A recombinant *Listeria monocytogenes* vaccine expressing a model tumour antigen protects mice against lethal tumour cell challenge and causes regression of established tumours

ZHEN-KUN PAN[1], GEORGIOS IKONOMIDIS[1], AUDREY LAZENBY[2], DREW PARDOLL[3] & YVONNE PATERSON[1]

[1]*Department of Microbiology and the University of Pennsylvania Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6076, USA*
[2]*Department of Pathology, and* [3]*Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA*
Correspondence should be addressed to Y.P.

***Listeria monocytogenes* is an intracellular organism that has the unusual ability to live in the cytoplasm of the cell. It is thus a good vector for targeting protein antigens to the cellular arm of the immune response. Here we use a model system, consisting of colon and renal carcinomas that express the influenza virus nucleoprotein and a recombinant *L. monocytogenes* that secretes this antigen, to test the potential of this organism as a cancer immunotherapeutic agent. We show that this recombinant organism can not only protect mice against lethal challenge with tumour cells that express the antigen, but can also cause regression of established macroscopic tumours in an antigen-specific T-cell-dependent manner.**

Listeria monocytogenes is a Gram-positive, facultative intracellular bacterium that has the unusual ability to live in the cytoplasm of the cell[1]. *L. monocytogenes* enters the host cell and is taken up in a phagosome but, unlike most other intracellular bacteria, it escapes into the cytoplasm of the cell by disrupting the phagosomal membrane, primarily through the action of a secreted virulence factor, listeriolysin O (LLO)[1–5]. It thus targets its own antigens to both the MHC class I and class II pathways of the infected cell for antigen presentation, a rare property relative to most viruses and bacteria, and thus induces strong cellular immune responses[1,4–6]. In previous studies we have verified that stable recombinants of *L. monocytogenes* that express foreign viral and bacterial antigens can elicit strong $CD8^+$ T-cell responses to that antigen[7–9]. This opens up the exciting possibility of using this organism as a cancer vaccine.

It is now well accepted that tumour cells express antigens that can be recognized by the immune system with the generation of antigen-specific $CD8^+$ cytotoxic T cells (CTLs) and $CD4^+$ T-helper-cell responses[10–15]. These antigens may include mutated self antigens, re-expressed embryonic antigens, tissue specific differentiation antigens or viral antigens[10–15]. Here we show that a recombinant *L. monocytogenes* that secretes a tumour-specific antigen can not only protect mice against challenge with a tumoricidal dose of tumour cells that express the antigen, but can also cause regression of established macroscopic tumours, in an antigen-specific, T-cell-dependent manner. This therapeutic response is dependent on the generation *in vivo* of both $CD4^+$ and $CD8^+$ effector T cells.

The ability of *L. monocytogenes* to confer prophylactic and therapeutic immunity to tumour challenge was assessed by using a previously described[8] *L. monocytogenes* recombinant (Lm-NP) that stably expresses and secretes a fusion protein consisting of the first 420 amino acids of LLO fused to the influenza nucleoprotein sequence 1–498 (NP) from influenza virus strain A/PR8/34. We have shown that Lm-NP effectively targets NP to the class I pathway for the induction of $CD8^+$ T cells[8]. The tumour cells chosen for study are CT26, an *N*-nitroso-*N*-methylurethane-induced murine colon tumour line[16] and the spontaneously arising renal cell carcinoma Renca[17]. Both cell lines are derived from BALB/c mice, express $H\text{-}2^d$ class I MHC molecules but not class II MHC and are highly tumorigenic. They were transduced with the same NP gene (A/PR8/34) that Lm-NP secretes by using a defective Moloney murine leukaemia retrovirus containing both the NP gene and a neomycin phosphotransferase gene.

Results

L. monocytogenes-NP can protect against tumour challenge

First, we tested the ability of Lm-NP to protect against tumour challenge (Fig. 1*a* and *b*). BALB/c mice were immunized with wild-type *L. monocytogenes* (Lm), sterile saline or the recombinant *L. monocytogenes* vaccine vector (Lm-NP). Two weeks after the last immunization, ten animals in each immunization group were challenged subcutaneously with 100 times the tumoricidal dose of CT26, Renca, CT26-NP or Renca-NP. As Fig. 1*a* shows none of the animals that received the Lm-NP vaccine showed any sign of Renca-NP growth. Six animals that received CT26-NP remained tumour-free, three had tumours of less than 5 mm and one had a tumour of 9 mm (Fig. 1*b*). All of the control groups of animals showed continuous tumour

