Science Translational Medicine
AAAS

Bacteria-Induced Gap Junctions in Tumors Favor Antigen Cross-Presentation and Antitumor Immunity

Fabiana Saccheri, *et al.*

Sci Transl Med **2**, 44ra57 (2010);
DOI: 10.1126/scitranslmed.3000739

Science Translational Medicine (print ISSN 1946-6234; online ISSN 1946-6242) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

TUMOR IMMUNOLOGY

Bacteria-Induced Gap Junctions in Tumors Favor Antigen Cross-Presentation and Antitumor Immunity

Fabiana Saccheri,[1] Chiara Pozzi,[1] Francesca Avogadri,[2] Sara Barozzi,[1] Mario Faretta,[1] Paola Fusi,[3] Maria Rescigno[1]*

(Published 11 August 2010; Volume 2 Issue 44 44ra57)

Antigen-presenting dendritic cells (DCs) trigger the activation of cytotoxic CD8 T cells that target and eliminate cells with the antigen on their surface. Although DCs usually pick up and process antigens themselves, they can also receive peptide antigens from other cells via gap junctions. We demonstrate here that infection with *Salmonella* can induce, in both human and murine melanoma cells, the up-regulation of connexin 43 (Cx43), a ubiquitous protein that forms gap junctions and that is normally lost during melanoma progression. Bacteria-treated melanoma cells can establish functional gap junctions with adjacent DCs. After bacterial infection, these gap junctions transferred preprocessed antigenic peptides from the tumor cells to the DCs, which then presented those peptides on their surface. These peptides activated cytotoxic T cells against the tumor antigen, which could control the growth of distant uninfected tumors. Melanoma cells in which Cx43 had been silenced, when infected in vivo with bacteria, failed to elicit a cytotoxic antitumor response, indicating that this Cx43 mechanism is the principal one used in vivo for the generation of antitumor responses. The Cx43-dependent cross-presentation pathway is more effective than standard protocols of DC loading (peptide, tumor lysates, or apoptotic bodies) for generating DC-based tumor vaccines that both inhibit existing tumors and prevent tumor establishment. In conclusion, we exploited an antimicrobial response present in tumor cells to activate cytotoxic CD8 T cells specific for tumor-generated peptides that could directly recognize and kill tumor cells.

INTRODUCTION

Dendritic cells (DCs), key players in the activation of T cells (*1*), are endowed with the ability to present exogenous antigens that have not been generated within DCs for the activation of T cells via the cross-presentation pathway. Cross-presentation is required for the initiation of effective antitumor T cell responses (*2*), and a repertoire of presented tumor peptides is crucial to activate T cells that will recognize and kill tumor cells (*3*). However, the antigen presentation machinery, and in particular the proteasome, differs between tumor cells and DCs (*4*). Thus, DCs can process and present peptides that are different from those presented by tumor cells, initiating a tumor-specific response that will not recognize the tumor (*4*).

Gap junctions (GJs) are channels that connect the cytoplasm of two adjacent cells (*5*). They allow the transfer of small molecules, including ions, second messengers, and metabolites, up to 1 kD (*5*). GJ intercellular communication (GJIC) participates in many physiological events such as cell cycle control, differentiation, cell synchronization, and metabolic coordination (*5*, *6*). GJs are formed by two hemichannels, called connexons, each made of six connexin proteins. There are at least 21 connexins, most of which are tissue-specific except connexin 43 (Cx43), which is ubiquitously expressed (*7*). Loss of GJIC is a common feature of many human tumors and can occur early during tumorigenesis (*8*, *9*). GJs also play a prominent role in the immune system (*7*). They are required for B and T cell differentiation, antibody secretion by B cells, T regulatory cell activity (*10*), and DC activation (*11*, *12*). GJs are also involved in antigen cross-presentation, where they allow the spreading of small linear peptides (up to 16 amino acids long) between neighboring cells (*13*), including apoptotic cells (*14*). The transferred peptides can be loaded onto major histocompatibility complex (MHC) class I molecules and presented on the surface of acceptor cells. This mechanism may be used to kill a ring of noninfected cells surrounding infected tissue for "sanitation" of the tissue. In addition, it may be used by the immune system to activate T cells specific for the infectious agent via transfer of antigenic peptides from infected cells to noninfected professional antigen-presenting cells (*13*). This pathway is also involved in killing of endothelial cells by tumor-specific cytotoxic T cells (CTLs) after transfer of peptides from tumor cells to endothelial cells in vitro (*15*). However, the in vivo relevance of GJ cross-presentation pathway and its exploitation in antitumor immunity have not been addressed.

Bacteria have been proposed as anticancer agents (*16*). The Gram-negative bacterium *Salmonella typhimurium* is particularly appealing for its ability to home preferentially to tumor sites (*17*). *Salmonella* can be used as a delivery vector for cytokines (*18*), chemokines (*19*), tumor antigens (*20*), and DNA-based vaccines (*21*, *22*). The intratumoral injection of *S. typhimurium* allows breaking ignorance and tolerance to melanoma. It acts both locally, by recruiting immune cells that lead to the elimination of the treated mass (*23*), and systemically, where it favors the development of an antitumor response via the cross-presentation of tumor antigens (*24*). Here, we examined how *S. typhimurium* infection facilitates the cross-presentation of tumor antigens and demonstrate that it can be used for generation of a DC-based tumor vaccine.

RESULTS

Bacteria induce the up-regulation of Cx43 in several tumor cell lines

The first requirement for the establishment of GJs between cells is the expression of connexins. Cx43 is ubiquitous and is implicated in im-

[1]Department of Experimental Oncology, European Institute of Oncology, Via Ripamonti 435, Milan 20141, Italy. [2]Department of Immunology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA. [3]Department of Biotechnology and Biosciences, University of Milano-Bicocca, Milan 20126, Italy.
*To whom correspondence should be addressed. E-mail: maria.rescigno@ifom-ieo-campus.it

mune responses (*7*) but is also down-regulated during melanoma progression (*25*). Indeed, GJIC is lost in many tumors, enabling the autonomous cell behavior of transformed cells (*9*). Here, we used the highly aggressive and low immunogenic B16F10 melanoma model (named B16 hereafter). We first tested whether B16 cells expressed Cx43. Although untreated B16 cells contained little Cx43, it was up-regulated after infection with *Salmonella*, as assessed by immunofluorescence and Western blot analysis at 24 and 48 hours (Fig. 1, A and B). The same up-regulation of Cx43 was observed when B16 cells were incubated with purified bacterial components, such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), and flagellin (Fig. 1C). A DC line, called DC1, treated with LPS was used as a positive control for Cx43 up-regulation (Fig. 1B). Because interferon-γ (IFN-γ) can also up-regulate Cx43 in immune cells (*12*), we tested whether IFN-γ could induce Cx43 in B16 cells. We found that IFN-γ not only up-regulated Cx43 but also synergized with the bacterial components (Fig. 1C). We then analyzed whether Cx43 was up-regulated in tumors infected with *Salmonella* in vivo. Mice were injected subcutaneously with 10^5 B16 cells in the right flank. After 10 days, when the tumor reached an area of 0.5 cm^2, tumors were infected with an avirulent strain of *S. typhimurium* (SL3261AT). One and 3 days later, tumors were resected, and Cx43 expression was analyzed by fluorescence-activated cell sorting (FACS) analysis. *Salmonella* treatment induced the up-regulation of Cx43 (Fig. 1D). We then analyzed whether IFN-γ expression was induced in vivo after intratumoral bacterial infection. IFN-γ was already detectable in infected tumors 1 day after infection, reaching a peak at 7 days (Fig. 1E). These results suggest that bacterial components and IFN-γ may synergize in vivo for the up-regulation of Cx43 and that noninfected cells could express Cx43 in the inflamed environment via the action of free bacterial components or IFN-γ. To evaluate whether this was a peculiar characteristic of the tumor cell line that we used, we extended the analysis to two additional murine melanoma cell lines (B16BL6 and C57B1) and to several human melanoma cell lines (WM-115, WM-266.4, SK-MEL-31, CHL-1, IGR-1, IGR-37, IGR-39, MEWO, and RPMI-7951). All tested murine tumors and half of the human cell lines showed behavior similar to that of B16 in response to bacteria, whereas the other human cell lines expressed higher basal levels of Cx43, which were either unchanged or down-regulated in response to bacteria (fig. S1). Thus, whereas tumor cells generally lose Cx43, treatment with bacteria and IFN-γ resulted in Cx43 up-regulation in all murine and half of the human melanoma cell lines that we tested.

