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RESEARCH ARTICLE

A recombinant E. coli vaccine to promote MHC class I-dependent antigen presentation: application to cancer immunotherapy

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We have examined the potential of recombinant Escherichia coli expressing listeriolysin O (LLO) to deliver tumour antigens to dendritic cells (DCs) for cancer immunotherapy. Using OVA as a model tumour antigen, we have shown in murine DCs that E. coli expressing cytoplasmic LLO and OVA proteins can deliver the OVA K^b-restricted epitope SIINFEKL for MHC class I presentation. In contrast, when E. coli expressing OVA alone were used, MHC class II presentation of the OVA 323-339 I-A^b-restricted peptide was predominant. When injected in vivo, DCs pulsed with E. coli expressing LLO and OVA induced production of cytotoxic T-lymphocytes capable of lysing an OVA-expressing melanoma cell line (B16-OVA) and resulted in suppression of tumour growth following challenge with B16-OVA. Immunisation of mice by direct injection of E. coli LLO/OVA provided a more potent anti-tumour response, resulting in complete protection in 75% of mice. Injection of live bacteria was not necessary as immunisation with paraformaldehyde-fixed E. coli LLO/OVA provided an even stronger anti-tumour response against B16-OVA. Altogether, our data highlight the potential of this system as a novel and efficient strategy for tumour immunotherapy.
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Keywords: *dendritic cells; E. coli; listeriolysin-O; cancer antigens; MHC*

Introduction

Dendritic cells (DCs) loaded with tumour antigens and administered as a vaccine have been shown to induce potent anti-tumour immune responses in animal models[1,2] and are currently being tested in early-phase clinical trials with some encouraging results.[3–7] Central to the induction of efficient anti-tumour immune responses is the generation of $CD8^+$ cytotoxic T lymphocytes (CTL). CTLs recognise MHC class I molecules in association with peptides derived from endogenous proteins[8] and therefore the induction of anti-tumour immunity relies on the efficient delivery of antigenic epitopes for MHC class I processing and presentation by DCs. Various strategies of loading DCs with antigen both *in vivo* and *ex vivo* have been shown to generate efficient $CD8^+$ T cell responses, including pulsing with peptides, tumour lysates, apoptotic bodies, infection with recombinant viruses, transfection of DNA and RNA, and DC-tumour cell hybrids (reviewed in Ref. 9) and recombinant proteins conjugated to iron beads or immunostimulatory DNA sequences.[10,11] Despite these successes, most of the methods currently used either require laborious production and purification techniques, access to tumour samples that are often difficult to obtain in useful quantities, or knowledge of the antigenic epitopes. In addition, a maturation stimulus is required as the injection of immature, antigen-loaded DCs could result in tolerance, rather than immunisation, in humans.[12] Clearly, there is a need for a simple, effective method to engineer activated DCs with tumour antigens loaded on MHC class I for tumour immunotherapy.

Bacteria and their components such as LPS and CpG motifs are some of the most potent inducers of DC maturation.[13–15] Recombinant *E. coli* have been used as a standard molecular biology tool for over three decades and are widely used for the production of recombinant proteins. Therefore, such bacteria could be promising candidates for the delivery of tumour antigens to DCs. Recently, it was demonstrated that the co-expression of listeriolysin O (LLO) with a model antigen, ovalbumin (OVA) in *E. coli* resulted in efficient MHC class I presentation of the OVA K^b-restricted epitope, SIINFEKL, subsequent to phagocytosis of the bacteria by a murine macrophage cell line.[16] In this system LLO, a pore-forming cytolysin from *Listeria monocytogenes*, is expressed as a bacterial cytoplasmic protein that is only released following the uptake of the bacteria by phagocytosis and their subsequent degradation in the phagocytic vesicles.

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LLO then perforates the phagosome, allowing release of its contents into the cytosol and access of co-expressed proteins for processing and presentation via the classical MHC class I presentation pathway.

In the present study, we demonstrate using OVA as a model tumour antigen that these observations can be extended to murine DCs, with the exception of an increased degree of LLO-mediated toxicity to the host cell. To investigate the ability of DCs pulsed with *E. coli* to mediate anti-tumour immunity we utilised the highly aggressive and poorly immunogenic B16 melanoma model[17] that had been transfected with OVA. We demonstrate that although vaccination with *E. coli*-pulsed DCs resulted in some suppression of tumour growth, direct injection of *E. coli* was a more potent vaccination strategy. Furthermore, *E. coli* killed by paraformaldehye fixation before vaccination could mediate in some animals complete protection against tumour challenge, suggesting the potential of this system as a novel, efficient strategy for tumour immunotherapy.

Results

MHC class I and II presentation mediated by LLO-expressing E. coli

To investigate MHC class I presentation following the addition of *E. coli* to DCs we utilised the B3Z T cell hybridoma,[18] which produces β-galactosidase and IL-2 upon recognition of the OVA peptide, SIINFEKL, in association with K^b. As shown in Figure 1a, DCs pulsed with *E. coli*/OVA were able to present SIINFEKL for MHC class I presentation to B3Z cells, but only at ratios of 100 bacteria per DC or higher. Expression of both LLO and OVA by *E. coli* resulted in a 100-fold increase in the efficiency of SIINFEKL/K^b presentation, with efficient presentation being observed at ratios as low as one bacterium per DC (Figure 1b). Presentation was maximal at ratios of 10 *E. coli*/LLO/OVA per DC and was consistently found to be similar to those observed following pulsing of DCs with saturating concentrations of SIINFEKL peptide (Figure 1c). At ratios of 100 *E. coli*/LLO/OVA bacteria per DC, class I presentation was dramatically reduced (Figure 1b). This is likely to be due to LLO-mediated toxicity, as the viability of the DCs was significantly reduced at this ratio (Figure 5b).

