Cancer Immunotherapy Based on Killing of Salmonella-Infected Tumor Cells

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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 in vivo infection of tumors and killing of infected tumor cells. Attenuated but still invasive, Salmonella typhimurium can be successfully exploited to invade melanoma cells that can present antigenic determinants of bacterial origin and become targets for anti-Salmonella-specific T cells. However, to fully appreciate the anticancer therapeutic properties of S. typhimurium, tumor-bearing mice need to be vaccinated against S. typhimurium before intratumoral Salmonella injection. Tumor infection when coupled to anti-Salmonella vaccination leads to 50% to 100% tumor-free mice with a better outcome on larger tumors. Invasive 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 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 Bifidobacterium, Clostridium, and Salmonella spp. has been analyzed (18). Salmonella enterica serovar 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 anticancer property (18–22). Moreover, invasive facultative intracellular bacteria such as 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 Salmonella, the mechanisms responsible for its efficacy were unknown.

In this study, we explored the mechanism(s) underlying the Salmonella-mediated antitumor effect in a mouse melanoma model. We chose the B16F10 melanoma model for its poor immunogenicity and because it displays strong immunoescape 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 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 antimelanoma effect of S. typhimurium: (i) S. typhimurium–infected malignant cells are recognized and killed by anti-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 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 Salmonella, it is necessary to amplify the pool of immune effector cells specific for S. typhimurium through vaccination. This allows recognition and killing of Salmonella-infected tumor cells.

Materials and Methods

Mice, cells, and bacterial strains. Five-week-old female C57/Bl6j mice were purchased from Harlan (Udine, Italy). The murine melanoma cell line B16F10 (a kind gift from Dr. P. Della Donna, Experimental
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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 BZ-T-cell hybridoma 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-3TsT 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 invA gene (SL1344 ΔinvA) and unable to form the TTSS, and two araO metabolically defective strains (SL3261AT ΔaroA j, SL3261AT ΔaroA j, SL3261AT ΔaroA j, SL3261AT ΔaroA j) and unable to form the TTSS, and two araO metabolically defective strains (SL3261AT ΔaroA j, SL3261AT ΔaroA j, SL3261AT ΔaroA j, SL3261AT ΔaroA j). Five recombinant variants of each strain were generated, expressing constitutively GFP or red fluorescence protein (TRP)-1 (kind gift from Dr. V. Hearing, Laboratory of Cell Biology, National Institutes of Health, Bethesda, MD) and with an antirabbit-CY5 antibody (PharMingen). Gentamicin protection assays were done on tumors resected 30 minutes after injection with SL3261AT or SL3261AT InvA. After resection, tumors were dissected, smashed through a cell strainer (Falcon, Como, Italy), and depleted of RBC. Cells were incubated for 2 hours at 37°C with 50 μg/mL gentamicin, lysed with sodium deoxycholate and plated as above. For flow cytometry analysis, tumor masses were injected with GFP-expressing SL3261AT or SL3261AT InvA. At the indicated time points, tumor cells were processed as above and stained either with rabbit-anti-ovalbumin and anti-immunoglobulin antibodies (Sigma), or with Gr1-phycoerythrin (PharMingen).

Analysis of leukocyte infiltration in the tumor mass. Tumors were injected with 106 SL3261AT, SL3261AT InvA 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 cytotoxicity analysis. Cells were stained with one of the following antibodies: anti-CD4-PE, anti-CD8-FITC, anti-CD25-PE, anti-CD120a-PE, anti-CD80-PE, anti-CD86-PE, anti-CD11b-PE, anti-Gr1-PE, anti-CD19-PE, anti-CD45-PE, anti-F4/80-PE, anti-IgG1-PE, anti-IgG2b-PE, anti-IgG2a-PE, anti-IgG3-PE, and anti-IgM-PE (all from PharMingen), 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 biotinylated antibodies and alkaline-phosphatase–conjugated streptavidin (all from PharMingen). To distinguish between melanin and histochemical stainings, cells were visualized by fluorescently labeled secondary antibodies (Jackson Immunoresearch). Presentation assay. B16F10 (106 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 × 104 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 8 × 106 splenocytes per well were plated in 24-well plates in complete RPMI supplemented with 10% FCS, 20 UI/mL rIL-2 (Proleukin, 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. Spleens of treated mice were collected 17 days after the first i.t. treatment and sectioned, smashed through cell strainer, and depleted of RBC. For cytotoxicity analysis, 106 cells were stained with anti-CD8-FITC antibody (PharMingen) and with TRP-2-K6 tetramer–PE-conjugated. As a negative control, a tetramer specific for β-galactosidase (β-gal)-2-K6 was also done for each spleen sample.

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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 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 number of dead and apoptotic cells (propidium iodide and Annexin V–positive cells) was evaluated by flow cytometry. Toxic effect exerted by the indicated S. typhimurium strains on B16F10 cells was evaluated 12 or 24 hours after infection of murine B16F10 and human IGR39 melanoma cells was analyzed by fluorescence microscopy. Cells were scored as dead or live (red + green), whereas intracellular bacteria are visible in red. Cells nuclei are stained in blue (4′,6-diamidino-2-phenylindole). Bars, 12 μm (left), 10 μm (middle), 15 μm (right). B, gentamicin protection assay. To evaluate the number of internalized bacteria, B16F10 cells were incubated with invasive (SL1344 and SL3261AT) or noninvasive (SL1344 InvA and SL3261AT InvA) S. typhimurium, lysed, and plated on TB agar. The number of colonies recovered for 10^3 B16F10 cells plated is reported. Representative of three independent experiments. C, toxicity assay. Percentage of dead and apoptotic cells (propidium iodide and Annexin V–positive cells) was evaluated by flow cytometry. Toxic effect exerted by the indicated S. typhimurium strains on B16F10 cells was evaluated 12 or 24 hours after in vitro coculture. As a positive control, cells were treated with puromycin or UV light. Columns, mean of three independent experiments.

