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ORIGINAL ARTICLE

Whole recombinant yeast vaccine induces antitumor immunity and improves survival in a genetically engineered mouse model of melanoma

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Malignant melanoma is one of the deadliest forms of skin cancer and its incidence is expected to rise over the next two decades. At present, there are no effective therapies for advanced melanoma. We have previously shown that administration of whole recombinant yeast expressing human MART-1 (hMART-IT) induces protective antimelanoma immunity in a B16F10 transplantable mouse model. In this study, we examine the effectiveness of the hMART-IT vaccine in a congenic strain of genetically engineered mouse model of melanoma, which recapitulates both the underlying genetics and the proper tumor microenvironment of naturally occurring melanoma. Subcutaneous administration of hMART-IT induced cytotoxicity against melanoma cells and antigen-specific production of Th1-specific cytokines by splenocytes. Weekly administration of hMART-IT significantly delayed the development of melanoma and prolonged the survival of mice compared with controls. Although histological analysis demonstrated diffuse infiltration of $CD4^+$ T cells and $CD8^+$ T cells, no reduction of regulatory T cells was observed, suggesting that hMART-IT cannot prevent immunotolerance in the tumor microenvironment. This study provides a proof of concept that genetically engineered mouse models lend valuable insights into immunotherapeutics being tested in the preclinical setting.
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Keywords: melanoma; genetically engineered mouse model; yeast-based vaccine

INTRODUCTION

Malignant melanoma is one of the deadliest forms of skin cancer and its incidence is expected to continue to rise over the next two decades.[1] Advances in surgery, chemotherapy and radiotherapy have reduced mortality to some degree, but the overall efficacy of these therapeutic modalities has been disappointing. Reducing morbidity and mortality from melanoma has therefore relied on primary prevention and early detection of disease. Recently, novel treatment approaches have been studied and offer potential therapeutic advantages over current strategies.[2–4]

We have previously shown that immunization of C57BL/6 mice with whole recombinant yeast transfected with hMART-1 effectively protects mice from subcutaneous challenge with a lethal dose of B16F10 mouse melanoma cells by inducing a MART-1-specific cytotoxic T-cell response and producing granulocyte/macrophage-colony-stimulating factor and interferon (IFN)-γ *in vitro*.[5] Although transplantable tumor models are valuable animal models for *in vivo* studies, they have several limitations to make them suboptimal for predicting immunotherapy efficacy in patients.[6] First, transplanted tumors are typically inoculated subcutaneously or intravenously and therefore do not grow in the anatomically appropriate site, resulting in an unnatural tumor microenvironment. Second, transplantable tumors generally progress very rapidly following inoculation, whereas spontaneous tumors usually develop more slowly through a gradual series of cellular changes from premalignant to malignant pathology. Consequently, the immune system of patients with naturally forming tumors becomes slowly acclimated to the presence of tumors, whereas the immune system of experimental animals with transplanted tumors is abruptly exposed. Third, heterogeneity of tumor cells, as is present in naturally occurring tumors, is difficult to artificially simulate in the transplantable tumor model. Accordingly, the tumor microenvironment in transplanted tumors is different from naturally occurring cancers, thereby resulting in altered patterns of angiogenesis and host immune response.

Genetically engineered mouse models of cancer are becoming useful systems for understanding the molecular and cellular pathogenesis of tumorigenesis and for evaluating anticancer agents *in vivo*.[7,8] These mouse models require genetic manipulations that faithfully recapitulate somatic events in human cancer cells. Melanomagenesis is associated with several somatic alterations in oncogenes and/or tumor suppressor genes.[9,10] Ink4a/Arf is a tumor suppressor protein encoded by *CDKN2A*, which acts through both p53 and Rb tumor suppressor pathways. Genetic analysis in familial melanoma patients has identified germline mutations in *CDKN2A*.[11] Furthermore, melanoma-associated oncogene activation most commonly involves N-ras and its effector pathways, Raf-MAPK/ERK kinase (MEK)-ERK (RAF-MEK-ERK) and PI3K-Akt. Activation of Akt3 promotes the development of melanoma by various mechanisms, including stimulation of cell proliferation and enhanced resistance to apoptosis.[12] Transgenic (TG) studies in mice have demonstrated that activated H-ras or N-ras mutations in melanocytes can lead to aberrant proliferation and malignant transformation.[13,14] Furthermore, mice

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with melanocyte-specific expression of activated H-ras or N-ras on an Ink4a-deficient background develop spontaneous cutaneous melanomas with high penetrance.[14–16] More recently, genetically engineered mouse melanoma models with inducible expression of *BRAF* or *KRAS* have been developed.[17,18] These mice do not need carcinogen treatment and have the same genetic alterations as those of human melanoma, thus serving as a clinically relevant animal model to test therapeutic efficacy.

Because genetically engineered mouse models are generally of a mixed genetic background, we established congenic strains to better suit immunotherapeutic experiments. FVB/N congenic strains of a TG mouse overexpressing melanocyte-specific H-ras^{G12V} were cross-bred with an Ink4a/Arf knockout (KO) mouse to generate an FVB/N congenic strain of TG/KO mice that is prone to develop spontaneous melanoma. Our TG/KO mice developed spontaneous melanomas with high penetrance (74%) at around 3−4 months of age, a timeframe that is amenable to experimental therapeutics. Using the TG/KO mice, we evaluated the efficacy of whole recombinant yeast expressing hMART-1 and showed that administration of yeast vaccine induces antitumor immunity against melanoma tumors and improves overall survival of tumor-bearing mice.