Cx43 up-regulation correlates with the generation of functional gap junctions

We next tested whether Cx43 up-regulation correlated with the formation of functional GJ pores. Infected or noninfected B16 cells were microinjected with a mixture of a GJ-diffusible dye (Lucifer yellow) and a GJ-nondiffusible dye (dextran–Texas Red, 70 kD) (*13*). After bacterial infection, Lucifer yellow was able to diffuse to adjacent cells (Fig. 2A) at a level comparable to that of the untreated NIH 3T3 cells used as a positive control (*26*). We then addressed whether similar intercellular communication was established with adjacent DCs. Twenty-four hours after the infection, infected and noninfected B16 tumor

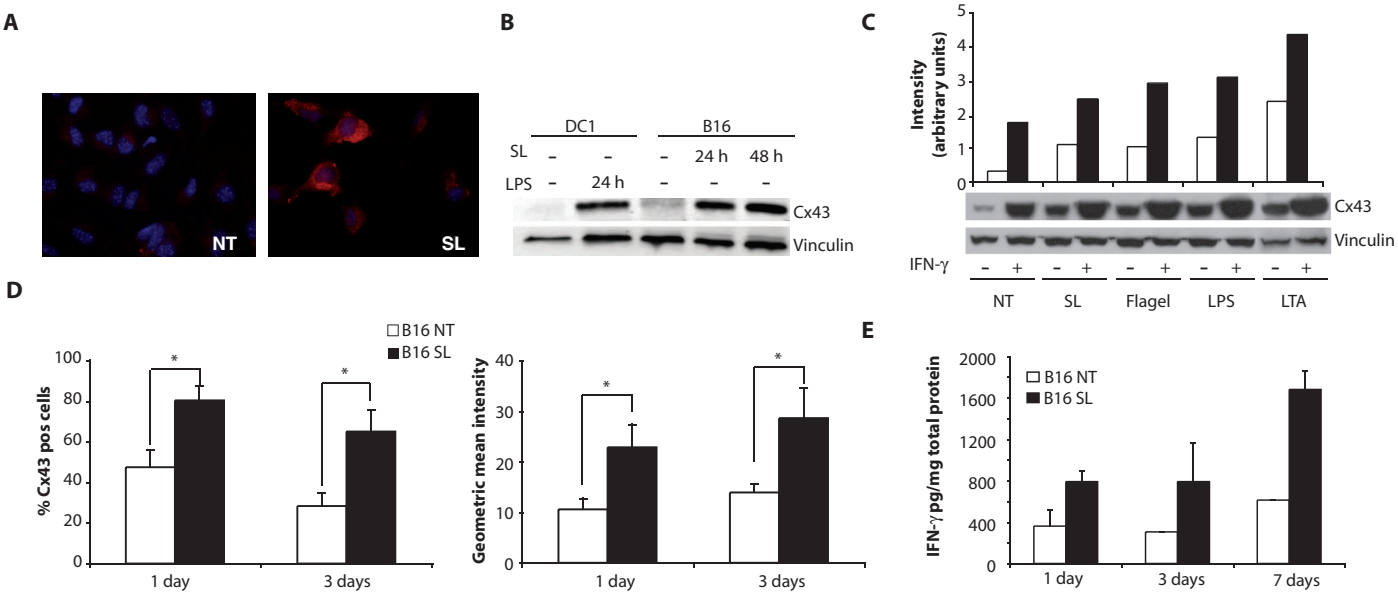

Fig. 1. Bacteria and IFN-γ up-regulate Cx43 expression in tumor cells. (**A**) B16 cells were left untreated (NT) or incubated with bacteria [*Salmonella* (SL)] for 2 hours. After 24 hours, Cx43 expression was evaluated by immunofluorescence (red, Cx43; blue, DAPI). (**B**) B16 cells or DC1 DCs were left untreated or incubated with bacteria (*Salmonella*) for 2 hours or LPS for 24 hours. Western blot after 24 and 48 hours with an antibody to Cx43 or to vinculin is shown. (**C**) B16 cells were untreated or incubated with *Salmonella* or bacterial products [flagellin (Flagel), LPS, and LTA] in the presence or absence of IFN-γ. Western blots after 24 hours with an antibody to Cx43 or to vinculin are shown. Bars show band quantification in the presence (black bars) or absence (white bars) of IFN-γ. (**D** and **E**) B16-established tumors were treated (B16 SL, black bars) or not (B16 NT, white bars) with *Salmonella*. (D) One and 3 days later, mice were killed, tumors were smashed, and cells were analyzed for Cx43 expression by FACS. Percentage of $Cx43^+$ cells (left) and geometric mean intensity (right) are shown. (E) One, 3, and 7 days after *Salmonella* infection, mice were killed, tumors were smashed, and IFN-γ production was measured by ELISA. Error bars, SD. Each experiment was repeated three or four times with similar results.

cells were labeled with the GJ-diffusible dye calcein-acetoxymethylester (calcein-AM), and LPS-treated DCs were labeled with the nontransferable dye 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO). We plated the cells separately but in close proximity in the same petri dish and monitored the transfer of dye from the B16 cells to DCs by real-time confocal videomicroscopy. As shown in movie S1 and in the sequence of frames collected from movie S1 (Fig. 2B), starting at 20 min, the transfer of the calcein dye was observed from tumor cells (white) to DCs (red), which gradually became yellow. The DCs were also able to transfer the dye among themselves, indicating that GJIC was forming among the DCs. To quantify the transfer of material occurring between tumor cells and DCs, we used a cytofluorimetric method to measure GJIC using the same dyes to distinguish donor from acceptor cells. After 1 hour of coculture, the number of $DDAO^+$calcein-AM^+ cells was significantly increased when tumor cells were pretreated with bacteria (Fig. 2C). This assay also allowed us to evaluate whether the transfer of the dye was indeed occurring via GJs because the GJ uncoupler heptanol, which is a pleiotropic lipophilic agent that blocks electrical cell-to-cell communication, completely abolished the transfer of calcein from B16 cells to DCs (Fig. 2C). This indicates that functional GJs can form between tumor cells and DCs for cell-cell communication and that this mechanism may be used for the transfer of antigenic material.

DCs present preprocessed tumor-derived peptide without the need of phagocytosis

To follow the transfer of antigenic material from infected tumor cells to DCs, we used a B16 cell line expressing the antigen ovalbumin (B16-OVA). We analyzed the appearance of the $OVA_{(257–264)}$ SIINFEKL peptide in association with MHC class I (K^b) molecules using the specific antibody 25-D1.16 (*27*) on the surface of DCs. To assess whether DCs acquired the peptide after phagocytosis of infected B16-OVA cells, we labeled the latter with the vital dye carboxyfluorescein diacetate succinimidyl ester (CFSE) and incubated the stained cells with DCs for 24 hours. We detected DCs positive for the 25-D1.16 antibody primarily in the fraction of cells that were negative for CFSE ($CD11c^+CFSE^-$ cells) and only after infection of tumor cells (Fig. 3A). The low percentage of K^bOVA-positive cells in the $CFSE^+$ DC fraction after incubation with B16 control is likely due to nonspecific binding of the antibody. This result suggests that phagocytosis of intact tumor cells or apoptotic bodies is not required for the exchange of the tumor-associated peptide from infected tumor cells to DCs. To address the nature of the transfer, we added heptanol to the cell culture. Heptanol completely abol-

