KBMA *Listeria monocytogenes* is an effective vector for DC-mediated induction of antitumor immunity

Mojca Skoberne,[1] Alice Yewdall,[1] Keith S. Bahjat,[2] Emmanuelle Godefroy,[1] Peter Lauer,[2] Edward Lemmens,[2] Weiqun Liu,[2] Will Luckett,[2] Meredith Leong,[2] Thomas W. Dubensky,[2] Dirk G. Brockstedt,[2] and Nina Bhardwaj[1]

[1]Cancer Institute, New York University School of Medicine, New York, New York, USA. [2]Anza Therapeutics, Concord, California, USA.

Vaccine strategies that utilize human DCs to enhance antitumor immunity have yet to realize their full potential. Approaches that optimally target a spectrum of antigens to DCs are urgently needed. Here we report the development of a platform for loading DCs with antigen. It is based on killed but metabolically active (KBMA) recombinant *Listeria monocytogenes* and facilitates both antigen delivery and maturation of human DCs. Highly attenuated KBMA *L. monocytogenes* were engineered to express an epitope of the melanoma-associated antigen MelanA/Mart-1 that is recognized by human $CD8^+$ T cells when presented by the MHC class I molecule HLA-A*0201. The engineered KBMA *L. monocytogenes* induced human DC upregulation of costimulatory molecules and secretion of pro-Th1 cytokines and type I interferons, leading to effective priming of Mart-1–specific human $CD8^+$ T cells and lysis of patient-derived melanoma cells. KBMA *L. monocytogenes* expressing full-length NY-ESO-1 protein, another melanoma-associated antigen, delivered the antigen for presentation by MHC class I and class II molecules independent of the MHC haplotype of the DC donor. A mouse therapeutic tumor model was used to show that KBMA *L. monocytogenes* efficiently targeted APCs in vivo to induce protective antitumor responses. Together, our data demonstrate that KBMA *L. monocytogenes* may be a powerful platform that can both deliver recombinant antigen to DCs for presentation and provide a potent DC-maturation stimulus, making it a potential cancer vaccine candidate.

Introduction

Melanoma is one of the most rapidly growing cancers worldwide, yet there is no satisfactory treatment either in the adjuvant setting or when it has advanced to metastatic disease. Several approaches to boost antitumor immune responses in melanoma have been attempted (1). While a few of these have successfully elicited high frequencies of melanoma-associated antigen–reactive T cells (2–4), their induction does not correlate with tumor regression. Complex and tailored therapies (e.g., adoptive T cell therapy or administration of anti-CTLA antibodies), have shown some success in reducing high tumor burden (5–7), albeit with high-grade toxicity. Therefore, alternative approaches are needed.

DCs are attractive cellular adjuvants that can increase host resistance to tumors (8). To optimally prime naive T cells, DCs must not only present high levels of MHC-peptide complexes but must also undergo activation and maturation. An ideal DC-targeting vaccine formulation, therefore, should simultaneously deliver antigens and activate DCs to enhance expression of surface MHC-peptide complexes and costimulatory molecules and production of cytokines that prime potent $CD8^+$ and $CD4^+$ T cell responses. To date, there is no standard, efficient, and cost-effective method that combines antigen loading and activation/maturation of DCs for clinical immunotherapy.

Listeria monocytogenes are Gram-positive facultative intracellular bacteria that are internalized by phagocytic and nonphagocytic cells. Previous studies have shown that self or heterologous antigens encoded by *L. monocytogenes* or the recombinant *L. monocytogenes* strains can access endogenous and exogenous antigen processing and presentation pathways to prime $CD8^+$ and $CD4^+$ T cells, respectively. Following internalization, *L. monocytogenes* gain access to the cytosol of APCs from the phagosome through expression of listeriolysin O (LLO). In addition, despite its preferential intracytoplasmic location, *L. monocytogenes* very efficiently prime $CD4^+$ T cell responses (9). These characteristics have made *L. monocytogenes* an attractive and efficacious platform in murine models of cancer and microbial infection. In humans, infection with live WT or recombinant *L. monocytogenes* activates DCs in vitro (10); however, for broad clinical application one must address pathogenicity while preserving immunogenicity of recombinant *L. monocytogenes* vaccines.

The potency of killed but metabolically active (KBMA) recombinant *L. monocytogenes* was recently demonstrated in an animal model of tumor immunity (11). KBMA *L. monocytogenes* are rendered noninfectious by the combined use of psoralen and long-wave UV light (UVA) and abrogation of nucleotide excision repair through deletion of the bacterial *uvrAB* genes. While KBMA *L. monocytogenes* cannot propagate, they remain metabolically active and express their genetic repertoire, including the heterologous

Nonstandard abbreviations used: Cr-51, Chromium-51; ELISPOT, enzyme-linked immunospot; HK, heat killed; Inl, internalin; KBMA, killed but metabolically active; LLO, listeriolysin O; *LmAg*, HLA-A*0201–restricted $CD8^+$ T cell epitopes of MP and melanoma-associated antigen MelanA/Mart-1 protein; *LmNY*, *L. monocytogenes* encoding full-length NY-ESO-1 protein; MC, a cocktail of IL-6, IL-1β, TNF-α, and PGE_2; moDC, monocyte-derived DC; MP, influenza matrix protein; S-59, synthetic psoralen DNA cross-linking agent; UVA, long-wave UV light.

Conflict of interest: T.W. Dubensky Jr., D.G. Brockstedt, K.S. Bahjat, P. Lauer, M. Leong, W. Luckett, W. Liu, and E. Lemmens are or were employees of Anza Therapeutics, which owns intellectual property covering the compositions and methods described in this manuscript. N. Bhardwaj was a former Science Advisory Board (SAB) member of Anza Therapeutics at the time of the study design. In addition, Anza Therapeutics employees hold stock options in the company. N. Bhardwaj is a coinventor of patents pertaining to human dendritic cell preparation.

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antigens they were engineered to express (11). Most importantly, KBMA *L. monocytogenes* induced cytolytic $CD8^+$ T cell responses to an endogenous tumor epitope and reduced tumor burden in a therapeutic CT-26 tumor model (10).

Several murine studies have provided us with evidence of the fundamental relationship between *L. monocytogenes* and endogenous DCs during an infection. Priming of naive $CD8^+$ T cells to *L. monocytogenes*–specific antigens is mediated by DCs (12). Contrary to previous assumptions, DCs are also required to maximally activate a recall response to various pathogens, including *L. monocytogenes* (13). These 2 events may be related to the fact that DCs are necessary for the efficient entry of *L. monocytogenes* into the spleen, in which T cell activation takes place (14). All together, this makes *L. monocytogenes* a promising candidate for development of human DC-targeting vaccines.

We report here that KBMA recombinant *L. monocytogenes* can be used as an antigen-loading vector to target human DCs. KBMA *L. monocytogenes* encoding tumor antigens induce DC maturation, secretion of Th1-polarizing cytokines and type I interferons, and expression of the recombinant tumor antigen and confer potent antigen priming activity to these APCs. Once infected with KBMA *L. monocytogenes* expressing full-length NY-ESO-1 protein, DCs presented antigens in the context of both class I and class II MHC molecules by donors with different MHC haplotypes. Furthermore, antigen-primed $CD8^+$ T cells acquired effector function and lysed a patient-derived melanoma tumor cell line. Thus, recombinant *L. monocytogenes* are a unique antigen delivery vehicle that can mature DCs for T cell priming, while simultaneously delivering both $CD8^+$ and $CD4^+$ T cell–restricted epitopes for presentation, independently of patient's MHC restriction.

Results

Deletion of selected virulence proteins preserves the DC activation potential of L. monocytogenes. The ability of *L. monocytogenes* to infect phagocytic cells, as well as to access the host cell cytosol following the escape from the phagolysosome, renders these bacteria a suitable vaccine platform for the delivery of antigens into the endogenous and exogenous pathways of antigen processing by DCs. It has been reported that WT *L. monocytogenes* (i.e., fully infectious and replicating *L. monocytogenes*) are extremely efficient at activating human monocyte-derived DCs (moDCs) (10). Their use, however, is contraindicated in humans. Therefore, we first tested whether highly attenuated strains of *L. monocytogenes*, which are suitable for clinical applications, retain their ability to efficiently activate DCs in vitro. The bacterial genes *actA, inlB*, and *hly* encode 3 important *L. monocytogenes* virulence determinants: ActA, which mediates actin polymerization; internalin B (inlB), which facilitates cell binding and invasion; and pore-forming LLO, which permits escape from phagolysosomes to cytosol. *L. monocytogenes* strains attenuated by deletion of either both the *actA* and *inlB* genes (Δ*actA*Δ*inlB L. monocytogenes*) or the *hly* gene (ΔLLO *L. monocytogenes*) were compared with WT *L. monocytogenes* in their ability to stimulate DCs to upregulate maturation markers, secrete proinflammatory cytokines, and stimulate allogeneic T cells. As anticipated, presence of LLO enhanced activation of DCs (Figure 1). Confirming previously reported data (15), deletion of the *hly* gene, to some extent, impaired the ability of DCs to upregulate the costimulatory molecules CD80 and CD83 (Figure 1, A and B) as well as the proinflammatory and pro-Th1 cytokines IL-1β (data not shown), TNF-α, and IL-12p70 (Figure 1C). However, the difference was only observed at lower MOI. Activation of DCs with Δ*actA*Δ*inlB L. monocytogenes* did not statistically significantly impair DC maturation when compared with the parent strain (Supplemental Table 1). In accordance with these findings, DCs infected with any of the attenuated strains induced proliferation of allogeneic T cells comparable to control DCs matured with a cocktail of inflammatory cytokines (a combination of IL-6, IL-1β, TNF-α, and PGE_2 hereafter referred to as MC) (Figure 1D). Thus, the ability to induce DC maturation was not impaired with the live-attenuated *L. monocytogenes* strain deleted of 2 virulence factors ActA and InlB.