RESULTS

Establishing TG/KO mice on FVB/N background

We generated a genetically engineered mouse melanoma model on an FVB/N congenic background by backcrossing a TG mouse and a KO mouse onto FVB/N strains and confirming that they are congenic via single-nucleotide polymorphism (SNP) marker genotyping. TG mice overexpressing melanocyte-specific H-ras^{G12V} were originally generated on a mixed genetic background (C57BL/6J (50%); CBA/J (50%)),[15] and were backcrossed onto FVB/N at the National Cancer Institute's (Frederick, MD, USA) MMHCC Repository for four to six generations. We further backcrossed the TG mouse onto FVB/N for three more generations to establish an FVB/N congenic strain. Ink4a/Arf KO mice were also originally generated on a mixed genetic background (C57BL/6J (80%); 129/Sv (18.7%); SJL/J (1.25%)).[15] These were also backcrossed onto FVB/N by the MMHCC for four to six generations followed by three more generations by us to establish the FVB/N congenic strain. We then cross-bred a TG mouse (FVB/N) with a KO mouse (FVB/N) to generate a TG/KO mouse that was prone to develop spontaneous melanoma.

DNA samples from 19 TG/KO mice were analyzed using 210 SNP markers on chromosome 1–19 by JAX Genome Scanning Services (Jackson Laboratory, Bar Harbor, ME, USA). All 19 samples were 100% FVB/N-like for all chromosomes, except for chromosome 4 near the *CDKN2A* gene. SNP markers on chromosome 4 at 84.2, 88.9, 100.1, 101 and 117.6 Mb were 129/Sv-like. When these five SNP markers were included in the analysis, 210 SNP markers were 97–98% FVB/N-like, confirming that our TG/KO mice were congenic.

TG/KO mice spontaneously develop MART-1-positive melanomas

Spontaneous development of melanomas on the ears, eyes, trunk and tail was noted in 20 of 27 (74%) TG/KO mice around 3–4 months of age (100.4 ± 4.8 days; Figures 1a–d). Among the remaining seven mice, two mice developed soft tissue tumors (lymphoma and fibrosarcoma) and five mice (18.5%) died suddenly of unknown causes, similar to previously reported observations.[15] Considering the role of CDKN2a as a *bona fide* tumor suppressor, it was suspected that some of these mice developed spontaneous tumors, such as endocrine tumors.[19–21] The melanomas from TG/KO mice were amelanotic and often ulcerated. Hematoxylin and eosin staining of the melanoma tumors showed a dermal infiltration of epithelioid and spindle cells with amphophilic cytoplasm and frequent cellular atypia (Figures 1e and f). Melanoma cells were rarely apparent in the epidermis. The expression of MART-1, tyrosinase, TRP-1, TRP-2 and gp100 mRNA was demonstrated by reverse transcription (RT)-PCR in melanoma lesions (Figure 2a). MART-1 protein expression in TG/KO mouse melanomas was confirmed using western blot (Figure 2b) and immunohistochemical staining (Figures 2c and d).

hMART-IT induces cytotoxicity against mouse melanoma in TG/KO mice

Because many tumors downregulate major histocompatibility complex (MHC) class I expression to escape from immune destruction, we first analyzed MHC class I expression of the target melanoma cells, 8B20 cells, by flow cytometry. Although MHC class I expression in 8B20 cells was downregulated at baseline, IFN-γ pretreatment induced upregulation of MHC class I to potentiate cell lysis by lymphocytes (Figure 3a). We then evaluated the ability of splenocytes from hMART-IT-vaccinated TG/KO mice to kill the IFN-γ-pretreated melanoma cells *in vitro*. Six-week-old TG/KO mice were treated with hMART-IT or phosphorus-buffered saline (PBS). Two weeks later, splenocytes were isolated, stimulated *in vitro* with hMART-IT, and tested for cytotoxicity against IFN-γ-pretreated 8B20 cells. No mice had visible melanoma at the time of harvest. Culturing splenocytes from hMART-IT-vaccinated mice with target cells for 4 h resulted in a dose-dependent lysis of 8B20 melanoma cells, whereas cytotoxicity was not observed from naive splenocytes (Figure 3b).

hMART-IT induces production of IL-2, tumor necrosis factor-α, IL-12 and IFN-γ *in vitro*

Supernatants were obtained from splenocytes after *in vitro* stimulation with hMART-IT and analyzed for cytokine production. Splenocytes from hMART-IT-immunized TG/KO mice produced significantly higher levels of interleukin (IL)-2 (Figure 4a), tumor necrosis factor-α (Figure 4b), IL-12 (Figure 4c) and IFN-γ (Figure 4d) than splenocytes from PBS-treated mice. These results demonstrate that hMART-IT induces the secretion of Th1 cytokines by splenocytes.

We then tested if the cytokine secretion elicited by hMART-IT was antigen-specific. Splenocytes from PBS- or hMART-IT-treated mice were stimulated with recombinant human H-ras protein (rHu-H-ras), recombinant human MART-1 protein (rHu-MART-1), hMART-IT or YVEC for 24 h. Splenocytes incubated with rHu-H-ras did not produce significant amounts of IL-12 or IFN-γ (Figures 4e and f). In contrast, rHu-MART-1 induced a dose-dependent secretion of IL-12 (14.80 ± 1.45 and $71.09 \pm 7.46\,pg\,ml^{-1}$ with 0.05 and $0.5\,\mu g\,ml^{-1}$ rHu-MART-1, respectively) by splenocytes from hMART-IT-treated mice, but in a lesser degree in splenocytes from PBS-treated mice. Splenocytes from hMART-IT-treated mice demonstrated increased secretion of IL-12 after incubation with hMART-IT ($79.42 \pm 10.30\,pg\,ml^{-1}$) and in a lesser degree after incubation with YVEC ($22.99 \pm 4.14\,pg\,ml^{-1}$), whereas those from PBS-treated mice secreted little IL-12 after incubation with hMART-IT ($2.81 \pm 1.26\,pg\,ml^{-1}$) or YVEC ($2.13 \pm 1.23\,pg\,ml^{-1}$). Similarly, rHu-MART-1 induced a dose-dependent secretion of IFN-γ (4404 ± 199 and $21339 \pm 2628\,pg\,ml^{-1}$ with 0.05 and $0.5\,\mu g\,ml^{-1}$ rHu-MART-1, respectively) by splenocytes from hMART-IT-treated mice, but in a lesser degree in splenocytes from PBS-treated mice. Splenocytes from hMART-IT-treated mice demonstrated increased secretion of IFN-γ after incubation with hMART-IT ($3290 \pm 428\,pg\,ml^{-1}$) and in a lesser degree after incubation with YVEC ($1050 \pm 106\,pg\,ml^{-1}$), whereas those from PBS-treated mice secreted little IFN-γ after incubation

