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ORIGINAL ARTICLE

Development of a live and highly attenuated *Listeria monocytogenes*-based vaccine for the treatment of Her2/neu-overexpressing cancers in human

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A chimeric human *Her2/neu* gene (ChHer2) harboring most of the known major histocompatibility complex class I epitopes of the HER2/neu oncogene was expressed as a fusion protein to a non-hemolytic fragment of listeriolysin O (LLO), by the highly attenuated *Listeria* vector *LmddA,* which lacks antibiotic selection markers and the ability to spread from cell-to-cell. This construct (ADXS31–164) was tested for immunogenicity and anti-tumor effects in mice. Despite being highly attenuated, ADXS31–164 proved to be efficacious in breaking immune tolerance toward the HER2/neu self-antigen. ADXS31–164 elicited strong T-cell immune responses in experimental animals. In tumors, ADXS31–164 caused a reduction in regulatory T cells (Treg) accompanied by an increase in the $CD8^+$/Treg ratio. Comparison of this vaccine with the conventional antibiotic resistant *Listeria* vector (*Lm*-LLO-ChHer2) shows that ADXS31–164 is more efficacious in delaying tumor growth in Her2/neu transgenic animals. Because of its well-defined attenuation mechanism and independence from antibiotic selection markers, ADXS31–164 is potentially more suitable for human use. These results support the future clinical development of this vaccine for the treatment of HER2/neu-overexpressing malignancies, such as breast, colorectal and pancreatic cancers.
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Introduction

Breast cancer is the second most deadly form of cancer in women in the United States, resulting in over 40 000 deaths annually.[1] HER2/neu overexpression can be detected in 25–30% of all breast cancers and is associated with aggressive disease, hormone resistance and a poor prognosis.[2] The *Her2/neu* oncogene is a potential target for immunotherapy as it is overexpressed in tumors, but has limited presence in other tissues, except for the heart.[3]

In the first approaches to target the HER2/neu antigen using *Listeria monocytogenes* (*Lm*), *Listeria*-based vaccines expressing small fragments of the extra and intracellular domains of the rat and human HER2/neu protein were constructed.[4,5] We also generated a *Listeria* vaccine (*Lm*-LLO-ChHer2) using a chimeric protein by fusing two of the extracellular and one intracellular fragments of the human HER2/neu, including most of the known major histocompatibility complex (MHC) class I epitopes of the protein.[5] All of these vaccines were shown to be immunogenic and efficacious in regressing pre-established tumors in FVB/N mice and delay the onset of spontaneous mammary tumors in HER2/neu-expressing transgenic animals.[5] Because of the high homology (~96%) between rat and human HER2/neu protein sequences and at least three known common H-$2D^q$ epitopes between the two proteins, these preliminary experiments suggested that *Lm*-LLO-ChHer2 could break the tolerance toward the HER2/neu self-antigen. However, the *Listeria*-HER2/neu vaccines developed, thus far, have been based on an attenuated *Listeria* platform, which used the antibiotic marker (*cat*), for *in vitro* selection of the recombinant bacteria in the presence of chloramphenicol.[5] In order to advance this vaccine toward human use, we have generated another recombinant *Listeria*-Her2/neu vaccine (ADXS31–164) using the highly attenuated *LmddA* vaccine vector, which has a deletion of the virulence gene *actA* and is devoid of antibiotic selection markers.[6] ADXS31–164 retains the plasmid for *Her2/neu* expression *in vivo* and *in vitro* by auxothrophic complementation of the *dal* gene, which is absent in the *LmddA* strain and required for the synthesis of D-alanine. ADXS31–164 expresses and secretes the chimeric HER2/neu protein fused to the first 441 amino acids of listeriolysin O (LLO). This study compares the immunogenicity and efficacy of this optimized vaccine for

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human use, with our previous chloramphenicol resistant version.[5]

Materials and methods

Materials

Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA) and DNA sequencing was carried out by Genewiz, South Plainfield, NJ. Flow cytometry reagents were purchased from Becton Dickinson Biosciences (BD, San Diego, CA). Cell culture media, supplements and all other reagents, unless indicated, were from Sigma (St Louise, MO). HER2/neu HLA-A2 peptides were synthesized by EZbiolabs (Westfield, IN). Complete RPMI 1640 (C-RPMI) medium contained 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 10% fetal bovine serum, penicillin/streptomycin and Hepes (25 mM). The polyclonal anti-LLO antibody was described previously[5] and anti-HER2/neu antibody was purchased from Sigma.

Mice and cell lines

All animal experiments were performed according to approved protocols by IACUC at the University of Pennsylvania or Rutgers University. FVB/N mice were purchased from Jackson laboratories (Bar Harbor, ME). The FVB/N HER2/neu transgenic mice,[7] which over-express the rat HER2/neu oncoprotein and the HLA-A2 transgenic mice (originally obtained from Dr Linda Sherman, the Scripps Research Institute, La Jolla, CA) were housed and bred at the animal core facility at the University of Pennsylvania. The NT-2 tumor cell line, which expresses high levels of rat HER2/neu protein, was derived from a spontaneous mammary tumor in these mice[7] and grown as described previously.[4] DHFR-G8 (3T3/neu) cells were obtained from the American Type Culture Collection and were grown according to the American Type Culture Collection recommendations. The EMT6-Luc cell line was a generous gift from Dr John Ohlfest (University of Minnesota, MN) and was grown in C-RPMI medium. Bioluminescent work was conducted under guidance by the Small Animal Imaging Facility at the University of Pennsylvania (Philadelphia, PA).