MHC class I presentation could be detected 30 min after incubation of DCs with *E. coli*/LLO/OVA, with maximal levels being achieved after co-incubation for 1 h. Longer co-incubation times of DCs for up to 4 h or overnight with either peptide or bacteria did not increase class I presentation levels (data not shown). Presentation decreased 24 h after pulsing with SIINFEKL peptide or *E. coli*/OVA and was essentially undetectable by 48 h (Figure 1a and c). By contrast, some MHC class I presen-

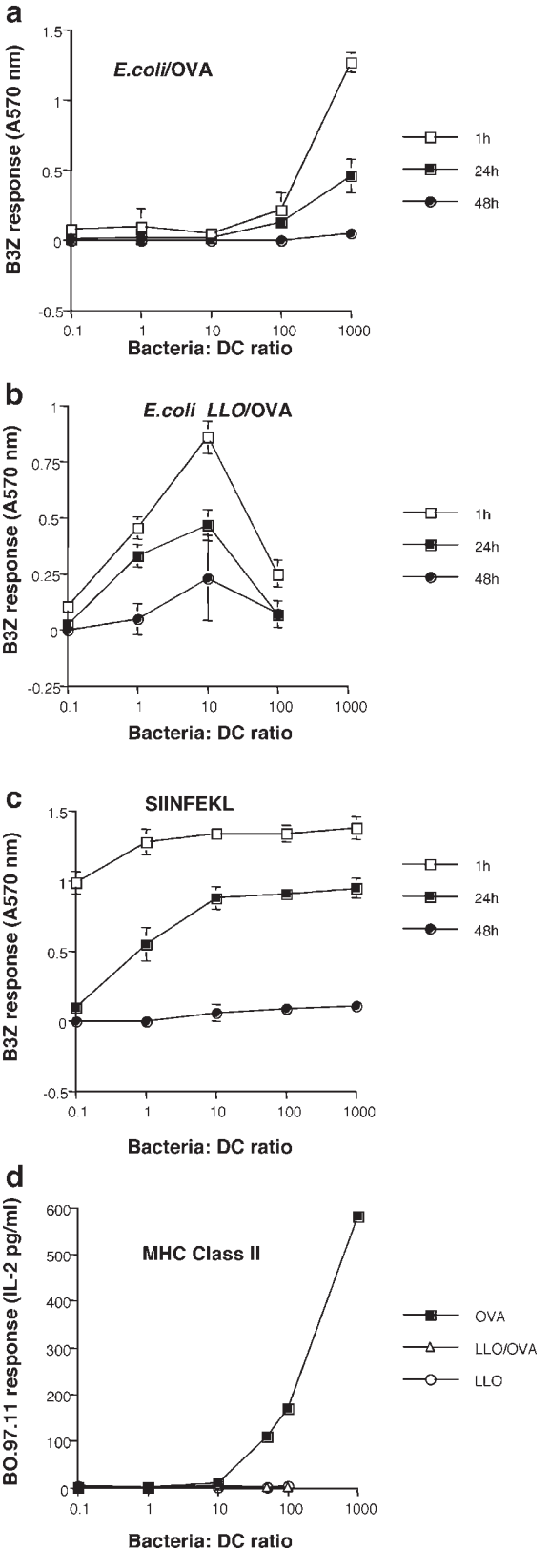

Figure 1 *E. coli/LLO-mediated delivery of OVA to DCs for processing and MHC class I presentation. DCs were incubated for 1 h with E. coli expressing OVA (a), or OVA and LLO (b) at various ratios, or were pulsed with SIINFEKL peptide (c). DCs were then washed and fixed in paraformaldehyde immediately, or after 24 or 48 h. Presentation of the SIINFEKL epitope on MHC class I was examined by co-incubation with B3Z hybridomas and measurement of β-galactosidase production after 16 h. Results are presented as the mean + s.d. of triplicate wells and are representative of three independent experiments. (d) MHC class II presentation on DCs mediated by E. coli. DCs were incubated with E. coli expressing OVA, LLO, or both for 1 h followed by washing. Presentation of the OVA 232-339/I-A^b complex was assessed by the addition of BO.91.11 hybridomas and incubation for 16 h. IL-2 production by the BO-97.11 hybridoma was quantitated by ELISA. Results are presented as the mean + s.d. of triplicate wells and are representative of three independent experiments.*