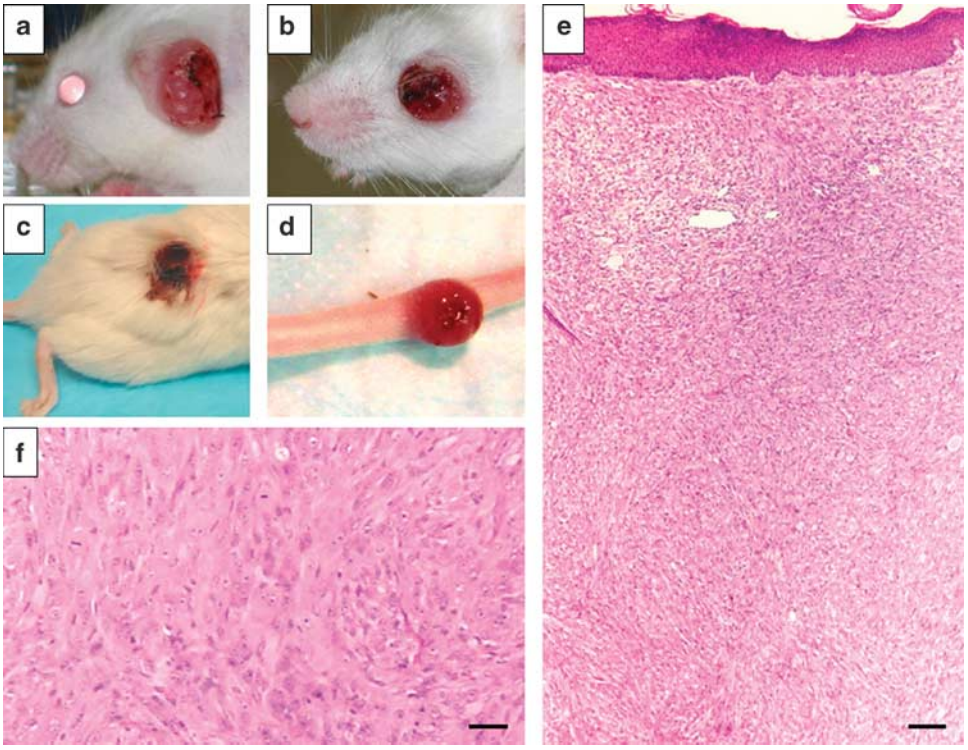

Figure 1 TG/KO mice on FVB/N background. Mice with melanocyte-specific H-ras^{G12V} expression on an Ink4a/Arf-deficient background spontaneously developed melanomas on the ears (**a**), eyes (**b**), torso (**c**) and tail (**d**) at 3–4 months of age. (**e** and **f**) Hematoxylin and eosin staining of melanomas. Scale bar represents 200 μm (**e**) and 80 μm (**f**).

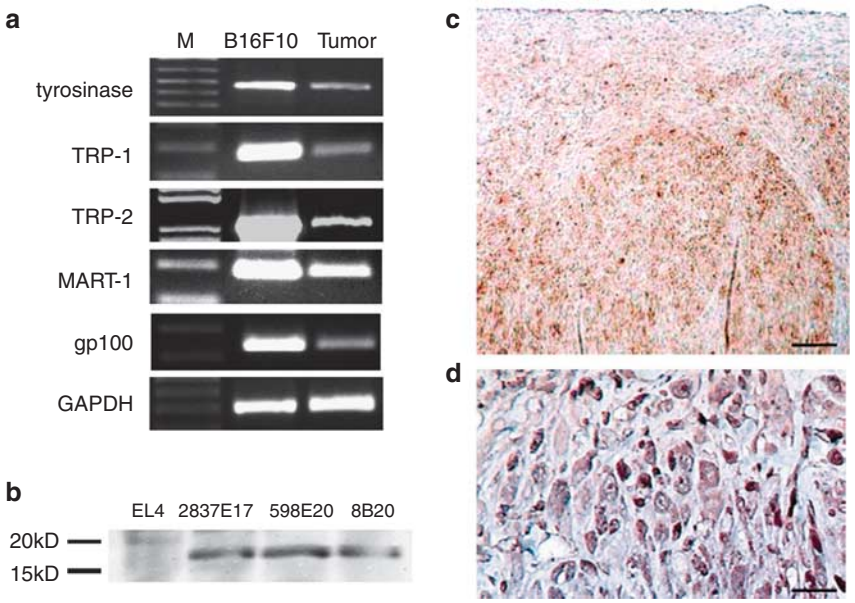

Figure 2 MART-1 expression in TG/KO mouse melanomas. (**a**) Expression of tyrosinase, TRP-1, TRP-2, MART-1 and gp100 mRNA in a TG/KO mouse melanoma tumor was evaluated using reverse transcription-PCR. Melanoma cell line B16F10 served as a positive control. M is a molecular weight marker. (**b**) Expression of MART-1 protein was evaluated by western blot using melanoma cell lines established from TG/KO mouse melanomas (2837E17, 598E20 and 8B20). EL4 cells served as a negative control. (**c** and **d**) Expression of MART-1 protein on TG/KO melanoma tumors using immunohistochemical staining. Scale bars represent 300 μm (**c**) and 30 μm (**d**).

with hMART-IT (2.19 ± 2.19 pg ml^{-1}) or YVEC (1.21 ± 1.21 pg ml^{-1}). These data suggest that vaccination with hMART-IT results in the MART-1-specific secretion of Th-1 cytokines.

