Cancer Immunotherapy Based on Killing of \textit{Salmonella}-Infected Tumor Cells

Francesca Avogadri, Chiara Martinoli, Liljana Petrovská, Claudia Chiodoni, Pietro Transidico, Vincenzo Bronte, Renato Longhi, Mario P. Colombo, Gordon Dougan, and Maria Rescigno

\textsuperscript{1}Department of Experimental Oncology, European Institute of Oncology; \textsuperscript{2}Immunotherapy and Gene Therapy Unit, Istituto Nazionale dei Tumori, Istituto di Chimica del riconoscimento molecolare, Consiglio Nazionale delle Ricerche, Milan, Italy; \textsuperscript{3}Centre for Molecular Microbiology and Infection, Department of Biological Sciences, Imperial College London, London, United Kingdom; and \textsuperscript{4}Department of Oncology and Surgical Sciences, University of Padova, Padova, Italy

**Abstract**

A major obstacle for the development of effective immunotherapy is the ability of tumors to escape the immune system. The possibility to kill tumor cells because they are recognized as infected rather than as malignant could help overcome immune escape mechanisms. Here we report a conceptually new approach of cancer immunotherapy based on \textit{in vivo} infection of tumors and killing of infected tumor cells. Attenuated but still invasive, \textit{Salmonella typhimurium} can be successfully exploited to invade melanoma cells that can present antigenic determinants of bacterial origin and become targets for anti-\textit{Salmonella}–specific T cells. However, to fully appreciate the anticancer therapeutic properties of \textit{S. typhimurium}, tumor-bearing mice need to be vaccinated against \textit{S. typhimurium} before intratumoral \textit{Salmonella} injection. Tumor infection when coupled to anti-\textit{Salmonella} vaccination leads to 50\% to 100\% tumor-free mice with a better outcome on larger tumors. Invasive \textit{Salmonella} also exert an indirect toxic effect on tumor cells through the recruitment of inflammatory cells and the cross-presentation of tumor antigens, which allow induction of tumor-specific immune response. This is effective in retarding the growth of untreated established distant tumors and in protecting the mice from subsequent tumor challenges. (Cancer Res 2005; 65(9): 3920-7)

**Introduction**

It has become apparent that despite the great specificity achieved with immunotherapy against cancers, a single modality is insufficient to eradicate such a complex disease (1). Many approaches have been developed to induce immunity against cancer and remarkably potent antitumor-specific cytotoxic responses have been achieved (2–5). However, most human and murine tumors do not regress and continue growing even in the presence of circulating tumor-specific T cells (5–8), because they have limited access to tumor masses (7), are rendered anergic or defective once inside the tumor (9, 10), or are unable to encounter their specific target due to down-regulation of antigen or MHC molecules (11, 12). Therefore, the immunosuppressive environment generated by the tumor can restrain the positive effects of potentiating the antitumor immune response. Recently, a number of studies have shown that it is possible to increase the visibility of the tumor by inducing an inflammatory state within the tumor microenvironment. This can be achieved by subjecting the tumor area to irradiation (6), radiofrequency ablation (13), or by injecting bacterial products like CpG containing DNA (14) or directly anaerobic bacteria such as \textit{Clostridium} spp. (15).

An interest in using bacteria as anticancer therapeutic agents dates back to the end of the 19th century, but this approach has been recently renovated with the advent of molecular biology (16, 17). The antitumor effect of different bacteria strains, including \textit{Bifidobacterium}, \textit{Clostridium}, and \textit{Salmonella} spp. has been analyzed (18). \textit{Salmonella enterica} serovar \textit{typhimurium} is particularly appealing as a cancer therapeutic agent because it is able to colonize preferentially tumor areas if given i.v. and because it has an intrinsic antitumor property (18–22). Moreover, invasive facultative intracellular bacteria such as \textit{Salmonella} have the significant ability to infect nonphagocytic cells via the expression of a type-three secretion system (TTSS), which facilitates penetration into host cells (23). Although many groups have studied the anticancer therapeutic effect of \textit{Salmonella}, the mechanisms responsible for its efficacy were unknown.

