Nontoxic radioactive *Listeria*^{at} is a highly effective therapy against metastatic pancreatic cancer

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No significant improvement in therapy of pancreatic cancer has been reported over the last 25 y, underscoring the urgent need for new alternative therapies. Here, we coupled a radioisotope, ^{188}Rhenium, to an attenuated (at) live *Listeria monocytogenes* (*Listeria*^{at}) using *Listeria*-binding antibodies, thus creating a unique radioactive *Listeria*^{at} (RL). We then demonstrated in a highly metastatic pancreatic mouse tumor model (Panc-02) that RL delivered radioactivity to the metastases and less abundantly to primary tumors in vivo, without harming normal cells. This result was possible because *Listeria*^{at} was efficiently cleared by the immune system in normal tissues but not in the heavily immune-suppressed microenvironment of metastases and primary tumor. Multiple treatments with low doses of the RL resulted in a dramatic decrease in the number of metastases (~90%) compared with control groups in the Panc-02 model. This is the first report of using live attenuated bacteria delivering a highly radioactive payload to the metastases, resulting in killing tumor cells in vivo without harming normal cells. The nontoxic RL treatment is attractive for clinical development as a therapy to prevent pancreatic cancer recurrence and metastases.

Pancreatic ductal adenocarcinoma, synonymous to pancreatic cancer, is the fourth leading cause of cancer deaths. The so-called silent killer is characterized by its metastatic behavior before the primary tumor can be detected, resulting in a 5-y survival rate of only 4%. Current cancer treatments—i.e., surgery followed by radiation and/or chemotherapy—are ineffective, particularly against liver metastases. Gemcitabine and erlotinib, Food and Drug Administration-approved drugs for pancreatic cancer treatment, improve median survival by ~6 mo in patients with advanced-stage disease (1–3), emphasizing the need for new alternative therapies for metastatic pancreatic cancer.

For any anticancer approach to be effective, it needs to target metastases and/or remaining tumor cells after primary therapeutic intervention. Indeed, in most cases, cancer therapy is now highly effective in eradicating primary tumors through combinations of surgery, radiation, and adjuvant therapy. The reason that cancer remains such a formidable health problem is its capacity to recur in the form of widespread metastases, often with a fatal consequence. In a previous study, we found that a highly attenuated bacterium, *Listeria monocytogenes* (*Listeria*^{at}), which was originally used to deliver tumor-associated antigens into antigen-presenting cells, also infected tumor cells in vitro and in vivo (4). Although *Listeria*^{at} was efficiently cleared by the immune system in the normal tissues within 3–5 d, immune suppression in the tumor microenvironment allowed these bacteria to accumulate in metastases and primary tumors and to kill tumor cells through high levels of reactive oxygen species (ROS) (4). Based on these results we hypothesized that *Listeria*^{at} could be used to deliver anticancer agents, such as therapeutic radionuclides (emitting cytocidal radiation such as beta-particles), specifically to the microenvironment of metastases and primary tumors and into tumor cells.

Targeted radionuclide therapy has proven successful in the treatment of several types of cancer and currently employs radiolabeled small molecules, monoclonal antibodies (Abs), peptides, and other tumor-targeting vehicles (5). The radioactive particles emitted by the radionuclides physically destroy the cancerous cells, and such therapies are not subject to multidrug-resistance mechanisms. There have been attempts to use targeted radionuclide therapy in form of radiolabeled tumor-specific Abs (radioimmunotherapy) for treatment of pancreatic cancer. However, radioimmunotherapy of pancreatic cancer has shown very modest results both preclinically (6–8) and in cancer patients with unresectable liver metastases (9). Obviously, new choices of targeting vehicles are needed to make targeted radionuclide therapy successful in the treatment of pancreatic cancer. In this regard, *Listeria*^{at} provides an attractive system for the delivery of radionuclides into the microenvironment of metastases and primary tumors. Here we show that live attenuated *Listeria*^{at} coupled with radionuclide ^{188}Rhenium (^{188}Re) is highly effective, particularly against metastases, in a mouse model of pancreatic cancer (Panc-02 model) without appreciable side effects.

Results

***Listeria*^{at} Multiplies in Metastases but Poorly in Primary Tumors and Not in Normal Tissues.** Metastatic Panc-02 tumors were generated in C57BL/6 mice. Subsequently, the tumor-bearing mice were injected with *Listeria*^{at} [0.5×10^7 colony-forming units (cfus)] intraperitoneally (i.p.), and their organs, primary tumors, and metastases were analyzed for the presence of live *Listeria*^{at} at various time intervals (1, 3, and 7 d after injection of the bacteria). As demonstrated in Fig. 1*A*, *Listeria*^{at} multiplied in the metastases between days 1 and 3, but not in normal tissues such as the spleen. Although a strong accumulation of *Listeria*^{at} was found in the primary tumors on day 1, no multiplication of *Listeria*^{at} was observed between days 1 and 3 or between days 3 and 7. Therefore, we also analyzed earlier time intervals (3, 6, and 72 h after injection of the bacteria) and found that *Listeria*^{at} did multiply to some degree between 3 and 6 h, but the number of cfu of *Listeria*^{at} decreased between 6 and 72 h in the primary tumor (Fig. 1*B*). The presence of *Listeria*^{at} in metastases, primary tumor, and tissues was confirmed by confocal microscopy (Fig. 1*C*). That *Listeria*^{at} could also multiply inside tumor cells is demonstrated here in vitro after 1, 3, and 6 h of incubation with the *Listeria*^{at} (Fig. 1*D*) and confirmed by confocal microscopy (Fig. 1*E*). Although after 1- and 3-h incubation usually one bacterium was found per tumor cell, after 6 h we detected four to six *Listeria*^{at} bacteria per tumor cell. Because gentamicin was used in the medium, the extracellular *Listeria*^{at} bacteria were dead, but the intracellular bacteria were alive. Special attention in this Panc-02 mouse tumor model was given to the pancreas. We found that the pancreas of tumor-bearing mice was heavily infiltrated with the Panc-02 tumor cells (Fig. S1*A*). *Listeria*^{at} strongly multiplied in the pancreas infiltrated with the tumor cells, but not in the pancreas of mice without