Vaccination with hMART-IT prolongs survival of TG/KO mice

To investigate if vaccination of TG/KO mice with hMART-IT protects against melanoma *in vivo*, animals were randomly assigned to cohorts

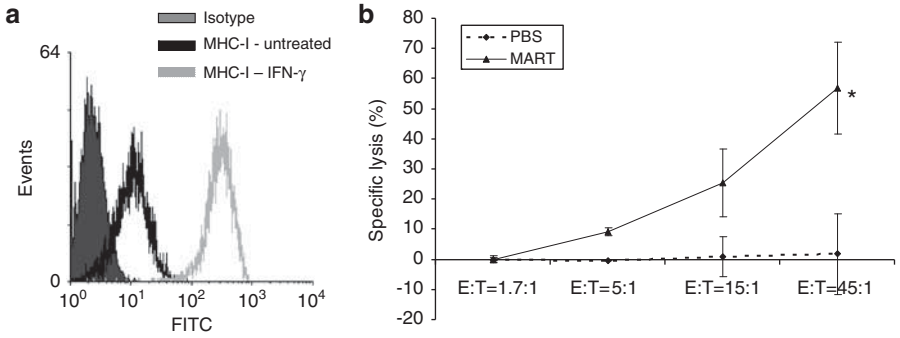

Figure 3 Splenocytes from hMART-IT-vaccinated mice induce cytotoxicity against melanoma cells *in vitro*. (**a**) Expression of MHC class I was determined by surface flow cytometry analysis from 8B20 cells untreated or treated with IFN-γ. (**b**) Splenocytes from PBS- and hMART-IT-treated mice were restimulated *in vitro* with hMART-IT, and used as effector cells. IFN-γ-treated 8B20 murine melanoma cells were used as target cells. Cytotoxic activity was determined using LDH release. E/T, effector to target cell ratio. Experiments were repeated three times using different sets of mice. Asterisk indicates significant difference ($P<0.05$ for the hMART-IT-treated mice compared with PBS-treated mice).

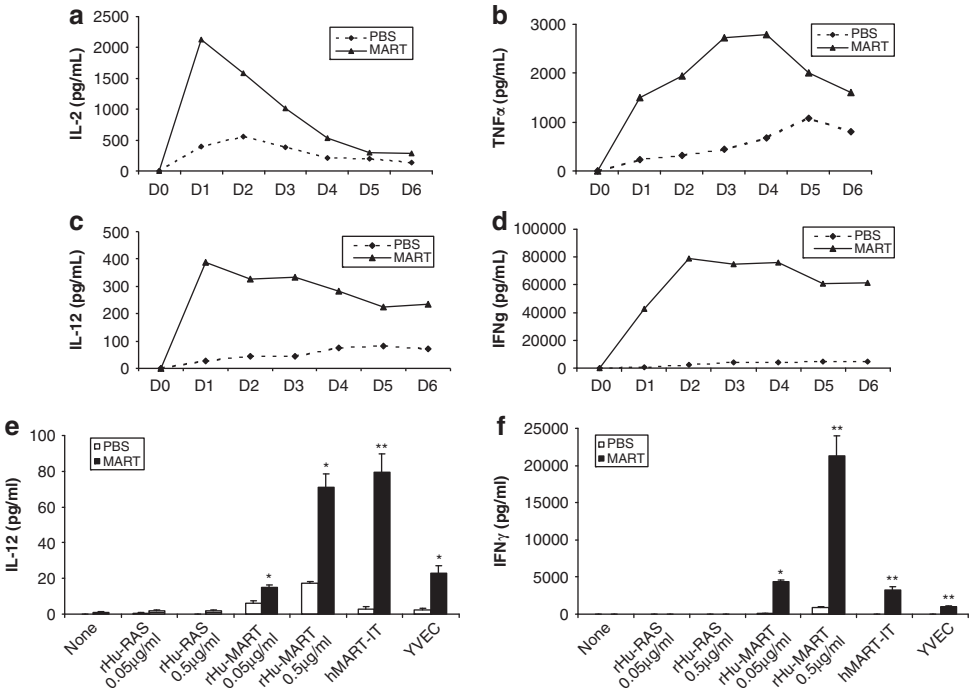

Figure 4 Splenocytes from hMART-IT-vaccinated mice produce Th1 cytokines when stimulated with hMART-1 or MART-1. (**a-d**) Splenocyte from PBS- and hMART-IT-treated mice were co-cultured with heat-inactivated hMART-IT at splenocyte/yeast ratio of 2:1 for 6 days. Supernatants were analyzed for IL-2 (**a**), tumor necrosis factor-α (**b**), IL-12 (**c**) and IFN-γ (**d**) production using the Bio-Plex mouse cytokine Th1/Th2 panel. Experiments were repeated three times using different sets of mice. The data are one of three representative experiments. (**e** and **f**) Splenocytes from PBS- and hMART-IT-treated mice were incubated with rHu-H-ras, rHu-MART-1, hMART-IT or YVEC for 24 h. Supernatants were collected and measured for IL-12 (**e**) and IFN-γ (**f**). The data represent mean ± s.e., $n=3$. The error bars are from one experiment performed in triplicate, and the experiments were replicated twice. $^*P<0.05$; $^{**}P<0.01$ compared with splenocytes from PBS-treated mice.

