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Research Article **Vaccines**

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In vivo antigen delivery by a *Salmonella typhimurium* type III secretion system for therapeutic cancer vaccines

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Bacterial vectors may offer many advantages over other antigen delivery systems for cancer vaccines. We engineered a *Salmonella typhimurium* vaccine strain to deliver the NY-ESO-1 tumor antigen (*S. typhimurium*–NY-ESO-1) through a type III protein secretion system. The *S. typhimurium*–NY-ESO-1 construct elicited NY-ESO-1–specific $CD8^+$ and $CD4^+$ T cells from peripheral blood lymphocytes of cancer patients in vitro. Oral administration of *S. typhimurium*–NY-ESO-1 to mice resulted in the regression of established NY-ESO-1–expressing tumors. Intratumoral inoculation of *S. typhimurium*–NY-ESO-1 to NY-ESO-1–negative tumors resulted in delivery of antigen in vivo and led to tumor regression in the presence of preexisting NY-ESO-1–specific $CD8^+$ T cells. Specific T cell responses against at least 2 unrelated tumor antigens not contained in the vaccine were observed, demonstrating epitope spreading. We propose that antigen delivery through the *S. typhimurium* type III secretion system is a promising novel strategy for cancer vaccine development.

Introduction

With the molecular identification of tumor antigens recognized by the human immune system, a number of cancer vaccine strategies targeting these antigens have been attempted (1–3). Although many of these strategies have resulted in the development of measurable immune responses, only a minority of treated patients has experienced clinical benefit such as tumor remission. Because of the weak clinical effectiveness of current cancer vaccine strategies, new immunogenic antigens and/or more effective adjuvant formulations, vectors, or vaccination methods are undoubtedly needed (4–6). Another obstacle for antigen-specific cancer vaccine strategies is that the identification of antigens expressed by the patient's tumor is required prior to determining the eligibility of the potential vaccine recipient. In fact, promising tumor-specific antigens discovered so far have expression frequencies limited to a subset of cancers, thus diminishing their potential usefulness as a single vaccine target as well as preventing many patients from meeting adequate eligibility criteria for potential trials.

NY-ESO-1 is a germ cell protein that is often expressed by tumor cells but not normal somatic cells (7). It was discovered by serological identification of antigens by recombinant expression cloning using the serum of an esophageal cancer patient (7, 8). The frequent finding of humoral and cellular immune responses against this antigen in cancer patients with NY-ESO-1–expressing tumors makes it one of the most immunogenic human tumor antigens known (9). However, the frequency of NY-ESO-1 expression in melanoma, lung, breast, ovarian, and bladder cancers is only 20–40%, and its expression pattern is often heterogeneous (7, 10).

A number of recent studies have highlighted the importance of stimulation of the innate immune system, in particular TLRs, to generate and maintain efficient acquired immune responses and presumably to block the suppressive activity of Tregs (5, 6, 11–15). Since pathogenic bacteria are endowed with the ability to stimulate the innate immune system, avirulent recombinant bacterial vectors have recently attracted attention for their potential use in cancer vaccine development (5, 6, 14, 16, 17). One of the most promising candidates among bacterial vectors is *Salmonella enterica* serovar Typhimurium (*S. typhimurium*). The simplicity of its administration, the ease of its genetic manipulation, and the availability of several virulence-attenuating mutations have made *S. typhimurium* a very versatile antigen delivery platform (18, 19). Its intracellular location within phagosomes, however, limits its ability to generate an MHC class I–restricted immune response. The recent adaptation of its type III secretion system to deliver heterologous antigens into the cytosol of infected cells has effectively overcome this limitation, and this approach has proved efficient for priming antigen-specific $CD8^+$ T cells against a variety of pathogens including influenza, lymphocytic choriomeningitis, and simian immunodeficiency viruses (19–21). In the present study, we constructed an avirulent recombinant strain of *S. typhimurium* endowed with the capacity to deliver the NY-ESO-1 tumor antigen (*S. typhimurium*–NY-ESO-1) through its type III secretion system into the cytosol of professional and nonprofessional APCs. We found that *S. typhimurium*–NY-ESO-1 efficiently elicited NY-ESO-1–specific $CD8^+$ and $CD4^+$ T cell responses in vitro in human PBMCs derived from cancer patients with preexisting immunity. Furthermore, oral administration of *S. typhimurium*–NY-ESO-1 to mice resulted in the regression of preestablished

Nonstandard abbreviations used: EBV-B, EBV-transformed B (cell); ELISPOT, Enzyme-linked immunospot; mERK2, mutated ERK2.

Conflict of interest: The authors have declared that no conflict of interest exists.

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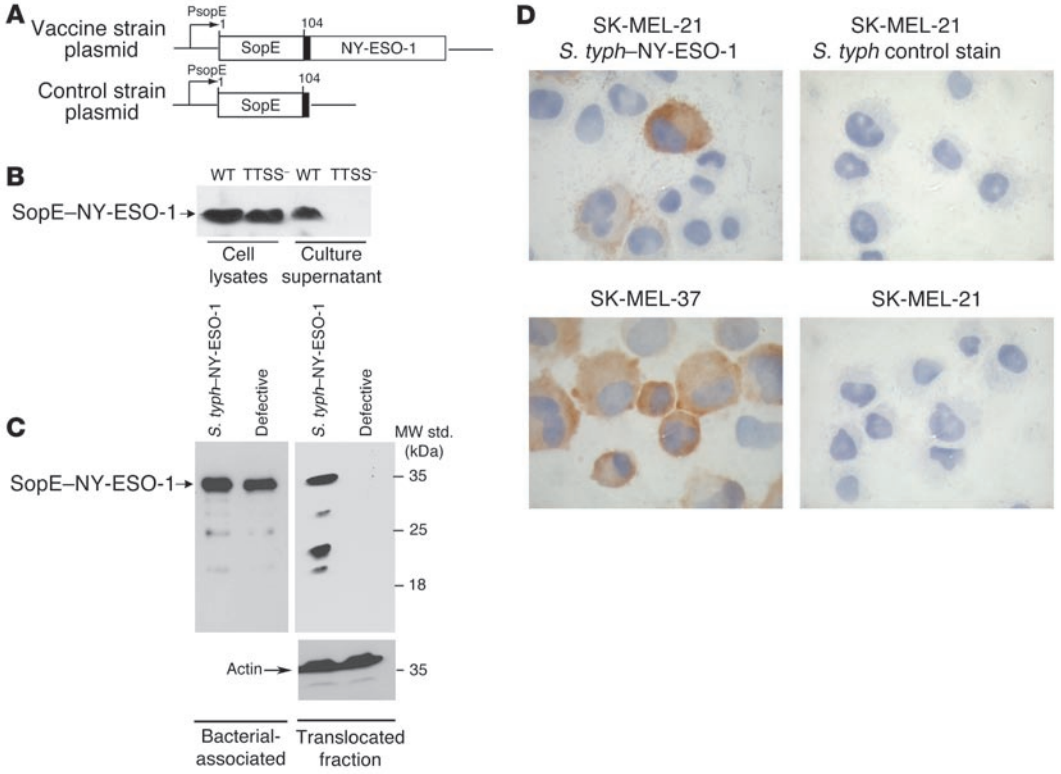

