

# Recombinant *Escherichia coli* Expressing Invasin Targets the Peyer's Patches: The Basis for a Bacterial Formulation for Oral Vaccination

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We have investigated the tropism of nonpathogenic recombinant invasive *Escherichia coli* in the gastrointestinal tract and the efficacy of this invasive *E. coli* as an oral vaccine for cancer immunotherapy. *E. coli* expressing invasins from *Yersinia pseudotuberculosis* selectively invade nonphagocytic cells in which  $\beta_1$ -integrin is expressed and accessible. Following internalization the *E. coli* are degraded in the phagosome. Coexpression of listeriolysin O (LLO) mediates release of the content of the bacteria into the cytosol of the invaded cell. *In vitro* and *in vivo* experiments demonstrated that gut epithelial cells failed to be invaded by invasive *E. coli*, due to a basolateral localization of  $\beta_1$ -integrin. By contrast, selective uptake of invasive bacteria from the intestinal lumen into Peyer's patches was observed *ex vivo*. Once in this structure, invasive *E. coli* colocalized with dendritic cells and possibly B cells. Oral administration of invasive *E. coli* coexpressing the model antigen ovalbumin and LLO from *Listeria monocytogenes* was able to elicit systemic protection against a lethal challenge of B16 tumor cells expressing ovalbumin. These data demonstrate the selectivity of invasins-mediated invasion to the Peyer's patches and indicate the potential of nonpathogenic, invasive *E. coli* as an oral vaccine with applications in immunotherapy.

**Key Words:** invasins, Peyer's patch, epithelium, oral vaccination, beta-1 integrin

## INTRODUCTION

Various bacterial pathogens utilize the gastrointestinal tract to gain systemic access during their life cycle, e.g., *Yersinia* [1], *Salmonella* [2], and *Listeria* [3]. This process involves the targeting of the M cells of the Peyer's patches. M cells are specialized antigen-sampling cells found in the follicular-associated epithelium overlying the Peyer's patches [4]. M cells transcytose antigens and microorganisms passing through the intestinal lumen to the underlying lymphoid tissue where specific immune responses can be initiated [4]. Therefore, the M cells can be described as the portals to the mucosal immune system. *S. typhimurium* and *L. monocytogenes* have been engineered to transfer plasmid-encoded or protein antigens and tumor-associated epitopes to antigen-presenting cells in the mucosal lymphoid tissue, resulting in specific cytotoxic T cell responses and protection against challenge with tumor cells expressing the antigens [5–14]. However, despite extensive attenuation [15,16], human clinical

trials using *Salmonella* have shown dose-limiting toxicity [16], which may restrict the efficacy and the ease of use of *Salmonella* in immunotherapeutic approaches. *Listeria* is a pathogenic bacterium that causes listeriosis and attenuation of *Listeria* may remove its invasive capacity, limiting its application as an oral vaccine for human therapy [17].

The tropism of *Yersinia* in the gastrointestinal tract is determined by different genes among which invasins is the major invasive factor. Invasins is a protein located at the surface of the bacteria [18]. It selectively binds to  $\beta_1$ -integrin on the surface of cells, triggering entry of *Yersinia* into host cells [18].  $\beta_1$ -Integrin is located at the basolateral side of intact gastrointestinal epithelial cells and is, therefore, inaccessible to *Yersinia* (*in vitro*) [19]. However, the M cells of the ileal Peyer's patches expose  $\beta_1$ -integrin at their apical side [20]. *Yersinia* specifically exploits this atypical  $\beta_1$ -integrin expression on the M cells to enter the mucosal lymphoid tissue and subsequently disseminate systemically [1,21].

We and others have demonstrated the potential of recombinant *Escherichia coli* expressing listeriolysin O (LLO) from *L. monocytogenes* to deliver antigens to antigen-presenting cells [22–25]. Using chicken ovalbumin (OVA) as a model tumor antigen, we have shown in murine dendritic cells (DCs) that *E. coli* expressing cytoplasmic LLO and OVA proteins can deliver the OVA K<sup>b</sup>-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<sup>b</sup>-restricted peptide was predominant. The mechanism is likely to involve an LLO-dependent release of the bacterial content into the cytosol followed by its processing by the proteasome and the presentation of the antigenic motifs onto MHC class I [22,23]. Immunization of mice by direct injection of *E. coli* LLO/OVA provided a more potent anti-tumor response, resulting in complete protection in 75% of mice [23].

*E. coli* have been engineered to express invasins and have been shown to invade nonphagocytic  $\beta_1$ -integrin-expressing mammalian cells leading to intracellular delivery of plasmids and recombinant proteins [26,27]. *E. coli* expressing invasins have also been shown to be efficient protein delivery to tumors to produce therapeutic effects *in vivo* in mice [27]. The aims of the current study are to characterize the tropism of *E. coli* expressing invasins in the gut and to assess the potential of recombinant invasive *E. coli* expressing LLO as an oral vaccine formulation.

## RESULTS

### Confluency Inhibits the Invasion of Caco-2 Cells by Invasive *E. Coli*

We co-incubated sparse Caco-2 cells or Caco-2 cells maintained at confluency for 2 weeks with various m.o.i. (multiplicities of infection, ratio of *E. coli*:mammalian cell) of *E. coli* BM2710 pGB2 $\Omega$ inv-hly/GFP (invasive *E. coli* harboring the plasmid pAT505 in which a prokaryotic promoter controls the expression of GFP). We performed FACS analysis to determine the percentage of GFP-positive cells 24 h after incubation with the *E. coli*. The vast majority of sparse Caco-2 cells co-incubated with *E. coli* BM2710 pGB2 $\Omega$ inv-hly/GFP at an m.o.i. of 100 were GFP positive, i.e., invaded by invasive *E. coli* expressing GFP (Fig. 1). By contrast, very few confluent Caco-2 cells were invaded, even at an m.o.i. of 1000 (Fig. 1). These data indicate that confluency inhibits the invasion.