with hMART-IT, YVEC or PBS vaccination. Cohorts received subcutaneous vaccination weekly starting at 6 weeks (42 days) of age. Mice were monitored for tumor development, tumor size and survival. Mice that developed ocular melanoma were killed and not included in the study. Final animal numbers investigated in the study were $n=36$, 30 and 35 for the treatment with hMART-IT, YVEC or PBS, respectively. Although the development of cutaneous melanoma tumors was delayed in hMART-IT-treated mice (110.56 ± 8.60 days) compared with PBS-treated or YVEC-treated mice (90.08 ± 4.93 days or 98.93 ± 6.12 days, respectively), the log-rank statistic was not significant ($P=0.052$). However, time from tumor development until death was significantly prolonged from 25.74 ± 3.18 days (PBS-treated mice) or 24.09 ± 3.10 days (YVEC-treated mice) to 39.41 ± 6.13 days (hMART-IT-treated mice; $P=0.034$ for log-rank test, Figure 5a). Likewise, overall survival of mice was significantly prolonged from 110.11 ± 5.90 days (PBS-treated mice) or 117.83 ± 5.70 days (YVEC-treated mice) to 140.28 ± 8.32 days (hMART-IT-treated mice; $P=0.001$ for log-rank test, Figure 5b). Tumor regression was not

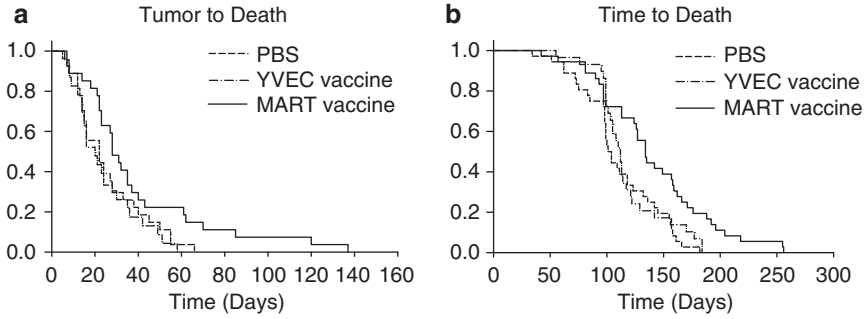

Figure 5 hMART-IT vaccination prolongs survival of TG/KO mice. TG/KO mice were inoculated subcutaneously into the lateral flank with hMART-IT (n=36), YVEC (n=30) or PBS (n=35) weekly since 6 weeks of age until death. Mice were assessed for tumor development, tumor size and survival once a week. Kaplan–Meier survival curves of time from tumor to death (**a**) and time to death (**b**) are shown. Log-rank test was used to test the difference in the survival curves. (**a**) Log-rank statistic test showed a significant difference among tumor-to-death curves (P=0.042 for hMART-IT-treated mice vs PBS-treated mice and P=0.021 for hMART-IT-treated mice vs YVEC-treated mice). (**b**) Log-rank statistic test showed a significant difference among survival curves (P=0.0012 for hMART-IT-treated mice vs PBS-treated mice and P=0.0056 for hMART-IT-treated mice vs YVEC-treated mice).

observed in any group. These results indicate that subcutaneous administration of hMART-1 significantly prolonged the survival of tumor-bearing TG/KO mice compared with naive mice and mice vaccinated with yeast transfected with an empty vector.

Splenocytes from tumor-bearing mice treated with hMART-IT retain cytototoxic activity *in vitro*

Although tumor growth was delayed and mice survived longer after hMART-IT vaccination, melanoma tumors continued to grow and regression was not observed. Thus, we investigated mechanisms of immunotolerance in hMART-IT-treated TG/KO mice bearing melanoma tumors. We first evaluated the ability of splenocytes from tumor-bearing mice to kill melanoma *in vitro*. Splenocytes from hMART-IT-treated TG/KO mice bearing melanoma tumors were isolated, stimulated *in vitro* with hMART-IT, and tested for cytotoxicity against IFN-γ-treated 8B20 cells. IFN-γ-treated B16F10 melanoma cells that express MART-1, but express the wrong MHC class I (that is, expressing H-2^b but not H-2^q), were used as a control target. Splenocytes from hMART-IT-treated TG/KO mice bearing melanoma tumors showed dose-dependent cytotoxicity against 8B20 melanoma cells, but not against B16F10 cells (Figure 6a), suggesting that cytotoxic activity of lymphocytes was retained in tumor-bearing mice.

hMART-IT-treated tumors show increased tumor-infiltrating $CD4^+$ T cells and $CD8^+$ T cells, but no reduction of $Foxp3^+$ cells

As tumor microenvironment may have significant effect in local immune defense,[22,23] we examined the presence of $CD3^+$, $CD4^+$, $CD8^+$ and $Foxp3^+$ cells within melanoma tumors using immunohistochemistry. Melanomas from mice vaccinated with hMART-IT demonstrated a significant increase in $CD3^+$, $CD4^+$ and $CD8^+$ cells within and around the tumor, compared with PBS-treated mouse melanoma (Figure 6b). However, the number of Foxp3-expressing cells was only mildly decreased in tumors from mice vaccinated with hMART-IT, as compared with PBS-treated mouse tumors. This finding may offer an explanation as to why hMART-IT vaccination only prolonged survival and did not ultimately protect animals from melanoma formation or death.