Figure 1

S. typhimurium type III secretion system delivers recombinant NY-ESO-1 to target cells. (**A**) Diagram of relevant plasmids used in these studies. PsopE, SopE promoter. (**B**) A plasmid expressing NY-ESO-1 fused to the type III secretion and translocation signals of SopE (SopE–NY-ESO-1) was introduced into a Δ*phoP* Δ*phoQ S. typhimurium* (WT) and an isogenic type III secretion-defective *invA* mutant (TTSS⁻). Whole cell lysates and cultured supernatants of these strains were then examined for the presence of the chimeric SopE–NY-ESO-1 protein by Western blotting as described in Methods. (**C**) Mouse CMS5a tumor cells were infected with *S. typhimurium*–NY-ESO-1 (*S. typh*–NY-ESO-1) or a translocation-defective strain, and the presence of SopE–NY-ESO-1 in the different fractions was examined as described in Methods. Blots containing the translocated protein fraction were reprobed for the host cell protein actin to verify equal loading of the samples. MW std., MW standard. (**D**) SK-MEL-21, a human melanoma cell line that does not express NY-ESO-1, was infected with *S. typhimurium*–NY-ESO-1 or the *S. typhimurium* control strain and examined by immunocytochemistry with anti–NY-ESO-1 mAb ES121. SK-MEL-37 served as a positive control of a human melanoma cell line with natural NY-ESO-1 expression.

NY-ESO-1–expressing tumors. Remarkably, administration of *S. typhimurium*–NY-ESO-1 at the local site of NY-ESO-1–negative tumors in the presence of preexisting NY-ESO-1–specific $CD8^+$ T cells also led to tumor regression. Animals that showed tumor regression developed $CD8^+$ T cell responses not only to NY-ESO-1, but also to tumor antigens not contained in the vaccine.

Results

Heterologous secretion and translocation into host cells of NY-ESO-1 tumor antigen by the S. typhimurium type III secretion system. The *S. typhimurium* type III secretion system encoded within the pathogenicity island 1 (SPI-1) has been previously used for the delivery of heterologous antigens (19). We constructed a *S. typhimurium* Δ*phoP* Δ*phoQ* avirulent strain carrying a plasmid that expresses a chimeric protein composed of the first 104 amino acids of the type III secreted protein SopE fused to reporter epitopes (see Methods) and the entire amino acid sequence of the NY-ESO-1 tumor antigen (*S. typhimurium*–NY-ESO-1). As a control, we utilized the same *S. typhimurium* Δ*phoP* Δ*phoQ* avirulent strain carrying an equivalent plasmid that expresses only the first 104 amino acids of the type III secreted protein SopE and reporter epitopes (see Figure 1A for a diagram of these plasmid constructs). The SopE–NY-ESO-1 chimeric protein was efficiently secreted into culture supernatants of the *S. typhimurium* vaccine strain, but not into supernatants of an SPI-1–encoded type III secretion–deficient mutant strain (Figure 1B). Furthermore, the SopE–NY-ESO-1 protein was delivered into the cytosol of the CMS5a mouse tumor cell line with an efficiency equivalent to that observed for other type III secreted proteins such as SopE itself (Figure 1C) (22). We next tested whether *S. typhimurium*–NY-ESO-1 could deliver the NY-ESO-1 chimeric protein to the cytosol of infected human melanoma cell line SK-MEL-21. As shown in Figure 1D, SK-MEL-21 cells infected with *S. typhimurium*–NY-ESO-1 — but not those infected with the *S. typhimurium* control strain, an identical strain carrying the control plasmid vector — showed substantial specific staining when examined by immunocytochemistry using an NY-ESO-1 mAb. Similar transfer of protein was observed after *S. typhimurium*–NY-ESO-1 infection of other cell types, including an epithelial cancer cell line (SK-LC-14; data not shown). These results indicate that *S. typhimurium*–NY-ESO-1 can efficiently deliver NY-ESO-1 through the *S. typhimurium* type III secretion system.

S. typhimurium type III secretion system delivers NY-ESO-1 to the MHC class I and class II antigen–presenting pathway. We examined

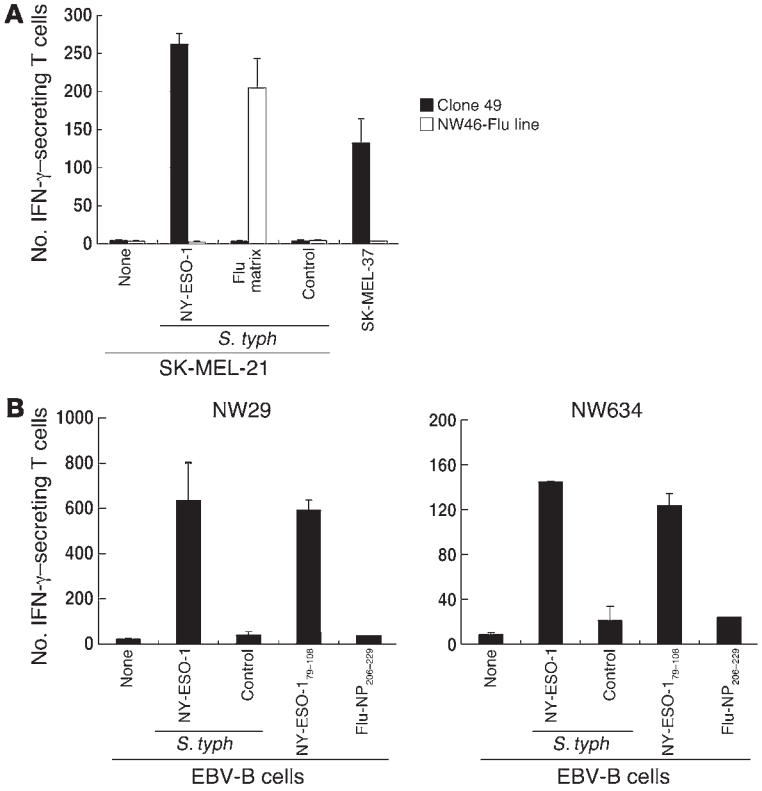

Figure 2
Antigens delivered by the *S. typhimurium* type III secretion system are efficiently presented to CD8+ T cells. (**A**) An HLA-A*0201–restricted CD8+ T cell clone specific for NY-ESO-$1_{157–165}$ (clone 49) and an HLA-A*0201–restricted CD8+ T cell line specific for Influenza matrix$_{58–66}$ (NW46-Flu) were cultured with SK-MEL-21 cells infected with *S. typhimurium*–NY-ESO-1, *S. typhimurium*–Flu matrix, or the *S. typhimurium* control strain, and specific IFN-γ secretion was assessed by ELISPOT assay. (**B**) CD8+ T cells derived from PBMCs of NY-ESO-1–expressing melanoma patients NW29 and NW634 were presensitized by $CD4^-CD8^-$ PBMCs pulsed with NY-ESO-$1_{79–108}$ peptide as described in Methods. The induction of CD8+ T cells was analyzed by ELISPOT assay for recognition of autologous EBV-B cells pulsed with peptides or infected with *S. typhimurium*. These experiments were performed independently 3 times with similar results. Data are mean ± SD.