Listeria *constructs and antigen expression*

Her2/neu-pGEM7Z was kindly provided by Dr Mark Greene at the University of Pennsylvania and contained the full-length human *Her2/neu* (hHer2) gene cloned into the pGEM7Z plasmid (Promega, Madison WI). The HER2/neu chimeric fragment (ChHer2) and *Lm*-LLO-ChHer2 constructs were generated as described.[5] *ChHer2* gene was excised from pAdv138[5,8] using *Xho*I and *Spe*I restriction enzymes, and cloned in frame with a truncated, non-hemolytic fragment of LLO in the *Lmdd* shuttle vector, pAdv134.[6] The sequences of the insert, LLO and *hly* promoter were confirmed by DNA sequencing analysis. This plasmid (pAdv164) was electroporated into electro-competent *actA, dal, dat* mutant *L. monocytogenes* strain, *LmddA*,[6] and positive clones were selected on Brain Heart infusion agar plates containing streptomycin (250 μg ml^{-1}). In some experiments, similar *Listeria* strains expressing hHER2/neu (*Lm*-hHer2) fragments were used for comparative purposes. These have been previously described.[5] In all studies, an irrelevant *Listeria* construct (*Lm* control) was included to account for the antigen-independent effects of *Listeria* on the immune system. *Lm* controls were based on the same *Listeria* platform as ADXS31–164, but expressed a different antigen such as HPV16-E7 or NY-ESO-1. Expression and secretion of fusion proteins from *Listeria* were tested as described previously.[8] Each construct was passaged twice *in vivo* as described previously.[9] For immunizations, *Listeria* vaccines were injected intraperitoneally at a dose of 1 to 5×10^8 colony-forming units as determined by bacterial titrations on Brain Heart infusion/S/agar titration plates.

Cytotoxicity assay

Groups of three to five FVB/N mice were immunized three times with 1-week intervals with *Lm*-LLO-ChHer2, ADXS31–164, *Lm*-hHer2 ICI$_{(679-1019)}$, *Lm* control or were left naïve. NT-2 cells were grown *in vitro*, detached by trypsin and treated with mitomycin C (250 μg ml^{-1} in serum-free C-RPMI medium) at 37 °C for 45 min. After five washes, they were co-incubated with splenocytes harvested from immunized or naïve animals at a ratio of 1:5 (stimulator/responder) for 5 days at 37 °C and 5% CO_2. A standard cytotoxicity assay was performed using europium-labeled 3T3/neu (DHFR-G8) cells as targets as previously described.[10] Europium released from killed target cells was measured after 4 h incubation using a spectrophotometer (Perkin Elmer, Victor 2) at 590 nm. Percent specific lysis was defined as (lysis in experimental group–spontaneous lysis)/(maximum lysis–spontaneous lysis).

Interferon-γ secretion by splenocytes from immunized mice

Groups of three to five FVB/N or HLA-A2 transgenic mice were immunized three times with 1-week intervals with ADXS31–164, a negative *Listeria* control or were left naïve. Splenocytes from FVB/N mice were isolated 1 week after the last immunization and co-cultured in 24-well plates at 5×10^6 cells per well in the presence of mitomycin C-treated NT-2 cells in C-RPMI medium. Splenocytes from the HLA-A2 transgenic mice were incubated in the presence of 1 μM of HLA-A2-specific peptides or 1 μg ml^{-1} of a recombinant His-tagged ChHer2 protein, produced in *Escherichia coli* and purified by a nickel-based affinity chromatography system (Qiagen, Valencia, CA). Samples from supernatants were obtained 24 or 72 h later and tested for the presence of interferon-γ (IFN-γ) using mouse IFN-γ enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences) according to manufacturer's recommendations.

Effect of ADXS31–164 on Tregs in spleens and tumors

Mice were implanted subcutaneously with 1×10^6 NT-2 cells. On days 7, 14 and 21, they were immunized with ADXS31–164, *LmddA* control or left naïve. Tumors and spleens were extracted on day 28 and tested for the presence of $CD3^+/CD4^+/CD25^+/FoxP3^+$ regulatory T cells (Tregs) and also $CD3^+/CD8^+$ T cells by fluorescence-activated cell sorting analysis. Briefly, splenocytes were isolated by homogenizing the spleens between two glass slides in C-RPMI medium. Tumors were minced using a sterile razor blade and digested with a buffer containing DNase (12 U ml^{-1}), and collagenase (2 mg ml^{-1}) in phosphate-buffered saline. After 60 min incubation at room temperature with agitation, cells were separated by vigorous pipetting. Red blood cells were lysed by red blood cell lysis buffer followed by several washes with C-RPMI-1640 medium containing 10% fetal bovine serum. After filtration through a nylon mesh, tumor cells and splenocytes were resuspended in fluorescence-activated cell sorting buffer (2% fetal bovine serum/phosphate-buffered saline) and stained with anti-CD3-PerCP-Cy5.5, CD4-fluorescein isothiocyanate, CD25-antigen-presenting cell antibodies followed by permeabilization and staining with anti-FoxP3-PE.[11] $CD8^+$ T cells were stained with CD8-fluorescein isothiocyanate and CD3-PerCP-Cy5.5. The ratio of $CD8^+$ T cells and Tregs was determined by dividing the frequency of $CD8^+$ T cells by the frequency of $CD4^+CD25^+$ $FoxP3^+$ Tregs, gated on $CD3^+$ T cells. Flow cytometry analysis was performed using four-color fluorescence-activated cell sorting calibur (BD Biosciences), and data were analyzed using cell quest software (BD Biosciences).