DISCUSSION

Previous work shows that mice with melanocyte-specific expression of activated H-ras on an Ink4a-deficient background develop spontaneous cutaneous melanomas after a latency of 5.5 months and with a penetrance of 42.8% (15 out of 35 mice).[15,16] In this study, the FVB/N congenic mouse model of the genetically engineered mice developed spontaneous melanomas after a latency period of 3–4 months and had a penetrance of 74%. The shorter latency and higher penetrance in our TG/KO mice may stem from phenotypic differences in inbred mouse strains. For example, C57BL/6J mice are known to develop a Th1 antitumor response and be refractory to the development of many tumors, whereas FVB/N mice are highly susceptible to chemically induced tumor formation with a high rate of malignant conversion.[24,25]

Although melanomas in the TG/KO mice were amelanotic, tumor cells expressed well-defined melanoma antigens, such as MART-1, S-100 and TRP-1. Thus, we evaluated the efficacy of immunotherapy using whole recombinant yeast expressing hMART-1 by utilizing the spontaneous mouse melanoma model. Yeast are avidly phagocytized by dendritic cells, thereby providing an efficient vector for delivering recombinant tumor antigens to the immune system.[26] Recombinant protein antigens that are naturally mannosylated by yeast have been shown to enhance MHC class I and II antigen presentation and T-cell stimulation when compared with non-mannosylated proteins.[27–30] In addition, yeast naturally possess adjuvant-like properties[31,32] and can be easily engineered to express multiple antigens. Furthermore, the whole yeast vaccine does not require precise knowledge of the antigenic epitopic sequence or their matched MHC class I or II, because full-length protein expressed in yeast is naturally processed and presented by dendritic cells. Indeed, despite the fact that MART-1 peptides recognized by T cells from FVB/N mice have not been defined, the cytokine production induced by hMART-IT was specific to the hMART-1 protein. Thus, the use of yeast systems will provide a unique immunostimulatory platform for the development of vaccines.

Furthermore, immunization of TG/KO mice with hMART-IT delayed melanoma tumor development and resulted in prolongation of survival from spontaneously arising melanoma when compared with PBS- or YVEC-treated mice. hMART-IT induced cytotoxicity and a strong Th1 cytokine response by increases in IL-2, tumor necrosis factor-α, IL-12 and IFN-γ *in vitro*, indicating a favorable environment for antitumor immunity. High-dose IL-2 administered intravenously has been shown to induce tumor responses in 16% of patients with unresectable melanoma, with 6% achieving a complete response.[33] Likewise, administration of IL-12 stimulates IFN-γ pro-

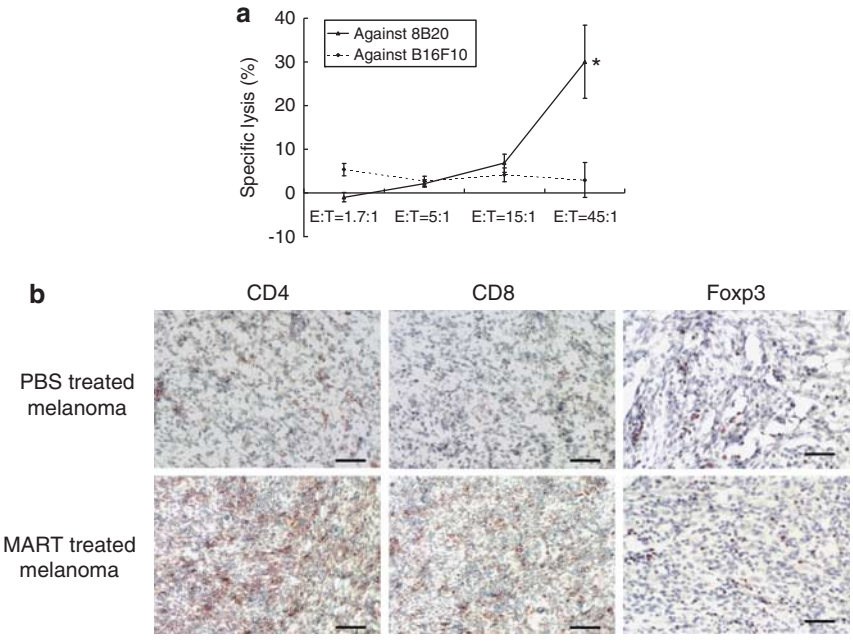

Figure 6 Analysis of tumor-bearing mice. (**a**) Splenocytes from hMART-IT-vaccinated tumor-bearing mice were restimulated *in vitro* with hMART-IT, and used as effector cells. IFN-γ treated 8B20 murine melanoma cells (H-2^q) and B16F10 murine melanoma cells (H-2^b) were used as target cells. Cytotoxic activity was determined using LDH release. E/T, effector to target cell ratio. Experiments were repeated twice using different sets of mice. Asterisk Indicates significant difference ($P<0.05$ for the cytotoxicity against 8B20 cells compared with that against B16F10 cells). (**b**) Expression of CD4, CD8 and Foxp3 in tumors from PBS-treated mice and those from hMART-IT-vaccinated mice using immunohistochemistry. Scale bar represents 100 μm.

duction and has produced tumor responses in an early phase 1 trial.[34] The combination of low-dose IL-2 with IL-12 administered intravenously has also been shown to produce modest antitumor effects in some patients through sustained elevation of IFN-γ.[35]

Despite the induction of cytotoxicity and Th1 cytokine production *in vitro* and slower tumor growth *in vivo*, melanoma tumors did not regress in hMART-IT-treated mice. The presence of diffuse infiltration of $CD4^+$ cells and $CD8^+$ cells in the tumors, as well as positive cytotoxic activity from splenocytes *in vitro*, suggests the induction of a T-cell-mediated immune response by hMART-IT in tumor-bearing mice and supports the finding that hMART-IT-treated animals exhibited a slower tumor growth. However, immunohistochemical analysis demonstrated the presence of $Foxp3^+$ cells in the tumors treated with hMART-IT. $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg cells) temper immunologic tolerance to self-antigens by suppressing antitumor immune responses. An increase in the number of functionally suppressive $CD4^+CD25^+$ cells has been observed in patients with metastatic melanoma[36] and nodal melanoma,[37] indicating that Treg cells may have a role in modifying the magnitude of the T-cell response in the patient. Sutmuller *et al.*[38] showed that $CD25^+$ depletion resulted in increased tumor rejection. They also demonstrated that antitumor treatment is strongly improved if vaccination is preceded by a depletion of $CD25^+$ Treg cells *in vitro*.