KBMA L. monocytogenes are stimulatory for human DCs. While heat-killed (HK) vaccines are safer to use, this is often at the expense of their immunogenicity. To further improve safety, we engineered a KBMA *L. monocytogenes* vaccine candidate on the Δ*actA*Δ*inlB*Δ*uvrAB L. monocytogenes* background as described previously (11). KBMA *L. monocytogenes* could no longer replicate, but they maintained metabolic activity, retained the ability to exit the phagolysosome, and escaped into the cytosol only where they colocalized with actin (Figure 2A). Therefore, recombinant antigen-expressing KBMA *L. monocytogenes*, in contrast to killed *L. monocytogenes*, have the potential to deliver antigens to the endogenous antigen processing pathway, a necessity for a successful vaccine vector, without the associated risks of a replicating live bacterium. The KBMA *L. monocytogenes* strain was compared with live, killed, and metabolically nonactive *L. monocytogenes* (inactivated with a high synthetic psoralen DNA cross-linking agent [S-59] concentration) with the same genetic background (Figure 2 and Supplemental Figure 1). The ability of KBMA *L. monocytogenes* to activate DCs was compared with well-described maturation stimuli LPS, poly I:C; or MC. Live Δ*actA*Δ*inlB*Δ*uvrAB L. monocytogenes* were as effective as 2 positive controls (LPS and MC) in terms of activating DCs (upregulation of typical maturation markers; Figure 2, B and C). Furthermore, the KBMA *L. monocytogenes* vaccine strain induced maturation of DCs to a similar extent as live bacteria, provided a higher MOI was used (10 vs. 1 MOI) (Figure 2, B and C). A similar pattern was observed with cytokines secreted by DCs upon infection (Figure 2, D–F). Live, KBMA *L. monocytogenes*, and LPS induced TNF-α, IL-6, and IL-12p70 to comparable levels and live and KBMA *L. monocytogenes* induced these to higher levels than HK *L. monocytogenes* (Figure 2D). Similar results were obtained with $CD11c^+$ DCs isolated from blood of healthy donors (Supplemental Figure 2). KBMA and live *L. monocytogenes* were equivalent inducers of MCP-3 and RANTES, 2 chemokines that recruit T cells (Figure 2E).

WT *L. monocytogenes* but not HK *L. monocytogenes* induce type I IFN transcription and secretion in mouse DCs (16). Similarly, secretion of type I IFN-α has also been detected following infection of murine macrophages with WT but not Δ*hly L. monocytogenes* strains (17). As shown in Figure 2F, we found that substantial amounts of IFN-α were produced by human moDCs upon exposure to live and KBMA *L. monocytogenes* but not HK or Δ*hly L. monocytogenes*, confirming the requirement for cytoplasmic entry of bacteria.

CCR7 is a chemokine receptor that recognizes the chemokines CCL19 and CCL21 produced in lymphatic vessel endothelial cells and T cell areas of lymph nodes and is therefore essential for trafficking of DCs to lymph nodes. Flow cytometry analysis revealed that infection with live and KBMA *L. monocytogenes* induced expression of CCR7 on DCs (Figure 2, G and H). The onset of CCR7 expression was rapid, commencing 6 hours after infection, with high levels maintained 24 hours after infection. The levels and kinetics of expression of CCR7 were comparable among LPS-matured DCs or DCs infected with live or KBMA *L. monocytogenes*

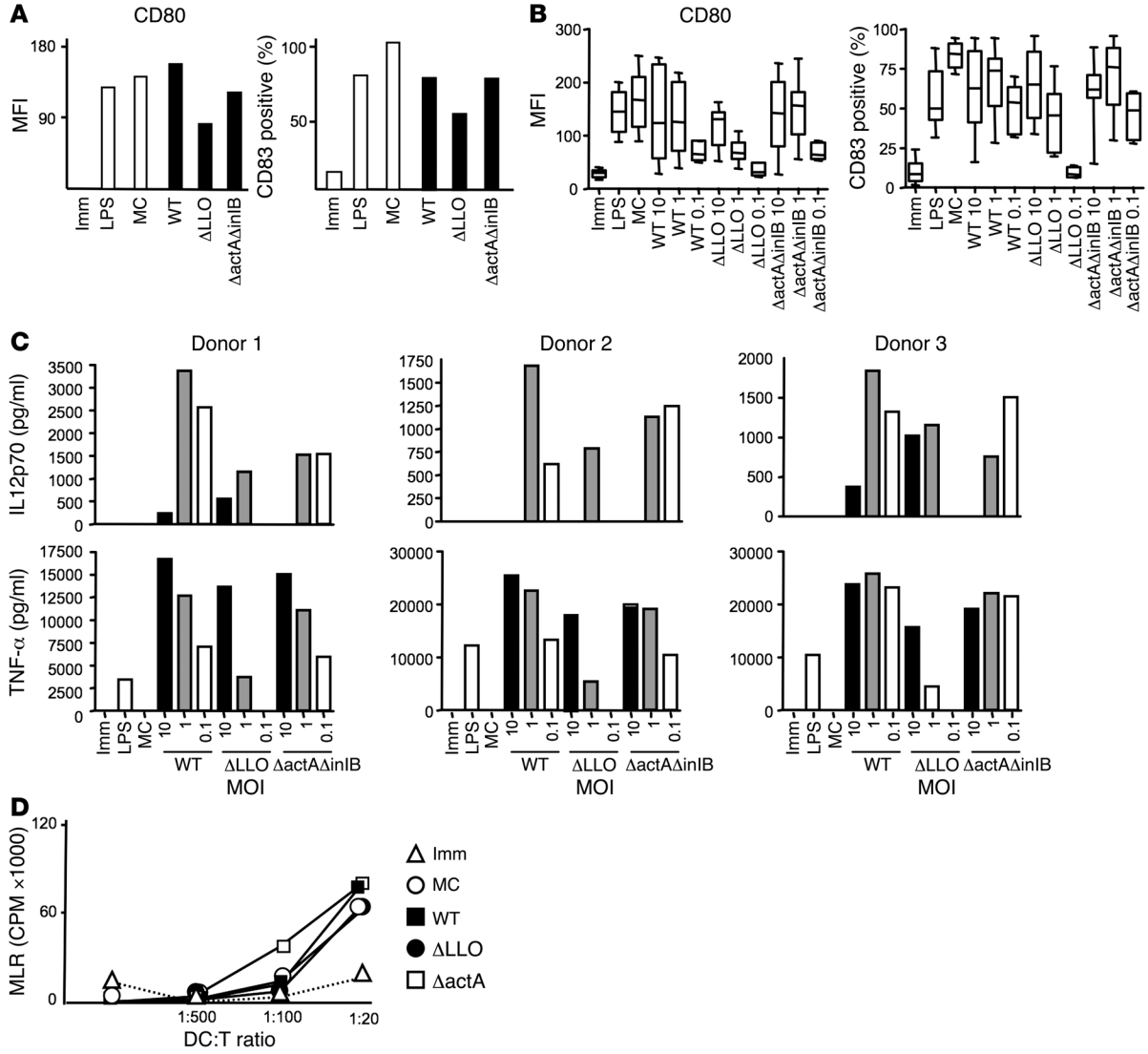

Figure 1

Deletion of virulence factors and immunogenicity of *L. monocytogenes*. Live-attenuated *L. monocytogenes* strains (*Lm*) deleted of either *hly* (ΔLLO) or *actA* and *inlB* (ΔactAΔinlB) were compared with WT *L. monocytogenes*, LPS, unstimulated (Imm), or a cocktail of inflammatory cytokines (MC) in their potency to mature human moDCs. (**A** and **B**) DCs were harvested 40 hours after infection with *L. monocytogenes* (MOI 1), and expression of CD80 and CD83 was assessed by flow cytometry. (**A**) Data for a single donor (**B**) and median value (horizontal line) and range (vertical line) with 25th and 75th percentiles (bars) of at least 7 individual results are shown. (**C**) At the same time supernatants from control and *L. monocytogenes*–infected DC cultures were collected (white bars, MOI 0.1 or controls; gray bars, MOI 1; black bars, MOI 10) for quantitation of IL-12p70 and TNF-α by cytokine bead array assay. Results of 3 representative donors are shown. (**D**) DCs were generated as above and cultured with allogeneic naive $CD4^+$ T cells for 4 days. T cell proliferation was measured by incorporation of radioactive thymidine during the last 16 hours. A representative experiment of 3 is shown.

strains and were superior to HK *L. monocytogenes*–infected DCs. Altogether, our data indicate that the coincubation of the KBMA *L. monocytogenes* vaccine strain with human DCs leads to full maturation of DCs and optimally equips them for priming.