### Transient and Reversible Disruption of the Tight Junction Can Restore the Invasive Properties of Invasive *E. Coli*

We grew Caco-2 cells to confluency on permeable supports and monitored the transepithelial electrical resistance (TEER) across the monolayers, as previously described by Ma *et al.* [28]. The apical side of the monolayers was exposed to various concentrations of

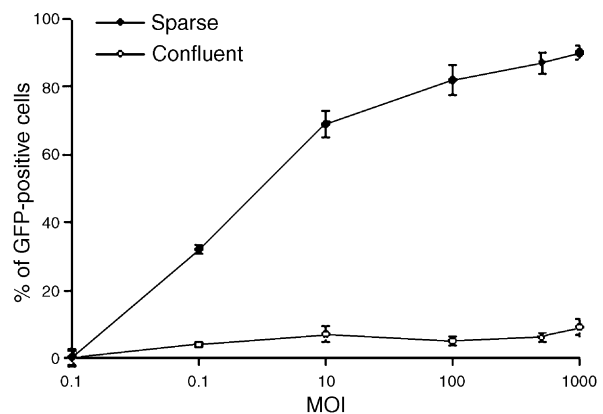


FIG. 1. Invasion of sparse and confluent Caco-2 cells by *E. coli* BM2710 inv-hly/Prok.GFP. Sparsely seeded or confluent Caco-2 cells were co-incubated with *E. coli* BM2710 inv-hly/Prok.GFP for 2 h, extensively washed, and refed with complete medium containing gentamycin. The percentage of GFP-positive cells was determined 24 h later by flow cytometry.  $n = 3$  wells of Caco-2 cells per condition, representative of two independent experiments.

ethanol, which has been shown to disrupt tight junctions transiently and reversibly [28]. To assess whether Caco-2 cell monolayers treated with ethanol could be invaded by invasive *E. coli*, we pretreated the apical side of Caco-2 monolayers with 8% ethanol (concentration shown to produce a nontoxic reversible reduction in the TEER across monolayers, data not shown), followed by co-incubation with invasive or noninvasive *E. coli* expressing GFP. FACS analysis of the cells of the monolayers 24 h postincubation showed that 52.5% of the cells incubated with invasive *E. coli* were GFP positive, as opposed to less than 1% when incubated with noninvasive *E. coli* ( $n = 3$ ,  $P = 0.0001$ ). To confirm these data, we compared the delivery of the LacZ gene by invasive *E. coli* to monolayers treated or not with ethanol to delivery by noninvasive *E. coli*. LacZ staining was not detected in untreated control Caco-2 cell monolayers (Fig. 2A) nor in monolayers incubated with noninvasive *E. coli*-LacZ treated or not with ethanol (Figs. 2B and 2C, respectively). Some  $\beta$ -gal staining in a very low proportion of cells was detected in ethanol-untreated monolayers exposed to invasive *E. coli*-LacZ (Fig. 2D). In accordance with the FACS data described above, we observed intense staining of  $\beta$ -gal in approximately 50% of cells in monolayers treated with ethanol and exposed to invasive *E. coli* expressing  $\beta$ -gal (Fig. 2E). These data clearly indicate that disruption of the tight junctions by ethanol allows invasion and protein delivery by invasive *E. coli* to occur.

### Tropism of Invasive *E. Coli* in the Murine Gastrointestinal Tract *ex Vivo*

To determine the tropism of recombinant, invasive *E. coli* in the gastrointestinal tract we used a perfusion organ bath to maintain viable murine small intestinal tissue. The tissue bath maintains tissue in Krebs–Henseleit

solution at 37°C and enables selective luminal perfusion of tubular organs to mimic *in vivo* oral administration. We perfused segments of murine ileal tissue containing Peyer's patches with *E. coli*-GFP (noninvasive) or *E. coli* inv-hly/GFP (invasive *E. coli* expressing GFP) for 1 h, followed by perfusion with Krebs–Henseleit containing gentamycin. We assessed entry of the *E. coli* into the Peyer's patches and epithelium by visualizing GFP (expressed by the *E. coli*) in frozen tissue sections. GFP was not detected in Peyer's patches or small intestinal tissue from ileal segments perfused with Krebs–Henseleit solution (not shown). We visualized fewer than five GFP dots per field of view within Peyer's patch tissue from ileum perfused with noninvasive *E. coli* (Fig. 3B), indicating that few noninvasive *E. coli* had gained entry into the Peyer's patches. The GFP signal was greatly increased in Peyer's patches from tissue perfused with invasive *E. coli* (Fig. 3E). We observed many GFP dots corresponding to the *E. coli* throughout the Peyer's patch tissue. Our data indicate that expression of invasin by *E. coli* dramatically enhances the transcytosis of *E. coli* from the intestinal lumen to the Peyer's patch. We detected very low numbers of GFP dots at the apical side of the small intestinal tissue perfused with either the noninvasive or the invasive *E. coli* (not shown), suggesting a marked tropism of invasive *E. coli* for the Peyer's patches.

#### Invasive *E. coli* Gain Access to the Peyer's Patch and Colocalize with Antigen-Presenting Cells

To determine whether Peyer's patch antigen-presenting cells (APC) had taken up the invasive bacteria, we prepared single-cell suspensions from Peyer's patches perfused with invasive *E. coli* expressing GFP and analyzed them by multicolor flow cytometry.