Tumor studies in HER2/neu transgenic animals

Six-week-old FVB/N rat HER2/neu transgenic mice (9–14 per group) were immunized six times with *Lm*-LLO-ChHer2, ADXS31–164 or *Lm* control as described above. They were observed twice a week for the emergence of spontaneous mammary tumors, which were measured using an electronic caliper, for up to 52 weeks. Escaped tumors were excised when they reached a size of 1 cm in average diameter and preserved in RNAlater (Qiagen) at −20 °C. In order to determine the effect of mutations in the Her2/neu gene on the escape of these tumors, genomic DNA was extracted using a genomic DNA isolation kit (Qiagen), and sequenced as described previously.[12]

Effect of ADXS31–164 on the growth of a breast cancer cell line in the brain

Balb/c mice were immunized once a week with ADXS31–164 or an irrelevant *Listeria* vaccine as described above. Each mouse received three immunizations before tumor implantation. EMT6-Luc cells were grown *in vitro* then injected into the brain of anesthetized mice at 5000 cells per mouse. Expression of HER2/neu by EMT6-Luc cells was detected according to a standard western blot protocol. EMT6-Luc cells produce the enzyme luciferase, and when they metabolize D-luciferin *in vivo*, the by-products are photons that are captured *ex vivo* using a Xenogen X-100 camera and displayed using a heat map. Imaging was performed on anesthetized mice on the indicated days. Pixel intensity is graphed as number of photons per second per cm^2 of surface area, which is shown as average radiance.

Statistical analysis

The log-rank χ^2-test was used for survival data and Student's *t*-test for the cytotoxic T lymphocyte and ELISA assays, which were performed in triplicates. A *P*-value <0.05 (marked as *) was considered statistically significant in these analyzes. All statistical analysis was carried out with either Prism software, V.4.0a (2006) or SPSS software, V.15.0 (2006). For all FVB/N rat Her2/neu transgenic studies we used 8–14 mice per group, for all wild-type FVB/N studies we used at least 8 mice per group, unless otherwise stated. All studies were repeated at least once except for the long-term tumor study in Her2/neu transgenic mouse model.

Results

Construction of ADXS31–164

Construction of the chimeric *Her2/neu* gene (ChHer2) was described previously.[5] Briefly, *ChHer2* gene was generated by direct fusion of two extracellular and one intracellular fragments of the HER2/neu protein by SOEing PCR method. The chimeric protein harbors most of the known human MHC class I epitopes of the protein.[13,14] The *ChHer2* gene was excised from the plasmid, pAdv138 (which was used to construct *Lm*-LLO-ChHer2) and cloned into *LmddA* shuttle plasmid, resulting in the plasmid pAdv164 (Figure 1a). There are two major differences between these two plasmid backbones. (1) Whereas pAdv138 uses the chloramphenicol resistance marker (*cat*) for *in vitro* selection of recombinant bacteria,[5,8] pAdv164 harbors the D-alanine racemase gene (*dal*) from *Bacillus subtilis* for auxotrophic complementation of the *LmddA* strain, which lacks the *dal–dat* genes required for the synthesis of D-alanine.[15] As D-alanine is an essential component of the cell wall, the plasmid must be retained *in vitro* and *in vivo* for bacterial replication and survival. This vaccine platform was designed and developed to address the Food and Drug Administration concerns about the antibiotic resistance of bioengineered *Listeria* vaccine strains. (2) Unlike pAdv138, pAdv164 does not harbor a copy of the *prfA* gene in the plasmid, as this is not necessary for *in vivo* complementation of the *Lmdd* strain. The *LmddA* vaccine strain also lacks the *actA* gene (responsible for the intracellular movement and cell-to-cell spread of *Listeria*), so the recombinant vaccine strains derived from this backbone are 100 times less virulent than those derived from the *Lmdd*, its parental strain.[6] *LmddA*-based vaccines are also cleared much faster (in less than 48 h) than the *Lmdd*-based vaccines from the spleens of the immunized mice.[6] The expression and secretion of the fusion protein LLO–ChHer2 from the ADXS31–164 strain was comparable with that of the *Lm*-LLO-ChHer2 in trichloroacetic acid-precipitated cell culture supernatants after 8 h of *in vitro* growth

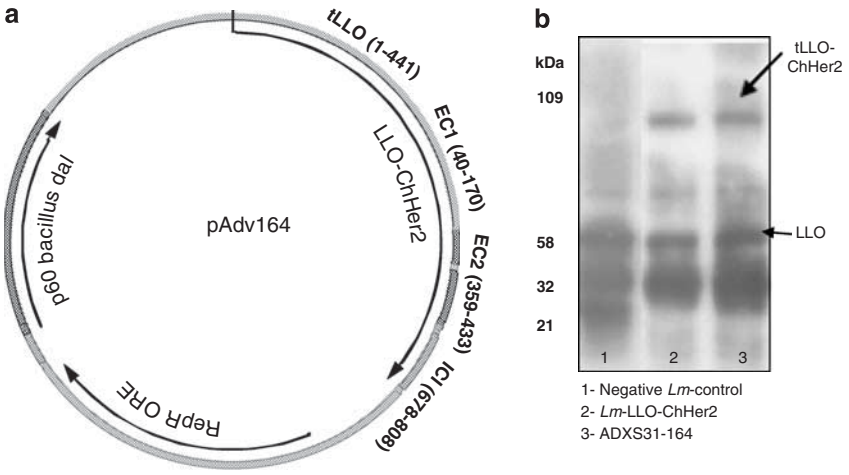


Figure 1 Construction of ADXS31–164. (**a**) Plasmid map of pAdv164, which harbors *Bacillus sabtilis dal* gene under the control of constitutive *Listeria* p60 promoter for complementation of the chromosomal *dal–dat* deletion in *LmddA* strain. It also contains the fusion of truncated listeriolysin O (tLLO)$_{(1-441)}$ to the chimeric human Her2/neu gene, which was constructed by the direct fusion of three fragments the Her2/neu: EC1 (aa 40–170), EC2 (aa 359–433) and ICI (aa 678–808). (**b**) Expression and secretion of tLLO-ChHer2 was detected in *Lm*-LLO-ChHer2 and *LmddA*-LLO-ChHer2 (ADXS31–164) by western blot analysis of the trichloroacetic acid-precipitated cell culture supernatants blotted with anti-LLO antibody. A differential band of ~04 kDa corresponds to tLLO-ChHer2. The endogenous LLO is detected as a 58 kDa band. Listeria control lacked ChHer2 expression.