KBMA L. monocytogenes encoding recombinant antigens access the endogenous pathway and stimulate antigen-specific $CD8^+$ T cells. To test the ability of KBMA bacteria to load antigen into the MHC class I pathway of APCs, Δ*actA*Δ*inlB*Δ*uvrAB L. monocytogenes* were constructed to express HLA-A*0201–restricted $CD8^+$ T cell epitopes of influenza matrix protein (MP) and melanoma-associated antigen MelanA/Mart-1 protein (*Lm*Ag). DCs infected with KBMA *Lm*Ag at MOI 100 were compared with DCs infected with live *Lm*Ag (MOI 10) (Figure 3A). DCs infected with empty vector served as negative controls. Forty hours after infection, the DCs were washed and cocultured with $MP_{58–66}$- or $MART1_{26–35}$-specific $CD8^+$ T cell clones for 24 hours. The number of cells synthesizing IFN-γ was enumerated by enzyme-linked immunospot (ELISPOT) assay. It should be pointed out that in this assay the number of responding T cell clones depends on multiple factors and not every clone will respond, even when DCs are optimally loaded with antigens (18). Activation of antigen-specific T cell clones was observed with HLA-A*0201$^+$ DCs infected with either

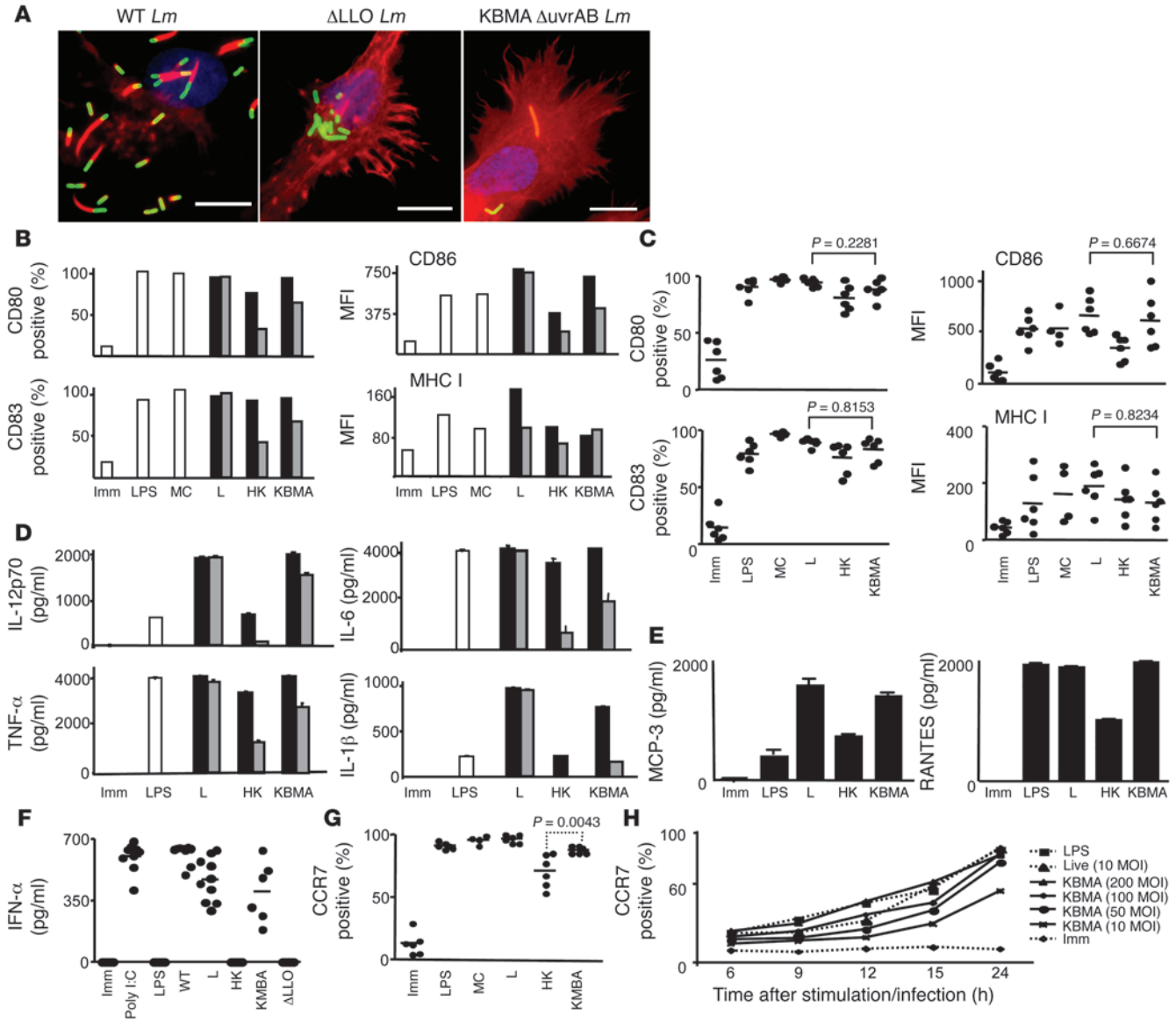

Figure 2

KBMA *L. monocytogenes* activate moDCs. (**A**) DCs were infected with *L. monocytogenes* strains, stained with antibodies to *L. monocytogenes* (green), phalloidin (red), and DAPI (blue). Visualization of polymerized actin and colocalization with the bacteria (yellow) confirms the presence of bacteria within the cytoplasm. Scale bar: 10 μm. (**B** and **C**) DCs were infected with live (L), HK, or KBMA *L. monocytogenes*. Unstimulated or DCs, exposed to LPS or MC, were used as controls. Expression of DC-maturation markers for a typical donor is shown (gray bars, MOI 1; black bars, MOI 10) (**B**). Results of multiple donors are shown in **C** (MOI 10). (**D** and **E**) Presence of cytokines (**D**) and chemokines (**E**) was measured by ELISA (gray bars, MOI 1; black bars, MOI 10). (**F**) DCs were infected at MOI 10 (WT, L) or MOI 200 (ΔLLO, HK, and KBMA *L. monocytogenes*). Immature, LPS, or poly I:C–stimulated DCs were used as controls. IFN-α in supernatants was measured. (**G**) DCs were infected with live, HK, or KBMA *L. monocytogenes* and left untreated or stimulated with LPS or MC. CCR7 was measured by flow cytometry after 40 hours. (**H**) DCs were infected with live or KBMA *L. monocytogenes*. CCR7 was measured at indicated time points. Numbers in parentheses indicate MOI used for infection. Nonstimulated or LPS-stimulated DCs were used as controls. In **C**, **F**, and **G**, values from individual donors (dots), mean values (lines), and Student's *t* test *P* values are shown. In **A**, **B**, **D**, **E**, and **H**, a representative experiment of at least 3 performed is shown. In **D** and **E**, error bars represent SD of triplicate culture wells.

live or KBMA *Lm*Ag, demonstrating that both live or KBMA *L. monocytogenes* can deliver antigen into the MHC class I pathway of antigen processing. No responses were observed either when HLA-A*0201$^+$ donor DCs were infected with the empty vector or when HLA-A*0201$^-$ donor DCs were infected with *Lm*Ag, thus confirming antigen specificity. Replication was not necessary for the presentation of recombinant antigens, since KBMA *L. monocytogenes* strains were able to induce effector responses from antigen-specific T cells. Both live and KBMA *L. monocytogenes* induced marked responses from T cells as early as 6 hours after infection. DCs infected with KBMA *L. monocytogenes* sustained expression over the first 12 hours, while those infected with live *L. monocytogenes*

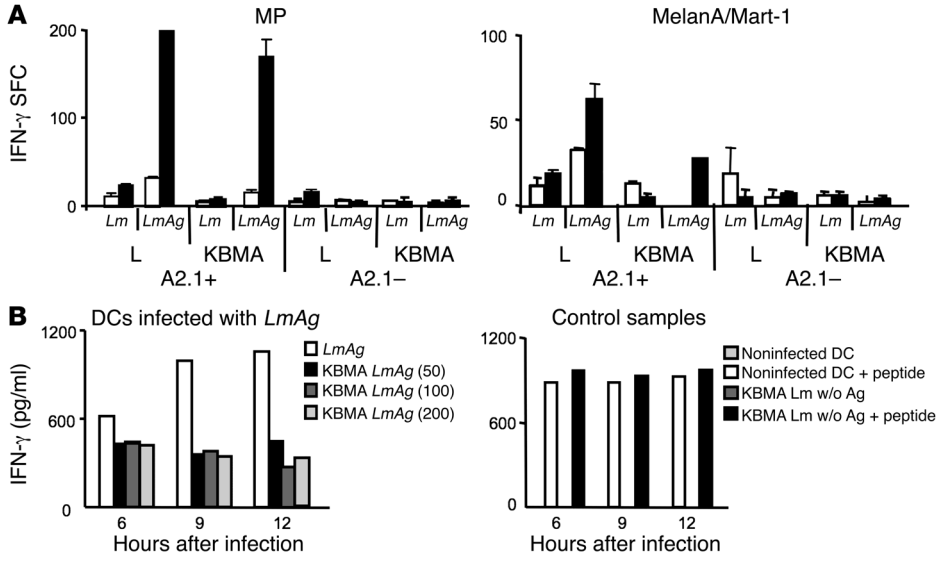

Figure 3

Live and KBMA *L. monocytogenes* successfully deliver recombinant antigens for presentation by MHC I molecules. (**A**) DCs from HLA A2.1$^+$ or HLA A2.1$^-$ donors were infected with recombinant *L. monocytogenes* Δ*actA*Δ*inlB*Δ*uvrAB* strain expressing *LmAg* or empty vaccine vector (*L. monocytogenes*) at MOI 10 (live) or MOI 100 (KBMA). After 40 hours, DCs were cocultured with 20,000 $MP_{58–66}$ or Mart-$1_{26–35}$–specific CD8$^+$ T cell clones in an IFN-γ–capturing ELISPOT assay. The number of spots corresponding to IFN-γ secreting T cells (SFC) is shown. Black bars represent responses of T cell–DC cocultures, and white bars represent DC-only cultures. Error bars represent SD of triplicate culture wells. (**B**) DCs were infected with live or KBMA *LmAg* (left panel) at the indicated MOI in 96-well plates. As a control, we used noninfected DCs, either unpulsed or pulsed with $MP_{58–66}$ peptide or DCs infected with *L. monocytogenes* that were either unpulsed or pulsed with $MP_{58–66}$ (right panel). At indicated times, DCs were fixed and MP-specific T cells were added to the wells. After 18 hours of T cell–DC coculture, supernatants were harvested and the amount of IFN-γ produced by T cells was determined by ELISA.

showed an increase in expression over time, likely related to replication of bacteria within the infected cell (Figure 3B). In both groups, expression of heterologous antigen remained detectable as demonstrated by the activation of antigen-specific T cells up to 40 hours after infection (Figure 3A). This period of antigen expression and recognition would encompass the time required for DCs to prime effector T cells in vivo (19).