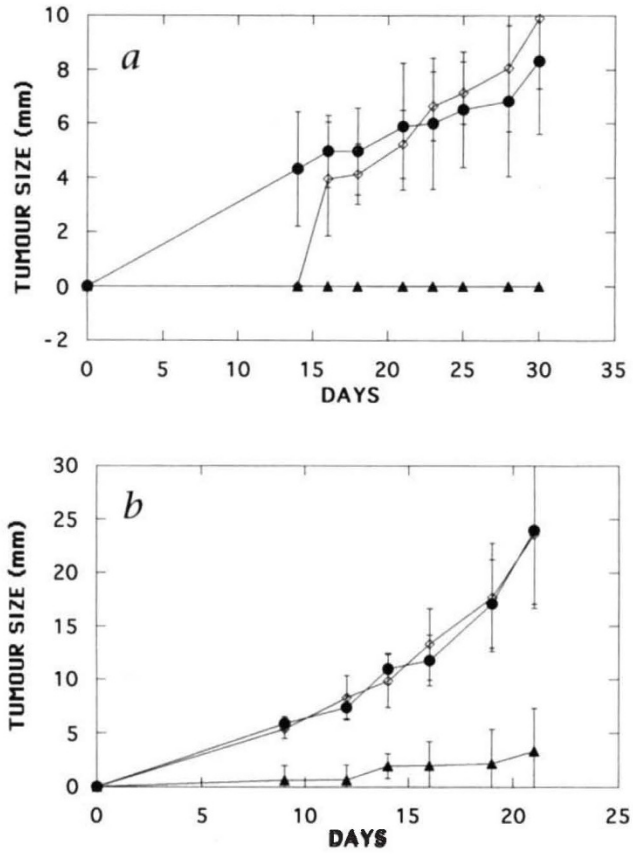

Fig. 1. Protection against tumour challenge with *L. monocytogenes*. *a*, Tumour growth in BALB/c mice challenged with Renca-NP. *b*, Tumour growth in BALB/c mice challenged with CT26-NP. Animals received either wild-type Lm (open diamonds), or the recombinant Lm-NP vector (filled triangles) or saline (filled circles) before challenge. Data are not shown for the corresponding control groups of animals that received either the parental Renca or CT26 tumour cell lines for which no difference in growth among the animals immunized with Lm, Lm-NP or saline was detected.

growth between 1.5 and 3.0 cm; growth of Renca and CT26 parental lines in animals immunized with Lm, Lm-NP and saline was equivalent (data not shown).

Lm-NP can cause regression of established tumours

In most animal studies, although tumour vaccines have been shown to protect against challenges with limited numbers of tumour cells, the vaccines have not been able to cure the animals of established tumours[18]. Previous attempts to treat macroscopic tumours by using either a whole tumour cell or recombinant vaccine approach have been unsuccessful. When therapeutic effects have been observed, they have generally applied only to microscopic and not to macroscopic tumours. We thus tested the therapeutic potential of our vaccine vector to limit tumour growth. Tumour cells (5×10^5) of either Renca-NP or CT26-NP were introduced into BALB/c mice subcutaneously. After measurable macroscopic tumours had grown in the mice, ten animals in each group received Lm-NP, Lm or no further treatment. Ten days later the mice were immunized again with either Lm-NP or Lm. Only the mice receiving the Lm-NP vaccine showed changes in tumour growth (Fig. 2*a* and *b*). In the case of the mice challenged with Renca-NP (Fig. 2*a*), only one out of ten mice showed any sign of visible tumour growth by four days after the last immunization, whereas control groups showed rapid progressive growth. This remission proved to be long-lasting and antigen-specific, in that the tumour-free mice showed no signs of regrowth for a further month, after which they were re-challenged on each flank with 5×10^5 cells of either Renca-NP or Renca. They showed no sign of Renca-NP growth, whereas Renca grew aggressively to the point where it was necessary to kill the animals.

In the case of animals challenged with the colorectal CT26-NP tumour (Fig. *2b*), we saw a significant slowing of tumour growth to a near standstill ($P < 0.001$ at day 17 and $P < 0.0001$ at day 19) for the animals that received the Lm-NP vaccine compared with the control groups. There was, however, no regression with CT26-NP as there was for the Renca-NP renal carcinoma, probably because CT26-NP is a much faster growing tumour than Renca-NP and is more unstable in its expression of NP. It seems probable, therefore, that CT26-NP tumours grown *in vivo* for three weeks in the absence of neomycin (G418) lose NP expression. To confirm this, CT26- NP tumours were explanted from BALB/c mice on days 12 and 28 after subcutaneous injection of 10^6 CT26-NP and tested for their ability to be lysed by secondary cultures of lymphocytes from mice immunized with vaccinia-NP. At a 60:1 effector:target ratio, the specific lysis of CT26-NP on day 28 was only 14% compared with 35% for tumour cells removed on day 12. At a 10:1 effector:target ratio, 8% of day 28 CT-26 cells were killed compared with 18% of day 12 tumour cells. Thus, it appears that expression of NP by CT26-NP is lost between day 12 and day 28 of *in vivo* growth.

The ability of an antigen-based therapy to eliminate macroscopic tumours in mice (Fig. 2*a*) is unprecedented in cancer immunotherapy in the absence of adjunctive therapy. For example, immune responses induced to the immunoglobulin idiotype of B-cell lymphomas were shown to cause regression of established tumours but only when combined with chemotherapy[19]. Currently, vaccinia virus is the only recombinant vaccine system that has been evaluated as a cancer vaccine[10]. The efficacy of a recombinant vaccinia virus that expresses NP as a vaccine against the tumours used in the current study was, therefore, evaluated. Although prior vaccination with this recombinant virus protected against subsequent challenge with Renca-NP (20 out of 20 mice protected) and CT26-NP (14 out of 20) when used to treat estab-

Table 1 Number of mice with detectable serum antibody levels against NP and LLO before and after Lm-NP immunization. Mice are grouped on the basis of their serum antibody titres.

Antisera/antigen	Number of mice with antibody levels of[a]			
	–	+	++	+++
Pre-immune/LLO	29	0	1	0
Pre-immune/NP	30	0	0	0
Post-immune/LLO	2	8	14	6
Post-immune/NP	11	19	0	0

[a]Levels of antigen-specific antibody in the serum samples were scored thus: titre (t) < 40 (–); 40 < t < 160 (+); 160 < t < 640 (++); 640 < t < 1280 (+++). In the case of the anti-NP responses, antibody levels were quantified by reference to an affinity-purified anti-NP monoclonal antibody; t = 160 (+) is equivalent to 1 μg ml^{-1} of anti-NP antibody.

lished tumours, there was no impact even on 5-day established microscopic tumours for either tumour line (0 out of 20 mice showed a change in tumour growth in each group). In addition, influenza virus was also incapable of influencing tumour growth in microscopic 5-day tumours (data not shown).

Antitumour effects of Lm-NP are not antibody mediated

The exquisite antigen specificity of the protection we observe in Figs 1 and 2 argues against protection being mediated by natural killer cells. The properties of *L. monocytogenes* are such that it does not induce a good humoral response[1]. Nevertheless, to assess the role of antibody in the antitumour response, we measured both the anti-NP and anti-LLO antibody levels in the mice that had received either Lm-NP or Lm. As Table 1 shows, the 30 mice that received the Lm-NP immunizations had serum anti-NP antibody levels of <1 mg ml^{-1} and low titres to both the natural *L. monocytogenes* antigen, LLO, and the recombinant NP. Thus, we feel that antibodies probably do not play a major role in the antitumour properties of Lm-NP.