Using a genetically engineered Hgf-$Cdk4^{R24C}$ melanoma mouse model, Tormo *et al.*[39] have shown that combined immunotherapy with vaccination and adjuvant injection delays melanoma growth, in concordance with our data. By further adding cyclophosphamide and adoptive transfer of tumor-specific T lymphocytes into these regimens, Kohlmeyer *et al.*[40] have shown that a combination chemo-immunotherapy results in complete regression of both primary and metastatic melanoma in the Hgf-$Cdk4^{R24C}$ melanoma mouse model. Cyclophosphamide induces T-cell homeostatic proliferation, abrogates Treg cells and resets dendritic cell homeostasis.[41–44] Adding chemotherapeutics to augment host immunity in our model may serve to enhance the immunologic response, possibly resulting in improvements in survival of mice. Furthermore, enhancing T-cell response by inhibition of cytotoxic T-lymphocyte-associated antigen-4 with ipilimumab has been shown to improve overall survival of stage III or IV melanoma patients in a Phase III trial.[4] Cytotoxic T-lymphocyte-associated antigen-4 is expressed on activated T cells and transmits an inhibitory signal to effector T cells.[45] Cytotoxic T-lymphocyte-associated antigen-4 is also expressed on Treg cells and is essential for Treg function.[46] Thus, the addition of ipilimumab or other T-cell modulators may serve to enhance the efficacy of the hMART-IT vaccine in melanoma treatment.

MATERIALS AND METHODS

Mice

Tyr-H-ras^{G12V} TG mice (FVB/N background) and Ink4a/Arf KO mice (FVB/N strain) were obtained from the National Cancer Institute.[15,47] They were further backcrossed onto FVB/N to establish FVB/N congenic strains. Presence of the transgene and KO alleles was assessed using genomic PCR.[15,47] The mice were cross-bred twice to generate TG mice with melanocyte-specific H-ras^{G12V} expression on an Ink4a/Arf-deficient background (TG/KO). Animals were kept under specific pathogen-free conditions, according to National Institutes of Health Animal Care Guidelines. Experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Colorado at Denver.

Genome scan

DNA was extracted from tail samples using REDExtract-N-Amp Tissue PCR Kit (Sigma, St Louis, MO, USA). SNP markers were selected from chromosomes 1–19 to distinguish FVB/N from C57BL/6;CBA/J;129/Sv by Jackson

Laboratory. Genotyping of 19 TG/KO mice was performed from each DNA sample by JAX Genome Scanning Service (Jackson Laboratory).

Yeast engineering

Yeast cells were engineered to express human MART-1, as previously described.[5,48,27] An expression vector was generated by inserting human MART-1 complementary DNA into pYEX-BX yeast vector (Amrad Biotech, Baronia, Australia). The vector was transfected into *Saccharomyces cervisiae*, W303α (ATCC, Manassas, VA, USA). The expression of MART-1 protein is under control of the copper-inducible-CUP1 promoter. Copper sulfate (0.5 mM, Sigma) was added to yeast cultures during log-phase growth and the expression of human MART-1 was confirmed by western blot. Yeast expressing human MART-1 was designated hMART-IT. Yeast transfected with pYEX-BX without MART-1 was designated YVEC and used as a vector control. Yeast were heat inactivated (56 °C, 1 h) and stored at −70 °C until use.

Cell culture

B16F10 murine melanoma and EL-4 murine thymoma cell lines were purchased from ATCC. Cells 8B20, 598E20 and 2837E17 were established from melanoma tumors spontaneously arising in TG/KO mice by dissociating tumors with collagenase I and hyaluronidase (Sigma) for 2 h and culturing dissociated cells. All cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (Summit Biotech, Ft Collins, CO, USA), L-glutamine (Sigma) and penicillin/streptomycin (Mediatech, Herndon, VA, USA).

Reverse transcription-PCR

Whole RNA was isolated from tumors, skin and spleen of TG/KO mice as well as B16F10 cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using the Reverse Transcription System kit (Promega, Madison, WI, USA). PCR was performed with 30 cycles using following primer sequences: Tyrosinase-F: 5′-GGCCAGCTTTCAGGCAGAGGT-3′; Tyrosinase-R: 5′- TGGTGCTTCATGGGCAAAATC-3′; TRP-1-F: 5′-GCTGCAGGAGCCTTCTTTCTC-3′; TRP-1-R: 5′-AAGACGCTGCACTGCTGGTCT-3′; TRP-2-F: 5′-GGATGACCGTGAGCAATGGCC-3′; TRP-2-R: 5′-CGGTTGTGACCAATGGGTGCC-3′; MART-1-F: 5′-CGCTCCTATGTCACTGCTGA-3′; MART-1-R: 5′-GGTGATCAGGGCTCTCACAT-3′; gp100-F: 5′-CGGATGGTCAGGTTATCTGGA-3′; gp100-R: 5′-TGGTGAAGGTTGAACTGGC-3′; glyceraldehyde-3-phosphate dehydrogenase-F: 5′-CCATGACAACTTTGGCATTG-3′; glyceraldehyde-3-phosphate dehydrogenase-R: 5′-CCTGCTTCACCACCTTCTTG-3′.