KBMA L. monocytogenes encoding full-length NY-ESO-1 protein delivers antigens for presentation by MHC I and MHC II molecules. Ideally, cancer vaccines should prime both CD4$^+$ and CD8$^+$ T cells. Immunogenic epitopes have been identified for many cancer-relevant antigens, mostly for HLA A2.1–specific CD8-restricted epitopes, while CD4-restricted epitopes have been identified for various MHC haplotypes (20, 21). When individual CD4- and CD8-restricted epitopes are combined into vaccines, only a very small group of patients that receive these treatments have the required combination of MHC class I and class II molecules to elicit desired responses. Thus, whole proteins are a better alternative, especially when targeted specifically to professional APCs. NY-ESO-1 is a cancer-testis antigen, detectable in up to 30%–40% of metastatic melanomas. It is expressed by a variety of cancers but not in adult somatic tissues, making it an attractive target for immunotherapies (22–24). We have shown previously that when administered with adjuvants, NY-ESO-1 protein induces both humoral and cellular immune responses (25). Here we tested whether KBMA attenuated *L. monocytogenes* that express the NY-ESO-1 protein are an efficient adjuvant and delivery system.

For the purpose of the experiment, we first generated NY-ESO-1–specific CD8$^+$ and CD4$^+$ T cell clones. The CD8$^+$ clones were HLA A2.1 restricted while CD4$^+$ clones were DR (clone no. 15, clone no. 18) or DQ (clone no. 30) restricted. HLA A2.1$^+$ or HLA DR B4*0101$^+$ DCs were infected either with KBMA Δ*act*Δ*inlB*Δ*uvr L. monocytogenes* encoding full-length NY-ESO-1 protein (*LmNY*) or its empty vector control (*L. monocytogenes*). CD4$^+$ or CD8$^+$ NY-ESO-1–specific T cell clones were then added for overnight culture, after which IFN-γ in supernatants was measured. As shown in Figure 4, KBMA *LmNY*-infected DCs activated both NY-ESO-1–specific CD4$^+$ and CD8$^+$ T cell clones. While CD8$^+$ T cell responses were comparable among the 6 donors tested, CD4$^+$ T cell responses varied in magnitude in a donor-specific manner. However, all observed responses were NY-ESO-1–specific and statistically significant when compared with responses elicited by empty vector (Figure 4 and Supplemental Figure 3A). Interestingly, DCs infected with KBMA *L. monocytogenes* elicited a more pronounced response from the NY-ESO-1–specific CD8$^+$ T cell clones than the DCs infected with live *L. monocytogenes* when both strains were used at the highest MOI, which does not yet to a large extent impair DC viability (MOI 100 vs. MOI 10). To confirm that presentation of NY-ESO-1 epitopes was not limited to in vitro generated DCs but would take place also when heterogenous APC populations are targeted, PBMCs or monocytes were isolated from blood, infected with *LmNY*, and used as stimulators for NY-ESO-1–specific T cell clones. These infected APCs were DR or DQ restricted and were cocultured with HLA matched clones overnight when we evaluated NY-ESO-1–specific clonal responses by measurement of IFN-γ in culture supernatants (Supplemental Figure 3, B and C). We confirmed that in addition to moDCs, heterogenous PBMC populations and monocytes isolated from blood efficiently processed and presented the full-length NY-ESO-1 protein encoded by *L. monocytogenes*.

In summary, our data demonstrate that both CD8- and CD4-restricted epitopes are efficiently processed and presented to T cell clones by APCs when the full-length protein is delivered by the *L. monocytogenes* vector.

KBMA L. monocytogenes–infected DCs prime melanoma antigen-specific effector CD8$^+$ T cells. To be effective in vivo, KBMA *L. monocytogenes* vectors must confer antigen-specific T cell priming activity to DCs. Therefore, naive T cells from a healthy individual were cocultured with autologous DCs after infection with KBMA *Lm*Ag encoding the Mart-1 epitope. Every 9 days, the T cells were restimulated with KBMA *Lm*Ag–infected DCs for a total of 3 stimulations. As shown in Figure 5A, DCs infected with KBMA *Lm*Ag expanded $MP_{58–66}$–specific naive CD8$^+$ T cells. Before the onset of priming, no staining of naive T

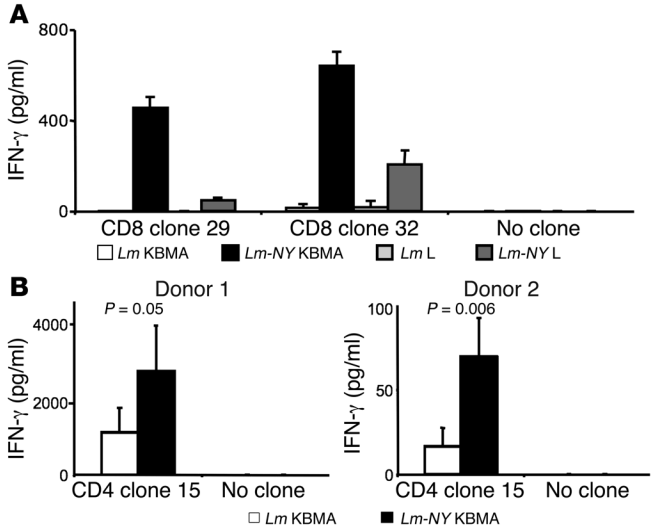

Figure 4
LmNY deliver antigens for presentation by MHC class I and class II molecules. Following infection with NY-ESO-1 protein encoding Δ*inlB*Δ*actA*Δ*uvrAB L. monocytogenes* (*LmNY*) or the empty vector control (*L. monocytogenes*) DCs were cocultured with either $CD4^+$ or $CD8^+$ NY-ESO-1–specific T cell clones for 18 hours. Supernatants were collected and IFN-γ was measured in an ELISA assay. Background IFN-γ secretion was determined by DC-only culture. Error bars represent SD of quadruplicate wells of DCs or T cell–DC culture. (**A**) HLA $A2.1^+$ DCs were infected and cocultured with 2 separate HLA A2.1–restricted NY-ESO-1–specific $CD8^+$ T cell clones. MOI for infection was 10 for live and 100 for KBMA *L. monocytogenes*. The experiment is a representative of 6 different donors tested. (**B**) DCs derived from 2 HLA DR B4*0101 restricted donors (shown are Donors 1 and 2 out of 3 donors tested) were infected with KBMA *L. monocytogenes* at MOI 100 and cocultured with NY-ESO-1–specific HLA DR B4*0101–restricted $CD4^+$ clone 15.

cells was apparent with visualization of tetrameric complexes of HLA-A*0201 and either $MP_{58–66}$ (Figure 5A, left panel) or Mart-$1_{26–35}$ (data not shown), verifying that the starting T cell population did not contain significant numbers of MelanA/Mart-1– or MP-specific cells.

We next evaluated whether live *Lm*Ag was more efficient at priming naive T cells to Mart-$1_{26–35}$ than KBMA *Lm*Ag in our in vitro system. DCs infected with live *Lm*Ag (MOI 10) or KBMA *Lm*Ag (MOI 50) were used to stimulate T cells as described above, and priming efficacy was tested with Mart-$1_{26–35}$ tetramers. A higher MOI was used for KBMA *Lm*Ag to compensate for this strain's inability to replicate inside the infected cell. Both strains were able to prime Mart-$1_{26–35}$-specific T cells at similar frequencies (Figure 5B, 4 left panels). The Mart-$1_{26–35}$ population from the culture stimulated by KBMA *LmAg*–infected DCs was enriched by using a limiting dilution assay of pooled wells in which Mart-$1_{26–35}$ tetramer-positive cells were detected (Figure 5B, 2 right panels). Specific antigen responses were measured by intracellular cytokine secretion. Only Mart-$1_{26–35}$ tetramer-positive cells responded to stimulation with Mart-$1_{26–35}$ peptide-pulsed T2 cells but not to HLA-A*0201–restricted HIV $Gag_{77–85}$ peptide-pulsed T2 cells that we used as a negative control (Figure 5C). In 2 different donors, most of the responding Mart-$1_{26–35}$ tetramer-positive cells produced several cytokines, including IFN-γ, TNF-α, and IL-2 (24% and 17% of Mart-$1_{26–35}$ tetramer-positive cells produced all 3 cytokines in both donors) (Figure 5D). Cytotoxicity of the Mart-$1_{26–35}$–specific T cells primed with KBMA *Lm*Ag was tested by using a chromium release assay. These cells lysed the MelanA/Mart-1 expressing $A2.1^+$ cell line Gmel but not the A2.1-888 melanoma line (Figure 5E). The lysis of Gmel was blocked when cells were pretreated with an anti-A2.1 antibody. Our data show that $CD8^+$ T cells primed by KBMA *Lm*Ag–infected DCs recognize native Mart-1 peptide and are able to execute their effector functions to destroy a melanoma tumor cell line.