whether NY-ESO-1 delivered by the *S. typhimurium* type III secretion system entered the MHC class I pathway and was presented to CD8+ T cells. SK-MEL-21 cells were infected with *S. typhimurium*–NY-ESO-1, an *S. typhimurium* endowed with the capacity to deliver a peptide derived from the influenza virus matrix protein (*S. typhimurium*–Flu matrix), or the *S. typhimurium* control strain (an identical strain carrying the control plasmid vector). The ability of the infected cells to present antigen to antigen-specific CD8+ T cells was then evaluated by enzyme-linked immunospot (ELISPOT) assay. The HLA-A*0201–restricted NY-ESO-$1_{157–165}$–specific CD8+ T cell clone, clone 49, was stimulated by *S. typhimurium*–NY-ESO-1–infected SK-MEL-21 cells (Figure 2A). In contrast, this T cell clone was not stimulated by SK-MEL-21 cells infected with *S. typhimurium*–Flu matrix, even though these infected cells were able to stimulate NW46-Flu, an HLA-A*0201–restricted CD8+ T cell line specific for Influenza matrix$_{58–66}$ (Figure 2A).

We also examined the antigen delivery capacity of the *S. typhimurium* type III secretion system using primary T cells from cancer patients. CD8+ T cells were purified from PBMCs of NY-ESO-1–expressing melanoma patients (NW29 and NW634) who had preexisting immunity against NY-ESO-1. Cells were presensitized by autologous $CD4^-CD8^-$ PBMCs pulsed with NY-ESO-$1_{79–108}$ peptide as described in Methods, and antigen-specific IFN-γ secretion of stimulated CD8+ T cells was analyzed by ELISPOT assay. The presensitized primary CD8+ T cells efficiently responded to *S. typhimurium*–NY-ESO-1–infected autologous EBV-transformed B cells (EBV-B cells) in a manner similar to cells pulsed with cognate peptide (Figure 2B). In contrast, autologous EBV-B cells infected with the *S. typhimurium* control strain or pulsed with a noncognate peptide did not show any measurable response.

We next asked whether the *S. typhimurium* type III secretion system was able to induce antigen-specific CD8+ T cells from PBMCs of cancer patients. CD8+ T cells derived from PBMCs obtained from patients NW29 and NW634 were presensitized by $CD4^-CD8^-$ PBMCs infected with *S. typhimurium*–NY-ESO-1 or the control strain, and antigen-specific IFN-γ secretion of CD8+ T cells was analyzed by ELISPOT assay or tetramer staining. Presensitization with $CD4^-CD8^-$ PBMCs infected with *S. typhimurium*–NY-ESO-1, but not with the control strain, induced NY-ESO-1–specific CD8+ T cells (Figure 3, A and B).

We also examined whether antigens delivered by the *S. typhimurium* type III secretion system could be presented to antigen-specific CD4+ T cells of cancer patients. NY-ESO-$1_{79–108}$ peptide–specific CD4+ T cells responded equivalently to autologous activated T cell APCs pulsed with cognate peptide and infected with *S. typhimurium*–NY-ESO-1 (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI28045DS1). Equivalent numbers of NY-ESO-$1_{79–108}$ peptide–specific CD4+ cells were obtained when CD4+ T cells from NW29 and NW634 were pre-sensitized by $CD4^-CD8^-$ PBMCs infected with *S. typhimurium*–NY-ESO-1 and pulsed with NY-ESO-1 peptide (Supplemental Figure 1, A and B). Taken together, these results indicate that antigens delivered by the *S. typhimurium* type III secretion system can be efficiently presented to CD8+ and CD4+ T cells.

Oral administration of S. typhimurium–NY-ESO-1 causes the regression of NY-ESO-1–expressing tumors in mice. We investigated the potential of *S. typhimurium*–NY-ESO-1 as a therapeutic cancer vaccine by examining its ability to cause the regression of NY-ESO-1–expressing tumors in a mouse model. BALB/c mice were inoculated with 2×10^6 CMS5a–NY-ESO-1 cells or 1×10^6 CMS5a parental cells. Seven days after tumor inoculation, animals received an oral administration of 1–2 × 10^8 CFU of *S. typhimurium*–NY-ESO-1 or of the control strain. To monitor the progression of tumor growth, the size of the tumors was measured 3 times per week. Administration of *S. typhimurium*–NY-ESO-1 or the control strain did not affect the growth of CMS5a tumors compared with untreated controls (Figure 4A). In contrast, administration of *S. typhimurium*–NY-ESO-1 resulted in the regression of CMS5a–NY-ESO-1 tumors (Figure 4A). The effect was dependent on the delivery of the NY-ESO-1 antigen, as mice

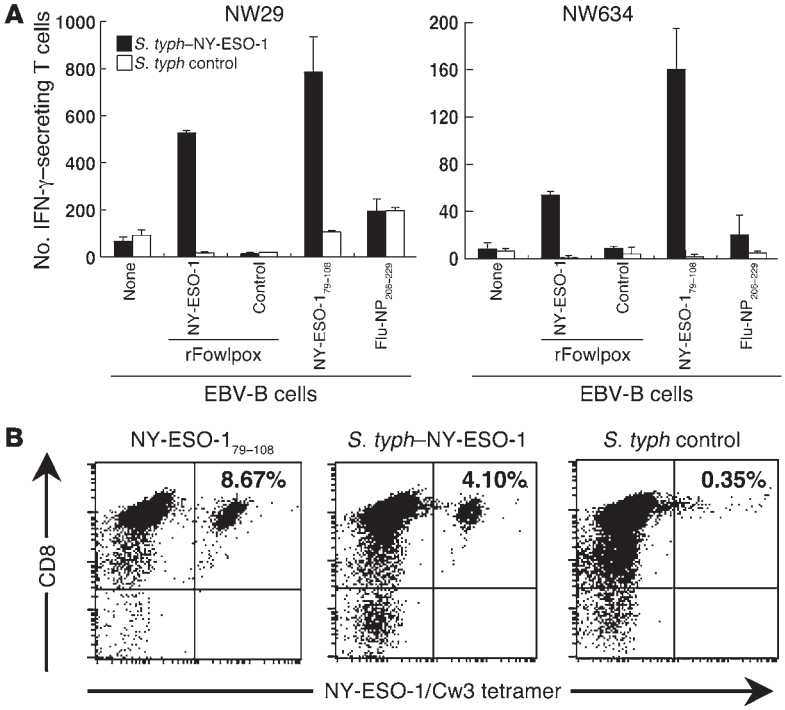

Figure 3
S. typhimurium type III secretion system induces antigen-specific primary CD8+ T cells from PBMCs. (**A**) CD8+ T cells derived from PBMCs of NW29 and NW634 were presensitized by CD4–CD8– PBMCs infected with *S. typhimurium*–NY-ESO-1 or the control strain as described in Methods, and induction of specific CD8+ T cells was analyzed by ELISPOT assay for recognition of autologous EBV-B cells pulsed with peptides or infected with recombinant Fowlpox (rFowlpox) virus. Data are mean ± SD. (**B**) NY-ESO-1–specific CD8+ T cells induced from NW29 were stained with NY-ESO-$1_{92–100}$/HLA-Cw*0304 tetramer–PE and anti-CD8–Tricolor and analyzed by flow cytometry. Experiments were performed independently at least twice with similar results. Percentages indicate frequency of tetramer-stained cells within CD8+ cells.

that received the *S. typhimurium* control strain did not show any measurable tumor regression (Figure 4A).