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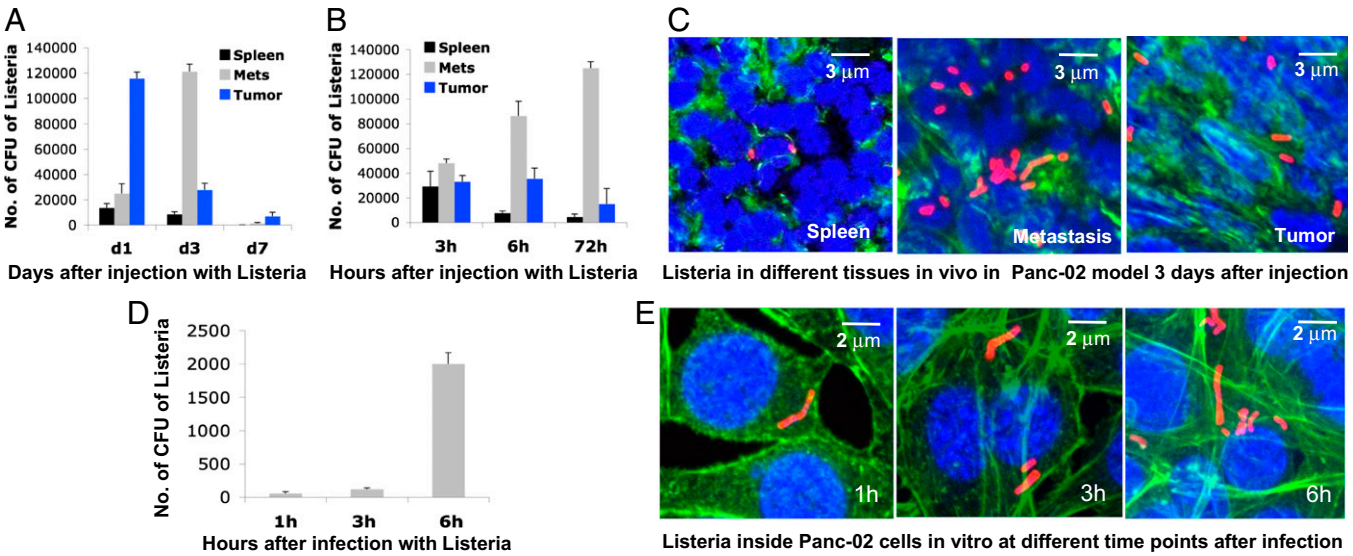

Fig. 1. *Listeria*^{at} multiplies in metastases and less abundantly in primary tumors but not in normal tissues. (*A* and *B*) Panc-02 tumor-bearing mice were injected i.p. with a high dose of *Listeria*^{at} (0.5×10^7 cfu) and analyzed for the number of cfu in spleen, tumor, and metastases at late (*A*) and early (*B*) time intervals. The error bars represent SEM. Data shown are the average of three independent experiments. Sample size was $n = 3$ mice per group. The number of *Listeria*^{at} cfu was determined per gram of tissue. (*C*) The presence of *Listeria*^{at} in these tissues was confirmed by confocal microscopy. (*D*) Listeria^{at} multiplies inside tumor cells. Panc-02 tumor cells were cultured with *Listeria*^{at} for 1 h, then further cultured in the presence of gentamicin, and finally terminated at various time points, to determine the number of cfu of *Listeria*^{at}. Data shown are the average of three independent experiments. The number of *Listeria*^{at} cfu was calculated per 10^6 cells per well. (*E*) The presence of *Listeria*^{at} inside Panc-02 tumor cells was confirmed by confocal microscopy. *Listeria*^{at} are red (Cy-3), nuclei blue (DAPI), and cytoplasm green (actin staining).

tumor cells (Fig. S1*C*), and this finding was confirmed by confocal microscopy (Fig. S1*B*).

Coupling with ^{188}Re and Stability of the Radioactive *Listeria*^{at} Complex. After demonstrating that *Listeria*^{at} selectively accumulated in metastases and primary tumors, we developed a radioactive *Listeria*^{at} (RL), with the goal of delivering the radioactivity selectively to the metastases. For this purpose we coupled ^{188}Re to *Listeria*^{at} using ^{188}Relabeled anti-*Listeria* Ab. ^{188}Re was chosen as radionuclide for *Listeria*^{at} because of its short half-life (16.9 h) compared to other beta emitters such as ^{131}I, ^{90}I, and ^{177}Lu, which allowed for the delivery of the radiation dose in a shorter amount of time and thus matching the aggressive growth of the pancreatic cancer. The Ab was radiolabeled with ^{188}Re using the methodology for labeling of the Abs with this radionuclide as described (10). The affinity and avidity of the Abs to *Listeria*^{at} is high. Briefly, 10^3 to 10^8 *Listeria*^{at} bacterial cells in 1 mL of PBS were incubated either with 1–40 μg of unlabeled Abs or with 60, 120, and 200 μCi of ^{188}Re-Abs for 1 h at 4 or 30 °C. RL generated with the 60, 120, and 200 μCi of ^{188}Re-Abs are designated here as RL-60, -120, and -200, respectively. Following the incubation, excess of ^{188}Re-Abs was removed by centrifugation, and the various RLs were resuspended in PBS and used for analysis.