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 OVA257-264 peptides on their cell surface in association with the MHC I Kb molecule. As a read-out system, we used the B3Z hybridoma that releases IL-2 after recognizing specifically the OVA-Kb3 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 released the bacteria intrasurally. 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 antimalanoma-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 Salmonella typhimurium derivatives infect tumor cells in vitro and are not toxic. A, in vitro infection of murine B16F10 and human IGR39 melanoma cells was analyzed by fluorescence microscopy. Cells were incubated with wild-type SL1344 or SL3261 InvA expressing S. typhimurium. Without permeabilizing the cells, only extracellular bacteria are accessible to FITC-conjugated anti-Salmonella antibody and stain yellow (red + green), whereas intracellular bacteria are visible in red. Cells nuclei are stained in blue (4′,6-diamidino-2-phenylindole). Bars, 12 μm (left), 10 μm (middle), 15 μm (right). B, gentamicin protection assay. To evaluate the number of internalized bacteria, B16F10 cells were incubated with invasive (SL1344 and SL3261AT) or noninvasive (SL1344 InvA and SL3261AT InvA) S. typhimurium, lysed, and plated on TB agar. The number of colonies recovered for 10^3 B16F10 cells plated is reported. Representative of three independent experiments. C, toxicity assay. Percentage of dead and apoptotic cells (propidium iodide and Annexin V–positive cells) was evaluated by flow cytometry. Toxic effect exerted by the indicated S. typhimurium strains on B16F10 cells was evaluated 12 or 24 hours after in vitro coculture. As a positive control, cells were treated with puromycin or UV light. Columns, mean of three independent experiments.
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The target cells loaded with 51Cr. The target cells tested were B16F10 cells, B16F10 representative of three individual experiments. Methods and restimulated activity of CTLs raised from mice immunized as described in Materials and Methods released by B32 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 2Cr. 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 versus the 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-treated B16F10 (P < 0.01). Points, mean of three different experiments; bars, ±SE.

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 infection 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 immune cells, whereas invasive derivatives were internalized only by the recruited phagocytes (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 cytotoxicity assay (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 post-infection. By contrast, the difference in the number of phagocytic cells infected by invasive and noninvasive Salmonella was not statistically significant.

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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 (vitiligo, Fig. 4C), indicating the generation of antimalanocyte-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).
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 (10⁷) 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 10⁷ 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 we 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. 1×10⁷ 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 cytfluorometry 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 1×10⁶ or 1.5×10⁶ 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 cm³, they were injected with 10⁷ 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 Salmonellae compared with PBS-treated controls (Fig. 6A; P<0.01), irrespective of the invasiveness of Salmonellae (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),
facilitates cross priming of endogenous tumor antigens, in line with a recent report showing that cross-presentation is occurring only when tumor antigens are presented outside of the tumor context (35). Indeed, when we evaluated the presence of tumor-specific T cells in the spleen of treated mice, we found a similar amount of CD8 T cells positive for the staining with TRP-2.Kb tetramer in the two situations (Fig. 6B). Interestingly, we found that treatment of large tumor masses with i.t. bacteria is far more efficient than treatment of small masses, leading to 100% tumor-free mice (compare Fig. 4A and D with Fig. 6A). Altogether, our data indicate that the i.t. injection of bacteria induces the development of tumor-specific CD8 T cells that are likely involved in the retardation of tumor growth at distant locations and in the induction of protective immunity against subsequent tumor challenges.

Discussion

We have described a novel approach of melanoma immunotherapy based on the property of intracellular bacteria, such as S. typhimurium, to infect host cells (23, 36). We have shown that S. typhimurium uses the TTSS machinery to infect melanoma cells; in fact, mutant strains which are defective in the TTSS, lose the ability to penetrate tumor cells, both in vitro and in vivo. Salmonella-infected tumor cells are not directly killed by S. typhimurium; rather, they present determinants of bacterial origin and become targets for anti-Salmonella-specific T cells; a feature that has not been previously described. We have shown that the property of tumor cells to alert the immune system of their “infected state” can be successfully exploited to induce complete regression of tumors, even very aggressive tumors like the B16F10 melanoma that is capable to subvert the immune response (24), and to promote tumor-specific immunity. This is effective both on distant untreated tumors and on subsequent tumor challenges.

The antitumor effect depends primarily on three factors: (a) the availability of anti-Salmonella-specific cytotoxic T cells, (b) the number of CFUs injected i.t., and (c) the size of tumors. In fact, the percentage of mice surviving and becoming tumor free increases from 20% to 50% when mice are vaccinated against Salmonella and receive invasive but not noninvasive Salmonellae. Therefore, we can envisage that anti-Salmonella-specific T cells that are induced during the vaccination are recruited in the inflamed tumor site where they kill infected tumor cells. This probably explains why in previous reports based on Salmonella as 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.

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