To gain insight into the mechanisms by which *S. typhimurium*–NY-ESO-1 induced tumor regression, we investigated the effect on tumor growth of the depletion of CD8+ or CD4+ T cells from animals that were inoculated with this strain. Injection of anti-CD8 mAb effectively blocked the therapeutic effect of *S. typhimurium*–NY-ESO-1 (Figure 4A). In contrast, injection of anti-CD4 mAb or an irrelevant control Ab did not affect the ability of *S. typhimurium*–NY-ESO-1 to induce tumor regression. Consistent with the involvement of CD8+ T cells in the therapeutic effect of *S. typhimurium*–NY-ESO-1, mice that were inoculated with this strain developed a large number of NY-ESO-1–specific CD8+ T cells. In contrast, mice that received the *S. typhimurium* control strain did not have any detectable levels of NY-ESO-1–specific CD8+ T cells (Figure 4B).

It has been previously shown that certain immunization strategies result in the development of an immune response against tumor antigens that are not contained in the vaccine but are present in tumor cells, a phenomenon known as epitope spreading (23, 24). Therefore, we investigated whether administration of *S. typhimurium*–NY-ESO-1 resulted in the generation of an immune response against antigens not contained in the vaccine but present in tumor cells. Mice harboring CMS5a–NY-ESO-1 tumors that received *S. typhimurium*–NY-ESO-1 developed antigen-specific CD8+ T cells against mutated ERK2 (mERK2) (25), an antigen present in CMS5a tumor cells (Figure 4B). In contrast, mice harboring CMS5a–NY-ESO-1 that were inoculated with the *S. typhimurium* control strain and mice with CMS5a parental tumors that received *S. typhimurium*–NY-ESO-1 or the control strain did not develop any response against mERK2 (Figure 4B). These results indicate that administration of *S. typhimurium*–NY-ESO-1 can induce immune responses against antigens expressed by the target tumor cells but absent from the vaccine and that these immune responses result in tumor regression.

S. typhimurium delivers antigen to target cells when administered intratumorally. The efficient in vitro antigen delivery by *S. typhimurium*–NY-ESO-1 prompted us to examine the ability of this vaccine strain to deliver antigen into tumor cells in vivo. *S. typhimurium*–NY-ESO-1 and the control strain were directly inoculated into palpable CMS5a tumors, which do not express NY-ESO-1. Inoculated tumors were then removed surgically and analyzed by immunohistochemistry for the presence of NY-ESO-1. NY-ESO-1 was detected in tumor cells inoculated with *S. typhimurium*–NY-ESO-1, but not in tumor cells inoculated with the *S. typhimurium* control strain. NY-ESO-1 was largely detected in areas surrounding necrotic foci (Figure 5). However, nonspecific staining was also observed in necrotic foci, which were a result of the inoculation (Figure 5). Although NY-ESO-1 was mainly detected in tumor cells, surrounding stroma containing fibroblasts was also positive for NY-ESO-1 (Figure 5). These results indicate that the *S. typhimurium* type III secretion system delivers antigen to the tumor site when locally administrated.

Local tumor antigen delivery by the S. typhimurium type III secretion system leads to epitope spreading and tumor regression. Given the efficient antigen delivery by *S. typhimurium*–NY-ESO-1 into tumor cells in vivo, we asked whether the *S. typhimurium* type III secretion system could be used to transfer NY-ESO-1 into tumor cells that do not express that antigen and render them susceptible to preexisting NY-ESO-1–specific CD8+ T cells. We therefore immunized BALB/c mice by gene gun with plasmids encoding NY-ESO-1 or with control vector plasmids twice in 2-week intervals. This immunization strategy resulted in the induction of NY-ESO-1–specific CD8+ T cells in mice immunized with NY-ESO-1 but not in mice immunized with control plasmids (Figure 6B). Immunized mice were then inoculated with 2×10^6 cells of the CMS5a-HE tumor cell line, which expresses tumor-specific antigens mERK2 and *c-erb*B-2/HER2/*neu* but does not express NY-ESO-1. Seven days after tumor inoculation, $0.5–1 \times 10^6$ CFU of *S. typhimurium*–NY-ESO-1 or of the control strain were injected directly into the tumor site, and tumor growth was examined 3 times per week. Administration of *S. typhimurium*–NY-ESO-1 not only prevented tumor growth in NY-ESO-1–preimmunized animals, but it also caused the regression of the incipient tumors (Figure 6A). In contrast, administration of the *S. typhimurium* control strain in NY-ESO-1–preimmunized mice and administration of *S. typhimurium*–NY-ESO-1 in control plasmid–preimmunized animals caused a slight