An important question was whether ^{188}Re killed the *Listeria*^{at} bacteria. To answer this question, serial dilutions of RL-60, -120, and -200 as well as controls, i.e., *Listeria*^{at} alone and *Listeria*^{at} with unlabeled Ab, were plated on agar and examined the next day for the number of *Listeria*^{at} colonies. Evidence that ^{188}Re did not kill the *Listeria*^{at} bacteria is shown in Fig. 2*A*—i.e., the number of *Listeria*^{at} colonies counted in the RL groups was not significantly different from the numbers of colonies counted in the control groups. Another question was whether ^{188}Re decreased the stability of the *Listeria*^{at}. Our *Listeria*^{at} strain (XFL-7) contained a *Listeria*^{at} chloramphenicol-resistant plasmid pGG34, whereas the *Listeria*^{at} itself is chloramphenicol sensitive. If the *Listeria*^{at} is unstable and loses its pGG34 plasmid, it will not grow on chloramphenicol. To analyze the stability of RL, serial dilutions of RL-60, -120, and -200 as well as of *Listeria*^{at} were plated on agar with chloramphenicol. A somewhat lower (but significant) number of colonies was observed with RL-200 [which contained the highest concentration of ^{188}Re-Ab (200 μCi)] compared with *Listeria*^{at} alone when cultured with chloramphenicol (Fig. 2*B*). We also analyzed whether the infection rate of Panc-02 cells with *Listeria*^{at} was altered by the radioactivity. For this purpose, Panc-02 cells were incubated with the various RLs for 1 h, followed by gentamicin treatment for 1 h, and then lysed and plated on agar. Interestingly, the infection rates of RL-120 and -200 for Panc-02 cells were significantly higher than of *Listeria*^{at} alone, whereas there was no significant difference between the *Listeria*^{at}-Ab complex and *Listeria*^{at} alone (Fig. 2*C*). This result suggests that the higher infection rate of RL might be caused by the radioactivity and not by the Ab. In summary, the highest infection rate of the Panc-02 cells was observed for RL-200. Based on these results, we chose to use a dose of 200 μCi of RL in all further experiments. Finally, we also tested the stability of the RL complex in serum and PBS. RL was incubated in serum or PBS for 0, 1, and 3 h at 37 °C and showed very low dissociation grades of RL—i.e., radioactive counts were found in the supernatant of the serum varying between 0.1% and 0.6% of the baseline radioactivity, and this result was not significantly different from the radioactivity in the supernatant of PBS (Fig. 2*D*).

Efficacy of RL in Metastatic Pancreatic Cancer in the Panc-02 Model. Preliminary experiments demonstrated that the injection of mice with a high dose of *Listeria*^{at} (0.5×10^7 cfu) once a week led to clearance of most of the *Listeria*^{at} bacterial cells between days 3 and 7 (Fig. 1*A*). However, for therapy, it is important to have a low level of live *Listeria*^{at} bacterial cells that can continuously deliver the radioactivity into the tumor cells, without inducing listeriosis. Therefore, we developed a unique treatment protocol for the RL. Mice were challenged with 2×10^6 Panc-02 tumor cells in the mammary fat pad on day 0. This process resulted in a small primary tumor in the mammary fat pad palpable at 5–7 d, and metastases were found predominantly in the portal liver (area of portal vein in the liver) and throughout the liver, accompanied by a strong production of ascites within 21 d. Three days after tumor cell injection, mice were injected i.p. daily with 10^4 cfu of RL/500 μL of saline (group 1), 10^4 cfu of *Listeria*^{at}/500 μL