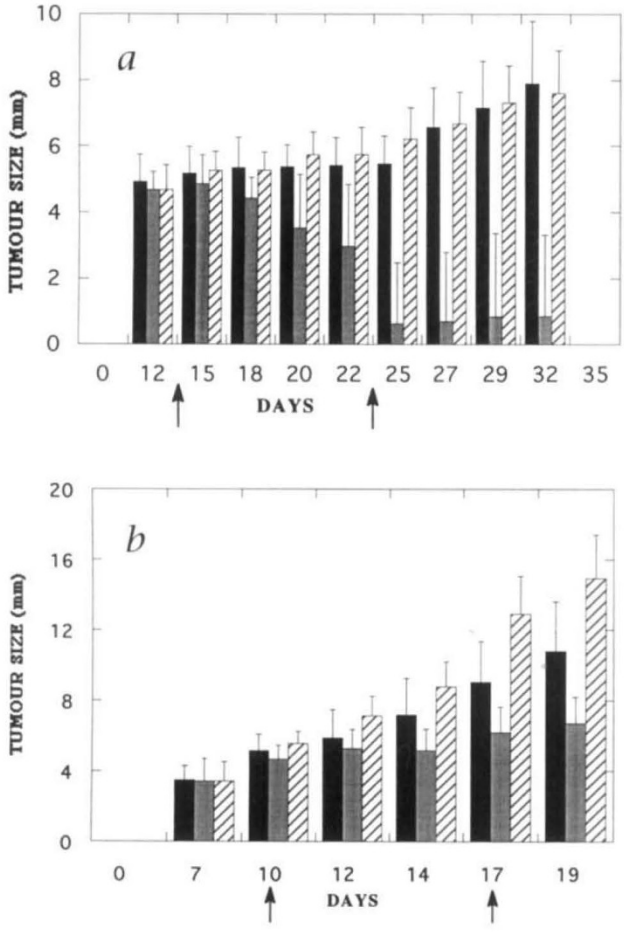

Fig. 2. Elimination of established Renca-NP tumour growth and inhibition of established CT26-NP tumour growth by the Lm-NP vaccine. Tumour cells (5×10^5) of either Renca-NP (*a*) or CT26-NP (*b*) were introduced subcutaneously into two groups of 30 mice. After measurable tumours had grown in the mice, ten mice in each group received Lm-NP (darkly shaded bars), ten mice received wild-type Lm (striped bars) and ten received saline (filled bars). The mice received immunizations on the days indicated by the arrows.

Tumour immunity is T-cell dependent

We next investigated the role of T cells in the Lm-NP-induced immunity to Renca-NP and CT26-NP. First, we performed a histologic analysis of NP-expressing tumours excized from Lm-NP immunized mice (Fig. 3). This revealed extensive lymphocyte infiltrates as well as macrophages and granulocytes (Fig. 3). Infiltrates were most extensive at the tumour borders (Fig. 3*b*), although immunohistochemical staining also revealed significant numbers of $CD3^+$ lymphocytes interspersed within the interstitium of the tumour (Fig. 3*d*), which were both $CD8^+$ (Fig. 3*f*) and $CD4^+$ (Fig. 3*e*). In contrast, NP-expressing tumours from Lm-immunized mice, as well as parental (NP–) tumours from Lm-NP-immunized mice, demonstrated only a mild peritumour lymphocyte-poor infiltrate (Fig. 3*a*) and no lymphocytes within the tumour interstitium (Fig. 3*c*). Thus, the infiltration of both $CD4^+$ and $CD8^+$ cells into the tumour was dependent on NP expression by both the tumour and the vaccine.

The infiltration of both $CD4^+$ and $CD8^+$ T cells suggested that both T-cell subsets might in fact play an effector role in the antitumour response. Both tumours are MHC class I^+/II^-, and class II expression on CT26 is not induced by cytokines such as interferon-γ (IFN-γ). Thus, $CD8^+$ cells might be expected to be the most critical effector cells. To verify that $CD8^+$ NP-specific T cells were cytotoxic for Renca-NP and CT26-NP, we next examined mice that had received the vaccine protocol for the presence of CTLs that could specifically lyse these tumour cell lines. Both NP^+ cell lines, but not the parental cell lines, were efficiently lysed by secondary CTL cultures from Lm-NP immunized mice (see Fig. 4) demonstrating the presence of cytotoxic T cells in the periphery of mice immunized with Lm-NP, which could have eliminated the tumour by a direct killing mechanism. Shown as a positive control, in Fig. 4, is the ability of these CTLs to kill the standard target cell P815 in the presence of the K^d-restricted NP epitope.

Finally, in order to evaluate the role for each T-cell subset as antitumour effectors *in vivo*, naive BALB/c mice were immunized with Lm-NP using the same protocol as used in Fig. 1. Ten days later, ten mice each were depleted of either $CD8^+$ cells, or $CD4^+$ cells by *in vivo* antibody administration, and ten were left with a complete T-cell repertoire. After T-cell depletion, the mice were challenged with CT26-NP. As a control, ten naive mice were also challenged. As Fig. 5 shows, the mice with a complete T-cell repertoire show protection against tumour growth induced by the Lm-NP vaccine, whereas depletion of the $CD8^+$ T-cell subset totally abrogated the vaccine effect. Indeed tumour growth in the Lm-NP immunized mice in which $CD8^+$ T cells were depleted was significantly increased ($P < 0.017$ at day 22) compared with the control (naive) group of mice. In addition, the mice in which the $CD4^+$ T-cell subset was depleted showed reduced protection indicating that $CD4^+$ T cells also play an important role in the control of tumour growth.

