PBS, CpG, PhoPc or PhoPcE7 cells were sacrificed at day 9 (left) or at day 15 (right) and cells recovered from cervix-vagina were analyzed by ex vivo IFNγ ELISPOT. The numbers of E7$_{49-57}$-specific IFNγ-secreting cells/10^5 cells are indicated. Horizontal bars represent mean responses. Significant differences are indicated by *p < 0.05 and ***p < 0.001 following one-way ANOVA and Tukey's post test (GraphPad Prism 5).

Table 2. Systemic E7-specific IFNγ-secreting cells /10^5 cells at day 9

IVAG immunostimulant	PBMC†	Spleen†	GLN†
PBS	296 ± 87	164 ± 32	24 ± 10
CpG	417 ± 82	103 ± 20	41 ± 8
PhoPc	159 ± 53	59 ± 14*	26 ± 8
PhoPcE7	108 ± 15	86 ± 19	22 ± 6

†Means ± SEM; *p< 0.05 as compared to intravaginal PBS.

cells (PBMCs), spleen and lymph node draining the genital tract (GLN) (**Table 2**) confirms that the effect of IVAG bacteria on E7-specific CD8+ T-cell recruitment was restricted to the GM, as similar or rather lower number of E7-specific IFNγ-secreting cells were measured among PBMCs as well as in the GLN and spleen. Our flow cytometry analysis also indicates that the percentage of TetE7+ CD8+ cells in the CV of E7-vaccinated mice that had received IVAG PhoPc or PhoPcE7 cells were increased ~15-fold as compared with IVAG PBS instillation and a similar fold-increase was measured for total CD8+ T cells (**Table 1**). This confirms that IVAG *Salmonella* after E7 vaccination mainly promote the recruitment of CD8+ T cells, including E7 vaccine-specific cells, from the periphery. The chemokines and/or selectines involved in this process will have to be investigated. However, it is noteworthy that, similarly to CpG, *Salmonella enterica* serovar Typhimurium can induce the upregulation of CCL3, CCL4, CXCL9, CXCL10, CXCL11 and E-selectin in the intestinal mucosa.[23,24] The fact that both CCL5 and CXCL10 are secreted by macrophages and dendritic cells upon *Salmonella* infection, while purified LPS or flagellin were less efficient in this respect,[25] may support our finding that live bacteria are superior immunostimulants than purified bacterial components. The ligands of CXCR3 and CCR5 reportedly increase in the GM 24h after IVAG infection with PhoPc and/or AroA bacteria, returning to steady-state levels 6 days later,[7] which may possibly correlate to the decreased T-cell recruitment we observed at day 15.

In conclusion, our data show that the IVAG instillation of *Salmonella* vaccine strains after vaccination can lead to a major

increase in vaccine-specific CD8+ T cell selectively in the GM, adding to the list of TLR agonists that may be used for cancer therapy.[26] Further experiments are needed to unravel the underlying mechanisms and determine the effects of this strategy on genital HPV-associated tumor regression. Interestingly, our preliminary data (not shown) suggest that bacterial immunostimulation may be useful for tumors located in other mucosal sites. As *Salmonella* appears to exert a multipronged antineoplastic effect,[27] the detailed characterization of these bacteria as local immunostimulants and antigen-delivery systems is warranted.

Disclosure of Potential Conflicts of Interest

Denise Nardelli Haefliger is an inventor on patent PCT/IB2009/051372: "Method and Vaccine for optimizing the specific immune responses." The remaining authors declared no conflict of interest.

Acknowledgments

This work was supported by the Swiss Cancer League (OCS 02304-082008 and KFS 2808-08-2011), the Swiss National Science Foundation (#310000-112406 and 31003A-135109) and the Fondation Emma Muschamp.

