

Generation of a prophylactic melanoma vaccine using whole recombinant yeast expressing MART-1

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Abstract: Malignant melanoma is a potentially deadly form of skin cancer and people at high-risk of developing melanoma will benefit from effective preventive intervention. Yeast can be used as an efficient vehicle of antigen loading and immunostimulation. *Saccharomyces cerevisiae* is not pathogenic to humans and can be easily engineered to express specific antigens. In this study, we have developed a melanoma vaccine using a yeast-based platform expressing a full-length melanocyte/melanoma protein to investigate its utility as a prophylactic melanoma vaccine in a transplantable mouse melanoma model. Yeast was engineered and expanded *in vitro* without technical difficulties, administered easily with subcutaneous injection, and did not show adverse effects, indicating its practical applicability and favourable safety profile.

Despite the lack of knowledge of dominant epitopes of the protein recognized by mouse MHC-class I, the vaccine protected mice from tumor development and induced efficient immune responses, suggesting that the precise knowledge of epitopic sequences and the matched HLA type is not required when delivering a full-length protein using the yeast platform. In addition, the vaccine stimulated both CD4⁺ T cells and CD8⁺ T cells simultaneously. This study provides a 'proof of principle' that recombinant yeast can be utilized as an effective prophylactic vaccine to target patients at high-risk for melanoma.

Key words: animal model – immunotherapy – melanoma – vaccine – Yeast

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Introduction

Malignant melanoma is the deadliest form of skin cancer and its incidence is rising fast (1). Patients who are at high-risk of developing melanoma include those with prior melanoma, dysplastic melanocytic nevi, large congenital nevi, large number of common acquired nevi, fair skin, familial melanoma and congenital disorders of DNA repair such as xeroderma pigmentosum (2,3). Currently, there is no good treatment for advanced melanoma and the best options for these high-risk populations are prevention and early detection.

Preventive measures such as sun avoidance and skin cancer screening are able to reduce incidence and mortality of melanoma. However, pharmacological and immunological approaches of melanoma prevention are disappointing despite progress in basic cancer research. Many experimental studies have shown that activation of both innate and adaptive immunity can prevent tumor development. While immunotherapy relies on activation of effector mechanisms to treat established tumors, cancer vaccines are being developed with an eye on prevention. As a

prophylactic cancer vaccine will be given to non-tumor-bearing individuals, it must fulfil the following characteristics in addition to efficacy: inexpensive and simple generation, practicable administration, applicability in diverse patient populations and a favourable safety profile.

Yeast has been shown to work as an efficient vehicle of antigen loading and immunostimulation (4). Administration of whole recombinant *Saccharomyces cerevisiae* yeast, expressing ovalbumin provoked antigen-specific cytotoxic T lymphocytes (CTLs) and T-helper cells *in vitro* and protected mice from ovalbumin-transfected lymphomas (5). Therapeutic immunization of mice with whole recombinant yeast expressing mammalian mutant Ras proteins caused the regression of pre-existing, carcinogen-induced, Ras mutation-bearing lung tumors in mice (6). *S. cerevisiae* is not pathogenic to humans and can be engineered to express specific antigens without technical difficulties. Recombinant yeast can be expanded easily *in vitro* and then administered to patients by subcutaneous injection. In contrast to other immunotherapeutic approaches (7), no expensive and time consuming isolation and propagation of patient-derived cells is required. Therefore, in the

current study, we investigated the utility of recombinant whole yeast expressing a melanocyte/melanoma antigen as a prophylactic melanoma vaccine. MART-1 (melanocyte/melanoma antigen recognized by T cells-1)/Melan-A was used as antigen as it is overexpressed in 80–90% of human melanomas (8,9). Using a transplantable mouse melanoma model, we found that subcutaneous administration of whole recombinant yeast expressing MART-1 provided protection against development of melanoma *in vivo* and elicited efficient immune responses involving both CD4⁺ and CD8⁺ T cells *in vitro*.

Methods

Mice

Seven to nine-week-old C57BL/6 female mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed under specific pathogen-free conditions according to National Institutes of Health Animal Care guidelines.

Cell culture

B16F10 murine melanoma and EL-4 murine thymoma cell lines were purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS; Summit Biotech, Ft Collins, CO, USA), L-glutamine and penicillin/streptomycin (Mediatech Inc., Herndon, VA, USA).

Flow cytometry

Cells were incubated with the following monoclonal antibodies for 30 min: phycoerythrin-conjugated anti-mouse CD45R B220 (clone RA3-6B2), fluoresceine isothiocyanate-conjugated anti-mouse CD4 (clone L3T4), phycoerythrin-conjugated anti-mouse CD8a (clone 53-6.7), phycoerythrin-conjugated anti-mouse NK1.1 (clone PK136), phycoerythrin-conjugated anti-mouse CD11c (clone N418) and fluoresceine isothiocyanate-conjugated anti-mouse H2K^b (clone AF6-88.5). Isotype-matched antibodies were used as controls. All antibodies were obtained from BD PharMingen (San Diego, CA, USA). Expression of cell surface markers was analysed by standard two-colour flow cytometry with a FC500 flow cytometer and CXP software (Beckman Coulter, Hialeah, FL, USA).