In a murine therapeutic tumor model, KBMA L. monocytogenes are more efficient as a vaccine if targeting APCs in vivo. In terms of targeting DCs for therapeutic treatment of tumors, there are 2 main strategies that can be employed. DCs can be matured and loaded with antigens ex vivo and then injected back into patients. This approach is well established, although DC-based immune therapies remain to be fully optimized. The second approach is to target DCs in vivo, either directly or via intermediate vector "carrier cells" that are cross-presented by DCs. Apart from being simpler and cheaper, this approach offers the advantage of targeting multiple DC types in addition to other APCs. Using *L. monocytogenes* as a vaccine vector makes the second approach even more appealing, since upon i.v. injection, *L. monocytogenes* spontaneously localize in T cell priming areas of the spleen (26) and deliver antigens for priming by resident DCs (12). It was shown previously that targeting APCs in vivo with KBMA *L. monocytogenes* encoding tumor antigen mediates priming to tumor antigens, breaks tolerance to the endogenous antigens, and controls tumor growth (11). We have now used a therapeutic vaccination model in CT-26 tumor-bearing mice. We have compared in vivo targeting to ex vivo loading of DCs using KBMA *L. monocytogenes* expressing the immunodominant T cell epitope AH1/A5. While ex vivo loading of DCs with KBMA *L. monocytogenes*–expressing AH1/A5 elicited a small AH1/A5-specific T cell response, the protective effect of the primed cells was insignificant (Figure 6). Both, DCs loaded with vaccine or empty vector mediated similar transient control of tumor growth compared with the untreated mice, likely dependent on stimulation of innate immunity. On the contrary, therapeutic immunization of mice with either live or KBMA recombinant *L. monocytogenes* induced protective immune responses against the tumor that expresses endogenous antigen (Figure 6B) and significantly prolonged survival when mice were followed for 100 days. In summary, KBMA *L. monocytogenes* expressing tumor antigen is a potent vaccine vector that primes protective T cell responses in mice, when its recombinant antigen is delivered to the in vivo resident APCs.

Discussion

Current therapeutic strategies to treat metastatic cancer have either shown limited success in tumor regression or were associated with high toxicities. Therefore, alternative approaches are urgently needed. DCs are attractive adjuvants in humans to increase host resistance to tumors. DCs acquire antigens through several mechanisms, efficiently process and present antigens to T cells (8), and have an excellent safety record in humans. However, new strategies are needed to optimize their immunogenicity, as there have been few reports demonstrating their ability to induce sustained, protective antitumor immunity in humans (27, 28). One such strategy is to use a system that efficiently targets DCs in vivo. In addition to possibly targeting more relevant in vivo resident DC subsets as well as other APCs, such strategies would also considerably lower the cost of the vaccine.

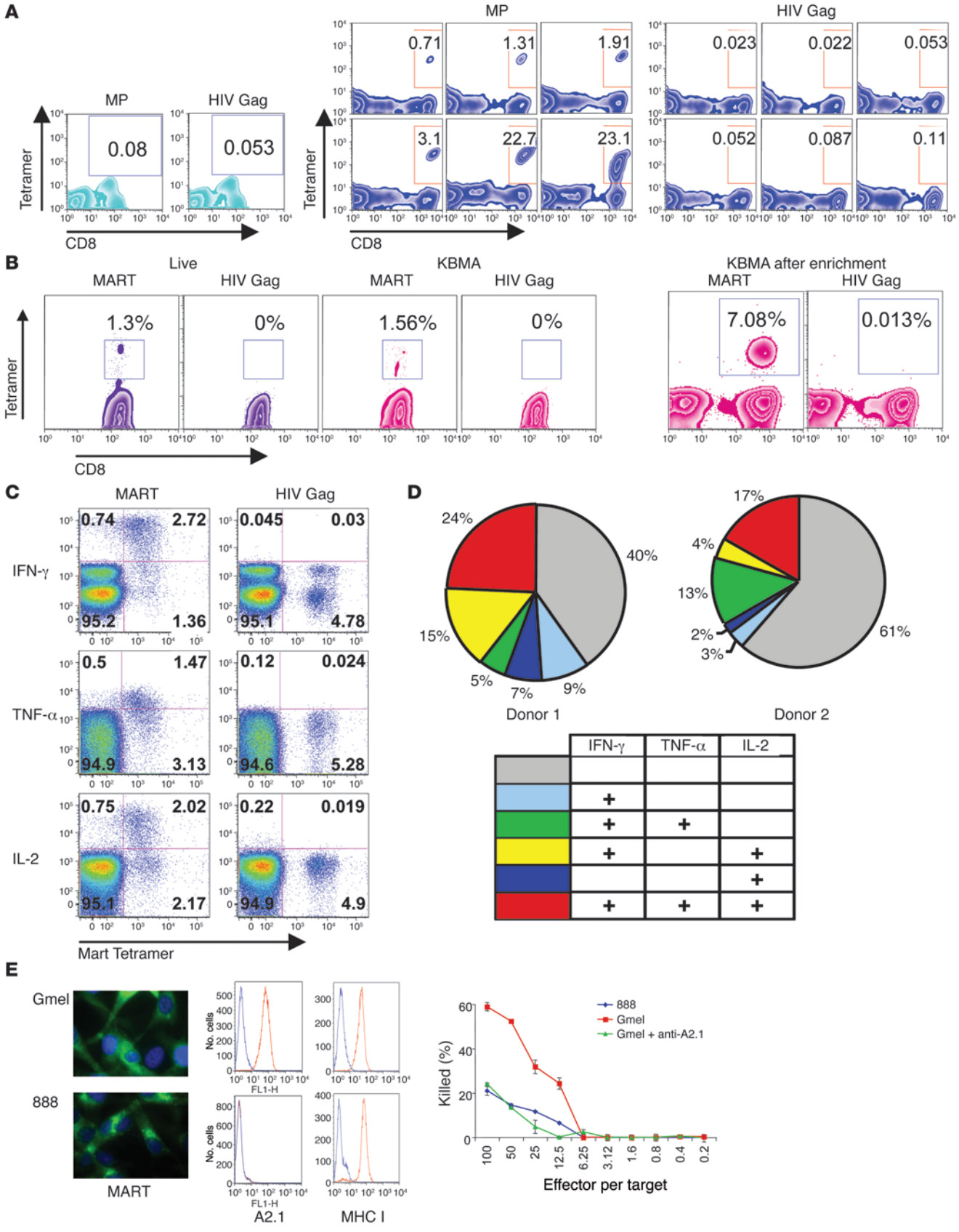
A
MP
HIV Gag
Tetramer
CD8
0.08
0.053
0.71
1.31
1.91
0.023
0.022
0.053
3.1
22.7
23.1
0.052
0.087
0.11
B
Live
KBMA
KBMA after enrichment
MART
HIV Gag
1.3%
0%
1.56%
0%
7.08%
0.013%
C
IFN-γ
TNF-α
IL-2
Mart Tetramer
0.74
2.72
95.2
1.36
0.045
0.03
95.1
4.78
0.5
1.47
94.9
3.13
0.12
0.024
94.6
5.28
0.75
2.02
95.1
2.17
0.22
0.019
94.9
4.9
D
24%
40%
15%
5%
7%
9%
Donor 1
17%
4%
13%
2%
3%
61%
Donor 2
IFN-γ
TNF-α
IL-2
E
Gmel
888
MART
No. cells
FL1-H
A2.1
MHC I
Killed (%)
Effector per target
888
Gmel
Gmel + anti-A2.1

Figure 5
Recombinant *L. monocytogenes* prime effector CD8+ T cells to the encoded recombinant antigens. HLA A*0201+ DCs were infected with the KBMA Δ*actA*Δ*inlB*Δ*uvrAB L. monocytogenes* strain expressing the HLA A*0201–restricted CD8+ T cell epitopes of MP and MelanA/Mart–1. (**A**) The naive T cell population lacks the MP-tetramer–positive cells (left panels). After stimulation, MP-specific CD8+ T cells were generated at various frequencies (each middle panel representing a single well). Lack of staining with HIV Gag tetramers assured specificity (right panels). The numbers indicate the percentage of tetramer-positive cells. (**B**) Both live and KBMA recombinant *L. monocytogenes* primed naive Mart-$1_{26–35}$–specific T cells at similar frequencies. Percentage of CD3-gated, CD8, and tetramer double-positive cells after priming (left panels) or after the enrichment with limiting dilution assay is shown (right panels). HIV Gag tetramers were used as a negative control. The population of cells enriched for Mart-1–tetramer-positive cells was used for the functional assays shown in **C**–**E**. (**C** and **D**) T cells were stimulated with T2 cells pulsed with Mart-$1_{26–35}$ peptide, and Mart-1–tetramer-positive cells were analyzed for secreted cytokines by intracellular staining. HIV Gag-tetramers were used as specificity controls. In **C**, the dot plots of 1, and in **D**, pie charts representing the spectrum of secreted cytokines by 2 tested donors are shown. In **C**, the numbers indicate the percentage of cells in each dot plot quadrant. (**E**) The same T cell populations were tested for cytotoxicity of melanoma cell lines. Gmel and 888 melanoma cell lines express MelanA/Mart-1 protein (left panel), but only Gmel is HLA A*0201–restricted (middle panels), which correlates with the latter's ability to be lysed by MelanA/Mart-1–specific T cells (right panel). Original magnification, ×100. Preincubation of Gmel with anti-HLA A*0201 antibodies inhibited lysis. A representative result for 1 of 2 donors is shown in **A**–**C** and **E**. In **E**, error bars represent SD of triplicate culture wells.