(Figure 1b), as a band of ~104 kDa was detected by an anti-LLO antibody using western blot analysis. The *Listeria* backbone strain expressing only LLO was used as negative control.

ADXS31–164 is as immunogenic as Lm-*LLO-ChHer2*

Immunogenic properties of ADXS31–164 in generating anti-HER2/neu-specific cytotoxic T cells were compared with those of the *Lm*-LLO-ChHer2 vaccine in a standard cytotoxic T lymphocyte assay. Both vaccines elicited a strong but comparable cytotoxic T-cell responses toward the HER2/neu antigen expressed by 3T3/neu target cells. Accordingly, mice immunized with a *Listeria* expressing only an intracellular fragment ($IC1_{679-1019}$) of HER2-neu fused to LLO[5] showed lower lytic activity than the chimeras, which may be due to the fact that the chimera contains more MHC class I epitopes than fragment $IC1_{679-1019}$. No cytotoxic T-lymphocyte activity was detected in naïve animals or mice injected with the irrelevant *Listeria* vaccine (Figure 2a). ADXS31–164 was also able to stimulate the secretion of IFN-γ by the splenocytes from wild-type FVB/N mice (Figure 2b). This was detected in the culture supernatants of these cells that were co-cultured with mitomycin C-treated NT-2 cells, which express high levels of HER2/neu antigen (Figure 5c).

Proper processing and presentation of the human MHC class I epitopes after immunizations with ADXS31–164 was tested in HLA-A2 transgenic mice. Splenocytes from immunized mice were co-incubated for 72 h with peptides corresponding to mapped HLA-A2-restricted epitopes located at the extracellular (HLYQGCQVV or KIFGSLAFL) or intracellular (RLLQETELV) domains of the HER2/neu molecule (Figure 2c). A recombinant ChHer2 protein was used as positive control and an irrelevant peptide or no peptide was used as negative controls. The data from this experiment show that ADXS31–164 is able to elicit anti-HER2/neu-specific immune responses to human epitopes that are located at different domains of the targeted antigen.

ADXS31–164 was more efficacious than Lm-*LLO-ChHer2 in preventing the onset of spontaneous mammary tumors*

Anti-tumor effects of ADXS31–164 were compared with those of *Lm*-LLO-ChHer2 in Her2/neu transgenic animals that develop slow growing, spontaneous mammary tumors at 20–25 weeks of age.[7] All animals immunized with the irrelevant *Listeria* control vaccine developed breast tumors within weeks 21–25 and were killed before week 33. In contrast, *Listeria*-Her2/neu recombinant vaccines caused a significant delay in the formation of the mammary tumors. On week 45, more than 50% of ADXS31–164 vaccinated mice (five out of nine) were still tumor free, as compared with 25% of mice immunized with *Lm*-LLO-ChHer2. At week 52, two out of eight mice immunized with ADXS31–164 still remained tumor free, whereas all mice from other experimental groups had already succumbed to their disease (Figure 3). These results indicate that despite being more attenuated, ADXS31–164 is more efficacious than *Lm*-LLO-ChHer2 in preventing the onset of spontaneous mammary tumors in Her2/neu transgenic animals.

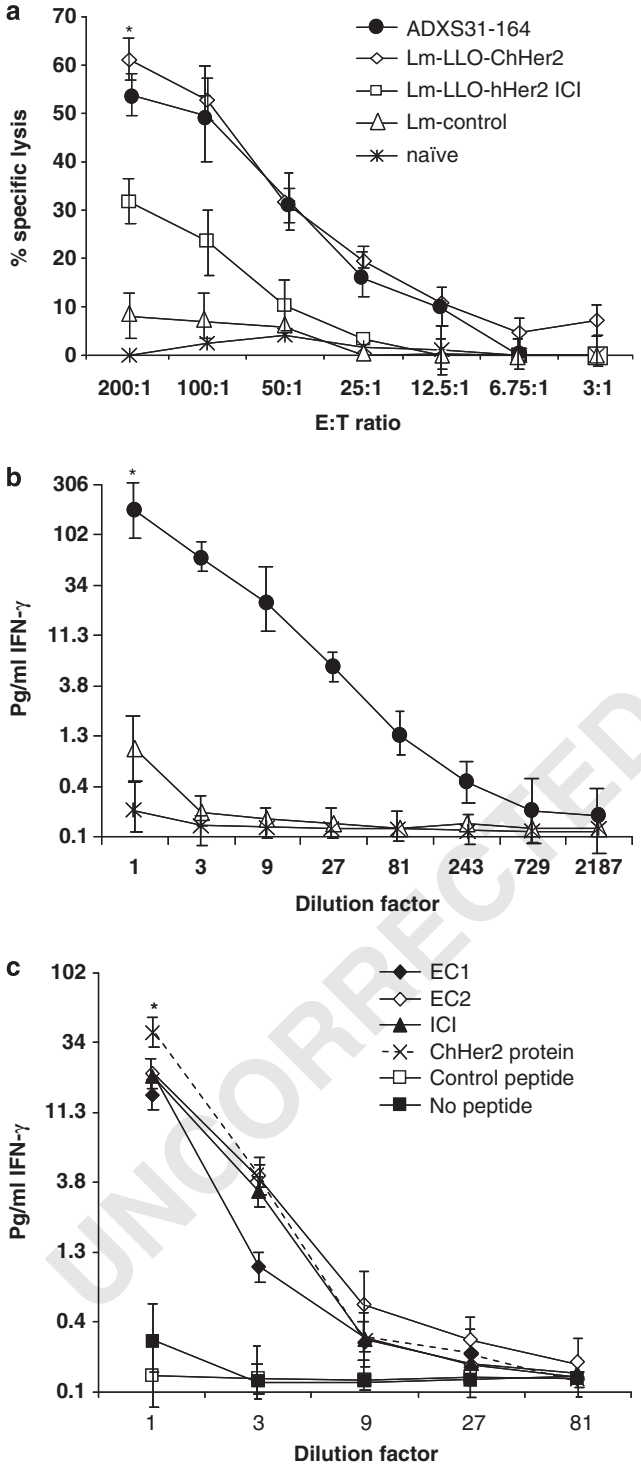
Mutations in Her2/neu gene upon immunization with ADXS31–164