In this study, we explored the mechanism(s) underlying the \textit{Salmonella}-mediated antitumor effect in a mouse melanoma model. We chose the B16F10 melanoma model for its poor immunogenicity and because it displays strong immunescape mechanisms compared with other tumor cell lines (24, 25). Immunotherapeutic strategies that are fully protective in other tumor settings are very ineffective on the B16F10 melanoma model, particularly when the latter is not genetically modified to increase its immunogenicity (24). We provide evidence that \textit{S. typhimurium} is a potent anticancer therapeutic agent even on the B16 melanoma model leading to 50\% to 100\% tumor-free mice in a therapeutic setting. We identified three mechanisms concurring in the antimalanoma effect of \textit{S. typhimurium}: (i) \textit{S. typhimurium}–infected malignant cells are recognized and killed by anti-\textit{Salmonella}–specific T cells; (ii) a massive recruitment of effector cells (both innate and adaptive immune cells) is induced at the site of infection; (iii) infection by \textit{Salmonella} induces the cross-presentation of tumor antigens and the establishment of systemic antitumor response. Moreover, we show that to fully appreciate the anticancer therapeutic properties of \textit{Salmonella}, it is necessary to amplify the pool of immune effector cells specific for \textit{S. typhimurium} through vaccination. This allows recognition and killing of \textit{Salmonella}-infected tumor cells.

**Materials and Methods**

\textbf{Mice, cells, and bacterial strains.} Five-week-old female C57/B16J mice were purchased from Harlan (Udine, Italy). The murine melanoma cell line B16F10 (a kind gift from Dr. P. Dellabona, Experimental...
Salmone
typhimurium as Anticancer Agent

Immunology Unit, Cancer Immunotherapy and Gene Therapy Program, DIBIT, H. San Raffaele Scientific Institute, Milano, Italy) and ovalbumin (OVA)-expressing EG-7 cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 50 μmol/L 2-mercaptoethanol. The human melanoma cell line IGR39 (ACC-236) was cultivated in DMEM supplemented as above. The B7-1 cell hybridomas specific for the H-2Kb restricted OVA epitope SIINFEKL was grown in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 5% FBS. Bovine cells were derived from bone marrows of C57/BL6j mice cultured in IMDM containing 10% FBS, supplemented as above plus 30% supernatant from granulocyte macrophage colony-stimulating factor-producing NIH-3T3 cells. Peritoneal macrophages were isolated after 5 days i.p. treatment with 4% thioglycollate, cultured in RPMI 1640 supplemented with 10% FBS and 2 mmol/L glutamine. Blast cells were obtained from spleenocytes of C57/BL6j mice, cultured for 5 days in RPMI complete medium supplemented with 10% FBS and 2 μg/mL concanavalin A (Sigma, Milano, Italy).

S. typhimurium derivatives were generated starting from wild-type SL1344 WT according to the methodology described by Datsenko and Wanner (26). Three strains were generated, one deficient in the gene (SL1344 Invα) and unable to form the TTSS, and two araO metabolically defective strains (SL3261AT Invα) of which one was also deficient in the invC gene (SL3261AT InvαC). Five recombinant variants of each strain were generated, expressing constitutively GFP or red fluorescence protein (DsRed, a kind gift of Dr. B. Glick, The University of Chicago, Department of Molecular Genetics and Cell Biology, 920 East 58th Street, Chicago, IL 60637, USA (27)), glutathione S-transferase (GST), GST-OVA, or GST-TRP2 fusion proteins, under transcriptional control of the promoter of LacZ gene.

Bacterial strains were grown at 37°C in Luria broth, supplemented with 10 μg/mL kanamycin (for S. typhimurium BA34 and SL3261AT Invα) and 50 μg/mL ampicillin (for all the strains carrying plasmid). Protein expression was induced by adding 1 mmol/L isopropyl-β-D-galactopyranoside to an A600nm = 0.5 bacterial cultures for 2 hours. Salmone
typhimurium toxicity. Single bacterial colonies were grown overnight and restated the next day at 1:10 of the original volume up to an A600nm = 0.6 corresponding to 1.2 × 10^6 CFU/mL. B16F10 cells and isolated peritoneal macrophages were incubated with bacteria for 1 hour, in a ratio of cell/bacteria ranging from 1:10 to 1:50, in the appropriate medium without antibiotics. Cells were washed with PBS and incubated in medium supplemented with 50 μg/mL gentamicin for 12 or 24 hours, to kill only extracellular bacteria. To evaluate cell death after bacterial infection, cells were treated with SYTOX green dye (Invitrogen) for 18 hours. Interleukin 2 (IL-2) secreted from the B3Z was measured as a positive control. The differences were considered statistically significant only when P < 0.05.

Evaluation of intracellular bacteria, in vitro and in vivo. B16F10 or IGR39 cells (10^4) were infected in vitro with DsRed-expressing S. typhimurium SL1344, SL1344 Invα, SL3261AT, or SL3261AT Invα as described above. After 4 or 18 hours, 10^6 cells were plated on coverslips and fixed in 1% paraformaldehyde. Samples were blocked with PBS 3% bovine serum albumin (BSA), incubated with rabbit-anti-Salmonella primary antibody (Valter Occhiena, Torino, Italy) and with an anti-rabbit-FITC–conjugated secondary antibody (PharMingen, Milano, Italy). Cells nuclei were stained with 4,6-diamidino-2-phenylindole. Alternatively, 10^6 cells were lysed with 0.5% sodium deoxicholate and serial dilutions were plated on TB agar to quantify the number of live intracellular bacteria.