Western blot

Cells EL4, 8B20, 598E20 and 2837E17 were suspended in RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, from Pierce, Rockford, IL, USA) and homogenized with an electronic homogenizer (VirSonic50, VirTis, Gardiner, NY, USA). Cell lysates were separated by 12% Tris-HCl SDS/polyacrylamide electrophoresis (Bio-Rad, Hercules, CA, USA) and transferred to a nitrocellulose membrane (Bio-Rad) at 200 mA for 90 min. Nonspecific binding was inhibited by incubation of nitrocellulose membrane in 0.2% Tris-buffered saline with Tween-20 with 5% nonfat milk for 1 h at room temperature. Membranes were incubated with mouse antihuman Melan-A/MART-1 (BioGenex, San Ramon, CA, USA) as the primary antibody and goat antimouse horseradish peroxidase-conjugate (Pierce) as the secondary antibody, followed by detection using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce), according to the manufacture's instructions.

Flow cytometry

Cells were incubated with fluorescein isothiocyanate-conjugated antimouse $H2K^q$ from BD Biosciences (San Diego, CA, USA) for 30 min. Isotype-matched antibody was used as a control. Expression of the cell surface marker was analyzed by standard two-color flow cytometry with a Beckman Coulter FC500 flow cytometer (Beckman Coulter, Hialeah, FL, USA) and Summit software (Dako Cytomation, Ft Collins, CO, USA).

Cytotoxicity assay

Six-week-old TG/KO mice were inoculated subcutaneously into the lateral flank with hMART-IT (5×10^7 yeast cells in 100 μl PBS) or PBS (100 μl, naive mice) weekly for 3 weeks. Two weeks after the last injection, spleens were collected and single cell suspensions were prepared. Splenocytes were co-cultured with hMART-IT at a splenocytes/yeast ratio of 2:1 for 6 days in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, penicillin/streptomycin and 50 μM β-mercaptoethanol and used as effector cells. Cells 8B20 were treated for 48 h with IFN-γ (50 U ml^{-1}, R&D Systems, Minneapolis, MN, USA) and used as target cells for T-cell cytotoxicity assays. Effector cells were resuspended to a final concentration of 6.75×10^6 cells ml^{-1}, and 50 μl were then mixed with target cells (effector to target ratios ranging from 1.7:1 to 45:1) and incubated at 37 °C in a humidified 5% CO_2 incubator for 4 h. Target cells and effector cells alone were used as controls. Cytotoxic activity of effector cells was determined using lactate dehydrogenase (LDH) release (Cyto-Tox 96 Non-Radioactive Cytotoxicity Assay kit, Promega) following the manufacture's instructions. Percent cytotoxicity was calculated using the following equation: Percentage-specific lysis=((experimental LDH release)−(target spontaneous LDH release)−(effector spontaneous LDH release))/((target total LDH release)−(target spontaneous LDH release))×100.

Cytokine analysis

Mice were injected subcutaneously with hMART-IT or PBS weekly for 3 weeks. Two weeks after the last injection, single cell suspensions were prepared from spleens. Splenocytes were co-cultured with heat-inactivated hMART-IT at a splenocytes/yeast ratio of 2:1. Supernatant was collected every 24 h for 6 days for cytokine profiles. Multiple simultaneous cytokine measurements were made using Bio-Plex mouse cytokine Th1/Th2 panel (Bio-Rad), according to the manufacturer's instructions. Briefly, splenocyte supernatant was incubated with spectrally addressed polystyrene beads coated with cytokine-specific antibodies, followed by incubation with biotinylated detection antibody and detection by streptavidin–phycoerythrin. The magnitude of the reaction was measured using a Luminex bead plate reader (Luminex, Austin, TX, USA), and cytokine concentrations were calculated by Bio-Plex Manager software (Bio-Rad). In some experiments, splenocytes were co-cultured with rHu-MART-1 (Prospec-Tany TechonoGene, Rehovot, Israel), rHu-H-ras (Oxford Biomedical Research, Oxford, MI, USA), YVEC or hMART-IT for 24 h, and supernatants were analyzed for cytokine profiles.

Vaccination protocol

From 6 weeks until death, TG/KO mice were inoculated subcutaneously into the flank with hMART-IT (5×10^7 yeast cells in 100 μl PBS), YVEC (5×10^7 yeast cells in 100 μl PBS) or PBS (100 μl, naive mice) weekly. Mouse skin was inspected weekly. Erythematous cutaneous tumors >2 mm in diameter were considered primary melanoma. Tumor volumes were measured weekly, and mice were euthanized when tumor volumes exceeded 2 cm^3 or when mice developed ocular melanoma.

Immunohistochemistry

Tumor samples were obtained from TG/KO mice vaccinated with hMART-IT or PBS. They were either fixed in 10% formalin and embedded in paraffin, or embedded in OCT Tissue Freezing Medium (Triangle Biomedical Science, Durham, NC, USA) and snap-frozen. Paraffin-embedded samples were sectioned, deparaffinized, preheated with heat/citrate buffer (Antigen Retrieval Solution, BioGenex) and incubated with anti-Melan-A/MART-1 (BioGenex), anti-CD3 (clone Cd3-12; Serotec, Raleigh, NC, USA) or anti-Foxp3 (Clone FJK-16 s, eBioscience, San Diego, CA, USA) at 4 °C overnight. Frozen tissue samples were cryosectioned, fixed in ice-cold acetone for 10 min and incubated with anti-CD4 (clone GK1.5, BD Bioscience) or anti-CD8 (clone XMG1.2, BD Biosciences) antibodies. Immunoreactions were visualized using avidin–biotin–peroxidase complex methods. Hematoxylin (Sigma) was used as a counterstain. Negative controls were incubated with isotype-matched antibodies.

STATISTICAL ANALYSIS

In vivo studies of survival were analyzed using SigmaStat software (Aspire Software International, Ashburn, VA, USA). Kaplan–Meier nonparametric

regression analysis was performed, and significance was determined using log-rank test. *In vitro* studies were analyzed by Student's unpaired *t*-tests. Differences were considered significant if $P<0.05$.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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