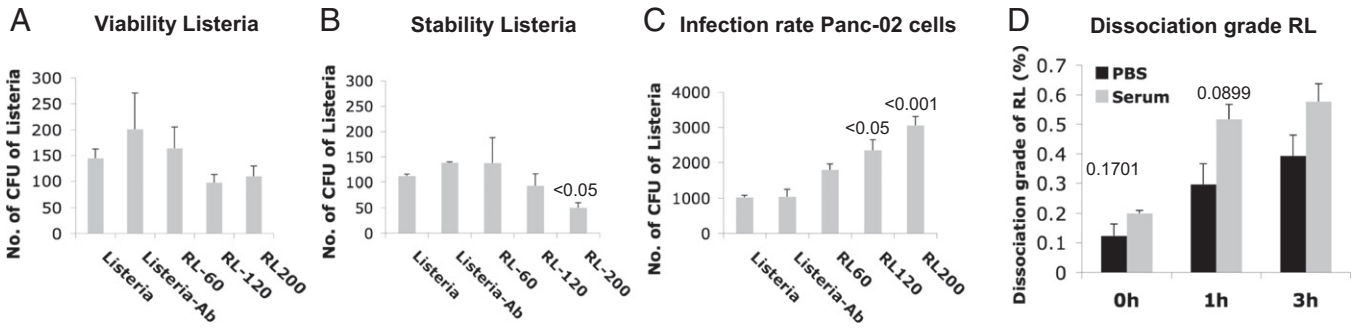

Fig. 2. Effect of ^{188}Re on *Listeria*^{at}. (*A*) *Listeria*^{at} bacteria were incubated with 60, 120, and 200 μCi of ^{188}Re-Abs (designated as RL-60, -120, and -200), plated on agar (LB only), and analyzed the next day for the number of cfu of *Listeria*^{at}, to analyze whether ^{188}Re kills *Listeria*^{at} bacteria. (*B*) To analyze the stability of RL, a similar experiment was performed on LB agar plates with chloramphenicol. (*C*) The effect of RL on the infection rate of Panc-02 was evaluated by incubating the cells with the various RLs in comparison with *Listeria*^{at} infection alone. The results were averaged and subjected to statistical analysis. All groups were compared with the *Listeria*^{at} group. $P < 0.05$ is statistically significant (ANOVA). The number of *Listeria*^{at} cfu was determined per 10^6 cells. (*D*) The stability of RL in serum was analyzed by incubating RL with serum or PBS (negative control) at 37 °C. After 0, 1, and 3 h, the RL bacteria were centrifuged, and the supernatant was analyzed for radioactive counts. The dissociation grade (percent) of the RL complex was determined by dividing free radioactivity in the supernatant and baseline radioactivity (10^7 cpm). Data shown are the average of two experiments. $P < 0.05$ is significant (unpaired *t* test). The error bars represent SEM.

of saline (group 2), 200 μCi of ^{188}Re/500 μL of saline (group 3), or 500 μL of saline (group 4) on days 3–9, followed by a rest period of 1 wk, followed by four injections with the same dose on days 16–19. All mice were euthanized on day 21 and analyzed for metastases and tumors. Although most of the metastases were predominantly present in the portal liver, metastases were also found in the pancreas, diaphragm, kidney, and mesenteric lymph nodes (MLNs). In addition, these metastases were eliminated by RL treatment. The metastases in Fig. 3*A* were a mixture of all metastases, but predominantly represent liver sites. RL decreased the number of metastases by ∼90% and *Listeria*^{at} alone by ∼50%, compared with the saline group or ^{188}Re (Fig. 3*A*). An example of the metastases in the portal liver of each group is shown in Fig. 3*B*. The effect on primary tumors was less pronounced but still significant. RL reduced the tumor weight by ∼64% and the *Listeria*^{at} by ∼20% compared with the saline group.

In the same experiment, we measured the amount of radioactivity that accumulated in metastases and normal tissues after the 11 treatments. We found that the radioactivity accumulated selectively in the metastases and much less in the primary tumors and normal organs—i.e., the radioactivity in the metastases was 4- to 15-fold higher compared with all other organs, except liver and kidneys (Fig. 3*C*). The radioactivity levels found in liver and kidneys were comparable to the metastases. We also measured the radioactivity as well as live *Listeria*^{at} 1 wk after the last treatment. It appeared that by that time point the radioactivity was not measurable anymore, and all *Listeria*^{at} were cleared by the immune system. Using medical internal radiation dose formalism, we have estimated that the dose delivered to the tumor by RL was ∼30 Gy, which is within a tumoricidal range for internal radionuclide therapy (5).

Safety Aspects of RL in Mice With and Without Tumors. Although both *Listeria*^{at} and ^{188}Re had mild side effects in human clinical trials in cancer patients (11–13), the combination of *Listeria*^{at} and radioactivity has never been tested for its safety in vivo. Therefore, we performed various experiments aimed at testing RL safety, such as biodistribution of RL, free ^{188}Re, and *Listeria*^{at}; pathology; and liver functions.

First, we measured the biodistribution of RL in all mouse tissues with and without Panc-02 tumors on days 1 and 7 after one i.p. administration of a high dose of RL (0.5×10^7 cfu) or with a comparable dose of free ^{188}Re. Day 1 was chosen to determine the biodistribution of RL in all tumor and normal tissues, and day 7 was chosen because we expected that, based on the half-life of ^{188}Re (17 h), the radioactivity should be below the detection level, which is important from a clinical point of view. One day after injection, all tissues were collected and measured for gamma radiation. Here, we demonstrate that radioactivity was present in all organs, including metastases and tumors on day 1 (Table 1). However, kidneys and liver contained much higher levels of radioactivity than the other organs or tumors and metastases (Table 1). A similar pattern was observed in mice without tumors, but lower levels of radioactivity were measured (Table 1). Free ^{188}Re was not detectable in all tissues of mice with or without tumors (Table 1). As expected, on day 7, no radioactivity was measurable in any of the tissues of mice with or without tumors because of the short half-life of ^{188}Re, i.e., 17 h (Table 1).