Discussion

In this article, we have shown that *L. monocytogenes* has the ability to deliver a foreign antigen to the immune system and to invoke cell-mediated immunity that is sufficiently strong to eliminate established tumours that express the same antigen.

The rejection of macroscopic tumours purely on the basis of an antigen-specific immune response and in the absence of adjunctive therapy is, to our knowledge, unprecedented.

The frequent failure of the immune system to respond to tumour antigens and the subsequent outgrowth of transformed cells in cancer has been thought to be due to the poor immunogenicity of naturally occurring tumours or tolerance mechanisms acting upon tumour-specific T cells[10,20]. This deficiency has been attributed to inadequacies in the $CD4^+$ T-cell response to tumour-specific antigens and can be remedied by transfecting the tumour cells with certain cytokine genes, such as those for the interleukins IL-2, IL-3, IL-4, IL-6, and IL-7, or granulocyte-macrophage colony-stimulating factor[10,20–23] or by costimulatory ligands[10,24], which either activate or bypass the requirement for T-cell help in mounting a protective tumour antigen-specific $CD8^+$ CTL response. These cosignals can also be provided by fusing tumour cells with activated B cells, resulting in an effective cancer vaccine[25]. However, an effector mechanism for $CD4^+$ T cells in the protective responses we observe for Lm-NP cannot be restricted to their role in priming antigen-specific $CD8^+$ T cells since, as seen in Fig. 5, they were depleted after the induction of antigen-specific cellular immune responses by Lm-NP, immediately before tumour challenge. Nor is a role for $CD4^+$ T cells in assisting antibody-dependent tumour protection probable, given the data shown in Table 1. The *L. monocytogenes*-induced helper response is known to be biased toward the T helper 1 subset as a consequence of IL-12 induction[26]. Thus one role of $CD4^+$ T cells could be to increase levels of IFN-γ, which could enhance expression of accessory molecules that are known to play a role in $CD8^+$ CTL lysis of tumour cells[10,24] and/or MHC molecules[27–29]. Escape of tumour cells from immune attack due to loss of MHC class I molecules has been frequently observed[28,29]. This has been attributed to a reduction in class I α-chain transcription[30,31] or to low levels of the antigen-processing proteosomes encoded by *LMP-7* and *LMP-2* and the peptide transporters *TAP-1* and *TAP-2* (ref. 27). Upregulation of these molecules by treating the tumours with IFN-γ reverses these deficits[27]. *L. monocytogenes* is known to induce strong T helper 1 $CD4^+$ T-cell responses, which will result in the release of IFN-γ. However, we observe a heavy infiltration of $CD4^+$ T cells only into the tumours that express NP from the mice that received the Lm-NP vaccine (Fig. 3*e*). This suggests that, rather than merely enhancing the ability of $CD8^+$ T cells to kill NP-expressing tumour cells, there is an antigen-specific antitumour role for $CD4^+$ T cells. Indeed, quantification of the T cells infiltrating the tumour shown in Fig. 3*d* indicates that about one-third of them are CD4 positive. We are currently investigating the role of *L. monocytogenes*-induced

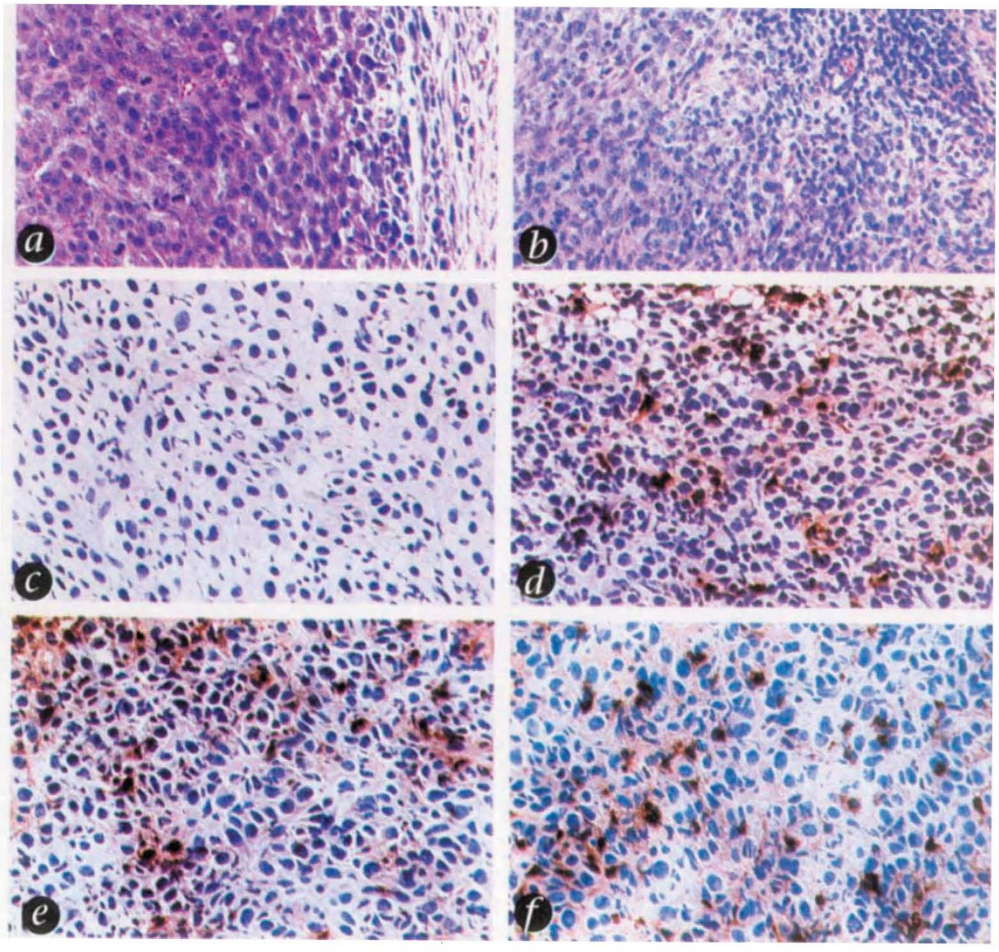

Fig. 3. Histologic analysis of CT26-NP tumours excized 23 days post challenge from mice immunized with Lm-NP and Lm. *a*, Lm-immunized mice, haematoxylin and eosin. *b*. Lm-NP-immunized mice, haematoxylin and eosin. *c*, Lm-immunized mice, CD3 immunoperoxidase stain. *d*, Lm-NP-immunized mice, CD3 immunoperoxidase stain. *e*, Lm-NP-immunized mice, CD4 immunoperoxidase stain. *f*, Lm-NP-immunized mice, CD8 immunoperoxidase stain. Magnification, ×400.