To evaluate bacteria internalization in vivo, tumor masses were injected with 10^8 S. typhimurium and then processed for confocal analysis, gentamicin protection assays or cytfluorometry as follows. For confocal analysis on tumor sections, tumor masses were injected with SL3261AT-DsRed or SL3261AT Invα-DsRed. Twelve or 36 hours later, tumors were resected, snap frozen in embedding medium (Tissue-Tek OCT compound, Sakura, Milano, Italy), and stored at −20°C until cutting. Cryosections (7 μm) were fixed in paraformaldehyde, blocked and permeabilized in PBS, 3% BSA, 0.1% triton, incubated with Kβ–FITC, rabbit-antitoxine–related protein (TRP)-1 (kind gift from Dr. V. Hearing, Laboratory of Cell Biology, National Institutes of Health, Bethesda, MD) and with an anti-rabbit-CY5 antibody (PharMingen). Gentamicin protection assays were done on tumors resected 30 minutes after injection with SL3261AT or SL3261AT Invα. After resection, tumors were dissected, smashed through a cell strainer (Falcon, Como, Italy), and depleated of RBC. Cells were incubated for 2 hours at 37°C with 50 μg/mL gentamicin, lysed with sodium deoxicholate and plated as above. For flow cytometry analysis, tumor masses were injected with GFP-expressing SL3261AT or SL3261AT Invα. At the indicated time points, tumor cells were prepared as above and either stained either with rabbit-anti-RPM and anti-Gr1-phocerythrin antibodies (Sigma), or with Gr1-phocerythrin (PharMingen).

Analysis of leukocyte infiltration in the tumor mass. Tumors were injected with 10^6 SL3261AT, SL3261AT Invα or PBS and resected 2, 4, or 6 days later. Half of the tumor mass was dissected and incubated in PBS containing collagenase (Roche, Milano, Italy), DNase and Hyaluronidase for 1 hour at 37°C, and cells were processed for cytfluorometry. Cells were stained with one of the following antibodies: anti-CD4-PE, anti-CD8-FITC, anti-panNK-FTTC, anti-CD11b-PE, anti-Gr1-Pe, anti-CD19-PE (all from PharMingen), anti-F4/80-PE (Caltag, Torino, Italy), and analyzed by FACScan (Becton Dickinson, Milano, Italy). The other half was snap frozen as above and sections (7 μm) were cut and fixed in acetone. Endogenous peroxidase activity was inhibited and sections were blocked in PBS, 20% FCS. Immunostaining was done with biontiylated antibodies and alcine
trophastases-conjugated streptavidin (all from PharMingen). To distinguish between melanin and histochemical stainings, cells were visualized by formaldehyde or histochemical stainings.

Presentation assay. B16F10 (10^4 cells per well) were plated in a flat-bottomed 96-well plate (Falcon) and infected with bacteria (1:12.5, 1:25, 1:50, 1:100 cells/bacteria ratios) for 1.5 hours. Cells were then washed and incubated in fresh medium containing gentamicin. Four hours later, 5 × 10^4 B3Z cells per well were added and incubated for additional 18 hours. Interleukin 2 (IL-2) secreted from the B3Z was measured by ELISA (R&D Systems, Milano, Italy), according to manufacturer's instructions.

Cytotoxicity assay. Effector cells were isolated from tumor-bearing mice treated as follows: mice were vaccinated with dendritic cells loaded in vitro with OVA-expressing SL3261AT at days 5 and 9 from B16F10 injection; at day 9, mice were treated intratumorally (i.t.) with OVA-expressing SL3261AT. A week later, mice were sacrificed and 5 × 10^7 splenocytes per well were plated in 24-well plates in complete RPMI supplemented with 10% FCS, 20 UI/mL rIL-2 (Promulkin, Chiron, Siena, Italy) and 1 μg/mL SIINFEKL peptide. Cultures were assayed 5 days later for CTL activity over a range of target/effector ratios (1:12.5, 1:25, and 1:50). Target cells were B16F10 cells, B16F10 cells infected for 18 hours with invasive or noninvasive OVA expressing SL3261AT, EG7 cells, blast cells.