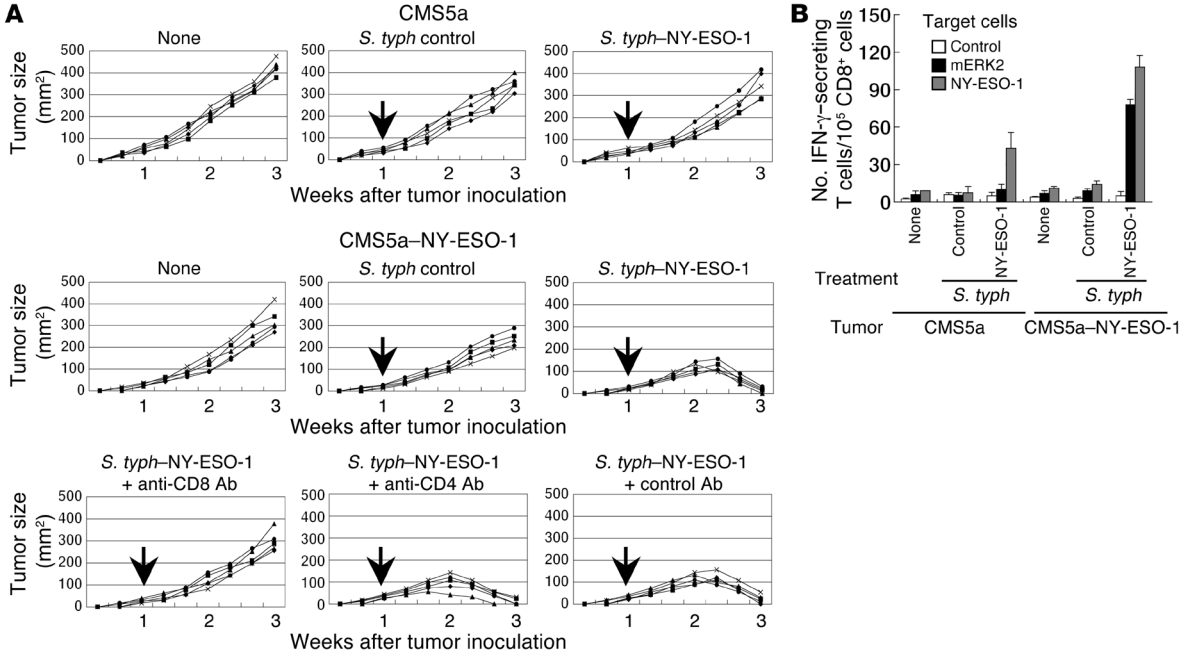

Figure 4
Oral administration of *S. typhimurium*–NY-ESO-1 causes tumor regression in mice bearing NY-ESO-1–positive tumors. (**A**) BALB/c mice were inoculated with 2×10^6 CMS5a–NY-ESO-1 cells or 1×10^6 parental CMS5a cells, and tumor growth was analyzed 3 times per week. Inoculation with $1–2 \times 10^8$ CFU of *S. typhimurium* by gavage needle was performed 7 days later. Some groups of mice were also injected intravenously with anti-CD4 or anti-CD8 mAb or control Ab in the form of 25-μl ascites every 5 days. Arrows indicate time points of *S. typhimurium* oral administration. Each line represents the tumor growth of an individual mouse. Tumor size was calculated as longitudinal diameter (mm) × horizontal diameter (mm). (**B**) $CD8^+$ T cells were purified from spleens of mice bearing CMS5a or CMS5a–NY-ESO-1 without or with administration of *S. typhimurium*–NY-ESO-1 or the control strain and analyzed for the number of specific IFN-γ–producing cells by ELISPOT assay. Data are mean ± SD. Experiments were performed independently at least twice with similar results.

initial reduction in the rate of tumor growth, but tumors finally went on to reach almost the same size as in untreated control animals (Figure 6A). Intratumoral administration of *S. typhimurium*–NY-ESO-1 into NY-ESO-1–preimmunized mice resulted in a marked increase in the levels of NY-ESO-1–specific $CD8^+$ T cells (Figure 6B). More importantly, these animals also developed $CD8^+$ T cell responses against mERK2 and *c-erb*B-2/HER2/*neu* antigens, which are expressed by the tumor cells but not present in the immunization constructs (Figure 6B). These effects were not observed in animals that received the *S. typhimurium* control strain or that had been preimmunized with control plasmids (Figure 6B). Again, epitope spreading was accompanied with tumor regression. Taken together, these results indicate that delivery of a heterologous antigen into tumor cells by the *S. typhimurium* type III secretion system renders these cells targets for preexisting $CD8^+$ T cells directed to the heterologous antigen, resulting in epitope spreading and tumor eradication.

Discussion

We have engineered a recombinant *S. typhimurium* strain that uses a type III protein secretion system to inject into host cells the NY-ESO-1 protein that is produced in the bacteria. As delivery of antigen through the type III secretion system was very specific, the possibility of nonspecific "piggybacking" of antigen was ruled out (Figure 1). We examined the capacity of this strain to elicit in vitro NY-ESO-1–specific $CD8^+$ and $CD4^+$ T cell responses in human PBMCs, to eradicate NY-ESO-1–expressing tumors in mice, and to deliver the antigen to tumor cells in vivo. We showed that *S. typhimurium*–NY-ESO-1 effectively delivered NY-ESO-1 to human melanoma cells in vitro through its type III secretion system, and these cells were able to present this antigen to T cell clones and lines via the MHC class I pathway (Figure 1D and Figure 2A). Antigen presentation by *S. typhimurium*–NY-ESO-1–infected cells was more efficient than presentation by tumor cells naturally expressing NY-ESO-1 (Figure 2A), even though the number of NY-ESO-1–expressing cells was lower in *S. typhimurium*–NY-ESO-1–infected tumor cells than in tumor cells naturally expressing NY-ESO-1. This suggests very effective processing of antigens when delivered by the type III secretion system. T cell recognition of *S. typhimurium*–infected target cells was equivalent to that of peptide-pulsed cells (Figure 2B). *S. typhimurium*–NY-ESO-1–infected $CD4^-CD8^-$ PBMCs from patients that had NY-ESO-1–expressing tumors and preexisting NY-ESO-1 immunity efficiently elicited NY-ESO-1–specific $CD8^+$ and $CD4^+$ T cells. The capacity of *S. typhimurium*–NY-ESO-1 to elicit NY-ESO-1–specific $CD8^+$ and $CD4^+$ T cells was equivalent to that of recombinant Fowlpox virus or adenovirus vectors (Figure 3, Supplemental Figure 1, and data not shown). Peptide-pulsed APCs elicited a higher number of tetramer-positive $CD8^+$ T cells compared with *S. typhimurium*–NY-ESO-1–infected APCs, while the number of IFN-γ–secreting cells did not differ (Figure 2B and Figure 3). This suggests that *S. typhimurium* may activate effector cells more effi-

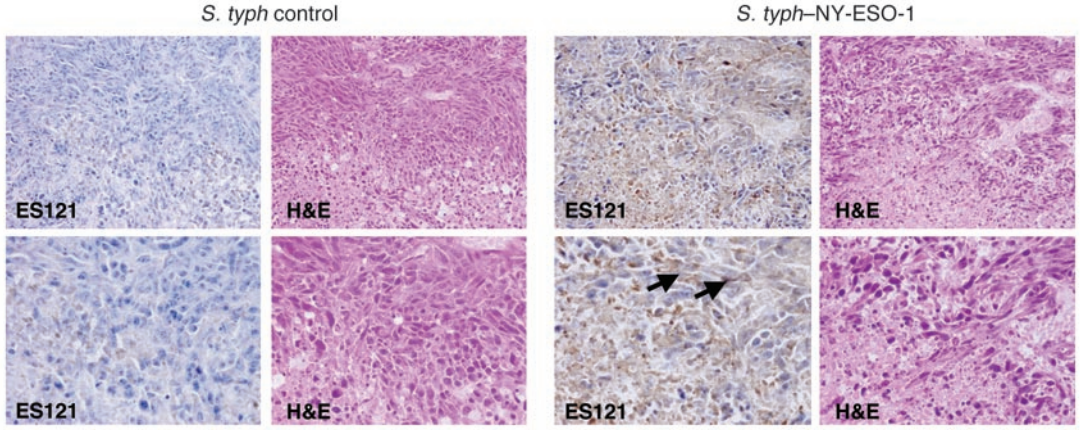

Figure 5
Intratumoral administration of *S. typhimurium* results in the delivery of antigen at the tumor site. BALB/c mice were inoculated with 1×10^6 parental CMS5a tumor cells. One week later, $0.5–1 \times 10^6$ CFU of *S. typhimurium*–NY-ESO-1 or of the control strain were directly inoculated to palpable tumors. After surgical removal, the presence of NY-ESO-1 at the tumor site was examined by immunohistochemistry as described in Methods. Arrows indicate NY-ESO-1–positive cells. Original magnification, ×10 (top row); ×40 (bottom row).

ciently than peptides, which lack costimulatory signals and consequently may activate IFN-γ–nonsecreting $CD8^+$ cells as reported previously (26). We did not observe any inhibition of T cell proliferation with *Salmonella* in our experimental conditions, though it was recently reported that direct contact–dependent inhibition occurred in longer-term cultures (27). Taken together, our results indicate that the *S. typhimurium* type III secretion system is an effective NY-ESO-1 antigen delivery platform in humans.