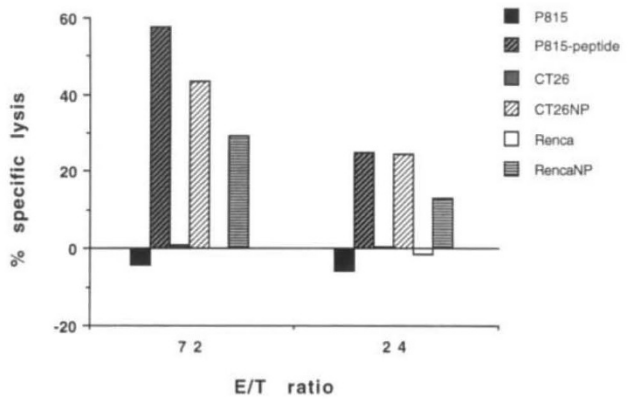

Fig. 4 CTLs generated by immunizing BALB/c mice with Lm-NP can kill the tumour cells CT26-NP and Renca-NP that express NP *in vitro*. Target cells were Renca, Renca-NP, CT26, or CT26-NP. As positive and negative controls for the lytic ability of the Lm-NP-specific CTLs, P815 cells, with and without the K^d-restricted NP peptide, were also used as targets. Plots of per cent specific lysis at two effector:target ratios (72:1 and 24:1) are shown.

$CD4^+$ T cells in antigen-specific inhibition and regression of tumour growth. Given that vaccinia-NP, which is known to induce strong $CD8^+$ T-cell responses had a far weaker antitumour response, it is probable that it is the collaboration of antigen-specific $CD4^+$ T helper 1 and $CD8^+$ CTLs induced by *L. monocytogenes* that generates potent enough responses to protect against tumour challenge and to destroy established tumours.

The use of *L. monocytogenes* as a cancer therapeutic is predicated on the presence of natural tumour-specific antigens to which a cellular immune response can be directed. However, a number of candidate T-cell-dependent antigens have already been defined for certain tumour types[10]. In addition, the powerful technique of sequencing naturally processed peptides from MHC class I molecules[12,32] isolated from tumour cells is likely to identify many more in the future. In particular, two groups of shared tumour antigens have been identified in human melanoma. MAGE-1 is the prototype for one group, which in normal adult tissues is expressed only in the testes[33]. The second group involves melanocyte-specific differentiation antigens such as tyrosinase. Tyrosinases have recently been shown to be a target for $CD4^+$ as well as $CD8^+$ melanoma-reactive T cells[10–15]. Tissue-specific differentiation antigens shared by the tumour may in fact be quite appropriate targets for immunotherapy of cancers derived from dispensable tissues.

Some of the most important human cancers against which antigen-specific immunotherapy may be efficacious are the virus-associated cancers such as human papilloma virus (cervical cancer), Epstein-Barr virus (Hodgkin's and nasopharyngeal cancers) and hepatitis (hepatoma). It is now recognized that 10–20% of cancer worldwide is virus associated. For example 90% of cervical cancers express the E6 and E7 oncoproteins derived from either HPV16 or HPV18 (refs 32,33). Indeed, some of the most important human cancers to which antigen delivery by *L. monocytogenes* could be applied are the virus-associated cancers, in which the viral antigens should be effective target antigens. The best example — cervical cancer — is the second leading cancer killer among women.

There are always safety concerns in using live vaccines for therapeutic use. However, *L. monocytogenes* is a relatively benign Gram-positive organism, which does not produce endotoxin and which is susceptible to a wide range of antibiotics including penicillin. In addition, when administered orally, the natural route of infection, we have shown that a recombinant *L. monocytogenes* can induce $CD8^+$ CTL responses in the periphery to the foreign antigen[7], thus opening up the exciting possibility of an oral anticancer therapy. We are currently exploring the potential of *L. monocytogenes* as an oral cancer vaccine using the model system described in these studies.

Methods

Cell lines. CT26, an *N*-nitroso-*N*-methylurethane-induced murine colon tumour line[16] and the spontaneously arising renal cell carcinoma Renca[17] are derived from BALB/c mice, express $H\text{-}2^d$ class I MHC molecules but not class II MHC and are highly tumorigenic. They were transduced with the same NP gene (A/PR8/34) that Lm-NP secretes by using a defective Moloney murine leukaemia retrovirus containing both the NP gene and a neomycin phosphotransferase gene as previously described[36,37]. Transductants were selected in G418 and retain the NP gene *in vitro* in the presence of G418. Expression of NP was confirmed in individual Renca-NP and CT26-NP clones by staining of permeabilized cells with an NP-specific monoclonal antibody and by their ability to be lysed by NP-specific CTLs (P. Golumbek, D. Pardoll, unpublished observations). The morphology of Renca-NP and CT26-NP is similar to that of the parental lines but the Renca-NP tumour grows more slowly than the parental line when injected subcutaneously into BALB/c mice. However, the expression of NP in Renca and CT26 does not enhance their immunogenicity or decrease their intrinsic tumorigenicity, and the minimal tumoricidal doses for Renca-NP and CT26-NP in BALB/c mice are identical to the parent lines (5×10^3 per mouse). Furthermore, animals in which Renca-NP and CT26-NP are growing do not have functionally detectable NP-specific CTLs. Thus, when expressed in the tumours, NP behaves indistinguishably from an endogenous tumour antigen.