Tetramer staining. Splenocytes of treated mice were collected 17 days after the first i.t. treatment and sectioned, smashed through cell strainer, and depleated of RBC. For cytfluorometry analysis, 10^6 cells were stained with anti-CD8-FITC antibody (PharMingen) and with TRP-2-Kβ tetramer-PE-conjugated. As a negative control, a tetramer specific for β-galactosidase (β-gal)-2-Kβ was also done for each spleen sample.
Results

Invasive Salmonella typhimurium is able to infect tumor cells. The ability of Salmonella to infect tumor cells was assessed in vitro by using invasive S. typhimurium (SL1344wt or SL3261AT) or InvA mutant derivatives (SL1344 InvA or SL3261AT InvA), deficient in the TTSS. The latter have reduced ability to penetrate host cells and are therefore a good control for the invasiveness assay. All strains were engineered to express the DsRed fluorescent protein as a marker for microscopy. B16F10 melanoma cells were incubated with DsRed-expressing S. typhimurium derivatives for 1 hour in medium without antibiotics and in medium containing gentamicin to block extracellular bacterial growth. To verify that the red S. typhimurium were inside the tumor cells, nonpermeabilized cells were stained with FITC anti-Salmonella antibody, which was accessible only to extracellular bacteria. We show that invasive S. typhimurium SL1344wt were able to infect tumor cells (red bacteria in Fig. 1A, left); by contrast, noninvasive S. typhimurium InvA were unable to efficiently invade tumor cells and stained in yellow (Fig. 1A, middle, due to the colocalization of red and green staining). The ability of the Salmonella derivatives to enter tumor cells was not restricted to the B16F10 cells as we could show a similar behavior with the IGR39 human melanoma cell line (Fig. 1A, right). The amount of internalized Salmonella per tumor cells was evaluated by plating a fixed amount of lysed tumor cells after the internalization assay. We could recover between 800 and 1,300 CFU per 10^3 B16 cells depending on the S. typhimurium derivative used (Fig. 1B). In contrast, very few Salmonella colonies were recovered after incubation of tumor cells with the S. typhimurium InvA noninvasive derivatives.

Salmonella is not directly toxic for tumor cells. It has been well documented that S. typhimurium is cytotoxic in macrophages (28, 29) through the activation of caspase-1 (30). To test whether S. typhimurium was per se toxic to tumor cells, we incubated the B16F10 melanoma cells in vitro with S. typhimurium for 12 or 24 hours. Salmonella did not induce any significant increase in the percentage of apoptotic or necrotic tumor cells, as judged by the similar percentage of Annexin V and propidium iodide–positive cells of infected and noninfected cells, compared with UV or puromycin induced B16F10 cell death (Fig. 1C). The toxicity of Salmonella was assessed on macrophages, which were readily killed by invasive Salmonella (Supplemental Fig. 1). Therefore, we can exclude that S. typhimurium exerted a direct intrinsic toxic effect on melanoma cells.

Infected tumor cells present antigenic determinants of bacterial origin. We then tested whether tumor cells processed and presented antigens from the internalized bacteria, in vitro. We incubated the B16F10 cells with S. typhimurium derivatives expressing the model antigen OVA and analyzed the ability of infected tumor cells to present OVA\textsuperscript{257-264} peptides on their cell surface in association with the MHC I K\textsuperscript{b} molecule. As a read-out system, we used the B3Z hybridoma that releases IL-2 after recognizing specifically the OVA-K\textsuperscript{b} complex. We detected IL-2 production only after incubation of the B3Z hybridoma with B16F10 cells previously infected with invasive but not with noninvasive OVA-expressing Salmonella (Fig. 2A). This indicates that only when the bacteria are actively entering the B16F10 melanoma cells, their proteins, including the heterologous OVA, are processed and presented on the tumor cell surface. Infected melanoma cells are also target of anti-OVA–specific cytotoxic killing. As we wanted to mimic the in vivo treatments, anti-OVA–specific CTLs were raised from tumor-bearing mice that were vaccinated with OVA-expressing Salmonella and received the bacteria intramuscularly. Only B16F10 cells infected with invasive but not with noninvasive OVA-expressing S. typhimurium (P < 0.01) were killed by anti-OVA–specific CTLs (Fig. 2B). A basal killing of noninvasive treated or nontreated B16F10 cells was observed. This is probably due to a contamination of antimelanoma-specific T cells that were raised during the treatments, as described below (Fig. 2B). The efficiency of anti-OVA CTLs was evaluated on the EG7 (OVA expressing) cell line. These results indicate that infected tumor cells present bacterial determinants on their cell surface and become target of anti-Salmonella–specific T cells.