Our data also indicate that the *S. typhimurium* type III secretion system is also an effective antigen delivery system in vivo. Oral administration of *S. typhimurium*–NY-ESO-1 into mice harboring NY-ESO-1–expressing tumors resulted in the development of antigen-specific $CD8^+$ T cells and the complete regression of the tumors. The vaccine strain used in these studies is not very efficient at reaching deeper tissues (28), suggesting that the immune response may well be largely primed at mucosal sites. Indeed, previous experiments carried out in monkeys with an equivalent strain strongly suggest this possibility (21). However, since low bacterial numbers can reach deeper tissues, systemic priming cannot be ruled out. Tumor regression was dependent on the induction of NY-ESO-1–specific $CD8^+$ T cells since (a) tumor regression was only observed in animals bearing NY-ESO-1–expressing tumors that received *S. typhimurium*–NY-ESO-1, but not in those that received the *S. typhimurium* control strain; and (b) depletion of $CD8^+$ cells from immunized animals abrogated tumor regression (Figure 4). Consequently, the antitumor activity of *S. typhimurium*–NY-ESO-1 is different from the previously reported antitumor activity of some avirulent strains of *S. typhimurium* that, when administered to mice, caused tumor regression. The previously described antitumor activity has been ascribed to direct toxic activity of *S. typhimurium* on tumor cells, which required the localization of the bacteria within the tumor tissue (29, 30). Under our experimental conditions, the orally administered *S. typhimurium* Δ*phoP* Δ*phoQ* strain does not reach tumor local compartments in substantial numbers. More importantly, the antitumor effect was not observed after administration of an identical control strain carrying the control plasmid vector without the gene encoding SopE–NY-ESO-1 chimeric protein (Figure 4).

The rapid and effective antitumor activity of *S. typhimurium* endowed with the ability to deliver a tumor antigen via its type III secretion is striking. It has been previously shown that stimulation of the innate immune system is essential to break immunological tolerance (14). TLR signals, which recognize microbial-associated molecular patterns existing in bacteria such as the *S. typhimurium* strain used in our study, are able not only to block the suppressive activity of $CD4^+CD25^+$ Tregs, but also to break CD8 tolerance even in the presence of $CD4^+CD25^+$ Tregs (11–13, 15). In our in vivo mouse model, expression of NY-ESO-1 by growing tumor cells alone did not elicit measurable numbers of antigen-specific $CD8^+$ T cells. However, upon oral administration of *S. typhimurium*–NY-ESO-1, animals developed antigen-specific $CD8^+$ T cells and the tumors underwent regression. It is possible that the demonstrated ability of *S. typhimurium* to strongly stimulate innate immunity may contribute to its ability to induce a strong $CD8^+$ response against NY-ESO-1 (16, 17).

Administration of *S. typhimurium*–NY-ESO-1 at the tumor site of animals primed with NY-ESO-1 DNA led to rapid eradication of tumors that did not initially express the NY-ESO-1 antigen. These results were surprising since (a) tumor eradication was strictly dependent on the delivery of NY-ESO-1 by the *S. typhimurium* type III secretion system and was not observed in animals receiving the *S. typhimurium* control strain; (b) tumor regression was dependent on the presence of $CD8^+$ T cells; and (c) tumor regression was not observed in unprimed animals (Figure 6). Administration of *S. typhimurium* at the tumor site caused some necrosis, but it was not sufficient in itself to eradicate the tumor (Figure 5 and Figure 6A). In this case, we believe that tumor regression was due, at least initially, to the recognition of tumor cells that had received NY-ESO-1 antigen via the *S. typhimurium* type III secretion system by preexisting antigen-specific $CD8^+$ T cells. This hypothesis is consistent with the observation that tumor regression was strictly dependent on the presence of preexisting antigen-specific $CD8^+$ T cells, since mice naive for NY-ESO-1 failed to prevent the rapid growth of CMS5 tumors. Even though NY-ESO-1 is an immunogenic antigen for mice, intratumoral treatment with *S. typhimurium*–NY-ESO-1 could not elicit specific T cells quickly enough to

