Dendritic Cells, Loaded with Recombinant Bacteria Expressing Tumor Antigens, Induce a Protective Tumor-specific Response[1]

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Abstract

Dendritic cells (DCs) are considered the most potent antigen-presenting cells and probably the only ones able to prime naive T cells. Indeed, DCs are distributed in tissues that interface the external environment, where they act as sentinels for incoming bacteria, viruses, and fungi. We have previously analyzed the capacity of DCs to interact with bacteria, and we have shown that bacteria can act as "Trojan horses," delivering heterologous proteins to DCs in a processed form that allows extremely efficient loading of both MHC class I and class II molecules. In this study, we have optimized the usage of recombinant bacteria as an antigen delivery system for DCs, with the aim to develop a new DC vaccination strategy in antitumor immunity. We have focused on a low immunogenic antigen, the tyrosinase-related protein-2 (Trp-2), a self-antigen expressed in mouse and human melanoma for which induction of antitumor immunity has proven to be very ineffective. We have given mice injections of either Trp-2/recombinant bacteria-loaded DCs or with bacteria alone engineered to express the Trp-2 melanoma antigen. We have shown that only DCs loaded with recombinant bacteria, but not with wild-type bacteria, were able to induce Trp-2-specific CTLs and immunity against the B16 tumor. Immunity was obtained in experiments of tumor vaccination as well as in experiments of tumor therapy. When therapy with bacteria-loaded DCs was performed in B16 tumor-bearing mice, 60% of the treated mice were tumor free 2 months after the initial tumor growth.

Introduction

Antigen delivery is a crucial field of research for vaccine development. Because antigen uptake, processing, and presentation dictate the type of induced T-cell response, a correctly designed delivery system offers a means of targeting antigens to the appropriate antigen-presenting cells. DCs[3], which are distributed in tissues interfacing the external environment, are ideally located for antigen uptake (1, 2). DCs professionally alert the immune system to the presence of invaders, initiate an immune response toward them, and may be regarded as target cells for vaccine development or as natural adjuvants (3, 4). The use of DCs as natural adjuvants in cancer therapy has been proposed recently (5), and clinical responses have been observed. Indeed, pilot clinical trials have shown, in some cases, complete or partial remission of the tumors in patients with melanoma, multiple myeloma, or lymphoma (6–9).

We have shown previously the capacity of live bacteria to induce the phenotypical and functional maturation of DCs (3, 4). In this study, we have optimized the usage of recombinant bacteria as an antigen delivery system for DCs, with the aim to develop a new DC vaccination strategy in antitumor immunity. We have established *in vitro* the best ratio and timing of infection of DCs with recombinant bacteria to achieve maximal presentation of the heterologous protein transduced in the bacterial strain. Thus, we have engineered an attenuated strain of *Salmonella typhimurium* (SL7207-AroA) to express tumor antigens in a form that allows extremely efficient loading of MHC class I molecules (10).

The recombinant bacteria expressed Trp-2, a self-antigen expressed in human and mouse melanoma. We have compared the antitumor activity of recombinant bacteria *versus* recombinant bacteria-loaded DCs in protection experiments against the B16 melanoma in a mouse model.

Materials and Methods

Cells and Reagents. The D1 cells are a long-term, growth factor-dependent, immature DC line that was originated from C57/BL6 spleen DCs (11). Culture medium was IMDM (Sigma) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Sigma), and 50 μM 2-mercaptoethanol (complete IMDM) with 30% supernatant from granulocyte/macrophage-colony stimulating factor-producing NIH/3T3 cells (R1 conditioned medium).

Recombinant Bacteria. Two constructs expressing the GST-OVA and GST-TRP2 fusion proteins under inducible LacZ prokaryotic promoter (ptac) were generated. A DNA segment coding for amino acids 47–275 of chicken egg albumin

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[3] The abbreviations used are: DC, dendritic cell; Trp, tyrosinase-related protein; IMDM, Iscove's modified Dulbecco's medium; GST, glutathione *S*-transferase; OVA, ovalbumin; IL, interleukin; CFU, colony-forming unit; WT, wild type; CTLL, IL-2-dependent CTL.

was amplified by PCR from pcDNA3 vector containing the *OVA* gene. In the amplification reaction, restriction sites for *Bam*HI and *Eco*RI were engineered at the 5′ and 3′ termini of the following primers: SENSE 224, 5′-CTT GGA TCC AAA GAC AGC ACC-3′; and ANTISENSE 224, 5′-GCT CGA ATT CTT CAG AGA CGC-3′.