Salmonella infects tumor cells in vivo. We examined the ability of immune cells to recognize and kill Salmonella-infected tumor cells in vivo. For this purpose, B16F10-bearing C57/B6 mice were treated i.t. with invasive and noninvasive derivatives of S. typhimurium. Both derivatives also harbored the attenuating mutation aroA (31) to prevent the mice being overwhelmed by...
the Salmonella infection. We confirmed that invasive *S. typhimurium* were able to infect tumor cells *in vivo* (Fig. 3A); tumor-bearing mice were injected i.t. with invasive or noninvasive DsRed-expressing *S. typhimurium*; tumor masses were resected and processed for confocal analysis. To distinguish between melanoma cells and tumor-infiltrating cells, sections were double stained for the melanoma-associated antigen TRP-1 (blue) and for MHC-I (green), which is expressed both by melanoma cells and infiltrating phagocytes. Consistent with our *in vitro* data, only invasive *S. typhimurium* was found inside tumor cells (Fig. 3A, top, arrows), whereas noninvasive derivatives were internalized only by the recruited phagocytes (Fig. 3A, bottom, arrowhead) shows an infiltrating phagocyte, characterized by high expression of MHC I molecules; three-dimensional reconstruction in Supplemental Fig. 2). The ability of invasive bacteria to infect tumor cells was confirmed by gentamicin protection assay on a fixed number of lysed cells extracted from tumor masses 30 minutes after *in vivo* infection (Fig. 3B) and by cytfluorometry (Fig. 3C), comparing the number of melanoma (Trp-1+) and phagocytic cells (Gr1+) positive for GFP-expressing bacteria. Although already 30 minutes after bacterial treatment, there was a clear difference in the infection properties of invasive and noninvasive bacteria; this was significant at 24 hours postinfection. By contrast, the difference in the number of phagocytic cells infected by invasive and noninvasive *Salmonella* was not statistically significant.

**Anticancer therapeutic effect of *Salmonella* treatments.** We evaluated the anticancer therapeutic properties of *Salmonella*. For this purpose, we compared the kinetic of growth of tumors infected either with invasive or with noninvasive derivatives, in mice vaccinated or not against *Salmonella*. We decided to vaccinate mice before i.t. bacterial application because it is unlikely that i.t. *Salmonella* injection would induce per se a quick and potent anti-*Salmonella*-specific response in naive mice. In fact, we have shown that *Salmonella* injected s.c. are confined to the injection site, thereby limiting the induction of adaptive

---

**Figure 2.** *S. typhimurium*–infected B16F10 cells are targets of anti-Salmonella CTLs. A, presentation assay. B16F10 cells were infected *in vitro* with *S. typhimurium* SL1344 or SL1344 ΔinVA expressing either the fusion protein GST-OVA or only GST and incubated with the B3Z T cell hybridoma. IL-2 released by B3Z cells after recognition of the H-2Kb/SIINFEKL complex. Representative of three individual experiments. B, cytotoxicity assay. Killing activity of CTLs raised from mice immunized as described in Materials and Methods and restimulated *in vitro* with SIINFEKL peptide, was assessed on target cells loaded with *35Cr*. The target cells tested were B16F10 cells, B16F10 cells preincubated with OVA-expressing invasive or noninvasive SL3261AT, blast cells, and EG7-OVA expressing cells. Percentage of lysis for each target indicated range of effector/target ratio is referred to 0% and 100% corresponding respectively to spontaneous lysis and detergent-mediated lysis values. Percentage of lysis of B16F10 cells infected with OVA expressing SL3261AT was significantly higher compared with noninfected or SL3261AT ΔinVA treated B16F10 (*P* < 0.01). Points, mean of three different experiments; bars, ±SE.