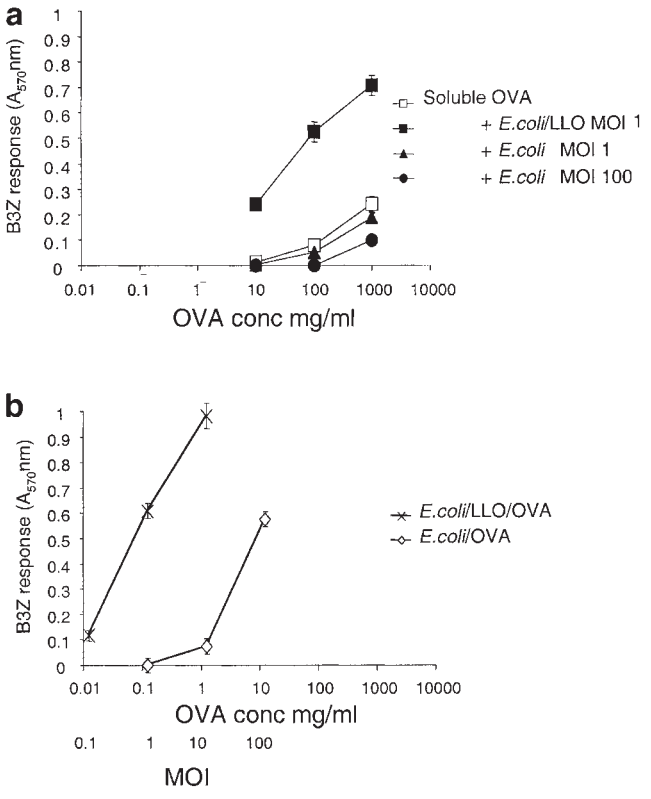

Figure 2 *The efficiency of E. coli-mediated delivery to MHC class I presentation compared with incubation with soluble OVA protein. (a) DCs were incubated with OVA co-incubated with E. coli, expressing or not LLO. (b) DCs were incubated with E. coli expressing OVA or co-expressing LLO and OVA at different MOIs. The amounts of OVA expressed by both bacteria were similar (as determined by Western blotting, not shown) and the relevant MOIs are presented in the Figure. Following incubation in these various conditions, cells were washed and B3Z hybridomas were added. Presentation of the SIINFEKL/K^b complex was quantitated by a LacZ assay after 16 h. Results are presented as the mean + s.d. of triplicate wells.*

tation could still be detected up to 48 h following infection with *E. coli*/LLO/OVA. Ratios as low as one bacterium per DC were sufficient to induce maturation of DCs as observed by the up-regulation of MHC class II molecules and costimulatory molecules CD80 and CD86 (data not shown).

To investigate MHC class II presentation by DCs incubated with *E. coli*, we utilised the BO.97.11 hybridoma, which secretes IL-2 upon recognition of the OVA 323-339 peptide in association with I-A^b. As shown in Figure 1d, *E. coli* expressing OVA could efficiently deliver the OVA 323-339 peptide for MHC class II presentation. However, co-expression of LLO completely abrogated this response, suggesting a complete redirection to the MHC class I presentation pathway.

To examine further the efficiency of *E. coli*/LLO-mediated MHC class I processing, we co-incubated DCs with soluble OVA protein alone or in combination with *E. coli* or *E. coli*/LLO. We first quantified the level of OVA expressed by *E. coli* by Western blotting and showed that *E. coli*/LLO/OVA and *E. coli*/OVA expressed equivalent amounts of OVA (not shown). Soluble protein is generally processed through the MHC class II presentation pathway and is not efficiently targeted for MHC class I processing.[19] In murine DCs, soluble OVA was very poorly processed by the MHC class I pathway as illustrated in Figure 2a. Co-incubation of DCs and soluble OVA with *E. coli* at either low (MOI 1) or high (MOI 100) bacteria to DC ratios did not increase the efficiency of MHC class I presentation. In contrast, co-incubation of soluble OVA with *E. coli* expressing LLO increased presentation at least 100-fold compared with OVA alone. These results suggest that LLO is necessary for the enhanced MHC class I presentation since this was only observed with *E. coli* expressing LLO. OVA, delivered as a recombinant protein in *E. coli*, was at least 10^3 times more efficient at inducing MHC class I presentation than mixing equivalent concentrations of OVA and bacteria together (Figure 2b compared with Figure 2a). Co-expressing OVA and LLO in *E. coli* resulted in a 10^5-fold

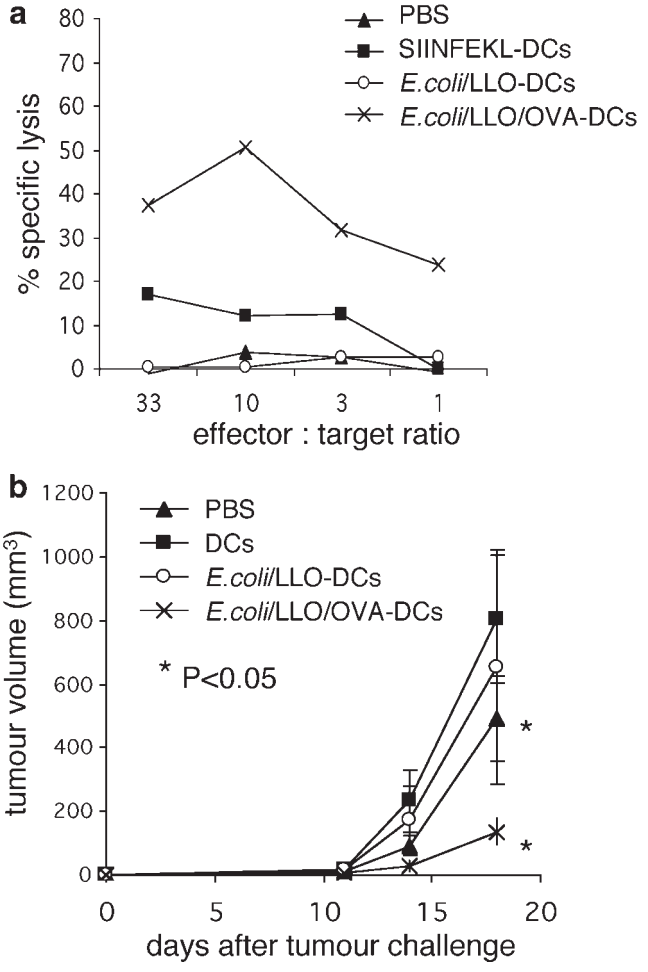

Figure 3 *Generation of anti-tumour immunity using E. coli-pulsed DCs. (a) Vaccination with DCs pulsed with E. coli/LLO/OVA induce OVA-specific CTL. DCs were pre-pulsed with either 5 μM SIINFEKL peptide or with E. coli/LLO or E. coli/LLO/OVA at a ratio of two bacteria per DC for 1 h. Mice received a single subcutaneous injection of 5×10^5 pulsed DCs on day 0. Splenocytes from two mice per group were isolated and pooled on day 9 and restimulated with B16-OVA in vitro for 5 days. Cytotoxicity against B16-OVA was assessed in a 4-h ^{51}Cr release assay. Results are presented as the mean of duplicate wells. (b) Vaccination with E. coli/LLO/OVA-pulsed DCs suppresses tumour growth. DCs were pulsed as in (a) and five C57BL/6 mice per group received subcutaneous injections of 5×10^5 DCs on days 0 and 7, followed by subcutaneous tumour challenge with 2×10^5 B16-OVA on day 14. Mice were killed once tumour area reached 1.44 cm^2. Results are presented as the mean tumour volume + s.e.m. and P values calculated using the Student's t test.*

increase in the efficiency of MHC class I presentation compared with soluble OVA and *E. coli* combined (Figure 2b compared with Figure 2a).