The majority of the cells obtained from Peyer's patches were CD45<sup>hi</sup> bone marrow-derived leukocytes and approximately 0.5% of the cells obtained from invasive *E. coli* expressing GFP-perfused tissue were GFP positive (Fig. 4B). No GFP<sup>+</sup> leukocytes were detected in cell suspensions perfused with Krebs–Henseleit solution (KHS) alone.

Approximately 2% of extracted Peyer's patch cells expressed high levels of CD11c (Fig. 4A), a marker of mouse dendritic cells [29]. Peyer's patch dendritic cells from tissue perfused with invasive bacteria were enriched for GFP<sup>+</sup> cells compared with the total leukocyte population ( $3.1 \pm 0.6\%$  of DC from *E. coli* BM2710 pGB2Qinv-hly/GFP-perfused tissue were GFP<sup>+</sup>; Fig. 4B), suggesting selective binding to and/or uptake into these APC. No GFP<sup>+</sup> DC were detected in cells from KHS-perfused tissue.

A small proportion (0.24%) of CD45R/B220-positive Peyer's patch cells from tissue perfused with invasive bacteria were also GFP<sup>+</sup> (Fig. 4B). CD45R/B220 is classically regarded as a marker of developing, mature, and activated murine B cells [30] and therefore the presence of CD45R/B220<sup>+</sup>GFP<sup>+</sup> cells may indicate uptake of bacteria by Peyer's

patch B cells. However, recent analysis has demonstrated that CD45R/B220 is also expressed by a subpopulation of DC, the plasmacytoid DC subset, found in mouse lymphoid tissue. Further analysis will be required to distinguish between bacterial uptake by B cells and plasmacytoid DC.

We attempted a similar experiment to assess the uptake of invasive *E. coli* by macrophages, using the rat anti-mouse F4/80 monoclonal antibody. However, we could not detect macrophages by flow cytometry in this experimental setting (data not shown).

#### Oral Vaccination of Mice against Subcutaneous Tumor Challenge

In a first set of experiments, we determined the maximum tolerated dose to be  $5 \times 10^7$  bacteria administered orally, by gavage, to C57/B6J mice. To determine whether the invasive *E. coli* could be used as an oral vaccine formulation, we gavaged C57/B6J mice on days 0, 7, and 14 with 5% sodium hydrogen carbonate (NaHCO<sub>3</sub>; to neutralize gastric acid) or  $5 \times 10^7$  invasive *E. coli* expressing the chicken OVA cDNA suspended in 5% NaHCO<sub>3</sub>. On day 21 we challenged the mice by subcutaneous injection of  $2 \times 10^5$  B16-OVA cells. Oral administration of invasive *E. coli* expressing LLO and OVA caused a reduction in the rate of tumor growth following subcutaneous challenge with B16-OVA cells compared to the control group, which was gavaged with NaHCO<sub>3</sub> (Fig. 5). The mean tumor volume at day 15 in mice gavaged with *E. coli* MC4100(DE3) inv-hly/OVA was 0.1026 cm<sup>3</sup> compared to 0.205 cm<sup>3</sup> in the control group gavaged with NaHCO<sub>3</sub> ( $P = 0.00686$ ). In separate experiments, noninvasive *E. coli* LLO/OVA, known to induce an efficient anti-tumor effect when injected subcutaneously [23], did not produce any reduction in the rate of tumor growth when administered orally (not shown).

#### DISCUSSION

In this study we investigated the tropism of nonpathogenic *E. coli* expressing invasin in the gastrointestinal tract. This tropism was hypothesized to be similar to that of *Yersinia pseudotuberculosis*, since the invasive property is conferred by expression of invasin from *Y. pseudotuberculosis*, which mediates endocytosis of bacteria by selectively binding to  $\beta_1$ -integrin on the surface of mammalian cells [18,31].

Invasion was observed in sparse cells but was largely inhibited in confluent cells (Fig. 1), in which  $\beta_1$ -integrin is strictly located below the tight junctions at the basolateral membrane, as in the intestinal epithelium [19]. Under these conditions, *Y. pseudotuberculosis* does not have access to  $\beta_1$ -integrin and Caco-2 cells are not invaded [19]. At low, noncytotoxic doses ethanol disrupts zona occludens-1 (a tight junctional protein) and perijunctional actin and myosin filaments, causing a struc-