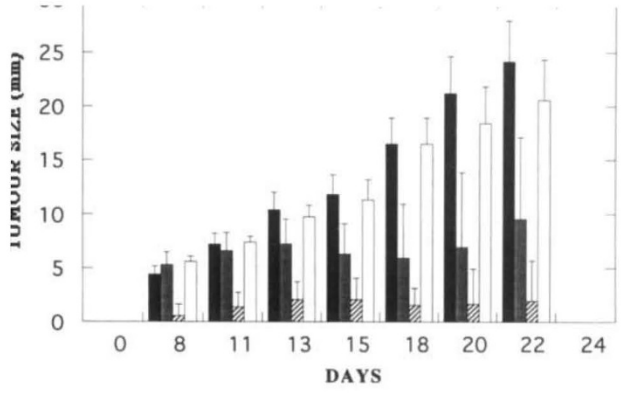

Fig. 5 Role of $CD4^+$ and $CD8^+$ T-cell subsets in protection against tumour challenge. Mice were immunized with Lm-NP using the same protocol as used in Fig. 1. Ten days after the last immunization, ten mice in each group were depleted of $CD8^+$ (filled bars) or $CD4^+$ T cells (darkly shaded bars) or were left with an intact T-cell repertoire (striped bars) and then challenged with CT26-NP as in Fig. 1. Ten naive mice that did not receive Lm-NP were challenged with CT26-NP on the same day as the test groups (white bars).

***L. monocytogenes* strains and propagation.** The *L. monocytogenes* strains used in this study are the haemolytic wild-type strain 10403S and DP-L2028, which was constructed from 10403S to stably express and secrete a fusion protein of LLO-NP (ref. 8). In this article, we refer to these strains as Lm and Lm-NP to draw attention to their antigenic difference. The construction of the *L. monocytogenes* recombinant DP-L2028 (Lm-NP) has been previously described[8]. Briefly, a DNA fragment encoding the full-length influenza NP (derived from plasmid pAPR502, a gift of P. Palese) was cloned as an in-frame fusion with the first 1,260 base pairs of *hly* and its promoter, along with upstream regulatory sequences. The construction resulted in an in-frame fusion plus the addition of two amino acids at the site of the fusion junction. The fusion and *prfA* gene were cloned into the shuttle plasmid pAM401 and introduced into a *prfA*-lacking strain of *L. monocytogenes* (DP- L1075) by electroporation to ensure its retention *in vivo*. The LLO-NP fusion protein was secreted and appeared, as predicted, at 105 kD on a western blot developed either with an anti-LLO polyclonal antiserum or an anti-NP monoclonal antibody. Further details can be found in ref. 8.

The dose lethal to 50% of animals tested (LD_{50}) of 10403S is ~3×10^4 in BALB/c mice and that of Lm-NP is 1–2×10^7. The bacteria are propagated in brain–heart infusion medium, broth and agar. Lm-NP is maintained at a concentration of 25 μg ml^{-1} of chloramphenicol in order to enforce the retention of the LLO-NP-containing plasmid.

Measurement of tumour growth. Tumour growth was measured using calipers every two days and was recorded as the narrowest and longest surface length. Values shown for tumour size (millimetre) are the mean of these two lengths per animal averaged over ten animals in each group. Error bars represent standard deviations. The *P* values quoted in the text for tumour growth differences between groups of mice were calculated using Statworks Student's *t*-test, unpaired and two sided.

Protection against tumour challenge with Lm-NP. BALB/c mice ($n = 120$) were divided into three groups of 40. One group was immunized with 0.1 LD_{50} (3×10^3 colony-forming units (CFU)) of wild-type Lm, one group was immunized with sterile saline and the third group was immunized with 0.1 LD_{50} (2×10^6 CFU) of the recombinant Lm-NP vaccine vector. After two weeks each group received a similar booster immunization. Two weeks after the last immunization, ten animals in each group were challenged subcutaneously on the left flank with either CT26 or Renca that had been transfected with the same influenza nucleoprotein gene that was used to transform the *L. monocytogenes* vector (CT26-NP or Renca-NP) or with the parental CT26 or Renca line. The number of tumour cells used for each mouse was 5×10^5, which is 100 times the tumoricidal dose.

Effects of Lm-NP on established tumour growth. Tumour cells (5×10^5) of either Renca-NP or CT26-NP were introduced subcutaneously to two groups of 30 mice. After measurable (5-mm) tumours had grown in the mice (day 10 for the mice that had received CT26-NP and day 13 for Renca-NP) they were divided into groups of ten. Ten mice received 0.1 LD_{50} of Lm-NP, ten mice received 0.1 LD_{50} of wild-type Lm, and ten received saline. The mice received boosting immunizations on day 17 for the CT26-NP group and day 23 for the Renca-NP group.

NP expression by CT26-NP after *in vivo* growth in BALB/c mice. CT26-NP tumours were grown in BALB/c mice by subcutaneous injection of 10^6 cells. These were explanted at days 12 and 28 and established *in vitro* for 7 days in the presence of G418 to prevent any further loss of NP. Their ability to be lysed by NP-specific lymphocytes was determined in a standard ^{51}Cr release assay[8] with NP-specific effector cells. CTLs were generated by immunizing BALB/c mice intraperitoneally with 2×10^6 plaque-forming units of vaccinia-NP. Spleen cells were removed 10 days later, stimulated for 7 days with irradiated CT26-NP and 10 U ml^{-1} IL-2, and purified by Ficoll-Hypaque centrifugation before being used as effector cells.