Regarding the TRP2 protein, we amplified the sequence coding for amino acids 1–516 from the Trp-2 sequence inserted in the pcDNA3 vector [kindly provided by Dr. P. Dellabona, Dibit, San Raffaele Hospital (HSR) Milan, Italy]. Again, we engineered a *Bam*HI and a *Xba*I restriction site at the 5′ and 3′ termini using the following primers: SENSE 224, 5′-CAC GGA TCC GAA GAC ATG GGC CTT G; and ANTISENSE 224, 5′-ATG CCC CGG GTC CTC AGT GTA TCT CTT GC.

Both PCR products were then inserted in the pGEX-4T1 cloning vector (Pharmacia), in-frame with the carbossilic terminus of the GST protein. Heterologous protein expression was evaluated by Western blot analysis on bacterial cell extracts.

Preparation of Recombinant Bacteria for Assays with DCs. *S. typhimurium* strain SL7207 was grown at 37°C in brain heart infusion, whereas recombinant SL7207-GST and SL7207-Trp-2 were grown in the same medium supplemented with 50 μg/ml ampicillin. Single colonies were grown overnight and restarted the following day at 1:10 of the original volume up to an absorbance at 600, equal to an absorbance of 0.5. Heterologous protein expression was then induced with 1 mM isopropyl-1-thio-βD-galactopyranoside for 2 additional h at 30°C.

Antibodies and Cell Surface Phenotype. The following monoclonal antibodies were purchased from PharMingen: H-2D^b (28-14-8), CD80/B7.1 (1G10), IA/Ed (2G9), and CD86/B7.2 (GL1). Before labeling experiments, FcR blocking was performed by incubating cells with 2.4G2 (rat antimouse Fcγ II/III receptor, CD32) supernatant. Staining was carried out according to standard immunofluorescence techniques, and flow cytometry analysis was performed with a FACScan (Lysis II and Cell Quest software; Becton Dickinson).

Processing and MHC Class I Presentation of Bacterial OVA Transduced by *S. typhimurium* in D1 Cells. DCs were incubated with recombinant OVA-expressing SL7207 bacteria (SL7207-OVA) or GST-SL7207 for 2.5 h in the absence of antibiotics, washed, incubated for an additional 4 h in medium containing gentamicin and tetracyclin, and fixed in glutaraldehyde (0.001%) before adding the B3Z T-cell hybridoma. IL-2 production by B3Z T-cell hybridoma was measured as [^{3}H]thymidine incorporation by IL-2-dependent CTLs.

Induction of CTLs *in Vivo* with D1-loaded Cells . Two weeks after immunization with D1 spleen cells, loaded either with SL7207-OVA or with SL7207-Trp-2, were restimulated *in vitro* for 3 days with the specific peptides for OVA (SIINFEKL) or Trp-2 (SVYDEFWL, 9-mer) and subsequently with IL-2 (100 units/ml) for 2 additional days. The cytotoxic activity of restimulated CD8 cells was tested on RMA-S cells loaded or not with 1 μM Trp-2 peptide or on OVA-expressing-RMA and RMA as a control. ^{51}Cr release was measured as a parameter for cytotoxic activity.

Tumor Vaccination with D1-loaded Cells. D1 cells were loaded with bacteria (at a bacteria:D1 ratio of 50:1) for 1 h at 37°C in medium without antibiotics. Cells were then washed and incubated for an additional hour in medium containing gentamicin (50 μg/ml) and tetracyclin (10 μg/ml) to kill both extracellular and intracellular bacteria. C57/BL6 mice received injections s.c. (200 μl) three times, either with 3×10^5 bacteria-loaded D1 cells or with 1×10^7 CFUs of WT or recombinant bacteria, at 7-day intervals, and 2 weeks later, mice were challenged with 2.5×10^4 B16 tumor cells contralaterally. Two-dimensional tumor sizes were monitored every 2 days.