Vaccination with E. coli/LLO/OVA-pulsed DCs induces anti-tumour responses

The ability of DCs loaded with *E. coli*/LLO/OVA to elicit anti-tumour immune responses *in vivo* was examined. In these experiments, DCs were pre-pulsed with either SIINFEKL peptide or with *E. coli*/LLO or *E. coli*/LLO/OVA at a ratio of two bacteria per DC for 1 h and injected subcutaneously into mice. Splenocytes were isolated after 9 days and restimulated *in vitro* for 5 days with B16 transfected with OVA (B16-OVA) before a ^{51}Cr-release assay to detect OVA-specific CTL activity. As shown in Figure 3a, vaccination with *E. coli*/LLO/OVA-pulsed DCs resulted in potent CTL activity against B16-OVA (37% specific lysis at an effector to target ratio of 33:1). This was higher than the specific lysis observed following vaccination with SIINFEKL peptide-pulsed DCs (17% lysis at effector to target ratio 33:1). Vaccination with PBS as a control or with *E. coli*/LLO-pulsed DCs failed to elicit any CTL activity against B16-OVA and no specific lysis was observed when control B16 (not expressing OVA) were used as targets (not shown).

To investigate whether DCs loaded with *E. coli*/LLO/OVA could elicit protection against tumour challenge, mice received subcutaneous injections of DCs pre-pulsed with either *E. coli*/LLO or *E. coli*/LLO/OVA at an MOI of 2, twice at 1-week intervals, and were challenged with the B16-OVA cell line 1 week later. Vaccination with *E. coli*/LLO/OVA-pulsed DCs resulted in a significant reduction in tumour volume by day 18 after tumour challenge ($P = 0.037$) compared with vaccination with PBS control, DCs alone, or DCs pulsed with *E. coli*/LLO (Figure 3b).

Direct injection of live E. coli/LLO/OVA protects against tumour challenge

To examine whether direct injection of live *E. coli*/LLO/OVA could result in anti-tumour immunity, mice were vaccinated with two intraperitoneal injections of live *E. coli*, *E. coli*/OVA or *E. coli*/LLO/OVA at 1-week intervals and challenged with B16-OVA 1 week later. As shown in Figure 4a, vaccination with *E. coli*/OVA resulted in a slight reduction in tumour volume com-

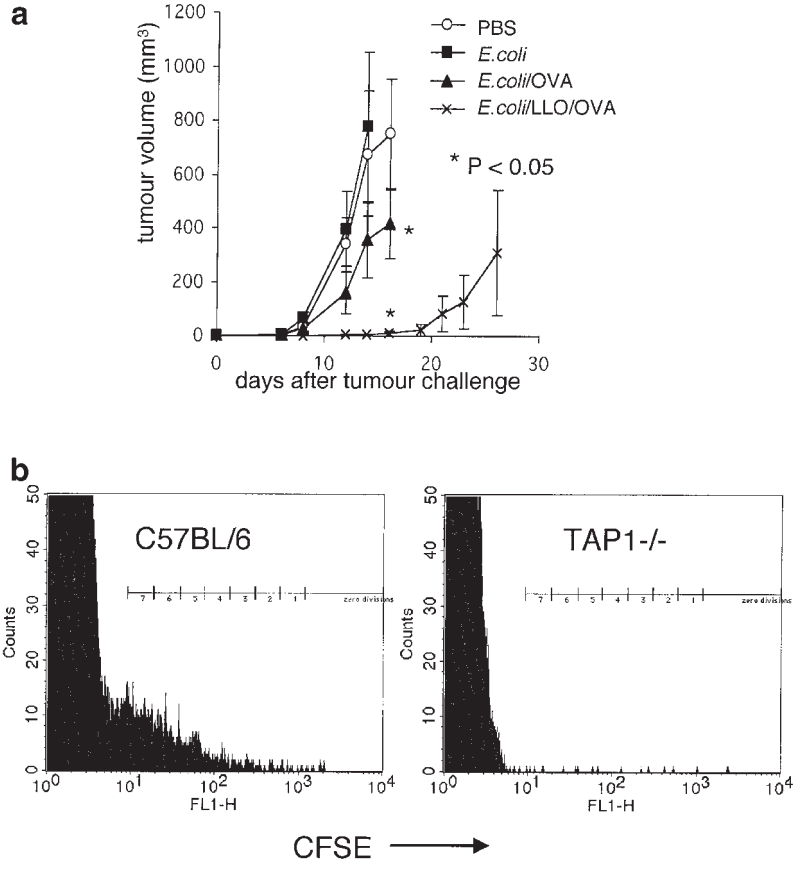

Figure 4 *Generation of anti-tumour immunity following direct injection of E. coli/LLO/OVA. (a) Vaccination with E. coli/LLO/OVA-pulsed DCs suppresses tumour growth. C57BL/6 mice (five per group) received intra-peritoneal injections of 10^8 E. coli, E. coli/OVA or E. coli/LLO/OVA on days 0 and 7, followed by subcutaneous tumour challenge with 2×10^5 B16-OVA on day 14. Results are presented as mean tumour volume + s.e.m., and are plotted for each group until at least one mouse had to be killed. Statistical analysis was performed using the Student's t test. Results are representative of three similar experiments. (b) E. coli/LLO/OVA stimulates proliferation of naive OVA-specific CD8$^+$ T cells in vivo. Splenocytes and draining lymph node cells from OT-1 transgenic mice were labelled with CFSE and adoptively transferred into C57BL/6 and TAP1−/− mice. Mice were vaccinated with 10^8 E. coli/LLO/OVA 24 h later. Draining lymph nodes were collected after 3 days, and proliferating T cells identified by staining with anti-CD8α and sequential halving of CSFE fluorescence by flow cytometry. Data shown are individual draining lymph nodes and are representative of lymph nodes from two mice per group.*