Yeast engineering

An expression vector encoding human MART-1 was generated by inserting human MART-1 cDNA (a kind gift from Dr Cassian Yee, Fred Hutchinson Cancer Research Center, Seattle, WA, USA), into pYEX-BX vector (Amrad Biotech, Baronia, Australia), and was transfected into *S. cerevisiae* (W303 α ; ATCC) (6,9). Transfectants were selected on supplemented yeast nitrogen base media lacking uracil (Becton

Dickinson, Sparks, MD, USA and MP Biomedicals, Irvine, CA, 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 blotting. The yeast expressing human MART-1 was designated as hMART-IT. Yeast transfected with pYEX-BX without MART-1 was designated as YVEC and used as a vector control. Yeast were heat inactivated (56°C, 1 h), aliquoted and stored at –70°C until use. Aliquots were thawed, washed twice in phosphate buffered saline (PBS) and resuspended in PBS prior to use *in vitro* and *in vivo*.

Western blotting

Yeast were resuspended and lysed in sample buffer (125 mM Tris pH 6.8, 4% sodium dodecyl sulfate (SDS), 10% glycerol, 0.006% bromophenol blue, 2% β -mercaptoethanol) containing proteinase inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA). Lysates were separated by SDS/polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with mouse anti-human Melan-A/MART-1 (BioGenex, San Ramon, CA, USA) as primary antibody and goat anti-mouse horseradish peroxidase-conjugate (Pierce, Rockford, IL, USA) as secondary antibody, followed by detection using SuperSignal Femto Kit (Pierce).

Vaccination protocol and tumor challenge

Mice were injected subcutaneously into the flank with hMART-IT, YVEC (5×10^7 suspended in 100 μ l PBS) or PBS (100 μ l) weekly for 3 weeks. Two weeks after the last injection, mice were challenged subcutaneously in the back with 1×10^4 B16F10 cells in 100 μ l PBS. Tumor development and progression were monitored every 2–3 days and mice were killed when tumor volumes exceeded 2 cm³.

Cytotoxicity assay

Mice were injected subcutaneously with hMART-IT, YVEC or PBS weekly for 3 weeks. Two weeks after the last injection, spleens were harvested and single cell suspensions were prepared. Splenocytes were co-cultured with hMART-IT at a splenocytes: yeast ratio of 2:1 for 5–6 days in cell culture medium supplemented with 50 μ M β -mercaptoethanol and used as effector cells. B16F10 cells untreated or pretreated with interferon (IFN)- γ (50 U/ml, 48 h; R&D Systems, Minneapolis, MN, USA) or EL-4 cells were used as target cells. Effector cells were mixed with target cells at varying effector: target (E: T) ratios and incubated for 4 h at 37°C. Cytotoxicity was determined using a commercial lactate dehydrogenase release assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega, Madison, WI, USA).

Cytotoxicity was calculated as per cent of specific lysis = $100 \times (\text{experimental-effector spontaneous-target spontaneous}) / (\text{target maximum-target spontaneous})$.

***In vitro* cellular subset depletion**

In selected studies, T-cell subsets were depleted using CD4 or CD8 microbeads and LD columns (Miltenyi Biotech, Auburn, CA, USA), following the manufacturer's instructions. Cells incubated in PBS without microbeads and isolated with LD columns served as positive controls (mock depletion). Flow cytometry analysis of depleted populations confirmed that more than 98% of relevant lymphocyte subsets were selectively depleted, whereas mock depletion was without effect.

Cytokine measurement

Mice were injected subcutaneously with hMART-IT, YVEC or PBS weekly for 3 weeks. Two weeks after the last injection, single cell suspensions were prepared from spleens. Splenocytes were co-cultured with hMART-IT at a splenocytes: yeast ratio of 2:1 for 5–6 days. Supernatants were measured for cytokine profiles using a microbead array assay and Bioplex Manager software (Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry

Tumor samples from C57BL/6 mice vaccinated with hMART-IT or PBS were either fixed in formalin and embedded in paraffin, or embedded in OCT embedding medium (Triangle Biomedical Sciences, Durham, NC, USA) and snap-frozen. Formalin-fixed samples were sectioned, deparaffinized, pretreated with heat/citrate buffer and incubated with antibody to CD3 (clone CD3-12; Serotec, Raleigh, NC, USA). Frozen samples were cryosectioned, fixed in ice-cold acetone for 5 min and incubated with antibodies to MelanA/MART-1 (BioGenex, San Ramon, CA, USA) or anti-mouse H2K^b (BD PharMingen). Immunoreactions were visualized using a horseradish-peroxidase-conjugated streptavidin-biotin method (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Haematoxylin was used as counterstain. Negative controls were incubated with isotype-matched antibodies.

Statistical analysis

In vitro studies were analysed using the Student's *t*-test. *In vivo* studies of tumor protection and survival were analysed using SigmaStat software. Kaplan–Meier non-parametric regression analyses were performed and significance was determined using the log rank test. Pairwise multiple comparison procedures (Holm–Sidak method) were performed to isolate each group. Differences were considered significant at $P < 0.05$.