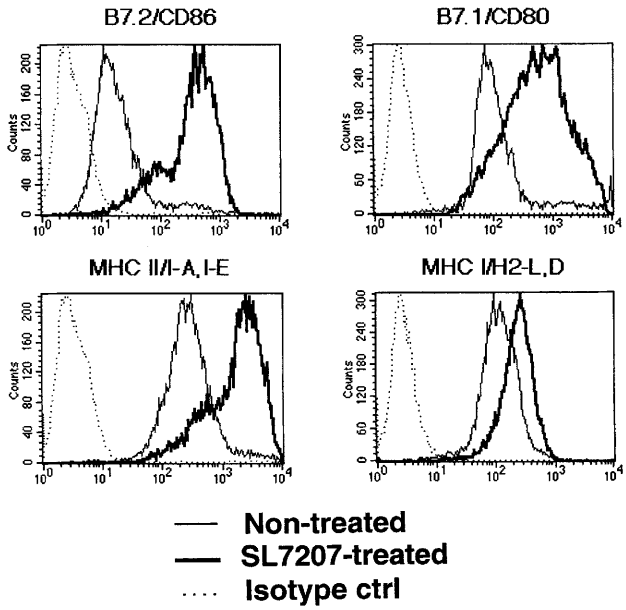

Fig. 1 DCs are activated by Gram-negative *S. typhimurium* AroA strain (SL7207). D1 cells were incubated with SL7207 for 18 h at a D1:bacteria ratio of 10:1. Up-regulation of costimulatory (B7.1 and B7.2) and MHC (I and II) molecules is shown by cytofluorimetry. *Thick line,* DCs activated with bacteria; *thin line,* untreated cells; *dotted line,* isotype control.

Tumor Therapy with D1-loaded Cells. D1 cells were loaded with bacteria (at a bacteria:D1 ratio of 50:1) for 1 hour at 37°C in medium without antibiotics. Cells were then washed and incubated for an additional hour in IMDM medium containing 5% FCS, gentamicin (50 μg/ml), and tetracyclin (10 μg/ml) to kill both intracellular and extracellular bacteria.

C57/BL6 mice were injected s.c. with 2.5×10^4 B16 tumor cells, and 4 and 8 days, later mice were treated with 3×10^5 bacteria-loaded DCs or with vehicle (IMDM). Two-dimensional tumor sizes were monitored every 2 days.

Results

DCs Are Activated by Gram-negative Bacteria and Can Present Heterologous Bacterial Antigens. Maturation of DCs is an essential process to gain APC functions. We have described previously that bacteria and bacterial products are among the most potent stimuli that induce DC maturation (3). Bacteria influence the transcriptional regulation of hundreds of genes involved in the activation of the antigen processing and presentation machinery. For instance, MHC I and II molecules, the PA28 proteasome subunit, as well as costimulatory molecules, are all up-regulated during bacteria-induced DC maturation (3). These modifications are dose dependent but already

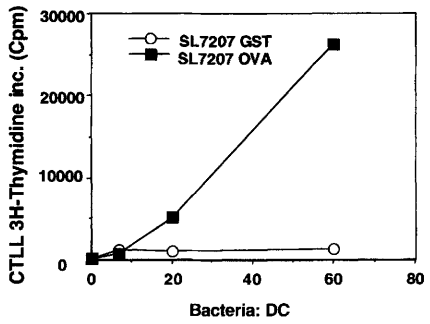

Fig. 2 DCs can process bacterial OVA derived from Gram-negative *S. typhimurium* AroA strain (SL7207) for MHC I presentation. DCs were incubated with recombinant OVA expressing SL7207 (SL7207-OVA, ■) or with WT SL7207 (SL7207-WT, ○) for 2.5 h in the absence of antibiotics, washed, and incubated for an additional 4 h in medium containing antibiotics. B3Z T-cell hybridoma was used as a read out for SIINFEKL OVA peptide presentation on MHC I molecules. IL-2 production was measured as [^{3}H]thymidine incorporation by CTLLs.