Mutations in the MHC class I epitopes of Her2/neu have been considered responsible for tumor escape upon immunization with small fragment vaccines[12] or trastuzumab (Herceptin),[16] a monoclonal antibody that targets

an epitope in the extracellular domain of Her2/neu. In order to determine if tumor escape after immunization with ADXS31–164 was also due to mutations in the *Her2/neu* gene, we extracted the genomic material from the slow-growing tumors in the Her2/neu transgenic animals and sequenced the corresponding fragments of the *Her2/neu* gene in the immunized and control mice. Mutations were not observed within the *Her2/neu* gene of any of the tumors extracted from vaccinated or naïve mice, suggesting alternative escape mechanisms (data not shown).

ADXS31–164 causes a significant decrease in intratumoral Tregs

Evidence suggests that effective anti-tumor responses after vaccination are associated not only with an increase in tumor infiltrating CD8$^+$ T cells but also decrease in the intratumoral suppressive immune cell populations.[17] To establish the mechanisms behind anti-tumor effects of ADXS31–164, the frequency of CD8$^+$ effector T cells in relation to Tregs in spleens and tumors of mice was determined. Splenocytes and intratumoral lymphocytes were isolated after three immunizations and stained for Tregs, which were defined as CD3$^+$/CD4$^+$/CD25$^+$/FoxP3$^+$ cells, although comparable results were obtained with either FoxP3 or CD25 markers when analyzed separately. The results indicate that immunization with the *Listeria* vaccines caused a considerable impact on the



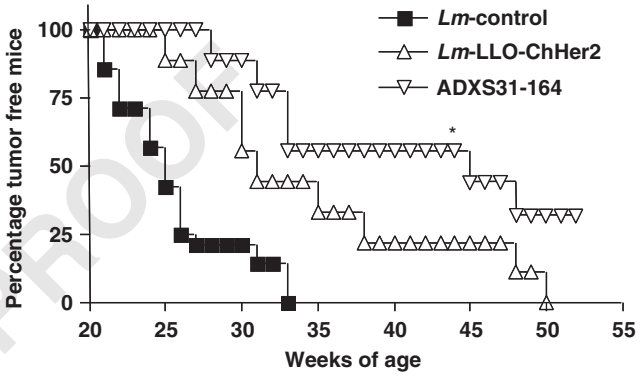


Figure 3 Tumor prevention studies for *Listeria*-ChHer2/neu vaccines. Her2/neu transgenic mice were injected six times with each recombinant *Listeria*-ChHer2 or a control *Listeria* vaccine. Immunizations started at 6 weeks of age and continued every three weeks until week 21. Appearance of tumors was monitored on a weekly basis and expressed as percentage of tumor-free mice. *$P<0.05$, $n=9$ per group.

Figure 2 Immunogenic properties of ADXS31–164. (**a**) Cytotoxic T-cell responses elicited by Her2/neu *Listeria*-based vaccines in splenocytes from immunized mice were tested using NT-2 cells as stimulators and 3T3/neu cells as targets. *Lm* control was based on the *LmddA* background that was identical in all ways but expressed an irrelevant antigen (HPV16-E7). (**b**) Interferon (IFN)-γ secreted by the splenocytes from immunized FVB/N mice into the cell culture medium, measured by enzyme-linked immunosorbent assay (ELISA), after 24 h of *in vitro* stimulation with mitomycin C-treated NT-2 cells. (**c**) IFN-γ secretion by splenocytes from HLA-A2 transgenic mice immunized with the chimeric vaccine, in response to *in vitro* incubation with peptides from different regions of the protein. A recombinant ChHer2 protein was used as positive control and an irrelevant peptide or no peptide groups constituted the negative controls as listed in the figure legend. IFN-γ secretion was detected by an ELISA assay using cell culture supernatants harvested after 72 h of co-incubation. Each data point was an average of triplicate data ± s.e. *P-value <0.001.