Second, we tested the biodistribution and clearance pattern of *Listeria*^{at} alone in mice, with or without Panc-02 tumors, on days 1, 3, and 7 after one i.p. injection of the high dose of *Listeria*^{at} (0.5×10^7 cfu). These three time intervals were selected because *Listeria*^{at} bacteria are able to multiply at different times in vivo as shown in Fig. 1 *A* and *B*, in contrast to the radioactivity that will only decrease after injection. On day 1, *Listeria*^{at} cfus were predominantly cultured from metastases and primary tumors (Table 2). *Listeria*^{at} multiplied strongly in the metastases between days 1 and 3, but not in the primary tumors, and the cfu of *Listeria*^{at} decreased in both metastases and tumor between days 3 and 7. We also analyzed the biodistribution and clearance at earlier time points (3, 6, and 72 h) and showed that the *Listeria*^{at} strongly multiplied in the metastases at all time points, but only to some degree in the primary tumors between 3 and 6 h (Table S1). A similar pattern of biodistribution of the *Listeria*^{at} cfu was observed with *Listeria*^{at} Ab (Table S2). In addition to the multiplication of *Listeria*^{at} in the metastases (less in the tumors), it cannot be excluded that some *Listeria*^{at} from the normal tissues migrated to the metastases and tumor tissues. *Listeria*^{at} again did not multiply in normal tissues, with the exception of the liver infiltrated with tumor cells. *Listeria*^{at} cfus were also found in the spleen at much reduced numbers, and some *Listeria*^{at} cfus were cultured from the rest of the tissues. On day 7, *Listeria*^{at} was cleared from all normal tissues, including the liver, whereas bacteria were still present in metastases and primary tumor, although at much lower levels than on day 1 or 3. In the mice without tumors, the cfu of *Listeria*^{at} were predominantly cultured from the spleen on day 1, but *Listeria*^{at} did not multiply in normal tissues, and *Listeria*^{at} bacterial cells were completely cleared from all tissues on day 7 (Table 2).

Finally, all tissues were analyzed for damage by histopathology after the 11 RL treatments in mice with pancreatic cancer. We were particularly interested in the normal liver and kidney because of the high radioactivity found in these tissues. One week after the last treatment, we observed no pathological damage by RL, ^{188}Re, or *Listeria*^{at} in the normal tissues, including liver or

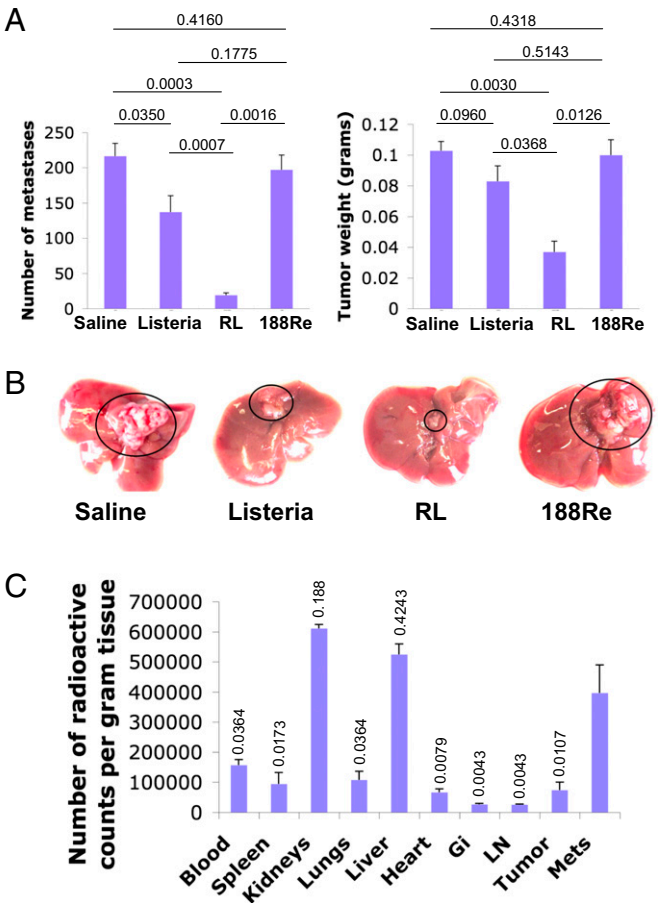

Fig. 3. Treatments with RL markedly reduced the number of metastases in the Panc-02 model. (*A*) Mice were therapeutically immunized 11 times with the low dose (10^4 cfu) of RL (*Listeria*^{at}-^{188}Re), *Listeria*^{at}, ^{188}Re, or saline and then euthanized 21 d later. Number of metastases and tumor weight was determined. Sample size was $n = 5$ mice per group. This experiment was repeated three times, and the results were averaged. $P < 0.05$ is statistically significant (Mann–Whitney). The error bars represent SEM. (*B*) From each group a representative is shown of metastases in the portal liver. (*C*) In the last of the three experiments, ^{188}Re was measured in all tissues of RL-treated mice 1 d after the last treatment by a gamma counter. The radioactive counts in each tissue were compared with the counts in the metastases using unpaired *t* test. $P < 0.05$ is statistically significant. The error bars represent SEM.

kidney (Table S3). We also analyzed the liver function by measuring for aspartate transaminase (AST) and alanine transaminase (ALT) in serum. One week after the last of 11 treatments with RL, *Listeria*^{at}, free ^{188}Re, or saline, liver functions were not altered by RL, ^{188}Re, or *Listeria*^{at} (Table S4).

Discussion

Unresectable liver metastases in pancreatic cancer patients are currently managed with cytotoxic treatment, which extends survival time by 6 mo only (1–3). Metastases are chemoresistant and cannot be removed by surgery or external radiation. In the present study, we demonstrate, in a highly aggressive mouse model of pancreatic cancer, that delivery of radionuclide ^{188}Re by a live attenuated *Listeria*^{at} bacterium resulted in a dramatic reduction in the number of metastases, compared with the control groups, correlating with an accumulation of ionizing radiation-emitting ^{188}Re in the metastases, without side effects.