**Figure 3.** Invasive *S. typhimurium* infects tumor cells *in vivo*. A, confocal analysis of tumor sections. Tumor masses were treated with DsRed expressing SL3261AT (top) or SL3261AT ΔinVA (bottom). Twelve hours later, tumor sections were stained with anti-TRP1 antibody (blue) to visualize tumor cells and with anti-H-2Kb antibody (green) to stain both tumor (arrows) and tumor-infiltrating cells (arrowheads). *S. typhimurium* is visible in red. Tumor cells are distinguishable from recruited immune cells because they express both TRP1 and low H-2Kb (blue and green). Noninvasive *S. typhimurium* are found only within tumor infiltrating cells, or attached to the outer membrane of tumor cells, whereas invasive *S. typhimurium* are found also within tumor cells. Bars, 10 μm. B, gentamicin protection assay. To avoid a massive recruitment of tumor-infiltrating cells, tumor masses were resected 30 minutes after bacteria injection. Tumors were processed and incubated in gentamicin containing medium, to kill extracellular bacteria. Cells were lysed and plated on TB agar. Recovered CFUs per 10^6 plated cells from each resected tumor are shown (individual points). Line, average number of colonies recovered from five different tumors. C, number of infected tumor cells (Trp-1^+) or infected Gr1^+ tumor-infiltrating cells was analyzed. Tumors were treated with GFP-expressing *S. typhimurium*, resected 6 or 24 hours later and processed for flow cytometry. Point, absolute number of infected tumor cells (•) or infected Gr1^+ cells (○) for each treated tumor mass. Left, *x* axis, number of infected GFP/Trp-1^+ tumor cells; right, *y* axis, number of infected GFP/Gr1^+ cells. Lines, average number of infected cells of four to six analyzed mice.
immune responses (32). Therefore, tumor-bearing mice were vaccinated at day 5 (when the tumor mass was visible) and day 9 from tumor injection with dendritic cells loaded with S. typhimurium (33, 34); as a negative control, mice were treated with unloaded dendritic cells (nonvaccinated mice). At day 7 (average tumor size = 0.01 cm²) and day 11, mice received i.t. 1 × 10⁸ CFU of invasive or invA mutant S. typhimurium or PBS as a control. Because dendritic cells are very powerful activators of the adaptive immunity, 2 days are sufficient to induce a potent anti-Salmonella–protective response, as attested by the ability of vaccinated mice to survive a lethal dose of bacteria (data not shown). We were expecting to detect significant tumor regression only when mice were both vaccinated against Salmonella and treated i.t. with invasive S. typhimurium. Indeed, this was the case and 50% of vaccinated mice survived when they received i.t. invasive S. typhimurium (Fig. 4A), whereas only 20% of vaccinated mice survived when treated with the invA strain (P < 0.01). Interestingly, of the mice that were vaccinated against S. typhimurium and that did not survive to tumor challenge those that received invasive Salmonella i.t. showed much slower kinetic of tumor growth (P < 0.05) compared with mice that received PBS i.t. (Supplemental Fig. 3). By contrast, control mice vaccinated with unloaded dendritic cells and receiving i.t. either invasive or noninvasive S. typhimurium derivatives showed a similar reduction in tumor growth (P = 0.6) leading to a maximum 20% survival compared with mice receiving PBS (Fig. 4B; P < 0.05). These data confirm our hypothesis that when tumor cells are infected by S. typhimurium they become target of anti-Salmonella–specific T cells. Indeed, when one of the two requirements is not met (i.e. when using noninvasive Salmonella that is unable to penetrate efficiently tumor cells or in the absence of an expanded repertoire of anti-Salmonella–specific T-cell response), the antitumor response is very limited. Interestingly, all of the surviving mice receiving invasive i.t. bacteria also showed signs of autoimmune reactions (vitrilgo, Fig. 4C), indicating the generation of antimelanocyte-specific responses. This is supported by the observation that 75% of mice surviving the first tumor challenge were protected against a second tumor challenge (data not shown).

Figure 4. Combination of vaccination and in situ injection of S. typhimurium drives dose-dependent protection against melanoma and induces autoimmune reactions. A, to evaluate the antitumor effect of in situ bacteria injection, C57/BL6J mice were injected s.c. in the left flank with 1.5 × 10⁶ B16F10 cells at day 0 and treated with two cycles of i.t. injection and two vaccination at the days indicated (arrows). Half of the mice (n = 24) were vaccinated with dendritic cells (DC) loaded with SL3261AT (A) and the other half (n = 24) was boosted with unloaded dendritic cells (nonvaccinated mice, B). Groups of eight mice, both vaccinated and not, were treated i.t. with PBS (○), SL3261AT (●), or SL3261AT invA (□). Tumor growth and mice survival was monitored every 2 to 3 days, measuring the x and y dimensions. Difference in tumor growth and survival curves between vaccinated mice treated with either invasive or noninvasive S. typhimurium is statistically significant starting from day 26 (*, P < 0.01). Difference in tumor growth and mice survival between vaccinated and nonvaccinated mice treated in situ with invasive S. typhimurium is statistically significant starting from day 29 (*, P < 0.05). Representative of two independent experiments. C, all of the mice surviving the treatment showed complete regression of the tumor mass and developed vitiligo in the area around the tumor injection (arrow). One mouse is representative of the whole group of mice. D, increasing the number of infected tumor cells results in better tumor protection. C57/BL6J mice were injected s.c. with B16F10 cells at day 0 and vaccinated at days 5 and 9 with dendritic cells loaded with SL3261AT. Groups of seven mice received a single i.t. injection at day 7 either of 10⁹ CFU SL3261AT, or SL3261AT invA, or of PBS. Tumor growth was monitored every 2 to 3 days. Percentage of survival was calculated and is reported beside each curve. Difference in tumor growth and mice survival in mice treated in situ with invasive or noninvasive S. typhimurium is statistically significant starting from day 22 (*, P < 0.01). A–D, day of treatment (arrows): vacc, DC vaccination; i.t., in situ bacteria or PBS treatment.
Increasing the number of infected tumor cells results in better tumor protection. To analyze the efficiency of killing of infected tumor cells, we decided to increase the number of infected cells by injecting i.t. 10 times more CFU (10^9) of *S. typhimurium*. Tumor-bearing mice were vaccinated twice with dendritic cells loaded with attenuated *S. typhimurium* and received one single i.t. injection of 10^9 CFU of either invasive or noninvasive *S. typhimurium* derivatives. One single dose of 10 times more CFU of invasive *Salmonella* lead to 66% of mice showing complete regression of tumor mass and being free from tumor (Fig. 4D). By contrast, i.t. injection of noninvasive *S. typhimurium InvA* mutant that was unable to infect tumor cells showed lower protection (28%; P < 0.01). The effect of the injection of noninvasive bacteria was higher than that of PBS and this is probably due to the recruitment of immune effector cells (see below). These results indicate that by increasing the number of infected tumor cells, one single dose of bacteria is sufficient to exert a similar or even better effect than two administrations, probably because a higher number of tumor cells are killed.