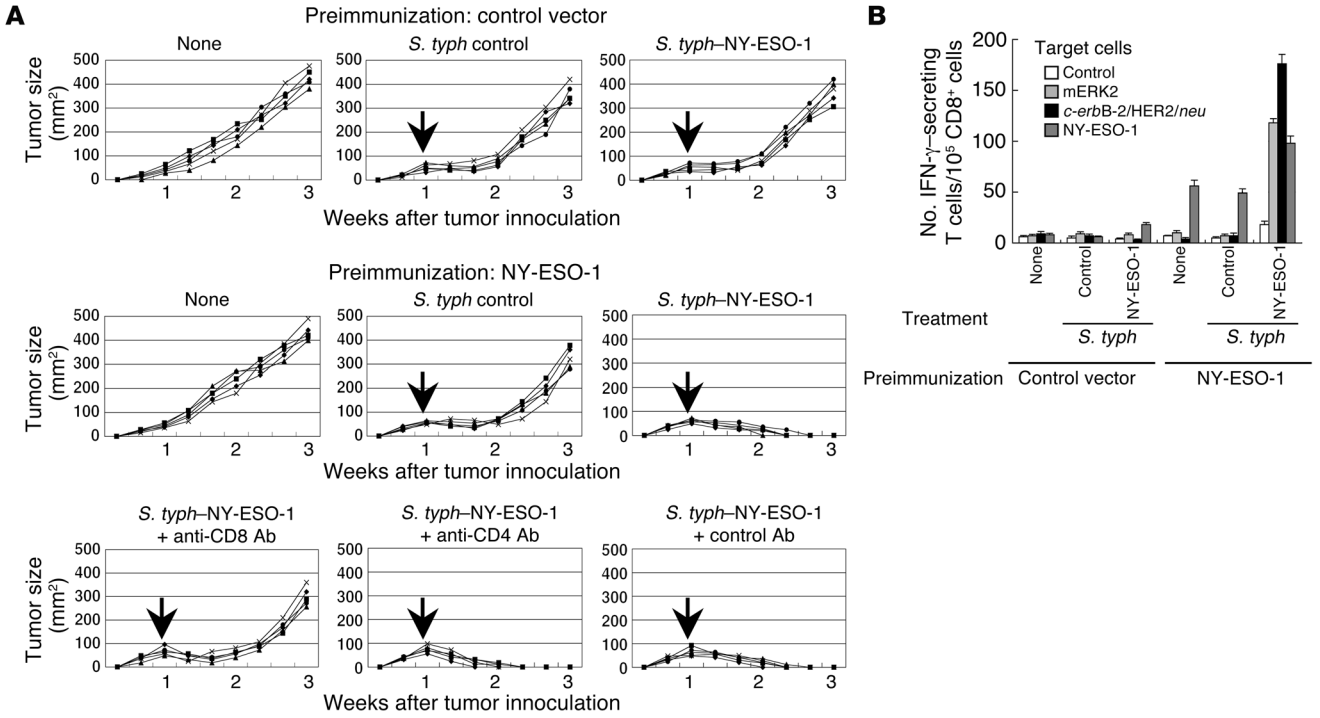

Figure 6

Local tumor antigen delivery by *S. typhimurium* type III secretion system causes regression of tumors in mice by epitope spreading. (**A**) BALB/c mice were immunized twice at 2-week intervals with plasmids encoding NY-ESO-1 or control vector by gene gun. These mice were inoculated with 2×10^6 CMS5a-HE tumor cells (NY-ESO-1–negative), and tumor growth was analyzed 3 times per week. After 7 days, injection of $0.5–1 \times 10^6$ CFU of *S. typhimurium*–NY-ESO-1 or of the control strain at the tumor site was performed. Some groups of mice were also injected intravenously with anti-CD4 or anti-CD8 mAb or control Ab in the form of 25-μl ascites every 5 days. Arrows indicate time points of *S. typhimurium* administration. Each line represents the tumor growth of an individual mouse. Tumor size was calculated as longitudinal diameter (mm) × horizontal diameter (mm). (**B**) $CD8^+$ T cells were purified from spleens from control vector– or NY-ESO-1–preimmunized mice bearing CMS5a-HE, either untreated or treated with *S. typhimurium*–NY-ESO-1 or the control strain, and analyzed for the number of specific IFN-γ–producing cells by ELISPOT assay. Data are mean ± SD. Experiments were performed independently at least twice with similar results.

provide protection in the absence of preexisting immunity to NY-ESO-1 (Figure 6A). Since undoubtedly not all tumor cells could have been targeted by *S. typhimurium*–NY-ESO-1, efficient tumor eradication most likely involved the generation of the $CD8^+$ T cell response against other tumor antigens not present in the vaccine. Consistent with this hypothesis, NY-ESO-1–preimmunized animals receiving *S. typhimurium*–NY-ESO-1 developed $CD8^+$ response against at least 2 other tumor antigens that were not present in the vaccine (Figure 6B), presumably by the previously observed phenomenon of epitope spreading (23, 24). In fact, we believe that epitope spreading is also essential for the rapid tumor regression observed after oral administration of *S. typhimurium*–NY-ESO-1 to animals carrying NY-ESO-1–expressing tumors, since in this case we also observed the development of $CD8^+$ T cells specific to antigens not present in the vaccine (Figure 6B). These data are in line with recent reports that tumor vaccine–induced CTLs not only act as effectors to kill tumor cells bearing the cognate antigen, but also generate conditions enabling the immune system to stimulate other tumor antigen–specific CTLs (31, 32).

S. typhimurium has the ability to invade nonphagocytic cells such as epithelial cells (33). As shown in Figure 5, the *S. typhimurium* type III secretion system injected NY-ESO-1 not only to tumor cells but to surrounding tissues as well. Tumor stromal cells might also be targeted and facilitate tumor eradication (34, 35). One concern to consider is undesired antigen delivery to normal tissue by *S. typhimurium* that could potentially prime autoimmune reactivity. Although after healing, mice treated with intratumoral injection of *S. typhimurium* did not exhibit any side effect except local inflammation, further observation might be required to completely rule out this possibility.

Our studies suggest what we believe to be a novel strategy for cancer immunotherapy using the *S. typhimurium* type III secretion system as an antigen delivery platform that would not require prior knowledge of the tumor antigen composition. This strategy would require the administration of a *S. typhimurium* strain engineered to deliver antigen(s) against which the patient would have preexisting $CD8^+$ T cells to tumor cells via its type III secretion system. Those antigens would not need to be expressed in tumor cells. In fact, it is conceivable that preexisting $CD8^+$ T cells against viral antigens such as influenza or EBV, of common occurrence in human populations due to vaccination and/or convalescent immunity, could be used for this purpose.

Methods

Patients and effector T cells. Patients NW29 and NW634 had NY-ESO-1–expressing melanoma as described previously (36, 37). PBMCs were collected by density gradient and stored in liquid nitrogen until use. All samples were collected after informed consent as a part of study approved

by the Ethics Committee of Landesärztekammer Hessen (Frankfurt, Germany). The HLA-A*0201–restricted $CD8^+$ T cell clone specific for NY-ESO-$1_{157–165}$, clone 49, was a kind gift of D. Valmori (Columbia University, New York, New York, USA) (38). The HLA-A*0201–restricted $CD8^+$ T cell line specific for Influenza matrix$_{58–66}$, NW46-Flu, was derived from melanoma patient NW46.

Mice. Female BALB/c mice were purchased from CLEA Japan and used at 7–10 weeks of age. Mice were maintained at the Animal Center of Mie University Graduate School of Medicine (Mie, Japan). The mouse experimental protocol was approved by the Ethics Committee for Animal Experimentation of Mie University Graduate School of Medicine.

Bacterial strains. The *S. typhimurium* Δ*phoP* Δ*phoQ* strain has been previously described (19). This strain was used to host plasmids expressing a chimeric protein consisting of the first 100 amino acids of SopE, which contains its secretion and translocation signals (39), fused to full-length NY-ESO-1 (*S. typhimurium*–NY-ESO-1) or to amino acids 58–66 of the influenza virus matrix protein (*S. typhimurium*–Flu matrix). The *S. typhimurium* control strain used in these studies was identical to the strains described above except that the plasmid expresses just the first 100 amino acids of SopE. All constructs contained 2 epitopes placed immediately adjacent to SopE$_{1–100}$: an M45 epitope tag from the adenovirus E4-6/7 protein (40) for chimeric protein detection, and an a mouse H-2^b haplotype class I–restricted epitope consisting of residues 366–374 from the influenza virus nucleoprotein. The control type III secretion–deficient strain used in Figure 1B and the translocation-deficient strain used in Figure 1C have been previously described (41, 42). All plasmids were constructed using standard recombinant DNA methodologies (41, 42). Analysis of culture supernatant proteins by the different vaccine constructs and translocation of type III secreted proteins into host cells were carried out as previously described (19) using an Ab directed to the M45 epitope tag present in the constructs.