pared with the PBS and *E. coli* controls. However, vaccination with *E. coli*/LLO/OVA resulted in a significant delay in tumour growth compared with the PBS and *E. coli* controls ($P = 0.0067$) so that by day 16 only one mouse out of five had started to develop a tumour, while all mice in the control groups had to be killed by this stage as their tumours had reached the maximum size allowed by the guidelines set by the institutional ethics committee (1.44 cm^2). 60% of mice vaccinated with *E. coli*/LLO/OVA survived at least 50 days with one in five remaining tumour-free for more than 60 days post-tumour challenge. Vaccination with *E. coli*/LLO/OVA was significantly more effective at reducing tumour volume than vaccination with *E. coli*/OVA ($P = 0.014$), suggesting that the presence of LLO is essential for the generation of anti-tumour immunity. This was OVA-specific and not due to non-specific effects of LLO alone since vaccination with *E. coli*/LLO did not affect tumour volume (data not shown). Rather, it appears that reduction in tumour volume was mediated by the redirection of OVA into the MHC class I presentation pathway as a result of the presence of LLO.

To more closely investigate the mechanism of tumour inhibition following direct injection of *E. coli*/LLO/OVA, we examined the ability of this vaccine to induce proliferation of naive $CD8^+$ T cells *in vivo* (Figure 4b). Splenocytes and lymph nodes from TCR-transgenic OT-1 mice, which express a TCR recognising the H-2^b/SIINFEKL complex, were labelled with CSFE and adoptively transferred into C57BL/6 mice. Mice received a single vaccination of *E. coli*/LLO/OVA 24 h later and the proliferation of the $CD8^+$ OT.1 cells was observed 3 days later by the decrease in CSFE fluorescence (around 10^3 fluorescent units for non-dividing cells, not shown), which sequentially halves for every round of cell division. Figure 4b shows $CD8^+$ OT.1 cells had undergone between three and seven rounds of division in response to direct injection of *E. coli*/LLO/OVA. No proliferation had occurred in control mice and no CFSE-positive cells were detected (not shown). In addition, no proliferation was observed following vaccination of TAP1−/− mice with *E. coli*/LLO/OVA (Figure 4b). These data suggest that vaccination with *E. coli*/LLO/OVA induces proliferation of naive OVA-specific $CD8^+$ T cells *in vivo*, and that presentation is mediated via the TAP-dependent MHC class I processing and presentation pathway.

Paraformaldehyde fixation of E. coli/LLO/OVA does not affect MHC class I presentation

One potential limitation of this system is the safety concern associated with the use of live bacterial vectors. To address this, we investigated whether killed *E. coli*/LLO/OVA would be as efficient at mediating MHC class I processing and presentation as their live counterparts. *E. coli*/LLO/OVA were fixed in 0.5% paraformaldehyde and loaded on to DCs, and MHC class I presentation of the SIINFEKL peptide was determined by response of B3Z cells. Paraformaldehyde fixation killed all bacteria (>99.99%) as determined by colony forming assays (data not shown). As shown in Figure 5a, fixation decreased the class I presentation at ratios of one bacterium per DC, but did not significantly decrease the level of MHC class I presentation at ratios of 10 bacteria per DC. At a ratio of 100 bacteria per DC an even higher level of MHC class I presentation from fixed bacteria was