It is known that *Listeria* has various mechanisms to infect cells in vitro and in vivo. *Listeria* can infect cells directly, such as macrophage-like cells through phagocytosis, or it can infect cells through a cell-to-cell spread mechanism characteristic for *Listeria*

Table 1. Biodistribution and clearance of RL

	Radioactive counts by RL on day 1, average ± SEM	
Tissue	Mice with tumors	Mice without tumors
Blood	44,137 ± 5,072	49,674 ± 6,849
Spleen	19,748 ± 3,769	24,746 ± 610
Kidney	300,895 ± 32,192	127,616 ± 11,583
Lungs	11,060 ± 740	21,083 ± 6,488
Liver	63,900 ± 20,483	64,561 ± 18,200
Heart	21,849 ± 19	25,551 ± 2,133
GI	19,113 ± 1994	14,682 ± 1,890
LN	34,243 ± 5,536	12,639 ± 1,244
Tum	25,835 ± 4,557	
Mets	33,681 ± 8,768	

C57Bl6 mice with or without Panc-02 tumors (Tum) and metastases (Mets) were injected with 0.5×10^7 cfu of RL or ^{188}Re and euthanized on days 1 and 7. The number of radioactive counts was determined in various tissues in triplicates, and the results of two experiments were averaged. $n = 5$ mice per experiment. The radioactivity by RL on day 7 and by ^{188}Re on days 1 and 7 was below the detection limit and therefore not shown. GI, gastrointestinal; LN, lymph nodes.

(14). In the present study, we found that *Listeria*^{at} directly infected pancreatic tumor cells (Fig. 1 *D* and *E*), and in an earlier study, it was found to also infect breast tumor cells (4). In addition, we found that *Listeria*^{at} infected myeloid-derived suppressor cells (MDSCs), and when injected into the tail vein of Panc-02 tumor-bearing mice, these MDSCs delivered the *Listeria*^{at} bacteria selectively to the metastases, where it could spread from cell to cell without being eliminated by the immune system; however, it was very poorly delivered to normal tissues such as the spleen (Fig. S2).

Because RL exhibits several mechanisms to kill tumor cells, we questioned which mechanism contributed the most to the strong effect on metastases in vivo. Based on a previous study in a metastatic breast tumor model (4T1) that received one preventive followed by two therapeutic treatments with *Listeria*^{at4}, we expected that *Listeria*^{at}-activated T cells should kill tumor cells in vivo in the Panc-02 model as well, because infected tumor cells become a target for *Listeria*^{at}-activated T cells and natural killer (NK) cells. However, after the 11 therapeutic treatments with low-dose RL in this Panc-02 model, CD8 T-cell (and NK cell) responses in vivo were weak and not statistically significant from the saline group (Fig. S3), suggesting a minor role of *Listeria*^{at}-activated immune responses in tumor cell destruction in vivo. As shown in this study, *Listeria*^{at} and RL infect Panc-02 cells (although not all of the tumor cells), and both can kill tumor cells through high levels of ROS (4, 15). In addition, extracellular *Listeria*^{at} or RL can kill tumor cells as well—i.e., through Listeriolysin O (LLO) secreted by *Listeria*^{at} inducing high levels of ROS inside the tumor cells (4) or through the crossfire effect of radioactivity generated by ^{188}Re (15). Although both had a significant effect on the metastases, the effect of RL was significantly higher than of *Listeria*^{at} alone. Based on these results, we concluded that the cytocidal ^{188}Re radiation mostly and *Listeria*^{at}-induced ROS less abundantly (but significantly) contributed to the destruction of tumor cells in the metastases, whereas *Listeria*^{at}-activated CD8 T and NK cells in vivo played a minor role in tumor cell destruction in this Panc-02 model.

A smaller effect of RL was observed on primary tumors. Radiation-induced damage is most effective in highly proliferating cells. Although tumor cells in the metastases are highly proliferating, in the primary tumors of this Panc-02 model, the tumor cells were largely dormant, and therefore metastases are a much better target for radiation-induced kill than primary tumors. To prove that radiation-induced damage is considerably less in nondividing cells than in highly proliferating cells, we tested the damaging effect of RL in cultures of Panc-02 cells with a high

Table 2. Biodistribution and clearance of *Listeria*^{at}

	cfu of *Listeria*^{at}, average ± SEM					
	Mice with tumors			Mice without tumors		
Tissue	Day 1	Day 3	Day 7	Day 1	Day 3	Day 7
Blood	53 ± 15.5	38 ± 4	bdl	362 ± 210	bdl	bdl
Spleen	13,645 ± 3,178	8,360 ± 2,145	bdl	189,000 ± 5,500	22,382 ± 9,467	bdl
Kidney	483 ± 120	130 ± 29	bdl	1,603 ± 584	263 ± 133	bdl
Lungs	381 ± 7	94 ± 14	bdl	376 ± 170	53 ± 28	bdl
Liver	274 ± 25	8,470 ± 2,135	bdl	5,398 ± 1,541	111 ± 23	bdl
Heart	821 ± 14	323 ± 4	bdl	27 ± 9	bdl	bdl
GI	bdl	bdl	bdl	bdl	bdl	bdl
LN	bdl	258 ± 57	bdl	1,217 ± 592	1,077 ± 373	bdl
Tum	172,283 ± 4,892	27,748 ± 5,222	7,037 ± 1,048			
Mets	38,118 ± 7,628	177,133 ± 9,831	1,287 ± 642			

C57BL/6 mice with or without Panc-02 tumors (Tum) and metastases (Mets) were injected with 0.5×10^7 cfu of *Listeria*^{at} and euthanized on days 1, 3, and 7. The number of *Listeria*^{at} cfu was determined in various tissues, and the results of two experiments were averaged. $n = 5$ mice per experiment. bdl, below detection limit; GI, gastrointestinal; LN, lymph nodes.