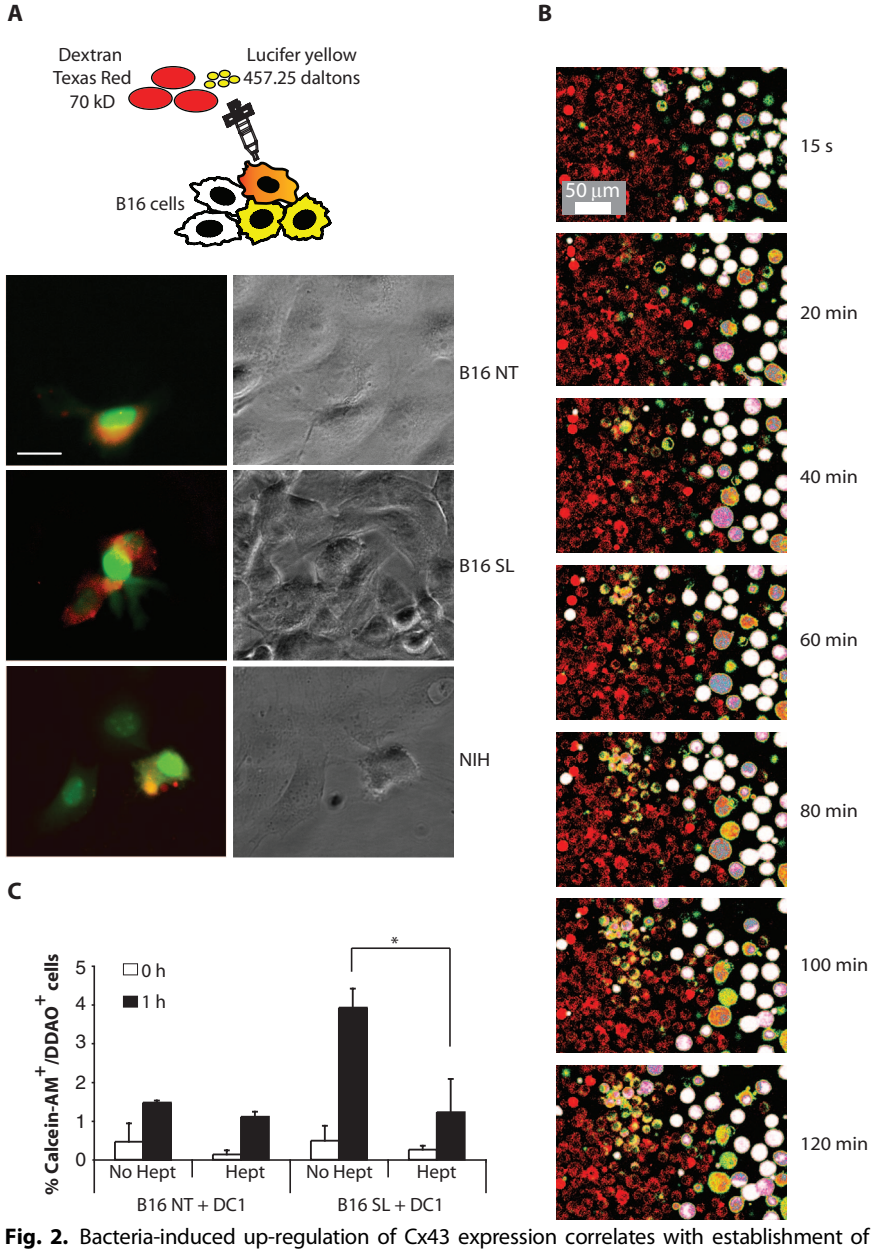

Fig. 2. Bacteria-induced up-regulation of Cx43 expression correlates with establishment of functional gap junctions. (**A**) B16 cells were infected (B16 SL) or not (B16 NT) in vitro with *Salmonella* and, after 24 hours, microinjected with a mix of the GJ-diffusible dye Lucifer yellow (green) and the nontransferable dye dextran–Texas Red (red, to mark microinjected cells). Cells were fixed immediately after microinjecting the last cell and observed for dye transfer by fluorescence microscopy. Untreated NIH 3T3 cells were used as positive control. Magnification, ×40. Scale bar, 20 μm. (**B**) Confocal microscopy analysis. B16 cells were infected with *Salmonella* and, after 24 hours, stained with calcein-AM (green/white), whereas DCs, previously treated with LPS for 1 hour, were stained with DDAO (red). A drop of each population was plated onto a microscope slide close to each other, and the cells were coincubated for 1 hour. The cells were analyzed by confocal microscopy for 2 hours. Representative single frames taken from the movie every 20 min are shown. (**C**) Only *Salmonella*-infected cells can communicate with DCs via a GJ-dependent mechanism. B16 cells were infected or not with *Salmonella*, pulsed with the GJ-diffusible dye calcein-AM, and incubated with DDAO-labeled DCs in the presence or absence of heptanol (Hept). One hour later, the percentage of cells double-positive for DDAO and calcein-AM, representing the DCs that received the dye from tumor cells, was evaluated by FACS (black bars). Error bars, SD. *$P < 0.05$. Each experiment was repeated twice with similar results.

ished the presentation of the peptide, indicating that the OVA peptide is likely transferred via GJIC (Fig. 3A). Because only linear peptides of up to 16 amino acids can be transferred via GJs, the exploitation of this pathway requires processing of the antigenic material within tumor cells. Thus, we examined whether proteasome-dependent degradation in tumor cells was necessary for the presentation of the OVA peptide by DCs. Pretreatment of B16-OVA cells with lactacystin, a cell-permeable, irreversible 20*S* or 26*S* proteasome inhibitor (*28*), prevented the exposure of the K^b-OVA complex on the DC surface (Fig. 3B). We then evaluated whether the presentation of peptides via GJ led to T cell activation and whether this mechanism was dependent on Cx43 expression by tumor cells. We generated a clone of B16-OVA cells that were stably silenced for the expression of Cx43. We used four different lentivirus constructs but only three were effective in silencing Cx43; hence, we used the noneffective one as a negative control. In Fig. 4A, the extent of Cx43 silencing is shown. We then tested T cell activation in terms of IFN-γ release using purified naïve OTI T cells that carry a T cell receptor (TCR) specific for the complex of K^b-$OVA_{(257–264)}$SIINFEKL peptide. We confirmed that pretreatment of B16-OVA cells with *Salmonella* favored the cross-presentation of OVA peptide by DCs (Fig. 4B). This presentation was partly dependent on Cx43 because its silencing in the B16-OVA cells reduced the capacity of DCs to activate OTI T cells (Fig. 4B). The residual activation of T cells may be due to alternative cross-presentation pathways or to incomplete silencing of Cx43 in the B16-OVA cells. Together, these data suggest that bacterial infection promotes the transfer of a processed peptide via a GJ-dependent mechanism.

Intratumoral bacterial injection increases the percentage of $Cx43^+$ DCs in lymph nodes

The transfer of GJ-diffusible dye occurs not only between tumor cells and DCs but also among DCs (movie S1 and Fig. 2B). These additional GJs could increase the number of DCs capable of presenting tumor-associated antigens in the draining lymph nodes. Hence, we tested the frequency of $CD11c^+Cx43^+$ DCs in lymph nodes that did or did not drain the tumor site; the tumor had been treated with *Salmonella* or phosphate-buffered saline (PBS) as a control. We observed that by 24 hours after infection, the frequency of $CD11c^+Cx43^+CD4^+$ DCs was already increased in both lymph nodes that drained the tumor site and those that did not, but only if the tumor site (or the skin) was infected with *Salmonella* (fig. S2A). At later time points (3 days), the $CD8^+$ subset of DCs expressing Cx43 was also increased in frequency (fig. S2A). This coincided with an increase in activated DCs, as shown by the up-regulation of the activation marker CD86 (fig. S2B). The activated DCs were those that expressed Cx43 (fig. S2C). We were puzzled to observe an increase in $Cx43^+$ DCs in non–tumor-draining lymph nodes because we previously showed that *Salmonella* remains confined to the infected site through the generation of a granuloma-like structure (*29*, *30*). However, we found an increased concentration of IFN-γ in the serum of mice whose tumors were infected (fig. S2D), possibly explaining the rise of $Cx43^+$ DCs in non–tumor-draining nodes, because IFN-γ can autonomously up-regulate Cx43. We did not see IFN-γ in the serum of *Salmonella*-infected mice that were not carrying tumors. This difference may be due to the nature of melanoma, which is highly vascularized, thereby giving IFN-γ a route to the bloodstream.

Cx43-dependent cross-presentation is the major mechanism of tumor-antigen cross-presentation in vivo

In our previous studies, we observed that intratumoral injection of *Salmonella* led to increased cross-presentation of tumor antigens by