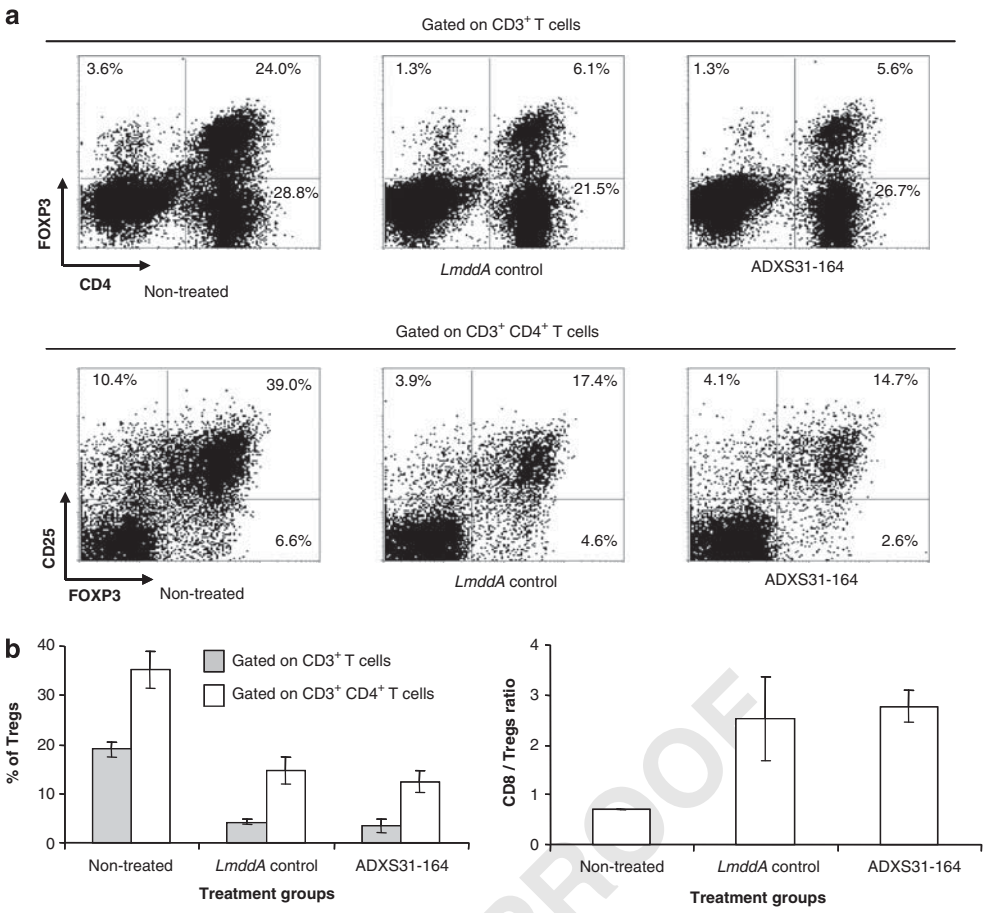


Figure 4 Effect of immunization with ADXS31–164 on the % of tumor-infiltrating T-regulated cells (Tregs) in NT-2 tumors. FVB/N mice were inoculated subcutaneously with 1×10^6 NT-2 cells and immunized three times with each vaccine at 1-week intervals. Tumors were harvested for 7 days after the second immunization. After isolation of the immune cells, they were stained for detection of Tregs by anti-CD3, CD4, CD25 and FoxP3 antibodies. (**a**) Dot plots of the Tregs from a representative experiment. (**b**) Frequency of CD25$^+$/FoxP3$^+$ T cells, expressed as percentages of the total CD3$^+$ or CD3$^+$CD4$^+$ T cells (left panel) and intratumoral CD8/Tregs ratio (right panel) across the different treatment groups. Data are shown as mean ± s.e.m. obtained from two independent experiments.

presence of Tregs in the tumors (Figure 4a). Whereas in average 19.0% of all CD3$^+$ T cells in untreated tumors were Tregs, this frequency was reduced to 4.2% for the irrelevant vaccine and 3.4% for ADXS31–164, a fivefold reduction in the frequency of intratumoral Tregs (Figure 4b). The decrease in the frequency of intratumoral Tregs in mice treated with either of the *LmddA* vaccines could not be attributed to differences in the sizes of the tumors. In a representative experiment, the tumors from mice immunized with ADXS31–164 were significantly smaller (mean diameter (mm) ± s.d., 6.71 ± 0.43, $n = 5$) than the tumors from untreated mice (8.69 ± 0.98, $n = 5$, $P < 0.01$) or treated with the irrelevant vaccine (8.41 ± 1.47, $n = 5$, $P = 0.04$), whereas comparison of these last two groups showed no statistically significant difference in tumor size ($P = 0.73$). The lower frequency of Tregs in tumors treated with *LmddA* vaccines resulted in an increased intratumoral CD8/Tregs ratio, suggesting that a more favorable tumor microenvironment can be obtained after immunization with *LmddA* vaccines. However, only the vaccine expressing the target antigen HER2/neu (ADXS31–164) was able to reduce tumor growth, indicating that the decrease in Tregs has an effect only in the presence on antigen-specific responses in the tumor. Notably, immunization with ADXS31–164 had no effect on the frequency of Tregs in the spleens, as compared with an irrelevant *Listeria* vaccine or the naïve animals (Supplementary Figure 1).

Immunization with ADXS31–164 can delay the growth of a metastatic breast cancer cell line in the brain

Mice were immunized with ADXS31–164 or irrelevant *Lm* control vaccines and then implanted intracranially with 5000 EMT6-Luc tumor cells expressing luciferase and low levels of HER2/neu (Figure 5c). Tumors were monitored at different times post-inoculation by *ex vivo* imaging of anesthetized mice. On day 8, post-tumor inoculation tumors were detected in all control animals, but in none of the mice in ADXS31–164 group (Figures 5a and b). ADXS31–164 could clearly delay the onset of these tumors, as on day 11 post-tumor inoculation all mice in negative control group had already succumbed to