L. monocytogenes are one of the recombinant microbial vector platforms that has proved to elicit protective responses in a number of experimental models, including infectious and malignant diseases (29–31). *L. monocytogenes* localize to T cell zones of spleen white pulp and efficiently targets DCs in vivo (26). Development of T cell anti–*L. monocytogenes* immunity depends on the presence of CD11c+ cells such as DCs (12, 26). Next to its remarkable immunogenicity and cost-effectiveness, several other features make *L. monocytogenes* an intriguing tumor vaccine candidate. These include the ability to break tolerance to self antigens (11, 32, 33) and the preserved efficacy of the recombinant *L. monocytogenes* vaccines despite preexisting immunity to the bacteria (34).

While only mild toxicity was observed when partially attenuated *L. monocytogenes* were administered to healthy human volunteers orally (35), it is not considered safe to administer a virulent form of a human pathogen to immunocompromised cancer patients. Efforts to reduce virulence of *L. monocytogenes* have focused on determinants that enable intracytoplasmic escape (LLO), replication, and intercellular spread without exposure to hostile extracellular defense components (ActA and InlB). Among the virulence determinants, LLO is likely one of the major factors in pathogenicity of *L. monocytogenes*; however, its deletion is also associated with a decrease in protectiveness of the T cells generated in infected mice (36).

Thus, to address the concerns inherent to delivering potentially toxic bacteria to humans, we constructed highly attenuated strains, lacking the proteins ActA and InlB, a strategy that reduces toxicity in mice by 1,000 fold (31). We show that Δ*actA*Δ*inlB L. monocytogenes* can still efficiently enter human DCs through InlB-independent phagocytic mechanisms and preserve DC-stimulatory capacity through their ability to mature these APCs. To ensure the safety of using *L. monocytogenes* as a vaccine, a further attenuating step was taken by limiting the ability of the bacteria to replicate. While HK *L. monocytogenes* are safe to use in mice, the primed CD8+ T cells of the memory repertoire do not acquire effector functions and are not protective from subsequent *L. monocytogenes* infection (37). Immunization of mice with a mixture of live and HK *L. monocytogenes* does not alter the phenotype of the CD8+ T cells primed by the HK *L. monocytogenes*, thus the absence of an inflammatory milieu cannot alone explain this intrinsic difference (26). Several approaches have been utilized to interfere with the replication of *L. monocytogenes* while preserving T cell–stimulatory capacity (38, 39). For clinical application, a well-controlled, cost-effective method with consistent inactivation of *L. monocytogenes* and full DC-stimulatory potential is preferred. We developed our candidate strain on a recently described vaccine platform that simultaneously takes advantage of the potency of live and the safety of killed microbes (KBMA *L. monocytogenes*) (11). KBMA vaccine strains were constructed by abrogating the capacity for nucleotide excision repair through deletion of the UV light resistance genes *uvrA* and *uvrB*. The gene deletion of *uvrAB* renders the bacteria exquisitely sensitive to photochemical inactivation through the combined treatment of S-59 and UVA. Because of their inability to repair the DNA cross-links, KBMA *L. monocytogenes* are unable to replicate and are thus functionally noninfectious (11). The very limited number of cross-links, however, preserves their metabolic activity, ability to exit the phagolysosome, and ability to escape into the cytosol. *L. monocytogenes* that are inactivated by high doses of S-59, with a correspondingly increased number of cross-links per genome, have abrogated replication and metabolic activity, similar to the recently described *L. monocytogenes* inactivated by irradiation (38).

In our studies, the *L. monocytogenes* with preserved metabolic activity supercede their nonmetabolic counterparts in DC-stimulatory potential (Supplemental Figure 1). The potent maturation stimulus provided by metabolically active *L. monocytogenes* to DCs is likely to be multifactorial. *L. monocytogenes* activate APCs through ligation of TLRs and through cytoplasmic TLR-independent pathways (17, 40–42). In addition, the induction of proinflammatory cytokines such as IL-1, TNF, IL-6, and type I IFN, factors known to activate immature DCs, are likely to amplify these effects.

To prove that highly attenuated KBMA could be used to target tumor antigens to DCs, we constructed recombinant *L. monocytogenes* that contain the MelanA/Mart-1 epitope or *L. monocytogenes* expressing full-length NY-ESO-1 protein under the LLO promoter. Our studies demonstrate that the proteins are efficiently processed within the infected DCs and MelanA/Mart-1 epitopes are expressed in sufficient amounts on the surface of DCs to prime MelanA/Mart-1–specific CD8+ T cells. KBMA recombinant *L. monocytogenes*–infected DCs induced cytolytic T cells that recognized native antigen expressed by patient-derived melanoma cells and secreted a panel of effector cytokines, including IFN-γ, TNF-α, and IL-2. Along with the HLA-A*0201–restricted MelanA/Mart-1 epitope, the recombinant *L. monocytogenes* also expressed an MP epitope. Supporting previous observations in the murine system (34), MelanA/Mart-1 priming was not hindered due to preexisting immunity to influenza (data not shown).

Unlike most viral vectors, *L. monocytogenes* constructs are not neutralized by preexisting *L. monocytogenes*–specific immunity. In addition, *L. monocytogenes* are not restricted by the length of heterologous sequence that can be inserted and thus allows the encoding of full-length proteins. This offers 2 advantages: (a) develop-

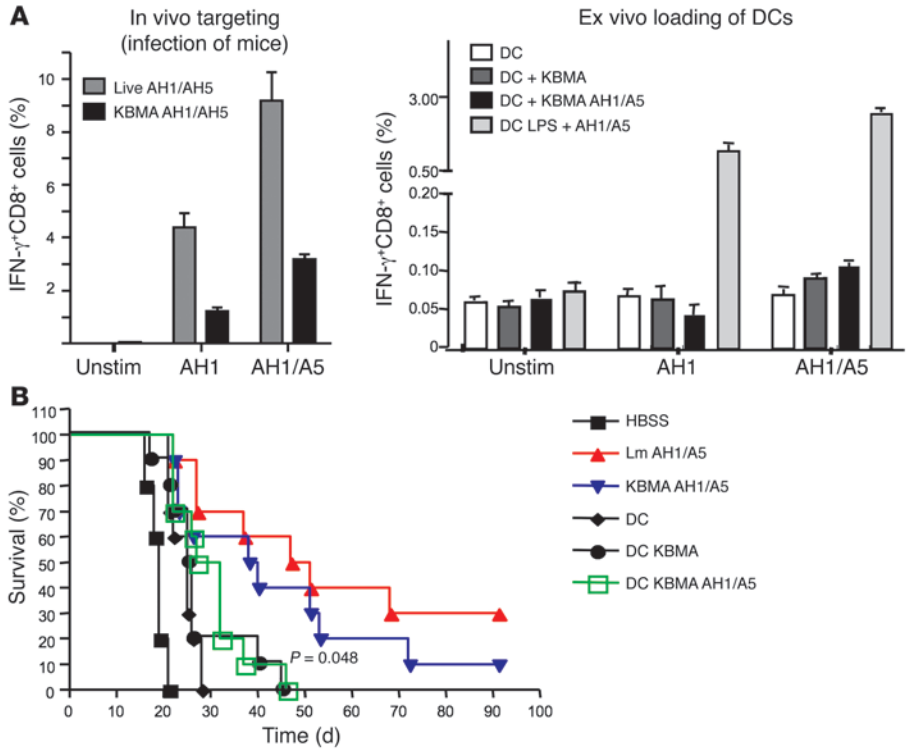

Figure 6
Targeting DCs with KBMA *L. monocytogenes*–expressing AH1/A5 in vivo controls CT-26 tumor growth. (**A**) Five mice per group were used for assessment of priming efficacy. The efficacy of T cell priming was compared between mice infected in vivo (left panel) and mice vaccinated with DCs infected in vitro (right panel). Mice were sacrificed 7 days after immunization, and the percentage of antigen-positive IFN-γ–secreting $CD8^+$ T cells upon in vitro stimulation with AH1 or AH1/A5 peptides in the presence of brefeldin A was analyzed by flow cytometry. Error bars represent SD of triplicate culture wells. (**B**) BALB/c mice were inoculated with CT-26 tumors and immunized 5 days later. They were randomized (10 per group) and vaccinated either with 1 of the indicated DC vaccines or with the live or KBMA *L. monocytogenes*–expressing AH1/A5. Survival of the mice was monitored over 100 days. *P* value of survival difference in mice vaccinated with DCs pulsed ex vivo with *L. monocytogenes*–expressing AH1/A5 versus mice infected with *L. monocytogenes*–expressing AH1/A5 is shown ($P = 0.048$). No significant difference was observed among mice vaccinated with live-attenuated or KBMA *L. monocytogenes* ($P = 0.328$).

ment of a vaccine or immune therapeutic that circumvents the problems of HLA restriction and (b) simultaneous priming of $CD4^+$ and $CD8^+$ T cells. The second is especially advantageous in tumor settings where $CD4^+$ T cell licensing is necessary for long-term protective $CD8^+$ T cell responses (43–45). We show that *L. monocytogenes* expressing full-length NY-ESO-1 protein efficiently delivered antigens for both MHC class I and class II presentation across different MHC restrictions.