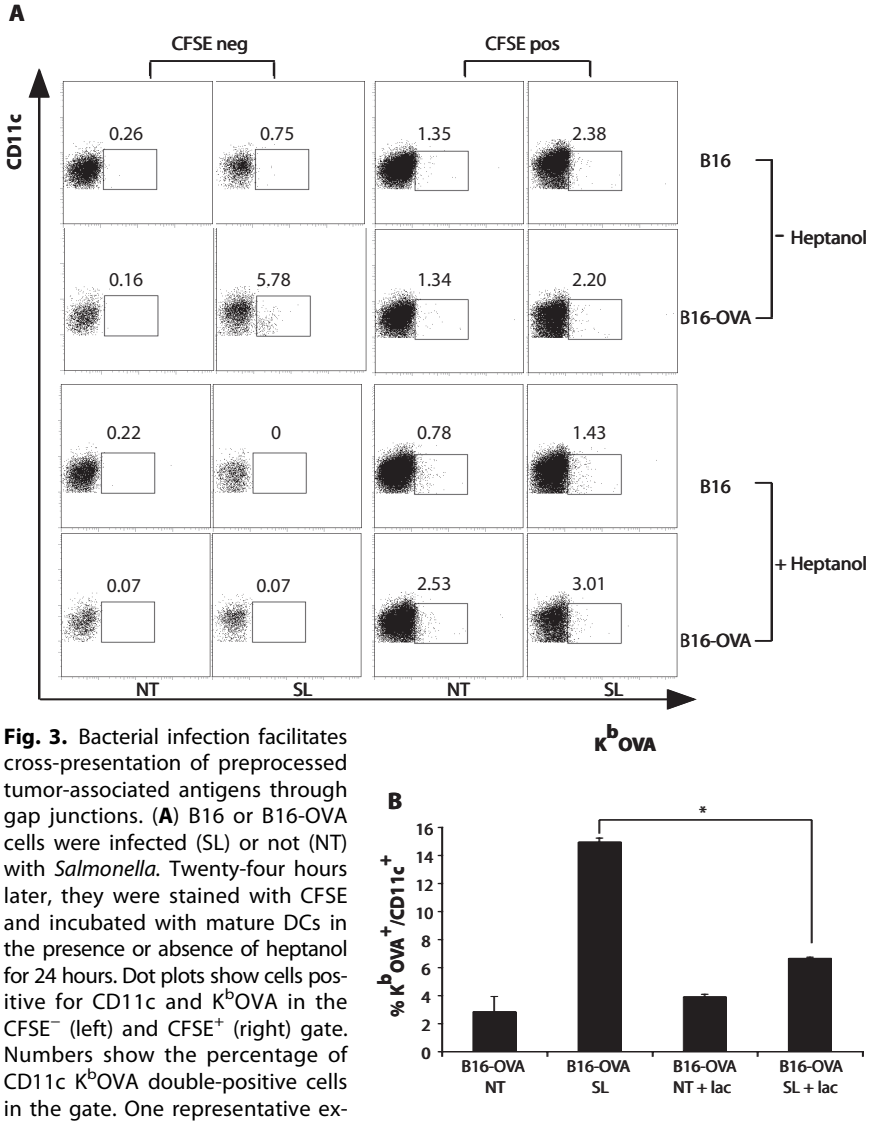

Fig. 3. Bacterial infection facilitates cross-presentation of preprocessed tumor-associated antigens through gap junctions. (**A**) B16 or B16-OVA cells were infected (SL) or not (NT) with *Salmonella*. Twenty-four hours later, they were stained with CFSE and incubated with mature DCs in the presence or absence of heptanol for 24 hours. Dot plots show cells positive for CD11c and K^bOVA in the $CFSE^-$ (left) and $CFSE^+$ (right) gate. Numbers show the percentage of CD11c K^bOVA double-positive cells in the gate. One representative experiment of three is shown. (**B**) Preprocessing in tumor cells is required for effective cross-presentation. B16-OVA cells were treated as above but in the presence or absence of the irreversible proteasome inhibitor lactacystin (lac). The percentage of K^bOVA/CD11c double-positive cells is shown. Error bars, SD. *$P < 0.05$. One experiment representative of two is shown. The percentage of $CD11c^+K^bOVA^+$ cells varied between experiments (compare A to B), but the trend was always similar.

DCs and to the induction of a systemic antitumor response that retarded the growth of distant, untreated masses (*23*, *24*). Because the intratumoral injection of *Salmonella* leads to a local inflammatory response and necrosis of tumor cells, many mechanisms of cross-presentation (*31*), including the capture of dying cells (*32*) or of released soluble proteins (*33*), could account for tumor antigen cross-presentation. Hence, we analyzed the contribution of a GJ-dependent cross-presentation pathway in vivo after bacterial infection. We hypothesized that if Cx43 was required to initiate a systemic antitumor response via GJs, its absence in the *Salmonella*-treated tumor would alter the growth of a distant Cx43 wild-type untreated tumor. We generated a cell line of B16 cells stably silenced for Cx43 until the end of the experiment (Fig. 5A). A B16 cell line transfected with a lentivirus carrying a short hairpin RNA (shRNA) ineffective in silencing Cx43 (Ctrl-B16) served as a control. B16 control (Ctrl-B16) or B16Cx43shRNA cells were introduced by inoculation into the left flank, and 3 days later, B16 wild-type cells were inoculated in the right flank. On days 11 and 15 after the first tumor challenge, *Salmonella*, or PBS as a control, was injected only in the tumor on the left side. The growth of the two tumors was followed over time. The tumors treated with bacteria regressed regardless of the presence or absence of Cx43 (Fig. 5B). This is not surprising because the initial killing of infected tumor cells is independent of the activation of an adaptive immune response to the tumor (*23*). In contrast, the growth of the distal untreated tumors was controlled only when the tumors injected with bacteria contained Cx43 (Fig. 5B). The antitumor response against the distal tumor was abrogated when the *Salmonella*-treated tumor was silenced for Cx43 (Fig. 5B). This suggests that Cx43 is the principal component of GJ in vivo and also that this structure plays a prominent role in *Salmonella*-induced tumor antigen cross-presentation. To confirm that the effect on the distant untreated tumor was mediated by the induction of a CTL response generated by DCs receiving tumor peptides from infected cells, we deleted CTLs using a neutralizing antibody to CD8 during the course of the experiment, following the schedule reported in Fig. 5B. An isotype immunoglobulin G (IgG) control antibody was used as a control. As expected, neutralization of $CD8^+$ T cells abolished the antitumor response in the distal untreated tumor (Fig. 5B, right panel). These experiments indicate that in vivo Cx43-dependent cross-presentation of tumor antigens in response to bacterial infection is dominant over other cross-presentation pathways and is required to generate an effective antitumor response.

DCs loaded in vitro with bacteria-treated tumor cells induce antitumor immunity

For many years, DCs have been proposed to be nature's adjuvants in cancer immunotherapy (*34*), and they have proven to induce strong immunological responses in treated patients. Nevertheless, the clinical effectiveness has been modest. One reason for this failure is that, because DCs have a proteasome different from that of tumor cells, they may generate peptides that are not readily produced within tumor cells. Hence, tumor cells do not present on their surfaces the peptides to which the CTLs are specific. A GJ-dependent mechanism of cross-presentation based on preprocessing of antigenic peptides within tumor cells may overcome this drawback. Thus, we compared the effectiveness of a GJ-dependent pathway of MHC class I loading with those generally used in the clinic (peptides, tumor lysates, and apoptotic bodies). We incubated DCs with purified peptides (GP100 and TRP2), tumor cell lysates obtained with freezing and thawing of B16 tumor cells, or ultraviolet (UV)–irradiated B16 cells. Additionally, we loaded DCs with infected or noninfected B16 cells silenced or not for Cx43. We purified the DCs (>98% purity) by positive selection and injected them subcutaneously after mitomycin C treatment to avoid the growth of any contaminating tumor cells in mice challenged 4 days earlier with B16 cells (in a therapeutic setting). We found that the only condition that led to an effective antitumor response was to use the DCs loaded with infected B16 cells (Fig. 6A). This pathway was effective on both small (<0.2 cm^2) and large (>0.4 cm^2) tumors and was dependent on Cx43 because the antitumor response was lost if the DCs were loaded with B16 cells that had been silenced for Cx43 (fig. S3). Because it was shown that transfer of peptides among DCs could also contribute to cross-presentation (*35*), we silenced Cx43 only in tumor cells to avoid inhibition of DC-DC peptide transfer.

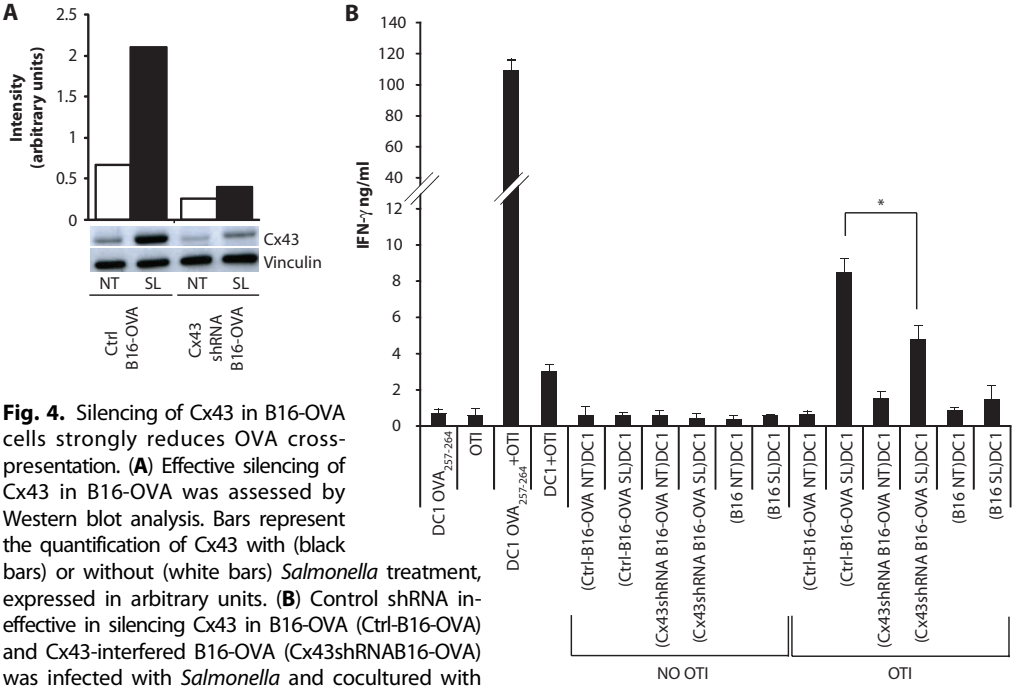

Fig. 4. Silencing of Cx43 in B16-OVA cells strongly reduces OVA cross-presentation. (**A**) Effective silencing of Cx43 in B16-OVA was assessed by Western blot analysis. Bars represent the quantification of Cx43 with (black bars) or without (white bars) *Salmonella* treatment, expressed in arbitrary units. (**B**) Control shRNA ineffective in silencing Cx43 in B16-OVA (Ctrl-B16-OVA) and Cx43-interfered B16-OVA (Cx43shRNAB16-OVA) was infected with *Salmonella* and cocultured with mature DCs in a 1:1 ratio. After 24 hours, DCs were purified by CD11c-positive selection and then treated with mitomycin C (25 μg/ml) to arrest the proliferation of possible remaining tumor cells. Cells were extensively washed and incubated in the presence (OTI) or absence (NO OTI) of OTI CD8 T cells with and without the $OVA_{(257–264)}$ peptide. After 48 hours, the supernatant was collected and the amount of IFN-γ was assessed by ELISA. Controls were DCs pulsed or not with the OVA peptide and incubated or not with OTI T cells. The experiment was repeated twice in triplicate. Error bars represent the SD of the triplicates.