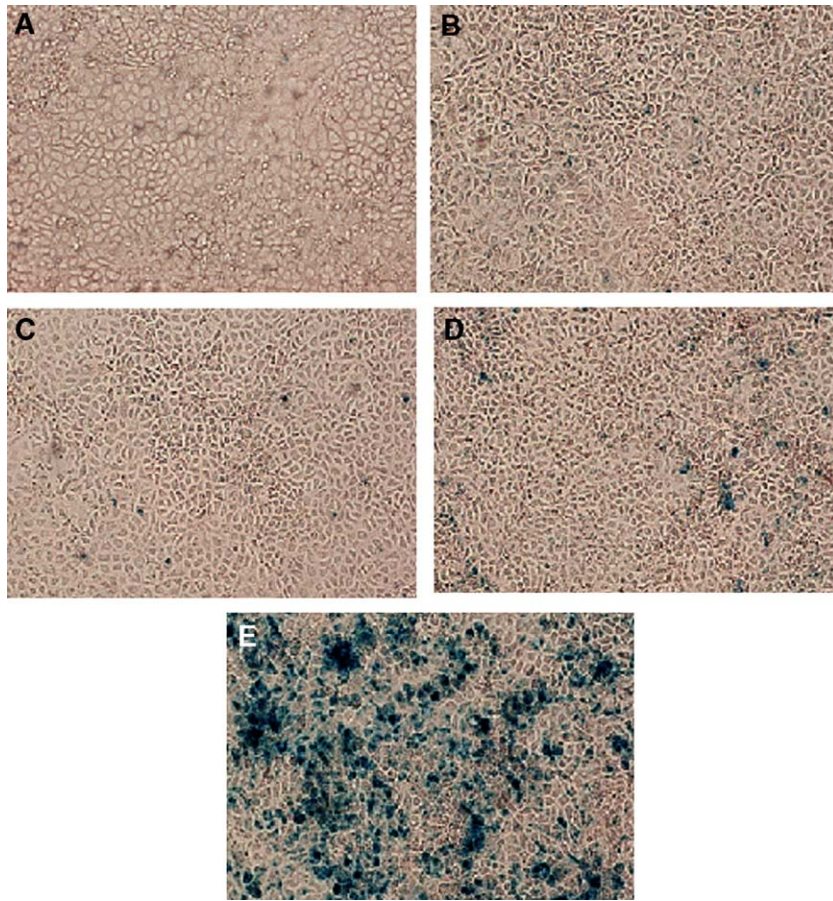


FIG. 2.

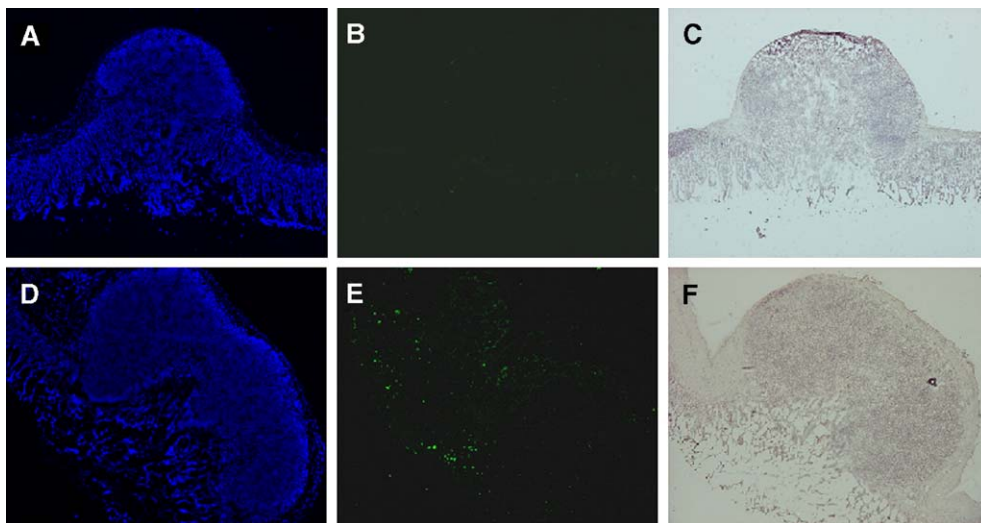
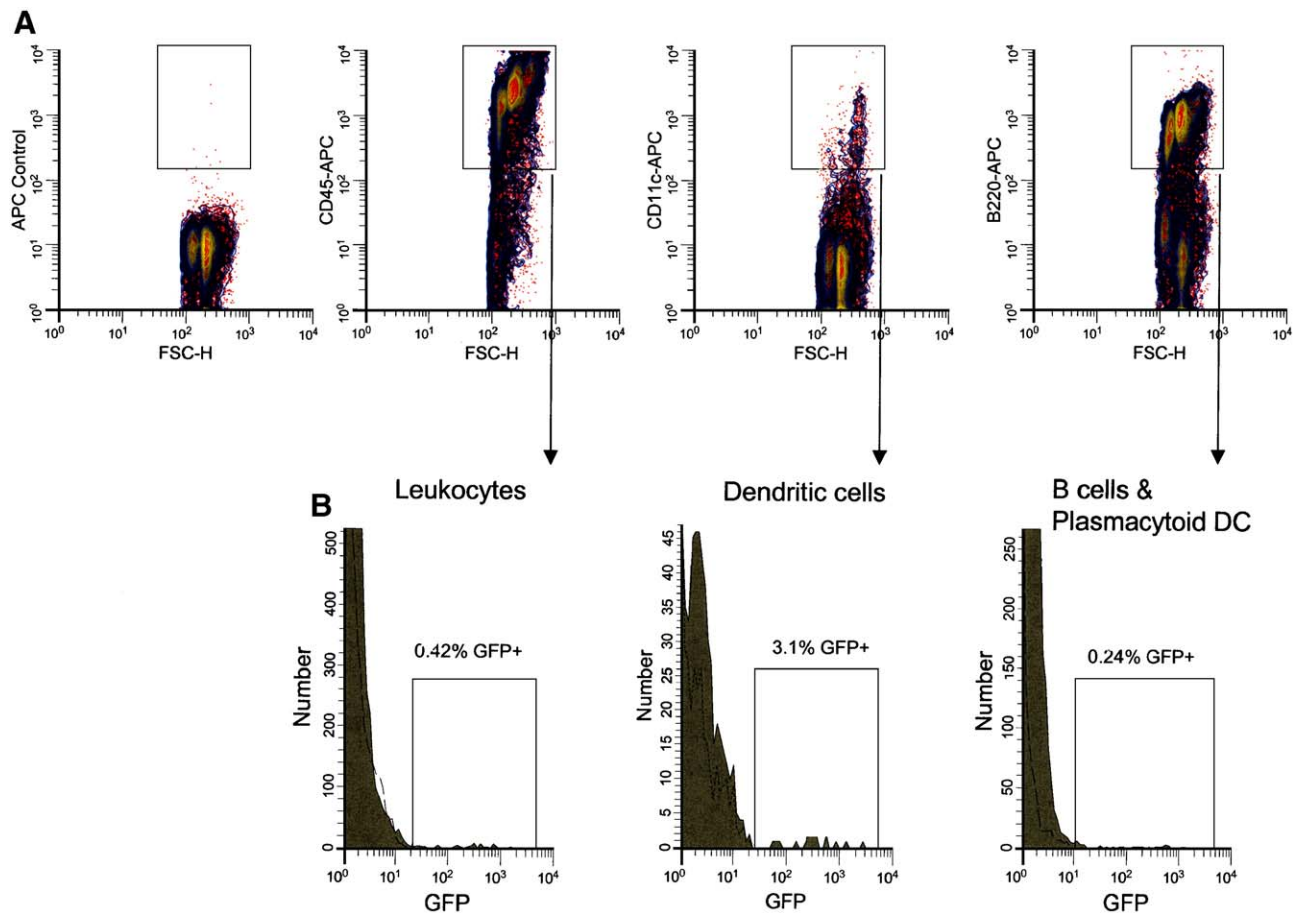