While ex vivo maturation and antigen loading of DCs is a well-established vaccine approach, which offers the advantage to monitor for vaccine quality before injection into patients, it has yet to prove its efficacy in patients with larger tumor burdens where tolerance to tumor antigens has been established. While prophylactic vaccination in an OVA-expressing tumor model by ex vivo loaded DCs mediated protective T cell responses to tumor (data not shown), we found that ex vivo loaded DCs had reduced ability to induce protective responses in a therapeutic vaccination model, using CT-26 tumor-bearing mice. In these experiments, initial immunization was also followed by 2 booster vaccinations. In contrast, targeting DCs in vivo by infecting mice with attenuated KBMA recombinant *L. monocytogenes* primed effector T cells that increased survival of tumor-bearing mice, an effect that was comparable to protection mediated by live *L. monocytogenes*. While there may be several reasons for the observed differences, the animal data indicate that vaccination strategies may need to be approached differently in tumor-free patients with high likelihood of recurrence versus patients with metastatic disease or unresectable tumors.

In summary, an ideal cancer vaccine would simultaneously induce $CD8^+$ and $CD4^+$ T cells. This can be made possible by using a vector that encodes a full-length cancer-relevant protein and preferentially targets APCs in vivo. Altogether, our studies show that KBMA Δ*actA*Δ*inlB*Δ*uvrAB* recombinant *L. monocytogenes* are a safe, efficient, and cost-effective method of antigen loading and activation/maturation of human DCs with great promise for successful clinical use.

Methods

Bacterial strains. The *L. monocytogenes* strains used in this study were derived from the WT *L. monocytogenes* strain DP-L4056 (46). *L. monocytogenes* strains with deletion of virulence proteins and *uvrAB* were prepared as described previously (11, 31). The pPL2 integration vector was used to construct recombinant *L. monocytogenes* encoding a single copy of OVA containing the HLA-A*0201–restricted T cell epitopes MelanA/Mart-1_{26-35} (ELA modified) and Flu MP_{58-66} and the H-K^d restricted AH1/A5 mouse T cell epitope or full-length NY-ESO-1 protein integrated adjacent to the $tRNA^{Arg}$ gene in the bacterial genome (11). The epitope-containing OVA construct is under the transcriptional control of the *hly* promoter and is secreted from the bacterium as a fusion protein to the amino terminal 62 aa of LLO. The MelanA/Mart-1_{26-35} and Flu MP_{58-66} epitopes were codon-optimized for *L. monocytogenes*, synthesized as complementary oligonucleotides with appropriate 5′ and 3′ overhangs, and added to the pPL2-LLO-OVA fusion construct by oligo ligation to unique *Ava*II and *Sap*I restriction sites within the OVA DNA sequence, respectively. For studies using viable *L. monocytogenes*, bacteria were grown at 37°C in brain-heart-infusion broth to log phase, washed once with PBS, and resuspended in 8% DMSO/PBS. Aliquots were frozen and stored at –80°C.

S-59/UVA inactivation of bacteria and metabolic activity. *L. monocytogenes* were grown in 50 ml of brain-heart-infusion broth at 37°C, with shaking to an OD_{600} of 0.5, and S-59 was added directly to the cultures for 1 additional hour. The bacterial cultures were transferred to culture plates and UVA irradiated at a dose of 6.5 J/cm^2 (FX1019 irradiation device; Baxter Fenwal). Photochemically treated bacteria were washed once with PBS and resuspended in 8% DMSO and stored at –80°C or used directly for experiments.

Media. RPMI-1640 (Cellgro) supplemented with 1 mM HEPES (Gibco) and 1% plasma (Valley Biomedical) was used for infection and culture of human DCs with *L. monocytogenes*. For ELISPOT assay and mixed lymphocyte reaction, media were supplemented with 20 μg/ml gentamicin (Gibco) and 5% pooled human serum (PHS; ValleyBiomedical) instead of 1% plasma. For maintenance of lines and for the Chromium-51 (Cr-51) release assay, cells were cultured in RPMI-1640 supplemented with 1 mM HEPES and 10% FBS (Gibco). Yssel's T cell medium (Gemini Bio-Products) supplemented with 5% PHS was used in the T cell priming assays. For priming of NY-ESO-1–specific T cells, we used complete IMDM media (Gibco) (IMDM with 5% PHS, glutamine, and nonessential amino acids; Sigma-Aldrich) supplemented with 100 units/ml penicillin, 0.1 mg/ml streptomycin, 150 IU/ml IL-2 (R&D Systems), and 10 ng/ml of IL-7 (R&D Systems). For NY-ESO-1–specific T cell–DC cocultures, complete IMDM supplemented with 20 IU/ml IL-2 and gentamicin was used.

Peptides. H-2K^d–restricted epitope AH1 or the altered AH1/A5 (SPSYAYHQF) were synthesized commercially (SynPep) and used as previously described (11). Flu $MP_{58–66}$ GILGFVFTL, MelanA/$Mart\text{-}1_{26–35}$ (ELA modified) ELAGIGILTV, and HIV $Gag_{77–85}$ SLYNTVATL peptides were synthesized by Genemed Synthesis Inc. A library of overlapping peptides spanning the full length of the NYESO protein was obtained from ProImmune Inc.

Generation of CD4$^+$ and CD8$^+$ NY-ESO-1 T cell clones. CD4$^+$ NY-ESO-1–specific clones were generated as described previously (47). Briefly, PBMCs were stimulated repetitively with the pool of overlapping peptides spanning the complete NY-ESO-1 aa sequence (P119–143, P139–160, the remaining peptides were 20mers overlapping by 10 aa). After 3 restimulations, antigen-specific T cells were enriched by IFN-γ capture assay (Miltenyi Biotec). The cells were then cloned by limiting dilution assay. We seeded 0.3 cells/well of 96-well plate and expanded cells polyclonally with PHA-L (Sigma-Aldrich). NY-ESO-1–specific cells were determined by measurement of intracellular IFN-γ upon stimulation with each of the NY-ESO-1 peptides in the presence of brefeldin A (Sigma-Aldrich). CD8$^+$ NY-ESO-1 cells were obtained from a patient that was vaccinated with NY-ESO-1 amino acids 157–165 (SLLMWITQV), with a V substituted for the naturally occurring C at position 165 for better HLA-A2 binding. NY-ESO-1–specific CD8$^+$ T cells were expanded by stimulation with $NY\text{-}ESO_{157–165}$ peptide in the presence of 1,000 IU/ml IL-2 for 10 days as previously published (48). APCs were then cloned and tested as described above.

Human PBMCs, DCs, APCs, and naive T cells. moDCs were generated from buffy coats (New York Blood Center) or leukaphereses (BRT Laboratories Inc.) as described previously (49); however, unless noted, addition of gentamicin to the media was omitted. Monocytes were obtained from buffy coats by CD14$^+$ magnetic selection (Miltenyi Biotech), and blood DCs were obtained by FACS sorting as described (18). T cells for priming were obtained from buffy coats, leukapheresis, or patient blood. Buffy coats were purchases from New York Blood Center, leukophereses were purchased from BRT Laboratories Inc., and usage of patients' blood was approved by New York University Institutional Review Board.

Infection of DCs with L. monocytogenes and immunofluorescence staining. Immature human DCs were seeded to 24-well plates at 10^6 cells/well or a 96-well plate at 1.5×10^5 cells/well and were incubated with *L. monocytogenes* for 1 hour at 37°C. Adherent cells were washed twice with RPMI containing 50 μg/ml gentamicin (Gibco) and kept in medium supplemented with 20 μg/ml gentamicin. In NY-ESO-1 experiments, DCs were infected in rotating tubes for 1 hour, washed, counted, and cocultured with T cells in U-bottom plates (Costar).

Human DCs were infected at an MOI of 1 or 10 for viable bacteria or an MOI of 20, 50, 100, or 200 for KBMA *L. monocytogenes* or Δ*hly L. monocytogenes* for 60 minutes at 37°C. Extracellular bacteria were removed by washing. For fluorescence microscopy, infected cells were incubated for 5 hours at 37°C on poly-L-lysine–coated coverslips in the presence of gentamicin, then fixed with 3.5% formaldehyde. Following permeabilization with TBS Triton X-100, DCs on coverslips were stained with rabbit anti–*L. monocytogenes* antibody (BD Diagnostics), followed by FITC-conjugated goat anti-rabbit (Vector Laboratories). Polymerized actin was visualized using phalloidin-rhodamine (red) (Molecular Probes) and coverslips were mounted using ProLong Gold antifade with DAPI (blue) (Molecular Probes). For detection of MelanA/Mart-1 expression in melanoma cell lines, adherent cells grown on chamber slides (BD) were fixed and stained intracellularly with Mart-1–specific antibodies (Novacastra) and FITC-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc.).

Assessment of human DC maturation. Following infection with *L. monocytogenes*, the DCs were cultured for 36 hours further unless otherwise noted. They were then stained with antibodies against CD80, CD83, CD86, HLA-A, -B, -C (all from BD Biosciences), or CCR7 (R&D Systems). The expression of the maturation markers was quantified according to MFI or percentage of positively stained cells on a FACSCalibur (BD Biosciences — Pharmingen).