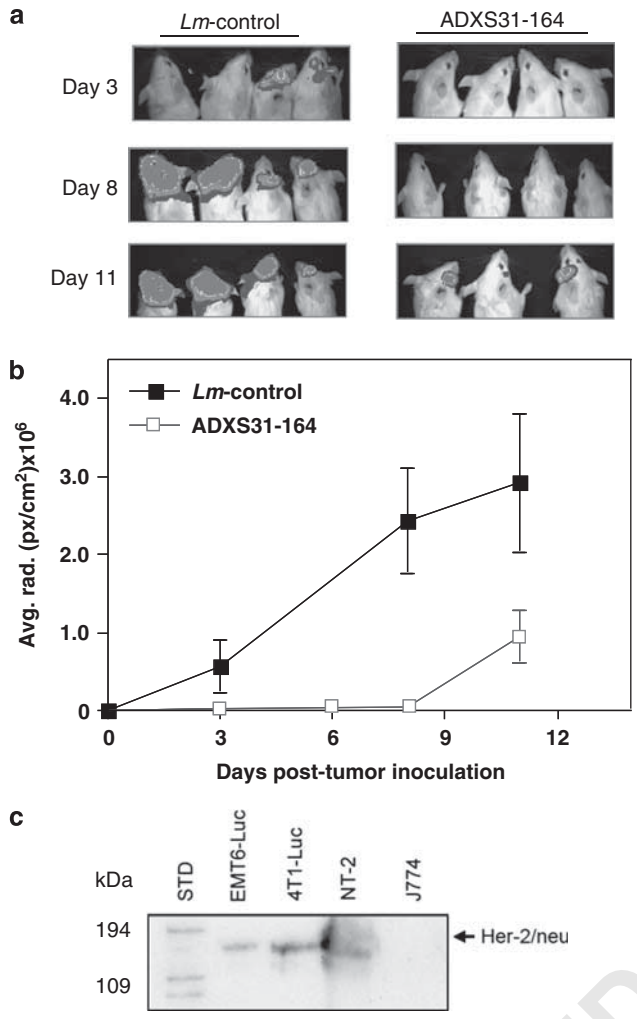


Figure 5 Vaccination with ADXS31–164 can delay the growth of a breast cancer cell line in the brain. Balb/c mice were immunized thrice with ADXS31–164 or a control *Listeria* vaccine. EMT6-Luc cells (5000) were injected intracranially in anesthetized mice. (**a**) *Ex vivo* imaging of the mice was performed on the indicated days using a Xenogen X-100 CCD camera. (**b**) Pixel intensity was graphed as number of photons per second per cm^2 of surface area; this is shown as average radiance. (**c**) Expression of HER2/neu by EMT6-Luc cells, 4T1-Luc and NT-2 cell lines was detected by western blots, using an anti-Her2/neu antibody. J774.A2 cells, a murine macrophage-like cell line was used as a negative control.

their tumors, but all mice in ADXS31–164 group were still alive, with only small signs of tumor growth. These results strongly suggest that the immune responses obtained with ADXS31–164 could possibly reach the central nervous system (CNS) and that *LmddA*-based vaccines might have a potential use for treatment of CNS tumors.

Discussion

Previously we constructed a number of *Lm*-LLO-Her2/neu vaccines that were shown to be very efficacious in treating established or spontaneous tumors in wild-type or Her2/neu transgenic animals.[5] The *Listeria* backbone used for those vaccines had a chromosomal deletion of the *prfA* gene, which controls the transcription of *Listeria* major virulence factors, such as LLO and ActA. This strain is completely avirulent unless the *prfA* is supplemented in *trans*. In our previous *Listeria* vaccine strains, *prfA* was complemented by expressing it from a plasmid that also harbored the antigen of interest and two *cat* genes, which conferred chloramphenicol resistance to the final vaccine strain.[8] Despite this complementation, the vaccine strains based on this background were still 10^4 times more attenuated than their parental *Listeria* strain, 10403s.[8] The attenuation of these strains is in part due to a mutated episomal *prfA* gene that restores virulence only partially. Furthermore, the high metabolic burden that the expression of a foreign antigen exerts on the bacterium is also an important mechanism of attenuation. For clinical use, not only high attenuation is important, but also the absence of resistance to antibiotics. For this reason, we developed a new vector (*LmddA*), which is devoid of antibiotic selection markers[15,18,19] and has a well-defined attenuation mechanism, that is, the chromosomal deletion of the *actA* gene.[6] We used the *LmddA* to develop other vaccines with potential clinical use and have shown that this *Listeria* backbone is equally or more efficacious for antigen delivery when compared with our previous vector.[6]

This strain also exerts a strong adjuvant effect, which is an inherent property of *Listeria*-based vaccines.[6] One manifestation of this adjuvant effect is the fivefold decrease in the number of the intratumoral Tregs caused by either the irrelevant *Listeria* or the ADXS-31–164 vaccines (Figure 4). Although the effect of immunization with ADXS31–164 was slightly more prominent on the reduction of intratumoral Tregs than that of the irrelevant *Listeria* in two independent studies, these differences were not statistically significant and might be explained solely by the smaller tumor sizes in the group receiving ADXS31–164. These results are in accordance with our previous observations using a recombinant *Listeria* vaccine for the prostate-specific antigen (*LmddA*-LLO-prostate-specific antigen) in a prostate-specific antigen-expressing murine tumors model.[6] One consequence of Treg reduction is an increase in the intratumoral CD8/Tregs ratio, which might result in less suppression of anti-tumor cytotoxic responses mediated by HER2/neu-specific CD8$^+$ T cells. In breast cancer patients receiving neoadjuvant chemotherapy, a higher intratumoral CD8$^+$/FoxP3 ratio has been correlated with a favorable response to the treatment, as compared with unchanged ratios in nonresponders.[17] Particularly, this treatment resulted in a decrease in FoxP3$^+$, but no change in CD8$^+$ and CD3$^+$ T-cell infiltrates, with a significant increase in cytotoxic markers, such as TiA1 and granzyme B.[17]

It is noteworthy that the *LmddA* vector expressing an irrelevant antigen (HPV16 E7) was also associated with a significant decrease in the frequency of Tregs in the tumors, likely as a consequence of innate immunity responses. The ability of bacteria to silence Tregs has been

already described. Interestingly, Treg cells from mice immunized with a recombinant *E. coli* strain expressing LLO, which provided them with the ability to escape from the phagosomes, were unable to suppress conventional T-cell proliferation.[20] Similarly, *Salmonella typhimurium*-induced Th1 cells were resistant to suppression by Treg cells, and this effect could be partially blocked by disruption of interleukin-6 or glucocorticoid-induced tumor necrosis factor receptor signals.[21] These results indicate that vaccines based on bacterial vectors might be advantageous in treating diseases associated with immunosupression such as cancer.