FIG. 3.



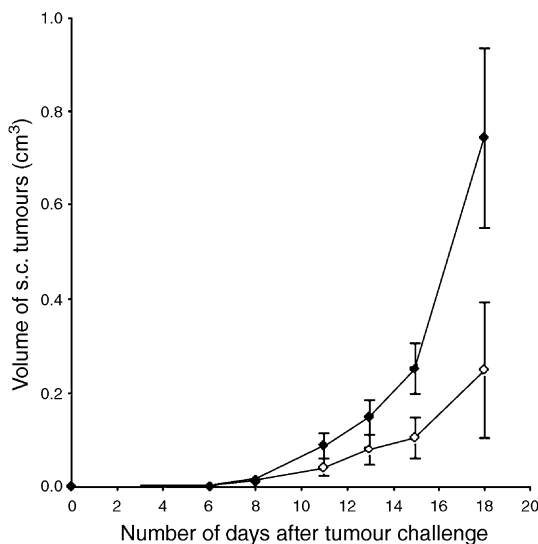
**FIG. 4.** Characterization of GFP-positive Peyer's patch cells extracted from small intestines perfused *ex vivo* with invasive *E. coli* expressing GFP. Following perfusion of small intestinal segments with *E. coli* BM2710 *inv-hly/Prok.GFP* or with Krebs–Henseleit solution alone, Peyer's patch cells were extracted and stained with a biotinylated anti-CD45, -CD11c, or -CD45R/B220 followed by streptavidin–allophycocyanin. Fluorescence was measured by flow cytometry and the data were analyzed using Winlist 5.0 software (Verity Software House, ME, USA). (A) Plots of forward scatter versus CD45 (gate: CD45<sup>hi</sup> leukocytes), CD11c (gate: CD11c<sup>+</sup> dendritic cells), or CD45RA/B220 (gate: B220<sup>+</sup> B cells and plasmacytoid DC). Boxed areas represent gated cell populations. (B) GFP fluorescence on the gated cell populations. Shaded histograms show fluorescence associated with cells from GFP<sup>+</sup> invasive *E. coli*-perfused tissue and the broken lines represent fluorescence of cells from tissues perfused with Krebs–Henseleit solution alone. The numerical values show the proportion of GFP<sup>+</sup> cells from bacteria-perfused tissues with reference to the control histogram.

tural opening of the tight junctions [28]. Invasive *E. coli*, as opposed to the noninvasive bacteria, can invade the cells of confluent Caco-2 cell monolayers that have been pretreated with ethanol (Fig. 2). These *in vitro* observa-

tions show that the tropism of invasive *E. coli* is strictly dictated not only by the expression but also by the accessibility of  $\beta_1$ -integrin on mammalian cells, in a paradigm very similar to that of adenovirus and the

**FIG. 2.** Invasion of confluent Caco-2 monolayers by invasive *E. coli* expressing  $\beta$ -gal. Confluent Caco-2 monolayers on coverslips were pretreated with serum-free medium (SFM) or SFM containing 8% ethanol, followed by co-incubation with SFM or *E. coli* BM2710 expressing  $\beta$ -gal for 2 h. Following extensive washing the monolayers were refed with complete medium containing gentamycin. Twenty-four hours later the monolayers were fixed and X-gal staining of  $\beta$ -gal activity was performed. (A) Untreated control monolayer. (B) SFM pretreatment, *E. coli* BM2710 *lacZ* (noninvasive). (C) 8% ethanol pretreatment, followed by *E. coli* BM2710 *lacZ* (noninvasive). (D) SFM pretreatment, followed by *E. coli* BM2710 *inv-hly/lacZ* (invasive). (E) 8% ethanol pretreatment, followed by *E. coli* BM2710 *inv-hly/lacZ* (invasive). The fields shown are representative of six monolayers from two independent experiments (original magnification  $\times 200$ ).

**FIG. 3.** Selective invasion of Peyer's patch tissue by invasive *E. coli* expressing GFP. C57/B6J mouse small intestines were maintained in an organ bath and perfused with Krebs–Henseleit solution, *E. coli* BM2710 *Prok.GFP* (noninvasive, A, B, C) or *E. coli* *inv-hly/Prok.GFP* (invasive, D, E, F). Peyer's patches and segments of small intestinal tissue were excised, snap frozen, and sectioned at a thickness of 5  $\mu$ m. Paraformaldehyde-fixed sections were stained with DAPI (A, D) or with hematoxylin and eosin (C, F). GFP (expressed by the *E. coli*) was visualized by fluorescence microscopy (B, E). Original magnification 40 $\times$ . The sections shown are representative of three small intestines from independent experiments.