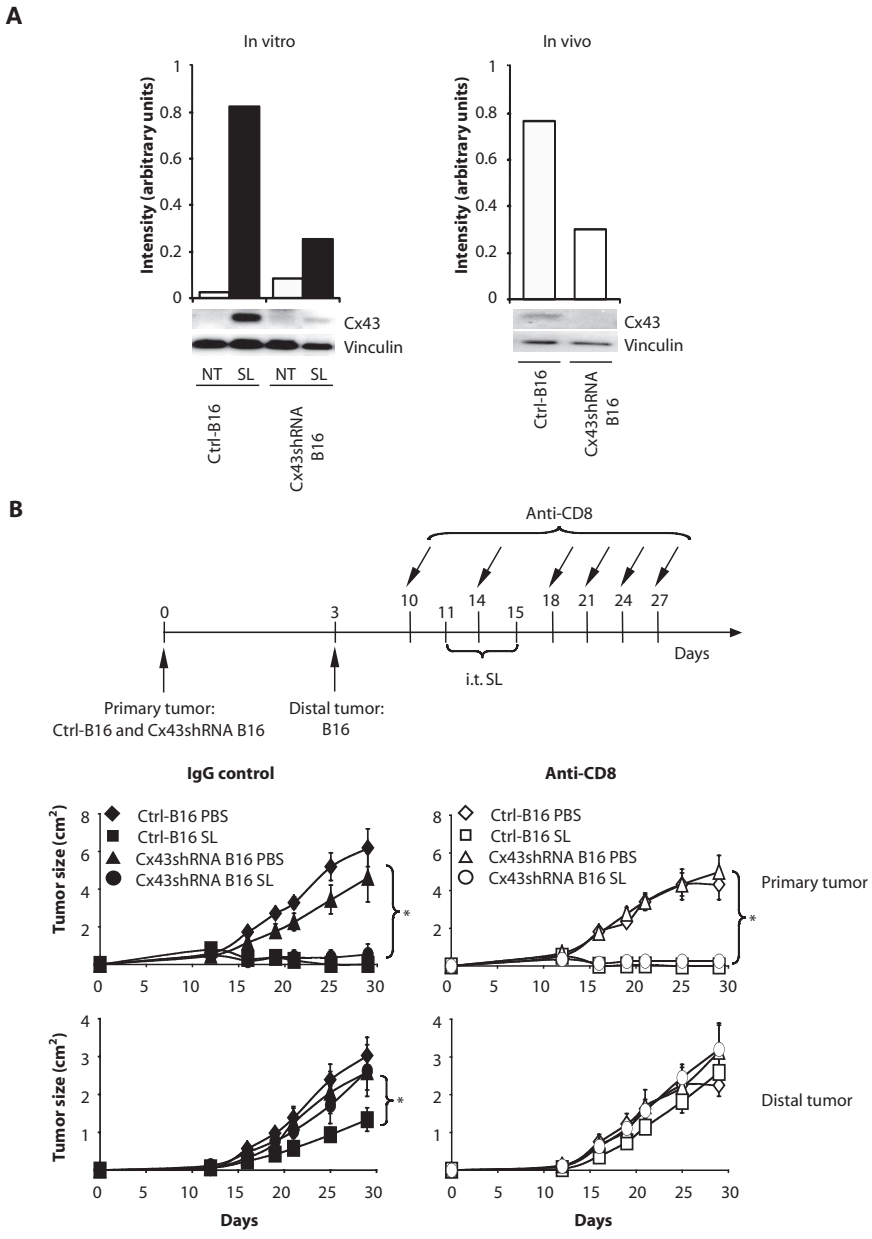

Fig. 5. Cx43 expression by tumor cells is required for the initiation of antitumor immunity. (**A**) Effective knockdown of Cx43 was determined by Western blot on control B16 (Ctrl-B16; with an shRNA ineffective in silencing Cx43) or on Cx43shRNA B16 cells infected (SL, black bars) or not (NT, white bars) with *Salmonella* in vitro (left) and on established B16Cx43shRNA tumors in vivo (right). Bars represent the quantification of Cx43 expressed in arbitrary units. (**B**) Mice received two tumor inoculations following the indicated schedule. B16 control or Cx43shRNAB16 cells were injected into the left flank for the generation of the primary tumor, whereas the distal tumor was formed by B16 wild-type cells injected in the right flank. Bacteria or PBS was injected only in the primary tumor. Groups of eight mice were injected intraperitoneally with neutralizing antibody to CD8 (right graphs) or isotype control (left graphs) at the days indicated in the schedule. The growth of the tumors is shown over time (upper graphs, primary tumors; lower graphs, distal tumors). Error bars, SE. *$P < 0.05$, when comparing the growth of the primary tumor when it was injected with *Salmonella* or PBS (upper graphs). *$P < 0.05$, when comparing the growth of the distal tumor when the primary tumor was either B16 control or Cx43shRNAB16, both injected with *Salmonella* (lower graph). One of three independent experiments is shown.

Even more pronounced was the effect observed in a preventive setting. If we vaccinated the mice with bacteria-treated B16 cells twice (on days 0 and 4) before the challenge with B16 cells (day 21), tumor growth was inhibited and 100% of the mice remained tumor-free and survived 50 days after tumor challenge (Fig. 6B). This effect was also dependent on Cx43 because it was abolished when we used Cx43-silenced B16 cells to load the DCs (Fig. 6B). When the tumor cells were injected at earlier time points (day 8 after vaccination), tumors grew, but more slowly, in mice receiving DCs loaded with bacteria-treated B16 cells; 50% of the mice survived (fig. S4). Note that tumor growth is shown until day 40 when mice carrying 4-cm^2 tumors were killed.

Together, these results indicate that the transfer of antigenic peptides from tumor cells to DCs through GJs is far more effective than standard pathways of DC loading in generating protective DC-based vaccines.

DISCUSSION

Here, we have provided evidence that it is possible to exploit an immune response to infection to inhibit tumor growth by bypassing tumor immune evasion mechanisms. Tumor cells that generally down-regulate GJIC and thereby escape control by the environment up-regulate Cx43 in response to infection with *Salmonella* or purified bacterial components such as LPS, flagellin, or LTA. The up-regulation of Cx43 in tumor cells is accompanied by the generation of functional GJ both between tumor cells and between tumor cells and DCs. These newly formed GJs can transfer processed antigenic material from tumor cells to the DCs for T cell activation. This is a dominant pathway of cross-presentation, which leads to the establishment of effective antitumor immunity in vivo. The preprocessing of the antigen by the tumor guarantees that DCs will present a repertoire of peptides that are also presented by tumor cells.

This feature was recently also described for apoptosis-dependent cross-presentation pathway of virally infected cells (*36*). It is likely that viruses also up-regulate GJ proteins in infected cells and, because apoptotic cells can establish GJs for intercellular communication (*14*), virus-infected apoptotic cells may also allow transfer of antigenic material to acceptor cells. Thus, the mechanism we have described here may be commonly used during those bacterial or viral infections that do not lead to apoptosis of the infected cells.