**Intratumoral bacteria injection facilitates recruitment of immune cells.** A possible explanation for the described intrinsic anticancer property of *S. typhimurium* may stand in its ability to recruit immune cells into an infected site, as recently described (32). Therefore, we tested whether *S. typhimurium* was able to induce recruitment of immune cells into the tumor site. We injected i.t. 1 × 10^9 CFU of either invasive or noninvasive *S. typhimurium*. At days 2, 4, and 6 from i.t. bacterial injection, mice were sacrificed and tumor masses were analyzed either by cytofluorometry or by immunohistochemistry to evaluate the magnitude of the tumor leukocyte infiltration. Already 2 days after *S. typhimurium* injection (regardless of their invasiveness), we detected a higher infiltration of innate immune cells, which consisted at day 4 primarily of cells of myeloid origin (80% CD11b^+), mostly granulocytes (75% Gr1hi, Fig. 5A). In contrast, we detected very little infiltrate of dendritic cells (CD11c^+, data not shown). T lymphocytes (both CD4^+ and CD8^+), and B lymphocytes (CD19^+) were recruited at later time points (6 days, Fig. 5B). Interestingly, at these time points no significant increase of natural killer (NK) cells was detected (Fig. 5B). Immunohistochemistry of *Salmonella*-treated tumor tissue confirmed the presence of a large leukocyte infiltration (Fig. 5C) with evident areas of necrosis (Fig. 5C, arrows) compared with control PBS-treated tissue. These results show that both invasive and noninvasive derivatives of *S. typhimurium* induce the recruitment of innate and adaptive immune cells.

**Bacteria injection in one lesion retards the growth of an untreated distant lesion.** We have shown that 75% of treated mice surviving a first tumor challenge were protected against a second challenge and had developed vitiligo (Fig. 4). This suggested the induction of systemic antimalanoma responses. Therefore, we explored whether in mice bearing two tumor masses, treatment of one lesion was affecting the growth of a distant one. Tumors were implanted by injecting 1 × 10^6 or 1.5 × 10^6 B16F10 cells s.c. on the left flank or on the back of mice, respectively. A lower amount of B16F10 cells was injected on the back to monitor the distant tumor growth for a longer time. When the left flank tumors reached the size of 0.25 cm^2, they were injected with 10^9 CFU of either invasive or noninvasive *S. typhimurium* derivatives and mice were vaccinated against *Salmonella*. The vaccination and the i.t. injections were repeated 4 days later. Remarkably, we found that whereas i.t. injection of invasive bacteria was again more effective than treatment with noninvasive bacteria (Fig. 6A), in both cases, systemic antitumor response was induced (Fig. 6A-B). In fact, the back tumors showed a reduced growth when the left flank tumors were treated with *Salmonella* compared with PBS-treated controls (Fig. 6A; P < 0.01), irrespective of the invasiveness of *Salmonella* (P = 0.3). This suggests that the recruitment of innate immune cells, which is similarly exerted by the two types of bacteria (Fig. 5A-B),

![Figure 5](https://cancerres.aacrjournals.org/content/65/9/3925/F5.large.jpg)