**FIG. 5.** Oral vaccination with invasive *E. coli* expressing LLO and OVA. C57/B6J mice (10 per group) were gavaged with 5% NaHCO<sub>3</sub> or *E. coli* MC4100(DE3) inv-hly/OVA on days 0, 7, and 14. On day 21 all mice were challenged by subcutaneous injection with  $2 \times 10^5$  B16-OVA cells. Results are presented as mean tumor volumes (cm<sup>3</sup>)  $\pm$  SEM and are shown up to day 18, at which point at least eight mice per group still remained in the experiment.

coxsackie and adenovirus receptor [32]. Therefore, if these *in vitro* data can be extrapolated *in vivo*, it is expected that cells of a normal gut epithelium would be unlikely to be efficiently invaded by invasin-expressing *E. coli*. By contrast these bacteria have the potential to target diseased mucosa with a disrupted epithelium that would render  $\beta_1$ -integrin accessible. Interestingly, experimental evidence supporting this hypothesis was obtained, with the observation that similar invasive *E. coli* could significantly reduce the severity of experimental colitis in mice [33].

M cells in the follicular-associated epithelium overlying the Peyer's patches have been described to be the only cell type in the intestinal epithelium in which  $\beta_1$ -integrin is accessible at the apical side [20]. Following *ex vivo* infection of mouse small intestine tissue with invasive *E. coli* expressing GFP, sections through Peyer's patches showed green fluorescence within the patches, indicating the invasive *E. coli* had been transcytosed and gained entry into the underlying lymphoid tissue (Figs. 3D–3F). Far fewer noninvasive *E. coli* were detectable in Peyer's patch tissue following *ex vivo* infection of small intestine tissue (Figs. 3A–3C), suggesting that expression of invasin by invasive *E. coli* had mediated the uptake of the majority of invasive *E. coli* into the Peyer's patch. Both noninvasive and invasive *E. coli* were observed in very low numbers at the apical side of sections of non-Peyer's patch small intestinal tissue (not shown). FACS analysis revealed that a very low percentage (approximately 1.4%) of epithelial cells from Peyer's patches and

small intestinal tissues was shown by flow cytometry to be GFP positive following *ex vivo* perfusion with invasive *E. coli* expressing GFP. Therefore, epithelial cells are unlikely to be a target of invasive *E. coli* in the gastrointestinal tract.

The cell types within the Peyer's patch that may take up transcytosed invasive *E. coli* [34] were characterized by flow-cytometric analysis of cells extracted from Peyer's patches of small intestinal tissue that were perfused *ex vivo* with invasive *E. coli* expressing GFP. Flow-cytometric analysis revealed that around 3% of CD11c-positive cells (i.e., dendritic cells) were also GFP positive (Fig. 4), emphasizing the potential of invasive *E. coli* as a vaccine. In addition, around 0.24% of CD45R/B220-positive cells were also GFP<sup>+</sup>, suggesting an uptake of invasive bacteria by Peyer's patches B cells.

Subcutaneous administration of recombinant *E. coli* coexpressing LLO and OVA has been shown to induce potent anti-tumor immune responses in mice [23]. These responses are thought to be initiated by dendritic cells taking up *E. coli* and presenting peptides derived from OVA on MHC class I molecules. Dendritic cells also sample antigen from the intestinal lumen [35,36]. The Peyer's patch is a major site of antigen sampling by DC [37] and at this site DC probably take up antigen acquired by overlying specialized M cells. However, DC in the lamina propria may also sample antigen directly by passing dendrites between epithelial cells [38] and this route of uptake may be particularly important in the terminal ileum [39]. Based on these studies and the demonstration that invasive *E. coli* are selectively taken up from the small intestinal lumen into Peyer's patch tissue and subsequently colocalize with CD11c-positive and CD45R/B220-positive cells, the hypothesis was made that invasive *E. coli* could deliver the model antigen OVA to the dendritic cells of the mucosal lymphoid tissue and thereby induce specific systemic immune responses against tumor cells expressing OVA. Oral administration of invasive *E. coli* expressing LLO and OVA induced suppression of tumor growth following subcutaneous challenge with B16 tumor cells expressing OVA (Fig. 5). Altogether, these data (Figs. 3, 4, and 5) suggest that sufficient numbers of invasive *E. coli* survive the harsh acidic conditions of the stomach, reach the small intestine intact functionally, are transcytosed into the lymphoid tissue, and deliver OVA to antigen-presenting cells.

Mucosal tolerance is tightly regulated and mucosal dendritic cells are thought to be tolerogenic in the steady state [40,41]. Recognition of microbial structures by pattern recognition receptors, such as Toll-like receptors, on dendritic cells and other cells and/or changes in the local cytokine environment lead to dendritic cell activation and maturation, enabling activation of T cells [42]. In this study invasive *E. coli* may have provided such changes in the cytokine milieu by signaling via Toll-

like receptors. Peyer's patch dendritic cells that had phagocytosed invasive *E. coli* may have been activated and matured by the *E. coli*, leading to migration to the mesenteric lymph node to present OVA-derived peptides to CD8 T cells. Dendritic cells *in vivo* have previously been shown to become activated when they take up bacteria following intragastric challenge with *Enterobacter cloacae* [37].