Tumors develop several immune evasion strategies that allow the generation of more aggressive cancers with reduced immune responsiveness (*37*). GJ down-regulation may be a mechanism of immune evasion that occurs early during tumorigenesis. This

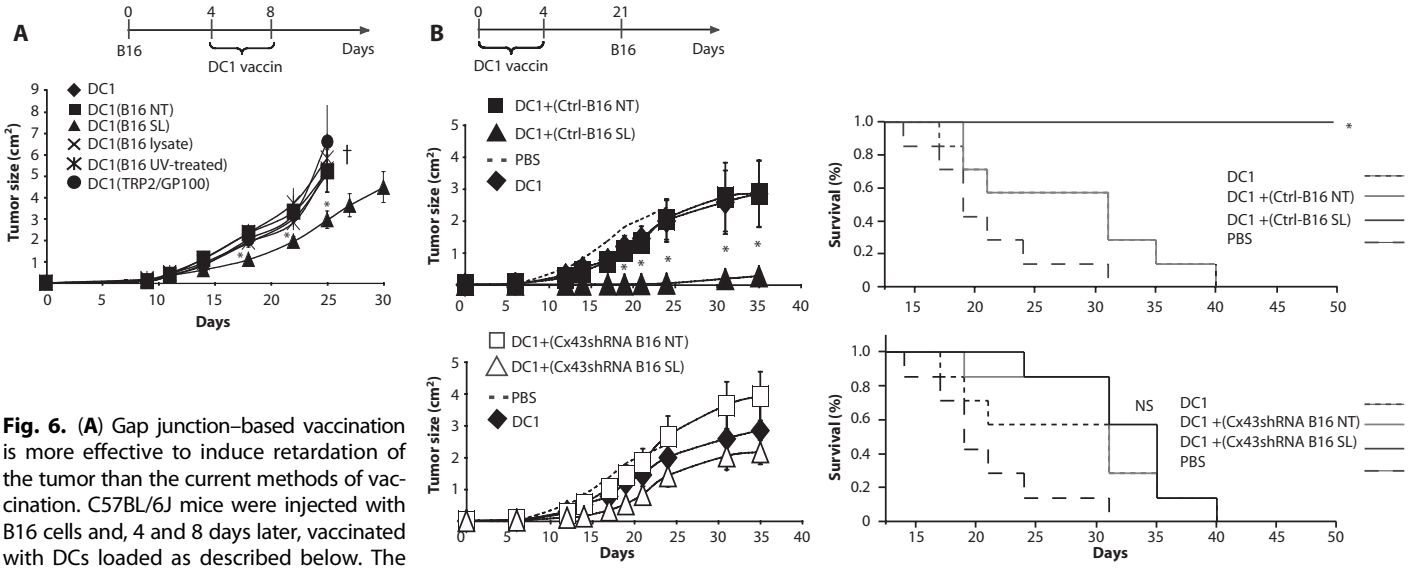

Fig. 6. (**A**) Gap junction–based vaccination is more effective to induce retardation of the tumor than the current methods of vaccination. C57BL/6J mice were injected with B16 cells and, 4 and 8 days later, vaccinated with DCs loaded as described below. The dagger indicates mice that died before the end of the experiment. Diamonds, unloaded DCs (DC1); triangles, DCs incubated with *Salmonella*-treated B16 cells (B16 SL); squares, DCs incubated with untreated B16 cells (B16 NT); Xs, DCs loaded with B16 lysate; asterisks, DCs loaded with B16 UV–treated cells; circles, DCs loaded with a mix of TRP2 and GP100 peptide (TRP2/GP100). (**B**) Loading of DCs with bacteria-treated tumor cells results in efficient antitumor vaccination in a preventive setting. On days 0 and 4, mice (n = 8 per group) were vaccinated with DCs loaded as described below or injected with PBS as control. Twenty-one days later, mice were injected with B16 cells. Upper graphs: PBS (dashed line), unloaded DCs (DC1, filled diamonds), and DCs incubated with *Salmonella*-treated (Ctrl-B16 SL, filled triangles) or untreated (Ctrl-B16 NT, filled squares) control B16 cells. Lower graphs: PBS (dashed line) and DCs loaded with bacteria-treated (DC1 + Cx43shRNAB16 SL, empty triangles) or untreated (DC1 + Cx43shRNAB16 NT, empty squares) Cx43shRNAB16. Error bars, SE. *P < 0.05, when comparing the growth of the tumors of mice vaccinated with DC1 + Ctrl-B16 SL versus DC1 + Ctrl-B16 NT. NS, not significant.

would inhibit tumor antigen cross-presentation and prevent the development of antitumor immunity. The Cx43 up-regulation in response to infection, however, would not be eliminated by selective pressure as the tumor develops and would therefore be retained. Upon infection, tumors would still be able to initiate the anti-infection program leading to Cx43 up-regulation and GJIC, as described here.

This cross-presentation pathway can be used ex vivo for the generation of potent DC-based vaccines. When we compared different pathways of tumor antigen cross-presentation such as direct loading with tumor peptides, tumor lysates, and apoptotic bodies, we found that the Cx43-dependent mechanism was the only one having a benefit in a therapeutic setting. In addition, when tested in a preventive experimental design such as would be appropriate for patients having undergone surgical resection of the tumor, vaccination with DCs loaded with infected tumor cells led to 100% of the mice being free of tumor at the end of the experiment.

It remains to be established whether this strategy could be translatable to humans and to other types of tumors and whether there would still be a benefit in tumors that did not show Cx43 up-regulation after infection. Given the ability of DCs to exchange material via GJ (*35*), this pathway could lead to the amplification of specific responses and could be widely applied.

MATERIALS AND METHODS

Mice, cells, and bacterial strain

Five-week-old female C57BL/6J and OTI OVA-TCR transgenic mice were purchased from Charles River and maintained in specific pathogen-free animal houses. Mouse studies were conducted according to the Italian law on approved experimental protocols.

The murine melanoma B16F10, B16F10OVA (called throughout the paper B16 and B16-OVA, respectively; a gift from P. Dellabona), and B16BL6 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 50 μM 2-mercaptoethanol (complete RPMI). B16-OVA was cultured in the presence of hygromycin B (100 μg/ml). The murine melanoma C57/B1 was grown in minimum essential medium (MEM) supplemented as above plus 1% nonessential amino acids (complete MEM). The human melanoma cell lines were cultured either in complete MEM supplemented with 1% sodium pyruvate {WM-115 [American Type Culture Collection (ATCC), CRL-1675], WM-266.4 (European Collection of Cell Cultures, 91061233), MEWO (Interlab Cell Line Collection, HTL97019), and SK-MEL-31 (ATCC, HTB-73)} or in Dulbecco's MEM supplemented as above {IGR-1 [German Resource Centre for Biological Material (DSMZ), ACC 236], IGR-37 (DSMZ, ACC 237), IGR-39 (DSMZ, ACC 239), RPMI-7951 (DSMZ, ACC 66), and CHL-1 (ATCC, CRL-9446)}. The murine DC line DC1 was generated in our laboratory following an established methodology (*38*).

S. typhimurium SL3261AT is an *aroA* metabolically defective strain on SL1344 background and is grown at 37°C in Luria broth.

In vitro infection with bacteria and treatment with pathogen-associated molecular patterns

Single bacterial colonies were grown overnight and restarted the next day to reach an absorbance at 260 nm (A_{600}) of 0.6 corresponding to 0.6×10^9 colony-forming units (CFUs)/ml. Murine and human melanoma cells were incubated with bacteria for 2 hours, at a cell-to-bacteria

ratio of 1:50, in the appropriate medium without antibiotics. Cells were washed with PBS and incubated in medium supplemented with gentamicin (50 μg/ml) for 12 or 24 hours to kill extracellular bacteria with or without IFN-γ (100 U/ml).

B16 cells and DC1 DCs were plated in six-well plates (2×10^5 cells per well) and grown for 18 hours. Cells were incubated with pathogen-associated molecular patterns (PAMPs) in complete medium with or without IFN-γ (100 U/ml) for 24 hours. The PAMPs used were LPS (1 μg/ml, Sigma), flagellin (0.1 μg/ml, Alexis), and LTA (10 μg/ml, Sigma).

Immunoblot analysis

Twenty-four hours after infection or treatment with PAMPs, melanoma cells were scraped in ELB buffer [250 mM NaCl, 0.5% NP-40, 50 mM Hepes (pH 7.0), 5 mM EDTA] containing 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and protease inhibitors. Cell lysates were run on SDS page and transferred onto Immobilon polyvinylidene difluoride membranes. Membranes were probed with a polyclonal rabbit antibody to Cx43 (Sigma), a monoclonal mouse antibody to Cx43 (Zymed), or a mouse antibody to vinculin (Sigma) overnight at 4°C and incubated with horseradish peroxidase–conjugated antibodies to mouse IgG (Calbiochem) or to rabbit IgG (Bio-Rad) for 1 hour. Visualization was carried out with enhanced chemiluminesence (GE Healthcare). Bands were quantified by densitometry with National Institutes of Health Image–based software Scion Image.

Immunofluorescence analysis

B16 cells were plated onto coverslips (5×10^4 cells per coverslip) and grown for 18 hours. Cells were infected with *Salmonella*. After 24 hours, the cells were fixed in 4% (w/v) paraformaldehyde in PBS for 15 min. Blocking and permeabilization were carried out by incubating the cells in PBS, 3% (w/v) bovine serum albumin, and 0.1% (w/v) Triton X-100 for 20 min. The cells were then stained with polyclonal rabbit primary antibody to Cx43 (1:2000, Sigma) and cyanine 3–conjugated secondary antibody to rabbit (1:400, Jackson ImmunoResearch Laboratories). Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Confocal microscopy was carried out with a Leica TCS-SP2 (Leica Microsystem).

shRNA-mediated knockdown

To generate stable clones of B16 and B16-OVA cells silenced for Cx43, we infected tumor cells with MISSION Lentiviral Transduction Particles specific for Cx43 (Sigma) following the recommended protocol. Four different lentiviruses were tested, of which three successfully knocked down Cx43. One of the successful lentiviruses and the unsuccessful lentivirus were cloned by limiting dilution and tested for silencing before and after bacterial infection. The unsuccessful clone was used as a negative control for the experiments (Ctrl-B16 and Ctrl-B16-OVA).