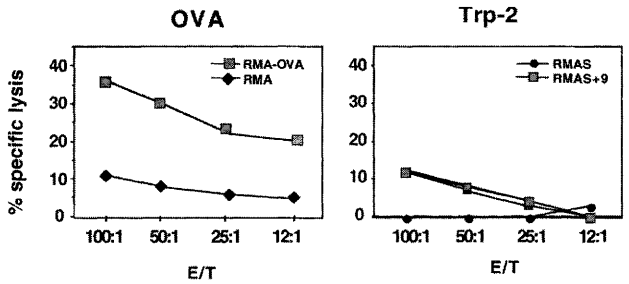

Fig. 3 Induction of OVA or Trp-2-specific CTLs *in vivo*. Spleen cells from mice immunized with D1 cells loaded with SL7207-OVA (*left*) or with D1 cells loaded with SL7207-Trp-2 (*right*) were restimulated *in vitro* with specific peptides (OVA, SIINFEKL; Trp-$2_{180-188}$, SVYDFFVWL, 9-mer). *Left panel,* cytotoxic activity of restimulated OVA-specific CD8 cells was tested on RMA cells expressing OVA (▨) or on RMA cells as a control (◆). *Right panel,* cytotoxic activity of restimulated Trp-2-specific CD8 cells was tested on RMA-S cells loaded with Trp-$2_{180-188}$: SVYDFFVWL (▨) or on unloaded RMA-S cells as a control (●). Two individual mice are plotted.

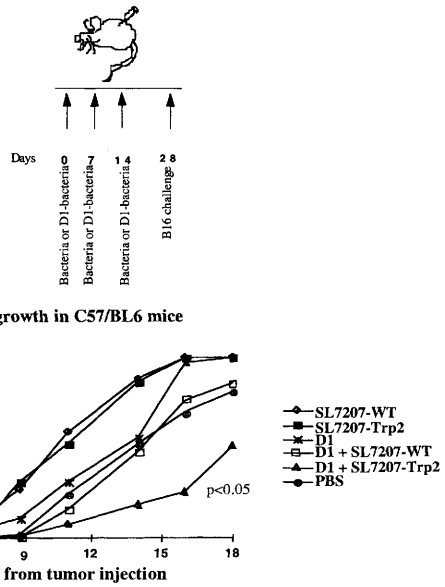

Fig. 4 Recombinant bacteria-loaded DCs partially protect mice from melanoma challenge. D1 cells were loaded with bacteria for 1 h at 37°C in medium without antibiotics; cells were then washed and incubated for an additional hour in medium containing gentamicin and tetracyclin to kill both extracellular and intracellular bacteria. Mice were injected three times with 3×10^5 cells or with 10^7 CFU of bacteria s.c. at 7-day intervals, and 3 weeks later, they were challenged with 2.5×10^4 B16 tumor cells contralaterally. Two-dimensional tumor sizes were monitored every 2 days.

maximal at a bacteria:DC ratio of 10:1 (Fig. 1) and are independent on the pathogenicity and on the class (Gram-positive *versus* Gram-negative) of bacteria tested (4).

Because the genetics of *S. typhimurium* are very well known and insertion of new genetic material is easy to perform, we have generated two recombinant bacterial strains of the auxotrophic *S. typhimurium* AroA strain SL7207. This strain is defective in the synthesis of aromatic amino acids; thus, after two to three divisions *in vivo,* it stops replicating. *S. typhimurium* AroA is still able to colonize mucosal epithelia of the mouse, but it is not lethal. Hence, we have expressed under-inducible LacZ promoter, a model antigen (chicken egg albumin, OVA), and the Trp-2, which is a tumor antigen of the melanoma B16, as fusion proteins with GST.