The advantages of using *E. coli* as therapeutic vectors are that many nonpathogenic strains exist and can be further attenuated, e.g., by mutation of diaminopimelic acid [26]. In addition, the delivery strategy of recombinant *E. coli* relies on lysis of the *E. coli* following uptake into mammalian cells [22,23]. Therefore, following administration of a known dose, the number of *E. coli* would decline, rather than escalating as in the case of replicating bacteria such as *Salmonella* or *Listeria*. This may be a safer, more predictable approach to utilizing bacteria in therapy. Paraformaldehyde fixation of *E. coli* is a safety measure that may be ideal to minimize potential toxicity of *E. coli* further *in vivo*. Paraformaldehyde fixation of recombinant *E. coli* has been previously shown to reduce the cytotoxicity associated with LLO expressed by *E. coli* to murine and human dendritic cells, while retaining the functions of antigen delivery and immunostimulation [23,24]. Paraformaldehyde fixation does not affect the efficacy of recombinant *E. coli* as a subcutaneous vaccine in mice [23] and preliminary data have demonstrated that paraformaldehyde fixation does not affect the invasion activity (R. Critchley-Thorne *et al.*, unpublished).

In summary, our data demonstrate that invasive *E. coli* can deliver antigens to the mucosal immune system, probably via the Peyer's patches, and induce systemic immunity in a tumor model. Invasive *E. coli* may be an ideal oral vaccine since they are nonpathogenic, are adapted to the gastrointestinal environment, and have the ability to deliver antigens to antigen-presenting cells, combined with natural adjuvant properties to promote cellular immune responses. This approach may enhance the efficacy of parenterally administered *E. coli* vaccines or may be suitable for inducing both mucosal and systemic immune responses in the treatment and prophylaxis of gastrointestinal pathologies.

## MATERIALS AND METHODS

**Bacteria.** The bacterial strains expressing GFP and  $\beta$ -galactosidase and plasmids used in this study have been previously described [22,26,27]. The attenuation of these bacteria reduces dramatically their ability to grow *in vivo*. The *E. coli* strain expressing OVA (MC4100 (DE3)) is a derivative of the K12 *E. coli* strain, harboring the DE3 bacteriophage, which contains the T7 RNA polymerase gene allowing IPTG-inducible expression of genes under T7 promoter control [22]. The invasive property was conferred by transformation with the plasmid pJP2, which encodes invasins and listeriolysin O under the control of the constitutive *tet* promoter. Plasmid pDP-E3615 encodes listeriolysin O, lacking its secretion signal sequence, under the control of the constitutive *tet*

promoter and also resistance to chloramphenicol [22]. Plasmid pDP-E3616 encodes 32-kDa truncated OVA cDNA under the control of the IPTG-inducible T7 phage promoter and confers resistance to kanamycin. Plasmid pTrc/HisA/OVA encodes 32-kDa truncated OVA under the control of the IPTG-inducible Trc promoter and also encodes resistance to ampicillin. The levels of expression of OVA achieved in *E. coli* have been previously reported [22,23]. The MC4100 (DE3) *E. coli* and plasmids described above were gifts from D. Higgins, Harvard Medical School (Boston, MA, USA).

**In vitro invasion experiments.** Caco-2 cells (obtained from the Cancer Research UK Cell Services collection) were seeded at a density of  $1 \times 10^4$ /cm<sup>2</sup> (sparse cells) in complete medium containing 10% fetal calf serum (FCS; Autogen Bioclear, Calne, UK) and allowed to attach overnight. To form confluent monolayers, cells were grown to confluency in DMEM containing 10% FCS at 37°C, 10% CO<sub>2</sub>, and maintained for 2 weeks. The mammalian cells were washed twice in serum-free medium (SFM) and then refed with SFM (containing 85  $\mu$ g/ml dap). Harvested *E. coli* BM2710 were added to the culture medium and the cells were incubated for 2 h at 37°C, 10% CO<sub>2</sub>. The cells were then washed three times with SFM and refed with complete medium containing 20  $\mu$ g/ml gentamycin. Gentamycin is an antibiotic that cannot permeate mammalian cells and, therefore, is used to kill extracellular bacteria, leaving intracellular bacteria unaffected [43].

**Biochemical assays.**  $\beta$ -Galactosidase activity was measured as previously described [27].

For lactose dehydrogenase release, samples of culture medium were taken from the apical side of Caco-2 cell monolayers grown on the permeable membranes. The culture medium samples were centrifuged at 1500 rpm and the supernatant was assayed for lactose dehydrogenase (LDH) activity using an LDH Cytotoxicity Detection Kit according to the manufacturer's instructions (Takara Biomedicals, Shiga, Japan). Production of formazan was assessed by measuring the optical density at 490 nm.

**Flow-cytometric analysis.** Caco-2 cells were harvested by centrifugation for 5 min at 1500 rpm at 4°C. The pellets were washed in PBS (for Caco-2 cells) or FACS buffer (PBS containing 2% FCS, 1 mM EDTA, 0.02% sodium azide—for Peyer's patch cells) and resuspended in PBS or FACS buffer. Fluorescence was measured using a FACSCalibur flow cytometer (Becton-Dickinson, CA, USA). The data were analyzed using CellQuest software (Becton-Dickinson), WinMDI version 2.8 software (J. Trotter, The Scripps Research Institute, La Jolla, CA, USA), or Winlist 5.0 (Verity Software House).