Mice treatments

B16 control cells (10^5) (Ctrl-B16; unsuccessfully silenced for Cx43) or 3×10^5 Cx43-interfered B16 cells (Cx43shRNAB16) were subcutaneously injected in the left flank of C57BL/6J mice (day 0), and after 3 days, 5×10^4 B16 wild-type cells were subcutaneously injected in the right flank. On days 11 and 15, 10^8 CFUs of *Salmonella* or PBS as a control were intratumorally injected in the left-flank tumors. The given dose of *Salmonella* was confirmed by plating bacterial dilutions on terrific broth agar plates. For the depletion of $CD8^+$ T cells, 100 μg of rat antibody to CD8 or isotype control antibody was injected in the peritoneum of the mice on days 10, 14, 18, 21, 24, and 27. Tumor growth was monitored by measuring the two visible dimensions with a caliper every 2 to 3 days. Statistical significance at each time point was calculated as described below.

For therapeutic vaccination, 10^5 B16 cells were subcutaneously injected into the left flank of C57BL/6J mice (day 0), and after 4 and 8 days (or 12 and 16 days in case of large tumors >0.4 cm^2), 3.5×10^5 DC1 DCs were subcutaneously injected into the right flank.

For preventive vaccination, 3.5×10^5 DC1 DCs were subcutaneously injected in the right flank of C57BL/6J mice (on days 0 and 4), and on day 4 (or day 21), 10^5 B16 cells were subcutaneously injected into the left flank.

The day before vaccination, Ctrl-B16 and Cx43shRNAB16 cells were infected in vitro with or without *Salmonella* and then cocultured with DC1 DCs in a 1:1 ratio. After 24 hours, DCs were purified by $CD11c^+$ selection [magnetic cell sorter (MACS), Miltenyi Biotec] and treated with mitomycin C (25 μg/ml) (Sigma) for 20 min at 37°C to arrest the proliferation of possible remaining tumor cells. For the comparison experiment of different vaccination protocols, DCs were also incubated with B16 lysate after three freezing and thawing cycles, B16 UV–treated cells, or a mix of TRP2 and GP100 peptide (1 μg/ml each).

Dye transfer assays

For microinjection assay, B16 cells were infected or not with *Salmonella*, and after 24 hours, 400 cells were microinjected under an inverted phase-contrast microscope (Axiovert 100, Zeiss) with a mixture of 2% Lucifer yellow CH (457.25 daltons, Sigma) and 1% tetramethylrhodamine dextran (70 kD, Molecular Probes) with an automated microinjection system at a pressure of 1200 hPa applied for 0.2 s.

For FACS assay, DCs were labeled with 10 μM DDAO (Molecular Probes) for 15 min at room temperature in the dark and then extensively washed with PBS. B16 cells were infected or not with *Salmonella* and, after 24 hours, labeled with 0.5 μM calcein-AM (Molecular Probes) in serum-free medium for 30 min at 37°C and then cocultured with DCs at a ratio of 2:1. Calcein transfer between tumor cells and DCs was evaluated by cytofluorimetry. The coculture of B16 or DCs was carried out in the presence or absence of 3.5 mM heptanol (Sigma).

For confocal assay, B16 cells were infected or not with *Salmonella* and, 24 hours later, stained with 3 μM calcein-AM in serum-free medium for 20 min at 37°C, whereas DCs, previously treated with LPS for 1 hour, were stained with 1 μM DDAO for 10 min at room temperature. After the staining, a drop of each population was plated in proximity to one another onto microscope slides and cells were coincubated for 1 hour. Dye transfer was visualized by a Leica SP2 Visible Laser Confocal Microscope. The movie was generated with Imaris software.

In vitro cross-presentation assay

B16 and B16-OVA cells were treated or not with *Salmonella* and, 24 hours later, stained with 5 μM CFSE for 20 min at 37°C in the dark and then washed with cold PBS. Where stated, B16 and B16-OVA cells were treated with 10 μM lactacystin (Sigma) overnight. Tumor cells were then added to DCs previously matured with LPS (1 μg/ml) and IFN-γ (100 U/ml) in a 1:1 ratio and left in coculture for 24 hours in the presence or absence of heptanol (3.5 mM). After 24 hours, cross-presentation of OVA peptide by DCs was analyzed by cytofluorimetry on $CD11c^+$ cells with an antibody that recognizes the OVA SIINFEKL peptide in association with MHC I (anti-K^bOVA, 25-D1.16).

Analysis of IFN-γ in the tumor mass

B16 control and Cx43shRNAB16-bearing mice were intratumorally injected with *Salmonella* or PBS as a control, and after 1, 3, and 7 days, tumors were removed and smashed in 1 ml of PBS containing 0.5% Triton X-100, incubated on ice for 1 hour, and centrifuged at 13,000 rpm at 4°C for 15 min. Supernatants were analyzed for the presence of IFN-γ by enzyme-linked immunosorbent assay (ELISA) (R&D Systems) according to the manufacturer's instructions.

Analysis of IFN-γ produced in vitro by OVA-specific CD8 T cells

B16-OVA control (Ctrl-B16-OVA) and Cx43-interfered B16-OVA (Cx43shRNAB16-OVA) were infected with *Salmonella* and cocultured with DCs previously matured with LPS (1 μg/ml) and IFN-γ (100 U/ml) in a 1:1 ratio. After 24 hours, $CD11c^+$ DCs were purified (MACS) and treated with mitomycin C (25 μg/ml) for 20 min at 37°C to arrest the proliferation of any remaining tumor cells. DCs (2×10^4) were cultured with 2×10^5 CD8 T cells purified from OTI mice with and without 1 μM $OVA_{(257–264)}$ peptide. After 48 hours, culture supernatant was assessed for IFN-γ by ELISA.

Statistical analysis

Student's paired *t* test was used to determine the statistical significance of the data. Significance was defined as $P < 0.05$ (two-tailed test and two-sample equal variance parameters). Statistic calculations and Kaplan-Meier survival curves were performed with JMP 5.1 software (SAS).

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/2/44/44ra57/DC1

Fig. S1. All of the murine and half of the human melanoma cell lines up-regulate Cx43 in response to *Salmonella*.

Fig. S2. Intratumoral bacterial injection increases the percentage of $Cx43^+$ DCs in lymph nodes.

Fig. S3. Loading of DCs with bacteria-treated tumor cells results in efficient antitumor vaccination in a therapeutic setting.

Fig. S4. Loading of DCs with bacteria-treated tumor cells results in efficient antitumor vaccination in a preventive setting.

Movie S1. Bacteria-induced up-regulation of Cx43 expression correlates with establishment of functional gap junctions.