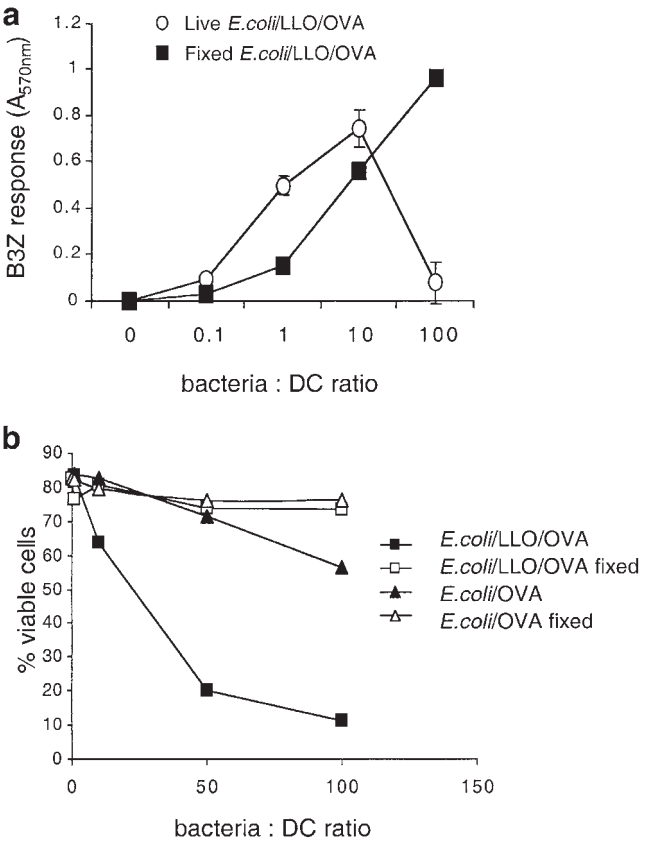

Figure 5 *Paraformaldehyde fixing of bacteria maintains MHC class I antigen presenting capacity while reducing LLO-mediated toxicity. E. coli/OVA and E. coli/LLO/OVA were fixed in 0.5% paraformaldehyde for 30 min followed by extensive washing. (a) DCs were incubated with live or fixed E. coli/LLO/OVA for 1 h followed by washing, and presentation of the SIINFEKL/K^b complex to B3Z was examined by β-gal production after 16 h. (b) DCs were incubated for 1 h with live or fixed E. coli/OVA or E. coli/LLO/OVA followed by washing, and DCs were allowed to recover for a further hour. Cell death was examined by propidium iodide staining and analysed by flow cytometry. Results are representative of at least two similar experiments.*

observed, while no significant presentation was observed following infection with live bacteria. This suggests that fixation does not abrogate the MHC class I processing properties of *E. coli*/LLO/OVA. Presentation to MHC class I mediated by *E. coli*/OVA was not affected by paraformaldehyde fixation (data not shown).

We next investigated whether the increase in MHC class I presentation after fixation was due to reduced toxicity. *E. coli*/LLO/OVA and *E. coli*/OVA were fixed in paraformaldehyde before loading on DCs and their toxicity was examined by propidium iodide staining and flow cytometry. As shown in Figure 5b, incubation with live *E. coli*/LLO/OVA resulted in high levels of toxicity to the DCs at ratios greater than 10 bacteria per DC. This was due to LLO as *E. coli*/OVA could be loaded at ratios of more than 50 bacteria per DC before any significant toxicity was observed. Interestingly, fixation of the bacteria with paraformaldehyde before loading on DCs resulted in a dramatic enhancement in the level of cell viability, with no toxicity observed even after loading at ratios as high as 50 bacteria per DC (Figure 5b). This is likely to be due to the partial inactivation of LLO to levels where it could still mediate perforation of the phago-

somes without exerting significant toxicity (data not shown). These results suggest that in addition to the safety advantage of using killed bacteria over live ones, fixing *E. coli*/LLO/OVA can significantly reduce toxicity while maintaining MHC class I antigen presenting capacity.

Vaccination with fixed E. coli/LLO/OVA protects against B16-OVA tumour challenge

We next examined whether DCs pulsed with fixed *E. coli* could still mediate anti-tumour immunity. As the toxicity of LLO on DCs was markedly reduced after fixing, up to 50 fixed bacteria per DC were loaded and vaccine schedules were performed as described in Figure 3a and b. DCs pulsed with fixed *E. coli*/LLO/OVA could elicit potent anti-tumour CTL responses that were more efficient than peptide-pulsed DCs, consistent with the results shown in Figure 3a (data not shown). Suppression of tumour growth was observed in 30% of animals and their survival time was almost double that observed for the control groups. However, this was not as efficient as vaccination with peptide-pulsed DCs, which resulted in protection of 60% of mice (data not shown).

Since direct injection of *E. coli* appeared to be a more effective vaccine strategy than pulsing DCs (Figures 3 and 4), we optimised the vaccination schedule and examined whether *E. coli*/LLO/OVA could still mediate anti-tumour immunity after fixation. Mice received three subcutaneous injections of fixed bacteria at 1-week intervals followed by a subcutaneous tumour challenge with B16-OVA 2 weeks after the last vaccination and were monitored for tumour growth and survival. As shown in Figure 6, vaccination with fixed *E. coli*, or fixed *E. coli*/LLO resulted in no significant effect on either tumour growth or overall survival compared with the PBS control, consistent with the results obtained following vaccination with live bacteria (Figure 4). Vaccination with fixed *E. coli*/OVA led to a delay in tumour growth in three out of eight mice (37.5%) and complete protection in another three mice for at least 90 days post-tumour challenge (Figure 6). Strikingly, vaccination with fixed *E. coli*/LLO/OVA led to a dramatic delay in tumour growth in two out of eight mice and complete protection in six out of eight mice (75%). These mice remained tumour-free for more than 90 days post-tumour challenge.

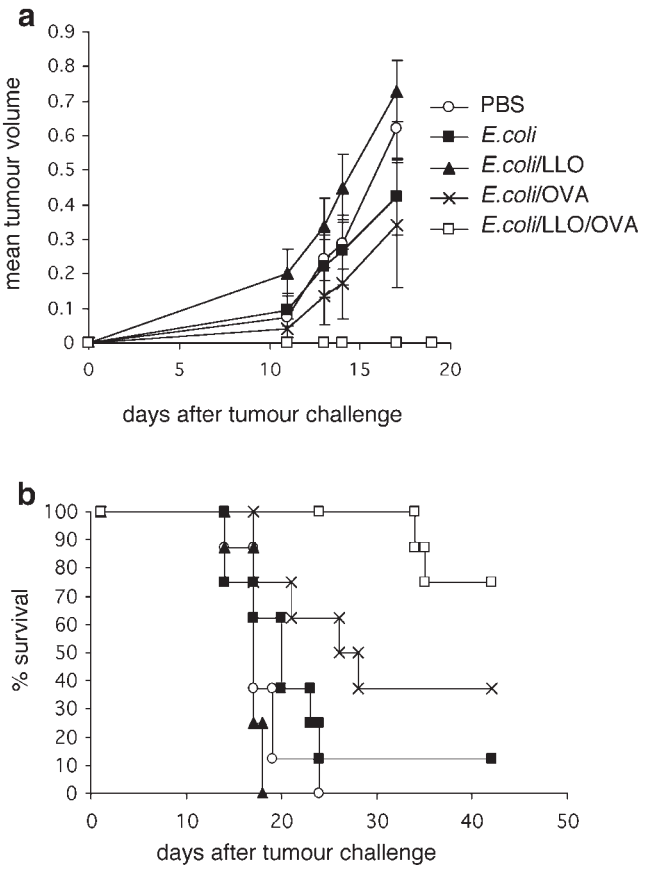

Figure 6 *Direct injection of fixed E. coli/LLO/OVA protects against tumour challenge with B16-OVA. C57BL/6 mice (eight per group) received three subcutaneous injections of 10^8 fixed bacteria at 1 week intervals, followed by subcutaneous tumour challenge with 2×10^5 B16-OVA 2 weeks after the last vaccination. Results are presented as (a) mean tumour growth until time points were at least one animal in the group had to be killed, and (b) the percentage of surviving mice, and are representative of two similar experiments.*