**Ex vivo infection of intestines.** Adult female C57/B6J mice were culled by cervical dislocation. The small intestines were removed, flushed, and tied into a perfusion organ bath (Hugo Saks Electronic Mayflower Tissue Bath, Germany). The tissue bath is specialized for studying perfused tubular organs such as intestines, blood vessels, and vas deferens. Individual solutions can be used for intraluminal perfusion and extraluminal superfusion. Krebs-Henseleit solution (NaCl 118.00 mmol/L, KCl 4.70 mmol/L, CaCl<sub>2</sub> 2.52 mmol/L, MgSO<sub>4</sub> 1.64 mmol/L, NaHCO<sub>3</sub> 24.88 mmol/L, KH<sub>2</sub>PO<sub>4</sub> 1.18 mmol/L, glucose 5.55 mmol/L, sodium pyruvate 2.00 mmol/L) was used to maintain the tissues. Carbogen (95% O<sub>2</sub> + 5% CO<sub>2</sub>; Boc Gases, Guildford, Surrey, UK) was used to adjust the pH of the KHS to 7.4. The organ bath and perfusion solutions were maintained at 37°C. Segments of small intestine were perfused intraluminally with KHS or *E. coli* suspended in KHS for 60 min. The tissue was then perfused with KHS containing 100  $\mu$ g/ml gentamycin for 30 min.

**Staining of small intestine and Peyer's patch tissue.** Samples of small intestinal and Peyer's patch tissue were immersed in isopentane for 20 min at room temperature. Tissues were removed from the isopentane and snap frozen in liquid nitrogen. The frozen tissue was sectioned at 5  $\mu$ m, air dried on glass slides, and incubated in 4% paraformaldehyde for 20 min at room temperature. The fixed sections were washed twice for 5 min in PBS, immersed in 30 nM DAPI (Molecular Probes, Leiden, Netherlands) for 3 min at 4°C protected from light, and washed a

further two times for 5 min in PBS. Sections were mounted using Permafluor Immunon aqueous mounting medium (Shandon, Pittsburgh, PA, USA) and were visualized using an Olympus BX51 fluorescence microscope (Olympus UK, Ltd., London, UK). Hematoxylin and eosin staining was performed as previously described [44].

**Isolation of epithelial cells and Peyer's patch cells from mouse small intestinal tissue.** Small intestinal tissue and Peyer's patches from C57/B6j mice were collected into RPMI Dutch modification medium (Sigma-Aldrich, Poole, UK) containing 10% heat-inactivated (HI) FCS. To remove mucus and fecal matter the tissues were transferred to Hanks balanced salt solution without calcium and magnesium (HBSS  $-Ca^{2+}$   $Mg^{2+}$ ; Gibco BRL, Crewe, UK) containing 1 mM dithiothreitol and incubated for 20 min at room temperature with occasional agitation. To remove and recover epithelial cells the tissues were washed twice with HBSS  $-Ca^{2+}$   $Mg^{2+}$  and incubated in HBSS  $-Ca^{2+}$   $Mg^{2+}$ , 1 mM EDTA at 37°C with gentle shaking for 30 min. The medium (containing the epithelial cells) was removed, passed through a 100- $\mu$ m nylon filter, and retained. The tissues were washed three times with HBSS  $-Ca^{2+}$   $Mg^{2+}$ . The media from washes were also passed through the filter and retained. The EDTA incubation and washes were repeated twice. The retained epithelial cells were centrifuged at 1500 rpm for 5 min at 4°C and stored on ice. To inactivate the EDTA the tissues were washed once in RPMI Hepes modification medium (Sigma-Aldrich) containing 10% HI FCS. To recover lamina propria cells the tissues were transferred to RPMI Hepes containing 1 mg/ml collagenase D (Roche Diagnostics, Mannheim, Germany), 0.2 mg/ml DNase I (Roche Diagnostics), and 2% heat-inactivated FCS and incubated at 37°C for 60 min with gentle agitation. The medium was passed through 100- $\mu$ m nylon filters and retained. The Peyer's patches were pressed through the filters using the barrel of a syringe and washed through with RPMI Dutch containing 10% HI FCS. The LPLs and epithelial cells were harvested by centrifugation at 1500 rpm for 5 min at 4°C, washed in RPMI Dutch containing 10% HI FCS, and centrifuged again.

For flow-cytometric analysis cells were washed in FACS buffer and labeled for 20 min with biotinylated antibodies to CD45, CD11c, or CD45R/B220 (BD Biosciences, Oxford, UK). After being washed twice by centrifugation in cold FACS buffer, the cells were labeled with streptavidin-allophycocyanin (BD Biosciences). Control labeling was performed with streptavidin-allophycocyanin alone. After washing, fluorescence data was acquired using a FACSCalibur flow cytometer (Becton-Dickinson). The data were analyzed using Winlist 5.0 (Verity Software House).

**Vaccination and tumor challenge.** *E. coli* were resuspended in 5%  $NaHCO_3$ . *E. coli* ( $5 \times 10^7$ ) were administered orally in a volume of 0.2 ml 5%  $NaHCO_3$  to female C57/B6j mice using autoclaved metal gavage needles. *E. coli* were administered three times at weekly intervals prior to tumor challenge with B16-OVA cells (obtained from the Cancer Research UK Cell Services collection), performed as previously described [23].

**Statistical analyses.** Statistical analysis of data was performed using the paired or unpaired two-tailed Student *t* test. The results of the analyses are expressed as *P* values. Statistical significance was defined as *P* < 0.05.

**Housing of animals.** C57/B6j mice were obtained from Harlan (Oxfordshire, UK) and kept in a germ-free environment with irradiated food and acidified water *ad libitum* at Cancer Research UK Biological Resources. Experiments were conducted after appropriate ethical approval and licensing was obtained in accordance with the United Kingdom Guidance on the Operation of Animals (Scientific Procedure) Act 1986 (HMSO, London, UK, 1990).

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