Measurement of cytokines. DCs cultured with various stimuli were incubated for 36 hours at 37°C before their culture supernatants were harvested and tested for the presence of the following cytokines: IL-6, IL-1β (both from R&D Systems), IL-12p70, TNF-α (both from BD Biosciences — Pharmingen), and IFN-α (PBL) and chemokines MCP-3 and RANTES (both from R&D Systems) by ELISA. IFN-γ (Biosource) was similarly measured in supernatants of T cell–DC cocultures. Alternatively, in selected experiments, cytokines produced by DCs were measured by FACS using human inflammatory Cytometric Bead Array (BD).

Antigen presentation. DCs were infected with either live (MOI 10) or KBMA (MOI 50 or MOI 100) *L. monocytogenes* (both *LmAg* expressing MP and Mart epitopes and *L. monocytogenes* without antigen) in a 24-well plate as described above and cultured for 36 hours at 37°C. The cells were harvested, counted, and cocultured with $MP_{58–66}$-specific T cell clones (50) in an IFN-γ capture ELISPOT plate (Millipore) for at least 16 hours. Subsequently, the plate was developed according the manufacturer's protocol, and IFN-γ spots were quantified with an automated counter (AID). For the antigen presentation kinetics assay, DCs were plated in the wells of a 96-well plate and infected with *L. monocytogenes* as described above. At 6, 9, and 12 hours after infection, the cells were fixed as described, and Flu-specific T cells were added directly to the wells containing the fixed DCs for 16 hours at which point the supernatants were harvested for an IFN-γ ELISA. In NY-ESO-1 experiments, DCs were infected for 1 hour in a rotating tube, treated with gentamicin, and put on ice for 30 minutes. CD4$^+$ or CD8$^+$ T cell clones were then added to DCs in U-bottom plates at a DC/T cell ratio of 1:3 for 18 hours at which point supernatants were collected and IFN-γ was measured by ELISA.

Cell fixation. In the antigen presentation kinetics assay, cells were fixed with 2% PFA (Sigma-Aldrich) for 10 minutes, washed with DMEM, and incubated with L-lysine for 20 minutes to neutralize the effects of PFA. L-lysine (Sigma-Aldrich) was prepared by diluting 36.54 g of L-lysine in 500 ml distilled water.

Priming. DCs infected with *L. monocytogenes* were cocultured with naive syngenic T cells derived from PBMCs by magnetic depletion (Miltenyi Biotech) of CD19-, CD14-, CD56-, and CD45RO-positive cells in the presence of IL-6 (1,000 IU/ml) (R&D Systems) and IL-12 (5 ng/ml). The T cells were restimulated every 9 days with the *L. monocytogenes*–infected DCs in the presence of IL-2 (10 U/ml) (Chiron) and IL-7 (5 ng/ml) (R&D Systems). A week after the third stimulation, the cells were analyzed for the presence of $Mart_{26–35}$ tetramer-positive cells. Antigen-specific T cells were then enriched by limiting dilution assay as described above with 10 cells seeded per well. Wells that contained more than 5% of specific cells were pooled and frozen in aliquots for further testing. For allogeneic priming (mixed lymphocyte reaction), PBMCs were depleted of CD19-, CD14-, CD56-, CD8-, and CD45RO-positive cells by magnetic beads (Miltenyi Biotech) and cocultured with allogeneic DCs.

Cell lines and blocking of HLA 2.1. Melanoma patient–derived cell lines, Gmel and 888, were used to test HLA-A2–restricted CTL function. Both express MelanA/Mart-1, but only Gmel is HLA-A2 positive, therefore 888 was used as a negative control. The T2 cells deficient in transporter associated protein were pulsed with peptides and used to stimulate HLA-A2–restricted $CD8^+$ T cells in the intracellular cytokine staining assay. Antibodies against human HLA A2.1 (clone BB7-2, HB82; ATCC) were used to show HLA-restriction of the CTL responses.

Generation of tetramers. PE-labeled tetrameric MHC-peptide complexes ($MP_{58–66}$/HLA-A*0201, MelanA/Mart-$1_{26–35}$/HLA-A*0201, and HIV $gag_{77–85}$/HLA-A*0201) were synthesized by the Vaccine and Cell Therapy Facility at New York University Medical Center as described previously (51). Plasmids encoding human β_2 microglobulin and HLA-A2 were a gift from Christian Munz of The Rockefeller University.

Tetramer and intracellular cytokine staining. T2 cells were pulsed with $Mart_{26–35}$ peptides or HIV $Gag_{77–85}$ peptides (both at 1 μg/ml) for 2 hours at 4°C in RPMI-1640. After extensive washing the peptide-pulsed T2 cells were cocultured with polyclonal T cells containing Mart-specific cells in the presence of 10 μg/ml of brefeldin A (Sigma-Aldrich) for 6 hours at 37°C. After the incubation, the cells were stained with a PE-conjugated tetramer of $Mart_{26–35}$ /HLA-A*0201 complexes, CD4 Alexa Fluor 700, CD8 PerCP-Cy5.5 and CD3 APC-Cy7 antibodies (BD Biosciences — Pharmingen). Next, the cells were fixed and stained intracellularly as previously described (52) with FITC-conjugated IFN-γ, PE-Cy7–conjugated TNF, and IL-2–APC antibodies (BD Biosciences — Pharmingen) and analyzed with an LSR flow cytometer.

Chromium release assay. Melanoma cell lines were labeled with Cr-51 (PerkinElmer) at a final concentration of 1 mCi/ml for 90 minutes at 37°C. After washing twice with RPMI-1640 supplemented with 10% FBS, they were cocultured with Mart-specific polyclonal T cells at various ratios for 6 hours. Cr-51 released into the supernatants was measured with a Wallac 1450 Microbeta counter (PerkinElmer). The percentage of cells killed was calculated by dividing specific release from maximum release, after first subtracting spontaneous release from both samples.

Mice and immunizations. Six- to eight-week-old female BALB/c (Charles River Laboratories) were handled according to US National Institutes of Health guidelines. All protocols requiring animal experimentation received prior approval from the Anza Therapeutics Animal Care and Use Committee. All vaccinations for immunogenicity and tumor studies were by i.v. injection using either 1×10^8 KBMA or 1×10^6 live attenuated *L. monocytogenes* or $1–2 \times 10^6$ DCs. Initial immunization was followed by 2 booster immunizations 14 days apart.

Generation and suspension of murine DCs. DCs were generated as described previously (53). Briefly, BM from tibias and femurs of BALB/c mice was cultured in RPMI-1640 plus 10% FBS and GM-CSF (20 ng/ml murine GM-CSF; R&D Systems). Ten days later, nonadherent cells were harvested and verified phenotypically to be myeloid DCs (MHC class II^{hi}, $CD11c^{hi}$, $CD86^{int}$). For loading with KBMA *L. monocytogenes*, immature DCs were incubated in suspension with KBMA *L. monocytogenes* for 1 hour at MOI of 20. Extracellular bacteria were washed away and cells placed back into suspension culture at 37°C. Fifteen hours later, cells were washed 3 times and resuspended in dPBS for injection.

Tumor studies. We i.v. implanted BALB/c mice with 2×10^5 CT-26 cells. We randomized mice 3 days later into experimental groups, 10 animals per group, and vaccinated them. Mice were injected with HBSS and vaccinated with 1 of the DC vaccines (DCs and DCs infected with KBMA Δ*act*Δ*inl*Δ*uvrAB L. monocytogenes*–expressing AH1/A5 or its empty KBMA vector control) or were inoculated i.v. with live or KBMA Δ*act*Δ*inl*Δ*uvrAB L. monocytogenes*–expressing AH1/A5 bacteria. AH1/A5 is the heteroclitic epitope of the AH1 epitope of tumor antigen gp70, naturally presented by the CT-26 tumor. Survival of the mice was monitored over 100 days. Mice were sacrificed if they started to show any signs of stress or labored breathing. Satellite mice (5 per group) were used for assessment of priming efficacy. Mice were sacrificed 7 days after immunization and the percentage of antigen-positive IFN-γ–secreting $CD8^+$ T cells, upon in vitro stimulation with AH1 or AH1/A5 peptides in the presence of brefeldin A, was analyzed by flow cytometry.

Statistics. Statistical significance among DCs infected by different *L. monocytogenes* strains were tested by 2-tailed Student's *t* test. *P* values of less than 0.05 were considered significant. Mean values of 5 mice were calculated to enumerate the responses to AH1 or AH1/A5 peptides. Tumor survival results were analyzed by Prism "Survival curve" statistical analysis.

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Address correspondence to: Mojca Skoberne or Nina Bhardwaj, New York University School of Medicine, 522 First Avenue, SML 1307, New York, New York 10016, USA. Fax: (212) 263-6729; E-mail: Mojcaskoberne@yahoo.de (M. Skoberne). Phone: (212) 263-5814; Fax: (212) 263-6729; E-mail: Nina.Bhardwaj@nyumc.org (N. Bhardwaj).

Keith S. Bahjat's present address is: Earle A. Chiles Research Institute, Robert W. Franz Cancer Research Center, Providence Portland Medical Center, Portland, Oregon, USA.

Mojca Skoberne's present address is: Genocea Biosciences, Cambridge, Massachusetts, USA.

Mojca Skoberne, Alice Yewdall, and Keith S. Bahjat contributed equally to this work.

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