**Figure 5.** *S. typhimurium* induces leukocyte recruitment inside the tumor. Recruitment of leukocytes after i.t. injection of *S. typhimurium* was assessed by flow cytometry (A-B), or by immunohistochemistry on tumor sections (C). Innate immune cells (CD11b^+; myeloid phagocytes, F4/80^+; macrophages; Gr1^hi; granulocytes) are already recruited 2 days after injection (A), whereas lymphocytes (both CD19^+ B cells and CD4^+ and CD8^+ T cells) are recruited later, 6 days after *in situ* treatment (B). C, fucsin staining of tumor sections for CD11b^+ cells confirmed the massive recruitment of phagocytes 2 or 4 days post-injection compared with PBS-treated tumors. Arrows, areas of necrosis.
sacrificed when one of the two tumors reached an area of 2 cm². The growth of untreated distant tumors was monitored every 2 to 3 days. Mice were injected i.t. with bacteria to induce tumor-specific CD8+ T lymphocytes (compare Fig. 4A). Interestingly, we found that i.t. treatment with bacteria, regardless of their invasiveness, leads to the generation of a “infected state” that is induced during the vaccination is recruited in the inflamed tumor site where they kill infected tumor cells. This effect is visible in previous reports based on Salmonella as an anticancer therapeutic agent, in the absence of vaccination, the antitumoral effect was limited and did not really differ when using invasive or noninvasive Salmonella derivatives (20). This is an important issue because it shows that when bacteria are actively infecting tumor cells and in the presence of an expanded pool of anti-Salmonella–specific T cells, the infectious phenomenon is more relevant. In accordance with this observation, increasing the concentration of i.t. injected bacteria that leads to a greater proportion of infected tumor cells, one single bacterial injection is sufficient to promote survival of 66% of treated mice. Finally, our results show that large tumors are more sensitive to i.t. bacterial injection coupled to vaccination, leading to 100% of mice that regress the tumor and become tumor free. The reasons for this effect are still under investigation, but a possible explanation may be that injection of large tumor masses results in a better targeting of the tumor area without affecting nearby healthy tissues.

Interestingly, we found that i.t. treatment with bacteria, regardless of their invasiveness, leads to the generation of a specific immune response to the melanoma-associated antigen TRP-2. This correlates with the development of autoimmune reactions (vitiligo), reduction of tumor growth at other locations, and protection to subsequent tumor challenge. Because we have shown that i.t. injection of Salmonella facilitates the recruitment of inflammatory cells, these may play a primary role in tumor clearance and in the induction of systemic antitumor responses. In fact, recruited immune cells, including granulocytes that have been shown necessary for tumor protection (37), could participate to tumor killing through Salmonella-induced release of nitric oxide (38). In addition, killing of Salmonella-infected tumor cells by NK cells, similarly to infected macrophages (39), cannot be excluded, as it has been shown that vaccination with mature dendritic cells induces activation of NK cells (40). During the clearance of killed tumor cells, tissue debris could be generated in large quantities, be taken up by endogenous antigen-presenting cells, and be presented to naive T cells for stimulation of tumor-specific T cells (41). Remarkably, local treatment of lesions potently affects the growth of untreated distantly located established tumors but does not lead to complete tumor regression. As reports from the literature have shown that i.v. administration of S. typhimurium is
safe in humans and leads to preferential accumulation in tumor areas (16, 21, 42, 43), it would be interesting to assess whether coupling of i.t. with i.v. injections of Salmonella might potentiate its therapeutic effect.

In conclusion, our approach shows that immunotherapies aimed at subverting immune escape mechanisms (5, 44, 45) that are independent on the kinetic of expression of tumor antigens (11) and that avoid ignorance of the tumor site are extremely powerful. We provide evidence that invasive S. typhimurium can infect malignant cells both in vitro and in vivo and that infected tumor cells similarly to any other cell type of the organism can alert the immune system of being infected by intracellular pathogens. This system could be virtually used to treat any type of malignancies regardless of the patients’ histotype and of the expression of particular tumor antigens thus avoiding individual-based therapies.

Acknowledgments

Received 8/19/2004; revised 2/7/2005, accepted 2/21/2005.

Grantsupport: Italian Association for Cancer Research and Italian Ministry of Health (Ricerca finalizzata).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

# Cancer Immunotherapy Based on Killing of *Salmonella*-Infected Tumor Cells

Francesca Avogadri, Chiara Martinoli, Liljana Petrovska, et al.


<table>
<thead>
<tr>
<th>Updated version</th>
<th>Access the most recent version of this article at: <a href="http://cancerres.aacrjournals.org/content/65/9/3920">http://cancerres.aacrjournals.org/content/65/9/3920</a></th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Material</td>
<td>Access the most recent supplemental material at: <a href="http://cancerres.aacrjournals.org/content/suppl/2005/05/04/65.9.3920.DC1">http://cancerres.aacrjournals.org/content/suppl/2005/05/04/65.9.3920.DC1</a></td>
</tr>
<tr>
<td>Cited articles</td>
<td>This article cites 45 articles, 18 of which you can access for free at: <a href="http://cancerres.aacrjournals.org/content/65/9/3920.full.html#ref-list-1">http://cancerres.aacrjournals.org/content/65/9/3920.full.html#ref-list-1</a></td>
</tr>
<tr>
<td>Citing articles</td>
<td>This article has been cited by 13 HighWire-hosted articles. Access the articles at: /content/65/9/3920.full.html#related-urls</td>
</tr>
</tbody>
</table>

E-mail alerts: Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions: To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions: To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.