Measurement of antibody titres. Thirty mice were bled before immunization. They then received Lm-NP according to the protocol described for the protection experiments. Sera samples were taken 8 days after the second immunization and titred by serial dilution, between 20× and 1,280×, for reactivity to LLO and NP by using an ELISA assay that can detect antibody levels down to 10 ng ml^{-1} (ABTS™ ELISA kit from Boehringer). LLO was purified from the culture supernatant of Lm-NP as previously described[38] and used at a concentration of 30 μg ml^{-1} to coat the ELISA plates. A/PR/8 virus was used as a source of NP and was dried down on ELISA plates at a concentration of 32 HAU per well. The plates were developed according to the manufacturers instructions.

To quantify the level of anti-NP antibody, serial dilutions of a known concentration of affinity-purified anti-NP monoclonal antibody were applied to the A/PR/8-coated plates. A plot of antibody concentration against optical density was linear up to 120 ng ml^{-1}. The values for levels of anti-NP antibody quoted in the text are calculated by reference to the linear phase of this standard curve.

Histological analysis. Haematoxylin and eosin sections were fixed in 10% formalin and embedded in paraffin wax. For immunoperoxidase staining, tissue blocks were embedded in OCT compound (Tissue-Tek, Miles, Elkhart, Indiana) and snap frozen in liquid N_2. Frozen sections were fixed in acetone, blocked with goat or rat serum, incubated for 45 min with primary monoclonal antibody, washed in phosphate-buffered saline and then stained with biotinylated secondary antibody (goat anti-rat for CD4 and CD8, goat anti-hamster for CD3). Localization of the antigen-antibody complexes was then done with Vectastain ABC (Vector laboratories) and diaminobenzidine developer. Primary monoclonal antibodies were 500A.2 (anti-CD3), GK1.5 (anti-CD4) and 2.43 (anti-CD8).

Analysis of cytotoxic T-cell activity against CT26-NP and Renca-NP. Mice were immunized with 0.1 LD_{50} of Lm-NP. Two weeks later the mice were killed, and secondary cultures were set up of spleen cells with syngeneic splenocytes and a synthetic peptide known to represent the K^d epitope of the NP protein (sequence 147–158), as previously described[8]. After four days in culture, the cytolytic activity was measured against CT26-NP, Renca-NP and the parental cell lines CT26 and Renca by using a ^{51}Cr release assay as previously described[8]. A positive control was included, P815, a mastocytoma tumour cell line known to be efficiently lysed by H-2^d-restricted NP-specific CTLs in the presence of the peptide. Released ^{51}Cr was calculated as (experimental c.p.m. – spontaneous c.p.m.)/(total c.p.m. – spontaneous c.p.m.) × 100%. All assays were performed in triplicate or quadruplicate. Plots of per cent specific lysis at two effector:target ratios (72:1 and 24:1) are shown. Other experiments in which the secondary cultures were stimulated with influenza (A/PR8/34)-infected splenocytes, instead of the NP peptide, gave similar results (data not shown). Immunization with wild-type *L. monocytogenes* did not induce effector cells that could lyse any of the tumour cell lines under the conditions used in these experiments (data not shown).

Depletion of T-cell subsets. Mice were depleted with antibody 2.43 specific for the CD8 molecule or with GK 1.5 specific for the CD4 molecule by immunizing daily intraperitoneally for four days with 0.5 mg of affinity-purified antibody as described[39]. After T-cell depletion, the mice were challenged subcutaneously with 5×10^5 CT26-NP cells per mouse. As a control, ten naive mice were also challenged with the same dose of CT26-NP (white bars). After chal-

lenge the $CD8^-$ and $CD4^-$ mice were maintained on 0.5 mg of 2.43 or GK 1.5 intraperitoneally at 3-day intervals. This protocol results in a depletion of $CD4^+$ or $CD8^+$ T cells of >98% (ref. 39).

Acknowledgements

We would like to thank Robyn Sutherland for helpful advice on T-cell subset depletions, Paul Golumbek for help with comparisons of Lm-NP to vaccinia-NP as a vaccine vector, and Daniel A. Portnoy for helpful discussions. Supported by a Pilot Project Award from the University of Pennsylvania Cancer Center to Y.P., who is a recipient of an American Cancer Society Faculty Research Award, and by National Institutes of Health grants to D.P.