(nonproliferating) and low (highly proliferating) density in vitro. After RL treatment, only 25% of the Panc-02 tumor cells at low density and 53% of Panc-02 cells at high density were alive the next day (Fig. S4). These results support the lesser effect of RL on the dormant tumors compared with the highly proliferating metastases.

The dramatic effect of RL on the metastases in the Panc-02 model was repeatedly found without inducing severe side effects on normal tissues. However, the radioactivity was high in the metastases and in liver and kidney. One could argue that the RL complex is unstable, but stability assays of RL in serum and PBS as control (0, 1, and 3 h at 37 °C) showed that the RL complex was highly stable and did not dissociate in the serum. Most likely, the radiolabeled *Listeria*^{at} are killed by the immune system and metabolized by the body. The processed radiolabeled proteins will end up in the liver and kidneys as the sites of their metabolism and excretion, respectively. Such a phenomenon is well known in radioimmunotherapy (15). Of clinical importance, neither *Listeria*^{at} nor radioactivity was detected in normal or tumor tissues 1 wk after the last treatment. Despite the accumulation of radioactivity in the liver and kidneys, pathological examination revealed practically no histologically apparent side effects in normal tissues, and liver functions, such as AST and ALT, appeared to be unaltered. This finding raises the question as to why RL, when accumulated in metastases as well as in normal kidneys and liver, destroys tumor cells, but not cells in normal kidney and liver. One possible explanation is that radiation-induced irreparable DNA damage is much higher for proliferating cells, like metastases, than in nondividing normal tissues. As mentioned above, a lesser effect of RL and *Listeria*^{at} was observed on primary tumors, because the proliferation of these dormant Panc-02 tumors is poor compared with the metastases. Finally, *Listeria*^{at} survives and replicates better, and produce higher levels of ROS in a hypoxic environment (16), like the metastases in this model.

The highly attenuated *Listeria*^{at} used in this study is different from wild-type *Listeria*, in the sense that the latter multiplies in hepatocytes in the liver or epithelial cells of the gastrointestinal tract (17, 18), whereas *Listeria*^{at} does not. In support of these results, we have already shown that a high dose of *Listeria*^{at} or ^{188}Re is less toxic in humans than chemotherapy (11–13). Currently, other *Listeria* constructs are available with reduced pathogenicity and used for different approaches. One example is the killed but metabolically active *Listeria* strain of Brockstedt et al. (19). This strain has reduced pathogenicity by deleting the actA (required for cell to cell spread, characteristic for *Listeria*) and the UV-sensitive uvrAB genes, without losing its potency to activate CD4 and CD8 T-cell responses. Another *Listeria* strain has been developed by Goebel and colleagues (20), which has been deleted for internalins InlA and InlB (ligands for entry receptors), but expressing *Stapylococcus aureus* antigen that can bind IgG Abs such as Herceptin and then induce internalization of the *Listeria* into Her2/neu-expressing breast tumor cells. Such an approach is highly promising, and a similar approach could be used to deliver radioisotopes with improved efficiency into tumor cells. In addition, others have shown the potential of bacteria for delivery of anticancer agents into tumor cells (21, 22).

In conclusion, our data demonstrate that live attenuated bacteria can deliver radioactivity to the metastases of pancreatic cancer in vivo, resulting in killing tumor cells without having severe side effects. Such an approach might start a unique era in the treatment of metastases from pancreatic cancer.

Materials and Methods

Mice. Normal female C57BL/6 mice aged 3 mo were obtained from Charles River and maintained in the animal husbandry facility of the Albert Einstein College of Medicine according to the Association for Assessment and Accreditation of Laboratory Animal Care guidelines, and according the guidelines of the Albert Einstein Institute for Animal Studies. All mice were kept under Biological Safety Level 2 conditions as required for *Listeria*^{at} treatments.

Cells and Cell Culture. The Panc-02 cell line is highly tumorigenic and metastatic and was derived in 1984 from a methylcholanthrene-induced ductal adenocarcinoma growing in a C57BL/6 female mouse (23) (provided by Chandan Guha, Albert Einstein College of Medicine). The Panc-02 cells were cultured in McCoy's medium supplemented with 10% (vol/vol) FBS, glutamine (2 mM), nonessential amino acids, sodium pyruvate (1 mM), Hepes (10 mM), and penicillin/streptomycin (100 U/mL).

***Listeria*^{at}.** In this study, *Listeria*^{at} (24) was used as the vehicle for the delivery of ^{188}Re to the tumor microenvironment. The *Listeria*^{at} plasmid pGG-34 expresses the positive regulatory factor A (prfA) and LLO (25). prfA regulates the expression of other virulence genes and is required for survival in vivo and in vitro (25). The background strain XFL-7 lacks the prfA gene and retains the plasmid in vitro and in vivo. The coding region for the C-terminal part of the LLO (cytolytic domain that binds cholesterol in the membranes) protein in the plasmid has been deleted, but *Listeria*^{at} is still able to escape the vacuole (25). Mutations have been introduced into the prfA gene and the remaining LLO (expressed by the pGG34 vector), which further reduced the pathogenicity of the *Listeria*^{at25}.