Dendritic cells loaded with salmonellae expressing either antigen were used to test presentation on MHC class I molecules both *in vitro* and *in vivo*. We first analyzed the presentation of OVA transduced by bacteria *in vitro*. D1 cells were incubated with recombinant OVA-expressing SL7207 bacteria (SL7207-OVA) or with GST-SL7207 (SL7207-GST) at different DC: bacteria ratios (Fig. 2). The capacity of DCs to process and present the "bacterial" OVA protein was assessed by the secretion of IL-2 by the B3Z hybridoma, which recognizes the SIINFEKL peptide in association with H2-k^b. The IL-2 produced was measured as [^{3}H]thymidine incorporation by CTLLs. To avoid unspecific proliferation of the CTLLs by cytokines secreted by DCs activated by bacteria, DCs were fixed in glutaraldehyde before adding the B3Z T-cell hybridoma. As shown in Fig. 2, D1 cells presented the OVA peptide to the B3Z hybridoma very well.

Bacteria-loaded DCs Can Induce Antigen-specific CTL Responses *in Vivo*. Because we confirmed the functionality of DCs *in vitro*, their ability to induce a CTL response *in vivo* was evaluated. DCs were loaded with *S. typhimurium* expressing either OVA (SL7207-OVA) or Trp-2 (SL7207-Trp-2; at a bacteria:D1 ratio of 50:1) for 1 h in medium without antibiotics. Cells were then washed and incubated for an additional hour in medium containing gentamicin and tetracyclin to kill both extracellular and intracellular bacteria. Bacteria-loaded D1 cells (3×10^5) were injected twice in mice s.c. Two weeks after the last injection, spleens were collected, and specific CTL responses were measured. Interestingly, we found that with this protocol of immunization, we could induce CTLs not only to a foreign antigen such as the OVA but also to the self-antigen Trp-2 (Fig. 3). Thus, presentation of bacterial antigens in asso-

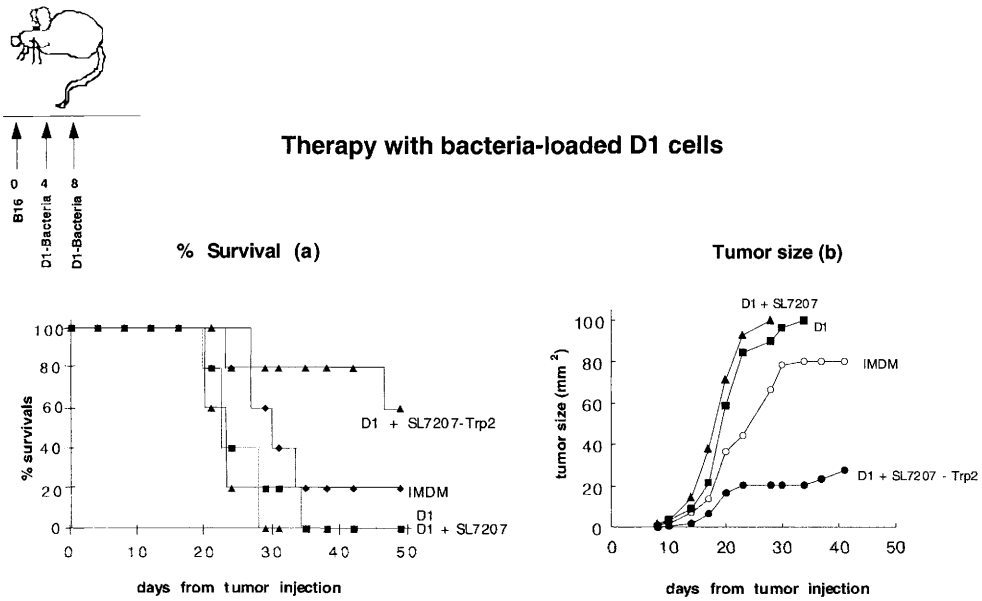

Fig. 5 Recombinant bacteria-loaded DCs limit tumor growth of pre-established melanoma. B16 tumor cells (2.5×10^4) were injected s.c. to C57/BL6 mice, and 4 and 8 days later, mice were treated with 3×10^5 bacteria-loaded DCs or with vehicle (IMDM). *a,* survival in mice was observed in 60% of the B16 tumor-bearing mice 50 days after treatment. *b,* tumor size. Only mice bearing a tumor and treated with DCs loaded with Trp-2-expressing bacteria (*SL7207-Trp2*) controlled the tumor size, whereas mice treated with DCs loaded with WT bacteria (*SL7207*) or untreated (*D1*) could not control the tumor growth.

ciation with class I molecules is not restricted to a particular heterologous antigen.