Discussion

In this paper we describe a simple, effective system for the delivery of tumour antigens to the MHC class I processing pathway of murine DCs. By utilising recombinant *E. coli* expressing cytosolic LLO and OVA as a model antigen, we could demonstrate MHC class I presentation that was at least 10^2 times more efficient than that observed with *E. coli* expressing OVA alone, and more than 10^5 times more efficient than incubation with soluble protein (Figure 2). In addition, incubation with *E. coli* resulted in efficient maturation of DCs as shown by the up-regulation of MHC class II, CD80 and CD86 molecules (data not shown). Immunisation of animals with DCs pulsed with *E. coli*/LLO/OVA resulted in the induction of anti-tumour CTL responses which were more potent than peptide-pulsed DCs, and led to a significant delay in tumour growth following challenge with the highly aggressive B16-OVA melanoma cell line (Figure 3).

Surprisingly, direct injection of *E. coli*/LLO/OVA resulted in more dramatic anti-tumour immunity than vaccination with pulsed DCs (Figure 4) and may represent a more versatile approach of utilising recombinant *E. coli* for tumour immunotherapy. Immunisation with *E. coli* expressing OVA alone did not significantly affect tumour growth, suggesting that the presence of LLO, and therefore MHC class I presentation, was critical to the development of the observed anti-tumour response. In addition, the ability of *E. coli*/LLO/OVA to induce proliferation of naive OVA-specific $CD8^+$ T cells following adoptive transfer into wild-type C57BL/6, but not TAP1−/− mice suggests that the anti-tumour response generated following direct injection was at least in part $CD8^+$ mediated and appeared to require processing and presentation via the TAP-dependent pathway.

The induction of more effective anti-tumour immunity following direct injection of *E. coli*/LLO/OVA compared with vaccination with bacteria-pulsed DCs may be due to only a very small proportion of injected DCs being able to migrate from the injection site,[20] resulting in less efficient responses. Alternatively the responses generated via either vaccine strategy may be mediated via different mechanisms. However, DCs are the only antigen presenting cells capable of inducing primary immune

responses,[21,22] suggesting that the *in vivo* generation of naive OVA-specific $CD8^+$ following direct injection of *E. coli*/LLO/OVA is likely to be DC-mediated. It is unclear, however, whether DCs are directly presenting antigens from *E. coli* or cross-priming T cells after acquiring antigen from other cell types.

A potential limitation of the system is the danger of utilising live bacteria as antigen delivery vehicles. In addition, the high degree of toxicity of LLO on murine DCs observed in the present study (Figure 5b) and by others[23] prevents efficient loading of DCs with bacteria. We were able to demonstrate that fixation of bacteria with paraformaldehyde before loading on DCs significantly reduced their toxicity without affecting their ability to present antigenic motifs to MHC class I (Figure 5a). More importantly, direct injection of fixed *E. coli*/LLO/OVA was able to afford complete protection against challenge with B16-OVA for at least 90 days in 75% of cases (Figure 6).

The *E. coli*/LLO system offers a number of advantages over current methods of targeting antigens for MHC class I presentation. Firstly, the system is easy, quick and cost-effective to produce and there is no need for DNA expression of antigen in the host cell or for laborious purification techniques. Secondly, the system is safer than other delivery methods using live vectors in that the *E. coli* K12 strain used is non-pathogenic and the delivery of antigen relies on killing of the bacteria by the antigen-presenting cell. Furthermore, fixing in paraformaldehyde as described here kills all bacteria, whilst still maintaining their ability to mediate anti-tumour immunity, thus providing an additional safety feature. Thirdly, incubation with bacteria induces activation and maturation of DCs, which is necessary for the efficient induction of T cell responses *in vivo*.[12] In addition, there is the capacity to target antigens for presentation to MHC class I, II, or both, depending on the presence or absence of LLO in the bacteria.

The *E. coli*/LLO system provides another unique advantage over other methods of delivery such as DNA transfection or viral infection in that full-length tumour-antigens can be expressed safely in bacteria as opposed to the host cell, even if they have unknown or potentially harmful functions. This has an added advantage in that antigenic epitopes do not need to be defined and treatment is less likely to be restricted to patients of specific HLA haplotypes. Many tumour antigens have already successfully been expressed as recombinant proteins in *E. coli*, demonstrating the simplicity of this technique.[24–26]

In addition to utilising this system as a vaccination strategy for tumour immunotherapy, infecting DCs with recombinant *E. coli*/LLO may represent an efficient method for identifying new tumour antigens from either potential cDNA candidates specifically expressed on tumours that could be identified by microarray analysis, or by screening tumour cDNA libraries against tumour-specific CTL. A similar approach has already been utilised to identify novel human $CD4^+$-restricted antigens from *Mycobacterium tuberculosis*,[27,28] and the addition of LLO would allow the identification of antigens recognised by CTLs.