^{188}Re, Anti-*Listeria* Abs, and Making RL. ^{188}Re was obtained from the ^{188}W/^{188}Re radionuclide generator (Oak Ridge National Laboratory, Oak Ridge, TN). The commercially available polyclonal Ab to *Listeria* (IgG1 isotype; BD Difco *Listeria* O antiserum Poly serotype 1,4; catalog no. 223021; this antiserum has high avidity and affinity for *Listeria*^{at} bacteria), and the isotype

matching control Ab to account for any possible nonspecific binding of the IgG to the *Listeria*^{at} have been radiolabeled with ^{188}Re using a described protocol for labeling proteins with this radioisotope (10). To make the RL, 10^3 to 10^8 *Listeria*^{at} bacteria in 1 mL of PBS were incubated either with 1–40 μg of unlabeled Abs or with 60, 120, and 200 μCi of ^{188}Re-Abs for 1 h at 4 °C or 30 °C. RL generated with the 60, 120, and 200 μCi of ^{188}Re-Abs are designated here as RL-60, -120, and -200. Following the incubation, excess ^{188}Re-Abs were removed by centrifugation, and the various RLs were resuspended in PBS and used for further work.

Tumor Challenge, Treatments, and Analyses. Tumor challenge, treatments, and analyses of tumors and metastases were performed as described with minor modifications (24). Briefly, Panc-02 tumor cells (2×10^6) were injected into the mammary fat pad on day 0. In the Panc-02 model, the primary tumor extends to the chest cavity lining, which is palpable 5–7 d after tumor cell injection, but primary tumors stayed relatively small, whereas metastases (visible by eye) predominantly developed in the portal liver, resulting in the production of ascites in the peritoneal cavity within ∼20 d. In addition, the pancreas was heavily infiltrated with Panc-02 tumor cells, but less frequently in the MLNs, diaphragm, spleen, and kidneys. Starting 3 d after tumor cell injection, mice were treated daily (days 3–9) i.p. with a low dose of *Listeria*^{at} (10^4 cfu per 500 μL of saline) ($LD_{50} = 10^8$), 200 μCi of RL (10^4 cfu per 500 μL of saline), 200 μCi of ^{188}Re (the same dose of ^{188}Re compared with *Listeria*–^{188}Re), or saline, followed by a rest period of 1 wk, and then followed by four more treatments on days 16–19 with 10^4 cfu of *Listeria*^{at}, RL, ^{188}Re, or saline. All mice were euthanized at day 21 and analyzed for tumor weight, frequency and location of metastases, and T- and NK-cell responses in blood and spleen.

Isolation of *Listeria*^{at} from Metastases, Tumors, and Normal Tissue. *Listeria*^{at} bacteria were isolated from metastases, tumors, and normal tissues as described (4). Briefly, mice with Panc-02 metastases and tumors were treated once with a high dose of *Listeria*^{at} (0.5×10^7 cfu) or 11 times with a low dose (10^4 cfu) and euthanized at various time points as indicated in the text. Metastases, tumors, and normal tissues were dissected and homogenized, plated on agar, and counted for *Listeria*^{at} colonies the next day. The number of *Listeria*^{at} cfu was calculated per gram of tissue.

Determination of Radioactive Counts in Tumor and Normal Tissues. Mice with or without Panc-02 metastases and tumors were immunized once with a high dose of *Listeria*^{at} (0.5×10^7 cfu) or 11 times with a low dose (10^4 cfu) and euthanized at various time points as indicated in the text. Metastases, tumors, and normal tissues of RL-vaccinated and control mice were dissected, weighted, and analyzed for gamma radiation in a gamma counter (Wallac). The number of radioactive counts was calculated per gram of tissue.

Infection of Tumor Cells in Vitro. The infectivity rate of the cell lines was assessed in vitro as described (4). Briefly, 1×10^6 cells per mL were infected with 10^7 (per well) of *Listeria*^{at}, for 1 h at 37 °C in culture medium as described above. After the incubation with gentamicin (50 μg/mL) for 1 h (killing all extracellular *Listeria*^{at} bacteria), cells were washed with PBS and lysed in sterile water, and serial dilutions were plated onto LB agar to determine the infection rate the next day.

Confocal Microscopy. Tissues from mice were snap-frozen in optimal cutting medium (OCT; TissueTek). Ten-micrometer sections were fixed with 3.7% formaldehyde for 10 min at room temperature (RT). In addition, Panc-02 tumor cells and MDSCs cultured with or without *Listeria*^{at} (1:1) were fixed in 3.7% formaldehyde. Cells were first permeabilized with 0.1% Triton X-100 in PBS for 5 min, followed by blocking (1% normal goat serum) and washing steps, and then incubated with the primary Abs for *Listeria* (IgG1 isotype; BD Difco *Listeria* O antiserum Poly serotype 1,4; catalog no. 223021) in PBS with 0.03% BSA and 0.1% Triton X-100 for 90 min at RT, followed by washing steps, and then incubated with the secondary Ab goat anti-rabbit IgG—Cy3-labeled (dilution 1:500) (Abcam) for 60 min at RT, followed by incubation with Alexa Fluor 488 Phallodin (40 U/mL; Life Technologies) for 20 min at RT to stain the cytoplasm. Slides were mounted with DAPI containing mounting medium (Vectashield) and analyzed on a Leica SP2 confocal microscope with a 63× oil immersion objective equipped with an AOBS system, using ImageJ software.

Statistical Analysis. To statistically analyze the effects of RL or *Listeria*^{at} on the growth of metastases and tumors, on immune responses, or on liver functions in the pancreatic mouse tumor model, unpaired *t* test, Mann-Whitney test, or ANOVA were used. Values of $P < 0.05$ were considered statistically significant.

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