DCs Loaded with *S. typhimurium* Expressing the Trp-2 Melanoma Antigen Induce Antitumor Immunity against B16 Melanoma. To correlate the generation of specific CTLs with antitumor immunity to B16 melanoma, C57/BL6 mice were vaccinated with D1 cells loaded with SL7207-WT or with SL7207-Trp-2 and challenged with the B16 melanoma. To determine the efficiency of antitumor immunity, mice were also immunized with bacteria alone, either SL7207-WT or SL7207-Trp-2. 3×10^5 cells or 10^7 CFUs of bacteria were injected s.c. three times at 7-day intervals, and 2 weeks later, mice were challenged with 2.5×10^4 B16 tumor melanoma cells contralaterally. Interestingly, injection of bacteria alone, both WT and Trp-2-expressing salmonellae, increased the rate of tumor growth as compared with vehicle-injected mice (Fig. 4). Conversely, D1 cells loaded with Trp-2-expressing bacteria, but not with WT-expressing bacteria, caused a significant delay in tumor growth in comparison with the groups of mice immunized with either vehicle or untreated D1 cells (Fig. 4). Thus, D1 cells loaded with recombinant bacteria, but not bacteria alone, are able to induce antitumor immunity against B16 melanoma.

DCs Loaded with *S. typhimurium* Expressing the Trp-2 Melanoma Antigen Are Therapeutic and Induce Protective Immunity against B16 Melanoma. To determine the efficiency of DC-based antitumor therapy in mice bearing the B16 melanoma, we have injected 2.5×10^4 B16 tumor cells s.c. to C57/BL6 mice, and 4 and 8 days later, mice were treated with 3×10^5 bacteria-loaded DCs or with vehicle (IMDM). As shown in Fig. 5*a,* survival in mice was observed in 60% of the B16 tumor-bearing mice after >50 days from treatment. Moreover, 33% of mice that survived, when rechallenged with B16 tumor cells, remained tumor free, thus indicating that a protective antitumor response had been generated (data not shown). Tumor size correlated with mice survival; only mice treated with DCs loaded with Trp-2-expressing bacteria (SL7207-Trp-2) controlled the tumor size (Fig. 5*b*), whereas mice treated with DCs loaded with WT bacteria (SL7207) or untreated (D1) could not control the tumor growth. Thus, D1 cells loaded with recombinant Trp-2 bacteria, but not WT bacteria are able to induce specific and protective antitumor immunity against pre-established B16 melanoma.

Discussion

Immature DCs accomplish a sentinel function by sampling and processing encountered antigens and by loading them on MHC molecules (3). Inflammatory mediators can induce a maturation process that triggers migration of DCs to secondary lymphoid organs, where they can present previously captured and processed antigens and prime T cells (12). For this central role of DCs in initiating an immune response, the use of DCs in therapy of cancer or of viral infections has been proposed recently (6–8). Assisting the immune system to mount a potent immune response toward antigens that are not foreign to the body, as tumor antigens, or to viral antigens is the aim of DC-based immune therapy. Pilot clinical trials using DCs loaded with tumor peptides are, indeed, very promising, showing, in some cases, complete or partial remission of the tumors in patients with melanoma (6, 8) or multiple myeloma (7).

Resistance to tumors is often associated with little infiltration of DCs and lymphocytes in tumor masses or with inactivation of DCs and T lymphocytes because of factors secreted by tumor cells, such as transforming growth factor-β and vascular endothelial growth factor (13, 14). DCs could be relevant for both activation of tumor antigen-specific T cells and for the recruitment of T cells at tumor sites through secretion of chemokines and cytokines. The activation state of DCs, which are to be used in immune therapy is crucial, because it has been shown that immature DCs could induce tolerance rather than

immunity (15). Thus, for a good success of DC-based therapy, DCs have to be activated and have to be able to present antigens on MHC class I molecules to induce cytotoxic responses.