Antigen-independent effects of *Listeria* on tumors have been attributed to a number of factors, including innate immune responses accompanied by a cascade of the cytokines and chemokines and upregulation of co-stimulatory molecules that are host's immediate response to the *Listeria* infection.[6] This adjuvant effect of *Listeria*-based vaccines is well orchestrated with its particular ability to deliver antigens into the antigen-presenting cells, and particularly to the intracellular space. Another mechanism for the anti-tumoral effects of *Listeria* was recently suggested by the discovery that the *Listeria* can infect and reside in the tumors and mediate the direct lysis of tumor cells.[22] Still other effects include *Listeria*'s ability to stimulate myelopoesis and the maturation of immature myeloid cells such as dendritic cell and their ability to facilitate the extravasation of activated immune cells into tumors.[23]

In addition to the role of innate immunity stimulated by *Listeria*-based vaccines, the delivery of a TAA by this vector has a crucial role in its strong and specific anti-tumor responses. Herein, we also show that ADXS31–164 exerts strong and antigen-specific anti-tumor responses with ability to break immune tolerance toward HER2/neu in transgenic animals. ADXS31–164 is highly attenuated and may have a better safety profile than the previous *Listeria* vaccine generation, as it is more rapidly cleared from the spleens of the immunized mice. This is in accordance with our previous reports, where another *LmddA*-based vaccine (*LmddA*-LLO-prostate-specific antigen) strain was demonstrated to be readily cleared *in vivo* in less than 48 h, even in the IFN-γ knockout mice.[6] However, this additional attenuation does not seem to affect the efficacy of the vaccine as ADXS31–164 resulted in a longer delay of tumor onset in transgenic animals than *Lm*-LLO-ChHer2, the antibiotic resistant and more virulent version of this vaccine. To our knowledge, this level of efficacy has rarely been reported by any other HER2/neu-targeted immunotherapeutic agent.

One of the hurdles in using Her2/neu as a vaccine target is its mutability. HER2/neu-positive tumors often escape the treatment with immunotherapeutic agents, such as Herceptin, in part due to escape mutations in those epitopes to which the immunotherapeutic agent is targeted.[12,16] We hypothesized that using a larger fragment of HER2/neu harboring additional MHC class I epitopes of the protein would prevent these escape mutations. This was a reasonable approach, which resulted in better therapeutic responses as compared with small fragment vaccines in Her2/neu transgenic animals.[5] Nonetheless, delayed tumor escape still occurred in 70% of the immunized animals. To prove our hypothesis, we sequenced the HER2/neu DNA fragments in the tumors that escaped treatment with ADXS31–164 and we did not find any point mutations or deletions in these regions. This is in contrast to previously published studies, in which smaller fragments of HER2/neu were expressed by *Listeria* vaccines,[12] where immune escape was associated with point mutations or deletions in known epitopes of this oncoprotein. However, other mechanisms responsible for tumor escape might be involved as it has been suggested in the literature, which might also have a role in the escape of HER2/neu-overexpressing tumors. Among these, downregulation of MHC class I cell surface expression[24,25] and/or of intercellular adhesion molecule 1, which is involved in homing of cytolytic effector T cells into the tumors,[26] have been reported in many solid tumors. Future studies will focus on the delineation of the role of these escape mechanisms and other factors such as the presence of suppressive immune cells in these slow-growing tumors. For example, the kinetics of Treg infiltration in long-term tumors was not tested herein and might have a role in the late tumor escape, once the acute effect of immunization with *Listeria* fades.

Another encouraging outcome from this study was that ADXS31–164 could inhibit the growth of Her2/neu-expressing tumors in the brain of immunized animals. Although the tumor model used herein was not derived from the CNS, it could still serve as a proof-of-concept of the ability of immunity elicited by *Listeria*-based vaccines to reach immune-privileged sites. These data support the idea of developing this platform toward other difficult and sequestered malignancies, such as gliomas or other neurological tumors. It has to be noted that as the immunization regimen used here followed a prophylactic model, the infiltration of the immune cells into the brain might have been facilitated by a leakage of the blood–brain barrier, possibly caused by the injection of the tumor cells directly into this compartment. However, the access of the CNS by immune cells, even in the absence of such disruption[27] and the prevention of CNS tumors upon immunization with other *Listeria*-based vaccines[28] have been shown by others.

In summary, ADXS31–164 was shown to be highly immunogenic, able to break immune tolerance toward the HER2/neu self-antigen and prevent tumor formation in Her2/neu transgenic animals. The new *Listeria* platform used as backbone for generating ADXS31–164 is more attenuated and eventually safer for the use in humans. Together with our previous work,[5] the data presented here establish the potential of a recombinant *Listeria* expressing a chimeric HER2/neu as a therapeutic vaccine for the treatment of Her2/neu-overexpressing solid tumors and warrants its future human testing. Advaxis has recently published the results of a phase I clinical trial using a recombinant *Listeria*-HPV16-E7 vaccine (ADXS11-001), in late stage cervix cancer patients, showing that a live attenuated *Listeria*-based vaccine can be safely administered to this patient population. The

low adverse effect profile observed in most patients and the beneficial responses with tumor reductions detected in 30% of evaluable patients support the potential of *Listeria* as an efficacious vaccine platform for human use.[29]

Conflict of interest

Advaxis Inc. is a for-profit vaccine and therapeutic company that has licensed or has an option to license all patents from the University of Pennsylvania that concern the use of *Lms* or listerial products as vaccines. The following authors have a financial interest in Advaxis Inc., and thus may have a conflict of interest: Drs PC Maciag, A Wallecha, S Rivera and V Shahabi. Dr MM Seavey declares no conflict of interest.

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