REFERENCES AND NOTES

1. R. M. Steinman, J. Banchereau, Taking dendritic cells into medicine. *Nature* **449**, 419–426 (2007).
2. P. van der Bruggen, B. J. Van den Eynde, Processing and presentation of tumor antigens and vaccination strategies. *Curr. Opin. Immunol.* **18**, 98–104 (2006).
3. A. Paschen, S. Eichmuller, D. Schadendorf, Identification of tumor antigens and T-cell epitopes, and its clinical application. *Cancer Immunol. Immunother.* **53**, 196–203 (2004).
4. L. Chapatte, M. Ayyoub, S. Morel, A. L. Peitrequin, N. Lévy, C. Servis, B. J. Van den Eynde, D. Valmori, F. Lévy, Processing of tumor-associated antigen by the proteasomes of dendritic cells controls in vivo T-cell responses. *Cancer Res.* **66**, 5461–5468 (2006).
5. B. J. Nicholson, Gap junctions—from cell to molecule. *J. Cell Sci.* **116**, 4479–4481 (2003).
6. G. Meşe, G. Richard, T. W. White, Gap junctions: Basic structure and function. *J. Invest. Dermatol.* **127**, 2516–2524 (2007).
7. J. Neijssen, B. Pang, J. Neefjes, Gap junction-mediated intercellular communication in the immune system. *Prog. Biophys. Mol. Biol.* **94**, 207–218 (2007).
8. T. J. King, J. S. Bertram, Connexins as targets for cancer chemoprevention and chemotherapy. *Biochim. Biophys. Acta* **1719**, 146–160 (2005).
9. M. Mesnil, S. Crespin, J. L. Avanzo, M. L. Zaidan-Dagli, Defective gap junctional intercellular communication in the carcinogenic process. *Biochim. Biophys. Acta* **1719**, 125–145 (2005).
10. T. Bopp, C. Becker, M. Klein, S. Klein-Hessling, A. Palmetshofer, E. Serfling, V. Heib, M. Becker, J. Kubach, S. Schmitt, S. Stoll, H. Schild, M. S. Staege, M. Stassen, H. Jonuleit, E. Schmitt, Cyclic adenosine monophosphate is a key component of regulatory T cell–mediated suppression. *J. Exp. Med.* **204**, 1303–1310 (2007).
11. E. Oviedo-Orta, W. Howard Evans, Gap junctions and connexin-mediated communication in the immune system. *Biochim. Biophys. Acta* **1662**, 102–112 (2004).
12. H. Matsue, J. Yao, K. Matsue, A. Nagasaka, H. Sugiyama, R. Aoki, M. Kitamura, S. Shimada, Gap junction-mediated intercellular communication between dendritic cells (DCs) is required for effective activation of DCs. *J. Immunol.* **176**, 181–190 (2006).
13. J. Neijssen, C. Herberts, J. W. Drijfhout, E. Reits, L. Janssen, J. Neefjes, Cross-presentation by intercellular peptide transfer through gap junctions. *Nature* **434**, 83–88 (2005).
14. B. Pang, J. Neijssen, X. Qiao, L. Janssen, H. Janssen, C. Lippuner, J. Neefjes, Direct antigen presentation and gap junction mediated cross-presentation during apoptosis. *J. Immunol.* **183**, 1083–1090 (2009).
15. H. Benlalam, A. Jalil, M. Hasmim, B. Pang, R. Tamouza, M. Mitterrand, Y. Godet, N. Lamerant, C. Robert, M. F. Avril, J. Neefjes, T. Tursz, F. Mami-Chouaib, C. Kieda, S. Chouaib, Gap junction communication between autologous endothelial and tumor cells induce cross-recognition and elimination by specific CTL. *J. Immunol.* **182**, 2654–2664 (2009).
16. D. Bermudes, L. M. Zheng, I. C. King, Live bacteria as anticancer agents and tumor-selective protein delivery vectors. *Curr. Opin. Drug Discov. Devel.* **5**, 194–199 (2002).
17. J. M. Pawelek, K. B. Low, D. Bermudes, Tumor-targeted *Salmonella* as a novel anticancer vector. *Cancer Res.* **57**, 4537–4544 (1997).
18. M. Loeffler, G. Le'Negrate, M. Krajewska, J. C. Reed, IL-18-producing *Salmonella* inhibit tumor growth. *Cancer Gene Ther.* **15**, 787–794 (2008).
19. M. Loeffler, G. Le'Negrate, M. Krajewska, J. C. Reed, *Salmonella typhimurium* engineered to produce CCL21 inhibit tumor growth. *Cancer Immunol. Immunother.* **58**, 769–775 (2009).
20. J. Fensterle, B. Bergmann, C. L. Yone, C. Hotz, S. R. Meyer, S. Spreng, W. Goebel, U. R. Rapp, I. Gentschev, Cancer immunotherapy based on recombinant *Salmonella enterica* serovar Typhimurium *aroA* strains secreting prostate-specific antigen and cholera toxin subunit B. *Cancer Gene Ther.* **15**, 85–93 (2008).
21. U. Pertl, H. Wodrich, J. M. Ruehlmann, S. D. Gillies, H. N. Lode, R. A. Reisfeld, Immunotherapy with a posttranscriptionally modified DNA vaccine induces complete protection against metastatic neuroblastoma. *Blood* **101**, 649–654 (2003).
22. A. G. Niethammer, R. Xiang, J. M. Ruehlmann, H. N. Lode, C. S. Dolman, S. D. Gillies, R. A. Reisfeld, Targeted interleukin 2 therapy enhances protective immunity induced by an autologous oral DNA vaccine against murine melanoma. *Cancer Res.* **61**, 6178–6184 (2001).
23. F. Avogadri, C. Martinoli, L. Petrovska, C. Chiodoni, P. Transidico, V. Bronte, R. Longhi, M. P. Colombo, G. Dougan, M. Rescigno, Cancer immunotherapy based on killing of *Salmonella*-infected tumor cells. *Cancer Res.* **65**, 3920–3927 (2005).
24. F. Avogadri, D. Mittal, F. Saccheri, M. Sarrafiore, M. Ciocca, P. Larghi, R. Orecchia, M. Rescigno, Intra-tumoral *Salmonella typhimurium* induces a systemic anti-tumor immune response that is directed by low-dose radiation to treat distal disease. *Eur. J. Immunol.* **38**, 1937–1947 (2008).
25. N. K. Haass, K. S. Smalley, M. Herlyn, The role of altered cell–cell communication in melanoma progression. *J. Mol. Histol.* **35**, 309–318 (2004).
26. C. J. Wei, R. Francis, X. Xu, C. W. Lo, Connexin43 associated with an N-cadherin-containing multiprotein complex is required for gap junction formation in NIH3T3 cells. *J. Biol. Chem.* **280**, 19925–19936 (2005).
27. A. Porgador, J. W. Yewdell, Y. Deng, J. R. Bennink, R. N. Germain, Localization, quantitation, and in situ detection of specific peptide–MHC class I complexes using a monoclonal antibody. *Immunity* **6**, 715–726 (1997).
28. G. Fenteany, R. F. Standaert, W. S. Lane, S. Choi, E. J. Corey, S. L. Schreiber, Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**, 726–731 (1995).
29. G. Rotta, E. W. Edwards, S. Sangaletti, C. Bennett, S. Ronzoni, M. P. Colombo, R. M. Steinman, G. J. Randolph, M. Rescigno, Lipopolysaccharide or whole bacteria block the conversion of inflammatory monocytes into dendritic cells in vivo. *J. Exp. Med.* **198**, 1253–1263 (2003).
30. G. Rotta, G. Matteoli, E. Mazzini, P. Nuciforo, M. P. Colombo, M. Rescigno, Contrasting roles of SPARC-related granuloma in bacterial containment and in the induction of anti–*Salmonella typhimurium* immunity. *J. Exp. Med.* **205**, 657–667 (2008).
31. T. A. Groothuis, J. Neefjes, The many roads to cross-presentation. *J. Exp. Med.* **202**, 1313–1318 (2005).
32. M. L. Albert, B. Sauter, N. Bhardwaj, Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* **392**, 86–89 (1998).
33. A. L. Ackerman, C. Kyritsis, R. Tampé, P. Cresswell, Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nat. Immunol.* **6**, 107–113 (2005).
34. J. Banchereau, A. K. Palucka, Dendritic cells as therapeutic vaccines against cancer. *Nat. Rev. Immunol.* **5**, 296–306 (2005).
35. A. Mendoza-Naranjo, P. J. Saéz, C. C. Johansson, M. Ramírez, D. Mandakovic, C. Pereda, M. N. López, R. Kiessling, J. C. Sáez, F. Salazar-Onfray, Functional gap junctions facilitate melanoma

antigen transfer and cross-presentation between human dendritic cells. *J. Immunol.* **178**, 6949–6957 (2007).
36. N. E. Blachère, R. B. Darnell, M. L. Albert, Apoptotic cells deliver processed antigen to dendritic cells for cross-presentation. *PLoS Biol.* **3**, e185 (2005).
37. G. P. Dunn, A. T. Bruce, H. Ikeda, L. J. Old, R. D. Schreiber, Cancer immunoediting: From immunosurveillance to tumor escape. *Nat. Immunol.* **3**, 991–998 (2002).
38. C. Winzler, P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V. S. Zimmermann, J. Davoust, P. Ricciardi-Castagnoli, Maturation stages of mouse dendritic cells in growth factor–dependent long-term cultures. *J. Exp. Med.* **185**, 317–328 (1997).
39. **Acknowledgments:** We thank M. Chieppa for technical assistance in confocal microscopy. **Funding:** M.R. was supported by grants from the Association for International Cancer Research, the Associazione Italiana per la Ricerca sul Cancro, the European Molecular Biology Organization Young Investigator Programme, the European Commission (7th framework programme), and the Italian Ministry of Health (Ricerca Finalizzata). **Author contributions:** F.S. did most of the experiments, with significant assistance from C.P. F.S. and C.P. analyzed all data. M.R. designed the study, analyzed all data and wrote the paper, with editorial input from F.S. and P.F. F.A. performed some of the experiments. S.B. and M.F. designed and performed microinjection experiments. **Competing interests:** The authors declare that they have no competing interests.

Submitted 16 December 2009
Accepted 23 July 2010
Published 11 August 2010
10.1126/scitranslmed.3000739

Citation: F. Saccheri, C. Pozzi, F. Avogadri, S. Barozzi, M. Faretta, P. Fusi, M. Rescigno, Bacteria-induced gap junctions in tumors favor antigen cross-presentation and antitumor immunity. *Sci. Transl. Med.* **2**, 44ra57 (2010).