Several genetic or nongenetic strategies have been described to deliver antigens into DCs. Viral (adenoviral and retroviral) as well as nonviral vectors have been used to transfect DCs or DC progenitors (16–19). Genetic modification of DCs guarantees that the tumor antigenic peptides are presented on MHC class I molecules for stimulation of cytotoxic T cells. In nongenetic strategies, the antigen can be delivered in the form of peptides (20–23) or recombinant proteins. The use of the peptide is restricted by HLA matching, whereas the soluble recombinant protein is only poorly presented on MHC I molecules. If instead the antigen is introduced via phagocytosis or receptor-mediated endocytosis, it can enter both MHC class I and II pathways of antigen presentation (10, 24, 25). In this study, we have proposed a new strategy to introduce exogenously produced antigens on MHC I molecules. As first described in macrophages (26), DCs can also present exogenous bacterial antigens on MHC class I molecules (10); thus, we have engineered bacteria to express genes codifying for tumor antigens.

Because the molecular mechanisms leading to the presenting DC phenotype are mostly unknown, we took advantage of a DC culture system, developed in our laboratory (11), which enabled us to grow mouse spleen immature DCs. This unique culture system has proven to be very useful for studying the DC maturation process induced by bacteria (10). We have shown previously that bacterial activation of DCs induces transient *de novo* synthesis of MHC class I molecules and an increase of their stability (10). The synthesis of MHC class I molecules is slow and reaches a peak at 18 h, which coincides with maximal DC activation. Moreover, the half-life of newly synthesized MHC class I molecules is increased 3-fold, changing from 3 to 9 h (10). Stabilization of MHC class I molecules would guarantee that antigen loaded-DCs could accomplish their final task of presenting the heterologous antigen to naive CTLs. This is particularly relevant in view of a therapeutic use of DCs in cancer immune therapy. Indeed, the stability of MHC class I molecules is unvaried when monocyte-derived DCs are treated only with bacterial products, such as lipopolysaccharide (27), suggesting that whole bacteria deliver signals leading to the full activation of DCs. Presentation of bacterial antigens is not a peculiarity of the strain of bacteria used, because we observed presentation on MHC I molecules when we engineered both Gram-positive (*Streptococcus gordonii*) or Gram-negative (*S. typhimurium* and *Escherichia coli*) bacteria with heterologous antigens.

Although many reports in the mouse have shown that DC-based vaccines are effective both in protection and in therapy against tumors, in most experiments foreign model antigens were used as tumor antigens. In our study, we have focused on Trp-2 because it is a low immunogenic self-antigen, shared in mouse and human melanoma (28), and induction of antitumor immunity has proven to be very ineffective. We have given mice injections of either Trp-2/recombinant bacteria-loaded DCs or with bacteria alone engineered to express the Trp-2 melanoma antigen. We have shown that DCs loaded with recombinant bacteria, but not with WT bacteria, were able to induce Trp-2-specific CTLs and antitumor immunity. Interestingly, when we compared the capacity of bacteria alone with bacteria-loaded DCs to induce antitumor immunity, we found that bacteria alone, both recombinant and WT, augment the rate of tumor growth. This result was unexpected, and it is of difficult interpretation. We can only speculate that because the bacteria were injected contralaterally, the infectious site was more powerful in the recruitment of activated T cells, depriving the tumor site of antigen-specific T cells. Interestingly, the efficacy of bacteria-loaded DCs was higher in a therapeutic setting against established B16 tumors. Two injections of DCs were sufficient to block tumor growth in 60% of mice. Moreover, 33% of these mice showed protection against rechallenge with B16 tumor cells, indicating that this therapeutic protocol can lead to immunological memory.

Overall, these results indicate that for a rational design of new vaccines aimed to stimulate DCs, bacteria are one of the best candidates as vectors. Although the protocols of immunization and antigen loading, as well as route of administration, still need some refinements, these data hold promises for the use of bacteria as Trojan horses in DC therapy of cancer.

Acknowledgments

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