In summary, the fixed recombinant *E. coli* described here combines non-specific adjuvant effects of bacteria with the specificity of tumour antigens loaded on MHC class I, leading to anti-tumour activity. This cost-effective technology could be the basis for unique 'off the shelf' formulations for the treatment of patients irrespective of their HLA-haplotypes.

Materials and methods

Mice

Female C57BL/6 mice were purchased from Harlan (UK) and were used between 6 and 8 weeks of age. TAP1−/− and OT-1 mice were kindly provided by Dr C Reis e Sousa (ICRF, London, UK).

Tissue culture

The T cell hybridoma cell line, B3Z, specific for the OVA 257-264 peptide (SIINFEKL) in the context of K^b,[18] was a gift from Dr N Shastri (University of California, Berkeley, CA, USA) and was maintained in RPMI containing 50 μM 2-mercaptoethanol and 10% foetal calf serum (FCS). BO-97.11 is a $CD4^+$ T cell hybridoma cell line specific for the OVA 323-339 peptide (ISQAVHAAHAEINEAGR) in the context of I-A^b,[29] and was a gift from Dr P Marrack (National Jewish Center for Respiratory Medicine, Denver, CO, USA), and was maintained in DMEM containing 10% FCS.

The B16 melanoma cell line transfected with OVA (B16-OVA) was a gift from Dr E Lord (University of Rochester, NY, USA) and was maintained in DMEM containing 10% FCS supplemented with 400 μg/ml G418.

DCs were cultured from bone marrow of C57BL/6 mice as previously described[30] and used for experiments on days 6 to 8 of culture.

Bacterial strains

All strains used in this study were derived from *E. coli* MC4100(DE3) and have been previously described.[16] DP-E3615 harbours plasmid pDP3615 containing the *hly* gene encoding LLO, lacking its secretion signal sequence, under the control of the constitutive *tet* gene promoter. DP-E3616 contains plasmid pDP3616 encoding the 32 kDa truncated OVA cDNA under the IPTG-inducible T7 phage promoter. DP-E3617 contains both pDP3615 and pDP3616 plasmids. All strains were grown to mid-log phase and stored as glycerol stocks. Expression of OVA was induced by 1 mM IPTG, as previously described.[16] Quantitation of OVA expression was evaluated by Western blotting. In some experiments *E. coli* were pre-fixed in 0.5% paraformaldehyde/PBS for 30 min RT followed by extensive washing in PBS before DC loading.

Reagents

OVA grade VI was purchased from Sigma (St Louis, MO, USA). OVA peptides 257–264 (SIINFEKL) and 323–339 (ISAVHAAHAEINEAGR) were synthesized by Cancer Research UK peptide synthesis laboratory.

Flow cytometry

Acquisition was performed on a Becton Dickinson FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analysed using CellQuest software. Cell death was analysed by staining cells with a solution of 100 μg/ml propidium iodide in PBS.

Antigen presentation assays

Measurement of MHC class I presentation of the SIINFEKL/K^b complex to B3Z hybridomas was perfor-

med as previously described.[16] DCs were seeded at 10^5 cells per well in flat-bottom 96-well plates in complete RPMI medium. *E. coli*, soluble OVA protein, or peptides were added at various concentrations. After 1-h incubation, plates were washed three times with PBS and 10^5 B3Z cells were added along with 100 μg/ml gentamicin for 16 h at 37°C, 5% CO_2. Plates were washed once with PBS and β-galactosidase activity was assessed by the addition of 100 μl of lysis buffer (PBS, 100 μM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP40 and 0.15 mM chlorophenolred-β-galactoside (CPRG, Calbiochem, La Jolla, CA, USA)). After 4 h at 37°C, 50 μl stop buffer (300 mM glycine, 15 mM EDTA) was added and the absorbance at 570 nm was read on a micro-plate reader. Presentation of the OVA 323-339/I-A^b complex to BO.97.11 cells was analysed in a similar manner, except that IL-2 production by the BO.97.11 hybridoma was measured from supernatants following culture for 16 h by ELISA.

Cytotoxicity assay

DCs were pre-pulsed with either 5 μM SIINFEKL peptide or with *E. coli* at a ratio of two bacteria per DC for 1 h. C57BL/6 mice received a single subcutaneous injection of 5×10^5 pulsed DCs on day 0. Splenocytes were isolated on day 9 and co-cultured with irradiated (20 Gy) B16-OVA or parental B16 *in vitro* for 5 days in complete RPMI at a ratio of 20 splenocytes per stimulator. Effecter cells were cultured with 10^4 ^{51}Cr-labeled B16 or B16-OVA targets at various effecter:target ratios for 4 h at 37°C. Specific lysis was assayed by removing 20 μl of supernatant and reading on a Topcount NXT counter (Packard, Meridien, CT, USA).

Tumour protection assays

DCs were pulsed with *E. coli* at a ratio of two bacteria per DC for 1 h followed by washing. C57BL/6 mice received subcutaneous injections 5×10^5 DCs on days 0 and 7 followed by subcutaneous tumour challenge with 2×10^5 B16-OVA on day 14. For direct injection experiments, mice received intra-peritoneal injections of 10^8 bacteria on days 0 and 7 followed by subcutaneous tumour challenge with 2×10^5 B16-OVA on day 14.

In vivo proliferation assay

Splenocytes and draining lymph node cells from OT-1 mice were labelled with 2 μM 5,6-carboxy-fluorescein succinimdyl ester (CFSE, Molecular Probes) as previously described[31] and 3×10^6 were adoptively transferred via the tail vein into C57BL/6 and TAP1−/− mice. Mice received subcutaneous injections of 10^8 *E. coli* 24 h later. Three days later the draining lymph nodes and spleens were removed, stained with anti CD8α-tricolour (Caltag, Burlingame, CA, USA) and 250 000 events collected and analysed by flow cytometry.

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