RECEIVED 6 FEBRUARY; ACCEPTED 4 APRIL 1995

1. Kaufmann, S.H.E. Immunity to intracellular bacteria. *Annu. Rev. Immun.* **11**, 129–163 (1993).
2. Cossart, P. *et al.* Listeriolysin O is essential for virulence of *Listeria monocytogenes*: Direct evidence obtained by gene complementation. *Infect. Immun.* **57**, 3269–3636 (1989).
3. Tilney, L.G. & Portnoy, D. A. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. *J. cell Biol.* **109**, 1597–1608 (1989) .
4. Berche, P., Gaillard, J. & Sansonetti, P. J. Intracellular growth of *Listeria monocytogenes* as a prerequisite for in vivo induction of T cell mediated immunity. *J. Immun.* **138**, 2266–2271 (1987).
5. Hahn, H. & Kaufmann, S.H.E. The role of cell mediated immunity to bacterial infections. *Rev. infect. Dis.* **3**, 1221–1250 (1991).
6. Kaufmann, S. H. E., Hug, E., Vath, U. & Muller, I. Effective protection against *Listeria monocytogenes* and delayed type hypersensitivity to Listeria antigens depend on cooperation between specific $L3T4^+$ and $Lyt2^+$ T cells. *Infect. Immun.* **48**, 263–266 (1985).
7. Schafer, R., Portnoy, D.A., Brassell, S.A. & Paterson, Y. Induction of a cellular immune response to a foreign antigen by a recombinant *Listeria monocytogenes* vaccine. *J. Immun.* **149**, 53–59 (1992).
8. Ikonomidis, G., Paterson, Y., Kos, F.J. & Portnoy, D.A. Delivery of a viral antigen to the class I processing and presentation pathway of *Listeria monocytogenes*. *J. exp. Med.* 180, 2209-2218 (1994).
9. Ikonomidis, G., Frankel, F.R., Portnoy, D.A. & Paterson, Y. *Listeria monocytogenes*: A novel live vaccine vector. in *Vaccines 95*, (ed. Brown, F., Charnock, R., Ginsberg, H. & Norrby, E.) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) (in the press).
10. Boon, T., Cerottini, J.-C., Van den Eynde, B. & van der Bruggen, P. Tumour antigens recognized by T lymphocytes. *Annu. Rev. Immun.* **12**, 337–365 (1994).
11. Coulie, P. *et al.* A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. exp. Med.* **180**, 35–42 (1994) .
12. Cox, A.L. *et al.* Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* **264**, 716–719 (1994) .
13. Gaugler, B. *et al.* Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J. exp. Med.* **179**, 921–930 (1994).
14. Topalian, S.L. *et al.* Human $CD4^+$ T cells specifically recognize a shared melanoma-associated antigen encoded by the tyrosinase gene. *Proc. natn. Acad. Sci. U.S.A.* **91**, 9461–9465 (1994).
15. Kawakami, Y. *et al.* Cloning of the gene coding for a shared human melanoma antigen recognized by autologous T cells infiltrating into the tumor. *Proc. natn. Acad. Sci. U.S.A.* **91**, 3515–3519 (1994).
16. Fearon, E.R., Itaya, T., Hunt, B., Vogelstein, B. & Frost, P. Induction in a murine tumor of immunogenic tumor variants by transfection with a foreign gene. *Cancer Res.* **38**, 2975–2980 (1988).
17. Murphy, G.P. & Hushresky, W.J. J. A murine renal cell carcinoma. *J. natn. Cancer Inst.* **50**, 1013–1025 (1973).
18. Schreiber, H. Tumor immunology. in *Fundamental Immunology* (ed. Paul, W.E.) chapt. 32 (Raven, New York, 1993).
19. Campbell, M.J., Esserman, L. & Levy, R. Immunotherapy of established murine B cell lymphoma: Combination of idiotype immunization and cyclophosphamide. *J. Immun.* **141**, 3227–3233 (1988).
20. Pardoll, D.M. Cancer vaccines. *Trends pharmacol. Sci.* **14**, 202–208 (1993).
21. Asher, A. *et al.* Murine tumor cells transduced with the gene for tumor necrosis factor-α *J. Immun.* **146**, 3227–3234 (1991).
22. Blankenstein, T. *et al.* Tumor suppression after tumor cell-targeted tumor necrosis factor gene transfer. *J. exp. Med.* **173**, 1047–1052 (1991).
23. Porgador, A. *et al.* Interleukin 6 gene transfection into Lewis lung carcinoma tumor cells suppresses the malignant phenotype and confers immunotherapeutic competence against parental metastatic cells. *Cancer Res.* **52**, 3679–3686 (1992).
24. Baskar, S. *et al.* Constitutive expression of B7 restores the immunogenicity of tumor cells expressing truncated MHC class II molecules. *Proc. natn. Acad. Sci. U.S.A.* **90**, 5687–5690 (1993).
25. Guo, Y. *et al.* Effective tumor vaccine generated by fusion of hepatoma cells with activated B cells. *Science* **263**, 518–520 (1994).
26. Hsieh, C.-S. *et al.* Development of T_H1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science* **260**, 547–549 (1993).
27. Restifo, N.P. Identification of human cancers deficient in antigen processing. *J. exp. Med.* **177**, 265–272 (1993).
28. Hui, K., Grosveld, F. & Festenstein, H. Rejection of transplantable AKR leukaemia cells following MHC DNA-mediated cell transformation. *Nature* **311**, 750–752 (1984).
29. Wallich, R. *et al.* Abrogation of metastatic properties of tumour cells by *de novo* expression of $H\text{-}2^k$ antigens following H-2 gene transfection. *Nature* **315**, 301–305 (1985).
30. Doyle, A. *et al.* Markedly decreased expression of class I histocompatibility antigens, protein and mRNA on human small-cell lung cancer. *J. exp. Med.* **161**, 1135–1151 (1985).
31. Lassam, N. & Jay, G. Suppression of MHC class I RNA in highly oncogenic cells occurs at the level of transcription initiation. *J. Immun.* **143**, 3792–3797 (1989).
32. Slingluff, C. L., Hunt, D.F. & Engelhard, V.H. Direct analysis of tumour-associated peptide antigens. *Curr. Opin. Immun.* **6**, 733–740 (1994).
33. van der Bruggen, P.C. *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* **254**, 1643–1650 (1991).
34. Cason, J., Khan, S.A. & Best, J.M. Towards vaccines against human papillomavirus type-16 genital infections. *Vaccine* **11**, 603–611 (1993).
35. Wu, T.-C.Immunology of the human papilloma virus in relation to cancer. *Curr. Opin. Immun.* **6**, 746–754 (1994).
36. Fetten, J.H., Roy, N. & Gilboa, E. A frameshift mutation at the NH2 terminus of the nucleoprotein gene does not affect generation of cytotoxic T lymphocyte epitopes. *J. Immun.* **147**, 2697–2705 (1991).
37. Huang, A.T.C. *et al.* Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science* **264**, 961–965 (1994).
38. Geoffroy, C. *et al.* Purification and characterization of an extracellular 29-kilodalton phospholipase C from *Listeria monocytogenes*. *Infect. Immun.* **59**, 2382–2388 (1991).
39. Kruisbeek, A. In vivo depletion of CD4 and CD8 specific T cells. in *Current Protocols in Immunology* (eds Coligan, J. E., Kruisbeek, A.M., Margulies, D. H., Shevach, E. M., Strober, W.) V. 1, 4.1.1-4.1.2 (Wiley, New York, 1994).