Tumors. Human melanoma cell lines SK-MEL-37, which expresses NY-ESO-1, and SK-MEL-21, which does not express NY-ESO-1, are both HLA-A*0201–positive. CMS5 is a 3-methycholanthrene–induced sarcoma cell line of BALB/c origin (43). CMS5a is a subcloned cell line obtained from CMS5, a tumor expressing mERK2 (25, 43). CMS5a–NY-ESO-1 and CMS5a-HE are cell lines derived from CMS5a stably transfected with NY-ESO-1 and *c-erb*B-2/HER2/*neu*, respectively (44). P1.HTR is a subline of mastocytoma P815 of DBA/2 origin (45).

Reagents. $CD8^+$ or $CD4^+$ T cells were depleted by the tail-vein administration of anti-CD4 (GK1.5, rat IgG2b) or anti-CD8 (19/178, rat IgG2b) mAb as described previously (46, 47). Synthetic peptides derived from the sequence of NY-ESO-$1_{79–108}$ (GARGPESRLLEFYLAMPFATPMEAELARRS) and influenza NP$_{206–229}$ (FWRGENGRKTRIAYERMCNILKGK) were obtained from NeoMPS (48). Recombinant Fowlpox vectors were obtained from Therion Biologics (37). A cDNA encoding NY-ESO-1 was cloned into pCAGGS-New (49), kindly provided by J. Miyazaki (Osaka University, Osaka, Japan), and purified using QIAGEN EndoFree Plasmid Mega Kit (QIAGEN).

S. typhimurium infection. *S. typhimurium* was grown as previously described (19). Target cells were infected with the different *S. typhimurium* strains for 1 hour at 37°C at MOI 40. Extracellular bacteria were killed by transferring the target cells to RPMI 1640 medium containing 100 μg/ml gentamicin (Sigma-Aldrich) and incubating at 37°C for 1 hour. Infected cells were subsequently used as APCs.

Immunocytochemistry and immunohistochemistry. Cytospin specimens of approximately 5×10^3 cells were air-dried for 20 minutes at room temperature and fixed with 10% buffered formalin for 20 minutes. Specimens were then rinsed 3 times with PBS and incubated with 1 μg/ml of the anti–NY-ESO-1 mouse mAb ES121 for 2 hours at room temperature. Dextran polymer–labeled system (EnVision+, Dako) was used for secondary detection. We used 3,3′-diaminobenzidine (DAB; BioGenex) as chromogen and counterstained it with hematoxylin. For immunohistochemical analyses, surgically removed tumor specimens were sliced into 2-mm thickness in the vicinity of the *S. typhimurium* injection site and then fixed in 10% paraformaldehyde for 7 hours at 4°C. Fixed specimens were dehydrated and mounted on paraffin. Sliced specimens of 2.5 μm were used. Immunohistochemistry was performed using the Histomouse-SP Plus Kit (Zymed Laboratories Inc.). Antigen retrieval was performed by heating deparaffined specimen at 105°C for 20 minutes in Dako high pH Target Retrieval Solution (Dako). Primary Ab ES121 at 2.5 μg/ml concentration was incubated for 16 hours at 4°C. DAB was used as chromogen. Hematoxylin counterstain was performed.

In vitro presensitization. In vitro presensitization was performed as previously described (36). Briefly, $CD8^+$ T cells were separated from PBMCs of cancer patients using magnetic beads (Dynabeads; Dynal) and seeded onto round-bottomed 96-well plates (Corning Inc.) at a concentration of 5×10^5 cells/well. As APCs for presensitization, PBMCs depleted of $CD4^+$ and $CD8^+$ T cells were pulsed with 10 μM of peptide overnight or infected with different *S. typhimurium* constructs. Expression of NY-ESO-1 was confirmed in $CD4^-CD8^-$ PBMCs following *S. typhimurium*–NY-ESO-1 infection. After irradiation, these cells were added to plates containing $CD8^+$ T cells at a concentration of 1×10^6 cells/well. After 20 hours, IL-2 (10 U/ml; Roche Diagnostics) and IL-7 (20 ng/ml; R&D Systems) were added. Subsequently, one-half of the medium was replaced by fresh medium containing IL-2 (20 U/ml) and IL-7 (40 ng/ml) twice per week.

Tetramer staining. Tetramer staining was performed as previously described (36). Briefly, presensitized $CD8^+$ T cells were stained with PE-labeled NY-ESO-$1_{92–100}$/HLA-Cw*0304 tetramers (prepared at the Ludwig Institute Core Facility by P. Guillaume and I. Luescher, Lausanne, Switzerland) for 15 minutes at 37°C before additional staining of Tricolor-CD8 mAb (CALTAG Laboratories) for 15 minutes at 4°C. After washing, results were analyzed by flow cytometry (FACSCalibur; BD Biosciences).

ELISPOT assay. The number of IFN-γ–secreting specific $CD8^+$ T cells was assessed by ELISPOT assays as previously described (36, 44, 46, 48). In human ELISPOT assays, flat-bottomed, 96-well, nitrocellulose-coated microtiter plates (Millipore) were coated with IFN-γ mAb (1-D1K; Mabtech). Presensitized T cells and EBV-B cells pulsed with peptides or infected with recombinant Fowlpox virus (100 PFU/cell) or the different *S. typhimurium* constructs (40 MOI) were added to each well and incubated for 24 hours. Spots were developed using biotinylated anti–IFN-γ mAb (7-B6-1-biotin; Mabtech), alkaline phosphatase–conjugated streptavidin (Mabtech), and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich) and counted with C.T.L. ImmunoSpot Analyzer and Software (version 2.08; Cellular Technologies Ltd.).

Mouse $CD8^+$ T cells were prepared from spleen cells by positive selection on a MACS column (Miltenyi Biotec). Target cells were P1.HTR transfected (ECM830; BTX Harvard Apparatus) with mRNA transcribed from plasmid DNA of NY-ESO-1, mERK2, *c-erb*B-2/HER2/*neu*, or EGFP using mMESSAGE mMACHINE T7 Kit (Ambion) as previously described (50). $CD8^+$ T cells (1×10^5) were cultured for 24 hours with 1×10^5 mitomycin C–treated P1.HTR transfected with each mRNA on 96-well nitrocellulose-coated microtiter plates (Millipore) coated with rat anti–IFN-γ mAb (R4-6A2; BD Biosciences — Pharmingen). Spots were developed using biotinylated anti–IFN-γ mAb (XMG1.2; BD Biosciences — Pharmingen), alkaline phosphatase–conjugated streptavidin (Mabtech), and alkaline phosphatase substrate kit (Bio-Rad) and were subsequently counted.

Immunization by gene gun. Gold particles coated with plasmid DNA (1 μg/injection) were prepared and delivered into the shaved skin of the abdomi-

nal wall of BALB/c mice by a Helios Gene Gun System (Bio-Rad) at a helium discharge pressure of 350–400 psi, as previously described (46, 47).

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