References

1. Bosch FX, Lorincz A, Muñoz N, Meijer CJ, Shah KV. The causal relation between human papillomavirus and cervical cancer. J Clin Pathol 2002; 55:244-65; PMID:11919208; http://dx.doi.org/10.1136/jcp.55.4.244.
2. Gissmann L, Nieto K. The therapeutic vaccine: is it feasible? Arch Med Res 2009; 40:493-8; PMID:19853190; http://dx.doi.org/10.1016/j.arcmed.2009.07.003.
3. Domingos-Pereira S, Decrausaz L, Derré L, Bobst M, Romero P, Schiller JT, et al. Intravaginal TLR agonists increase local vaccine-specific CD8 T cells and human papillomavirus-associated genital-tumor regression in mice. Mucosal Immunol 2012; In press; PMID:22968420; http://dx.doi.org/10.1038/mi.2012.83.
4. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med 2006; 354:610-21; PMID:16467548; http://dx.doi.org/10.1056/NEJMra052723.
5. Li Q, Cherayil BJ. Role of Toll-like receptor 4 in macrophage activation and tolerance during Salmonella enterica serovar Typhimurium infection. Infect Immun 2003; 71:4873-82; PMID:12933828; http://dx.doi.org/10.1128/IAI.71.9.4873-4882.2003.
6. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol 2001; 167:1882-5; PMID:11489966.
7. Echchannaoui H, Bianchi M, Baud D, Bobst M, Stehle JC, Nardelli-Haefliger D. Intravaginal immunization of mice with recombinant Salmonella enterica serovar Typhimurium expressing human papillomavirus type 16 antigens as a potential route of vaccination against cervical cancer. Infect Immun 2008; 5:1940-51; PMID:18332214; http://dx.doi.org/10.1128/IAI.01484-07.
8. Levine MM, Woodrow GC, Kaper JB, Cobon GS. Attenuated *Salmonella* as a live vector for expression of foreign antigens. New York: Dekker, 1997.
9. Garmory HS, Leary SE, Griffin KF, Williamson ED, Brown KA, Titball RW. The use of live attenuated bacteria as a delivery system for heterologous antigens. J Drug Target 2003; 11:471-9; PMID:15203915; http://dx.doi.org/10.1080/10611860410001670008.
10. Germanier R, Füer E. Isolation and characterization of Gal E mutant Ty 21a of Salmonella typhi: a candidate strain for a live, oral typhoid vaccine. J Infect Dis 1975; 131:553-8; PMID:1092768; http://dx.doi.org/10.1093/infdis/131.5.553.
11. Begier EM, Burwen DR, Haber P, Ball R; Vaccine Adverse Event Reporting System Working Group. Postmarketing safety surveillance for typhoid fever vaccines from the Vaccine Adverse Event Reporting System, July 1990 through June 2002. Clin Infect Dis 2004; 38:771-9; PMID:14999618; http://dx.doi.org/10.1086/381548.
12. Decrausaz L, Revaz V, Bobst M, Corthésy B, Romero P, Nardelli-Haefliger D. Induction of human papillomavirus oncogene-specific CD8 T-cell effector responses in the genital mucosa of vaccinated mice. Int J Cancer 2010; 126:2469-78; PMID:19816937.
13. Fraillery D, Baud D, Pang SY, Schiller J, Bobst M, Zosso N, et al. Salmonella enterica serovar Typhi Ty21a expressing human papillomavirus type 16 L1 as a potential live vaccine against cervical cancer and typhoid fever. Clin Vaccine Immunol 2007; 14:1285-95; PMID:17687110; http://dx.doi.org/10.1128/CVI.00164-07.
14. Miller SI, Kukral AM, Mekalanos JJ. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc Natl Acad Sci U S A 1989; 86:5054-8; PMID:2544889; http://dx.doi.org/10.1073/pnas.86.13.5054.
15. Ernst RK, Guina T, Miller SI. How intracellular bacteria survive: surface modifications that promote resistance to host innate immune responses. J Infect Dis 1999; 179(Suppl 2):S326-30; PMID:10081503; http://dx.doi.org/10.1086/513850.
16. Hoiseth SK, Stocker BAD. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature 1981; 291:238-9; PMID:7015147; http://dx.doi.org/10.1038/291238a0.
17. Hallez S, Brulet JM, Vandooren C, Maudoux F, Thomas S, Heinderickx M, et al. Pre-clinical immunogenicity and anti-tumour efficacy of a deleted recombinant human papillomavirus type 16 E7 protein. Anticancer Res 2004; 24:2265-75; PMID:15330171.
18. Crook T, Tidy JA, Vousden KH. Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. Cell 1991; 67:547-56; PMID:1657399; http://dx.doi.org/10.1016/0092-8674(91)90529-8.
19. Harmala LA, Ingulli EG, Curtsinger JM, Lucido MM, Schmidt CS, Weigel BJ, et al. The adjuvant effects of Mycobacterium tuberculosis heat shock protein 70 result from the rapid and prolonged activation of antigen-specific CD8+ T cells in vivo. J Immunol 2002; 169:5622-9; PMID:12421941.
20. Qian X, Lu Y, Liu Q, Chen K, Zhao Q, Song J. Prophylactic, therapeutic and anti-metastatic effects of an HPV-16mE6Delta/mE7/TBhsp70Delta fusion protein vaccine in an animal model. Immunol Lett 2006; 102:191-201; PMID:16242781; http://dx.doi.org/10.1016/j.imlet.2005.09.004.
21. Hopkins S, Kraehenbuhl JP, Schödel F, Potts A, Peterson D, de Grandi P, et al. A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization. Infect Immun 1995; 63:3279-86; PMID:7642256.
22. Paterson Y, Guirnalda PD, Wood LM. Listeria and Salmonella bacterial vectors of tumor-associated antigens for cancer immunotherapy. Semin Immunol 2010; 22:183-9; PMID:20299242; http://dx.doi.org/10.1016/j.smim.2010.02.002.
23. Godinez I, Haneda T, Raffatellu M, George MD, Paixão TA, Rolán HG, et al. T cells help to amplify inflammatory responses induced by Salmonella enterica serotype Typhimurium in the intestinal mucosa. Infect Immun 2008; 76:2008-17; PMID:18347048; http://dx.doi.org/10.1128/IAI.01691-07.
24. Guo L, Lim KB, Gunn JS, Bainbridge B, Darveau RP, Hackett M, et al. Regulation of lipid A modifications by Salmonella typhimurium virulence genes phoP-phoQ. Science 1997; 276:250-3; PMID:9092473; http://dx.doi.org/10.1126/science.276.5310.250.
25. Pietilä TE, Veckman V, Kyllönen P, Lähteenmäki K, Korhonen TK, Julkunen I. Activation, cytokine production, and intracellular survival of bacteria in Salmonella-infected human monocyte-derived macrophages and dendritic cells. J Leukoc Biol 2005; 78:909-20; PMID:16033811; http://dx.doi.org/10.1189/jlb.1204721.
26. Galluzzi L, Vacchelli E, Eggermont A, Fridman WH, Galon J, Sautès-Fridman C, et al. Trial Watch: Experimental Toll-like receptor agonists for cancer therapy. Oncoimmunology 2012; 1:699-716; PMID:22934262; http://dx.doi.org/10.4161/onci.20696.
27. Leschner S, Weiss S. Salmonella-allies in the fight against cancer. J Mol Med (Berl) 2010; 88:763-73; PMID:20526574; http://dx.doi.org/10.1007/s00109-010-0636-z.