Results

W303 α yeast is successfully transfected to express human MART-1

cDNA encoding human MART-1 was inserted into pYEX-BX vector and was transfected into W303 α yeast. The copper inducible expression of human MART-1 in yeast was analysed by Western blotting at various time points after the induction (Fig. 1). MART-1 expression was optimal at 8 h after the induction. The yeast expressing human MART-1 was designated as hMART-IT. Yeast cells transfected with the control plasmid pYEX-BX, designated as YVEC, did not have detectable expression of hMART-1 (data not shown).

hMART-IT inhibits B16F10 melanoma engrafting and increases survival of mice

To investigate whether hMART-IT can induce protective immunity against tumor challenge and/or prolong survival of mice after tumor challenge, C57BL/6 mice were treated with hMART-IT ($n = 11$), YVEC ($n = 9$), or PBS ($n = 11$) prior to subcutaneous challenge with MART-1-expressing B16F10 melanoma cells (10). While 91% of the mice treated with PBS developed tumors at the site of challenge within 35 days, 78% of the mice treated with YVEC and only 27% of the mice treated with hMART-IT developed tumors (Fig. 2). The log rank statistic showed a significant difference between survival curves ($P = 0.005$ for log-rank test, $P = 0.0012$ for hMART-IT-treated mice versus PBS-treated mice and $P = 0.009$ for hMART-IT-treated mice versus YVEC-treated mice). All mice that developed tumors died 1–3 weeks after the development of tumors. None of the mice treated with hMART-IT developed clinical signs of vitiligo, uveoretinitis, or systemic side effects. These results demonstrated that immunization of C57BL/6 mice with hMART-IT is effective in protecting mice from developing melanoma.

hMART-IT induces cytotoxic activity against mouse melanoma which is MHC class I-dependent and MART-1-specific

To determine if hMART-IT activates CTLs against mouse melanomas expressing MART-1, *ex vivo/in vitro* cytotoxicity

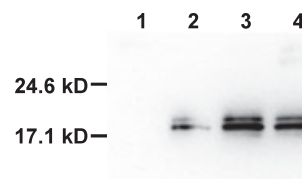


Figure 1. Expression of human MART-1 by hMART-IT yeast. hMART-IT yeast, transfected with an expression vector encoding human MART-1, were induced for various time periods: 0 h (lane 1), 4 h (lane 2), 8 h (lane 3) and overnight (lane 4). The expression of human MART-1 was confirmed by Western blotting.

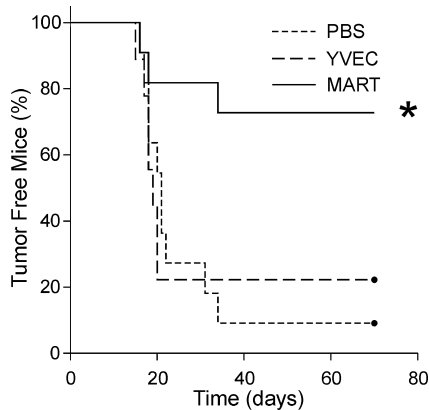


Figure 2. hMART-IT inhibits tumor engrafting. Kaplan–Meier curves for tumor development in C57BL/6 mice treated with phosphate-buffered saline (PBS) ($n = 11$), YVEC ($n = 9$), or hMART-IT ($n = 11$) and challenged with B16F10 melanoma cells. *indicates significant difference using Log rank test ($P < 0.05$ for the hMART-IT-treated mice compared with PBS-treated mice or YVEC-treated mice).

assays were performed. C57BL/6 mice were treated with hMART-IT, YVEC, or PBS. Two weeks later splenocytes were isolated, stimulated *in vitro* with hMART-IT, and tested for cytotoxicity against various target cells. Splenocytes from hMART-IT-immunized animals showed dose-dependent CTL activity against IFN γ -pretreated B16F10 melanoma cells expressing MHC class I (H-2^b) and mouse MART-1 (Fig. 3a). Cytotoxicity in YVEC-treated mice was not dose-dependent and was significantly lower than that in mice treated with hMART-IT ($P = 0.0069$ at an E:T ratio of 16:1 and $P = 0.00028$ at an E:T ratio of 32:1). CTL activity was not induced in mice treated with PBS. Splenocytes from hMART-IT-immunized animals did not lyse IFN γ -untreated B16F10 melanoma cells that do not express MHC class I (H-2^b) (Fig. 3b,d) or EL-4 murine thymoma cells that express MHC class I (H-2^b) but do not express MART-1 (Fig. 3c), indicating that cytotoxicity is MHC class I-dependent and MART-1-specific. Flow cytometry analysis demonstrated that the effector cell population consisted mainly of CD4⁺ T cells and CD8⁺ T cells (data not shown). B220⁺ cells, NK1.1⁺ cells and CD11c⁺ cells decreased to become <2% of all cells during the *in vitro* stimulation period (data not shown).

Both CD4⁺ and CD8⁺ T cells are required for the induction of cytotoxic activity against mouse melanoma

Next, we tested if CD4⁺ and/or CD8⁺ T cells were involved in melanoma-specific cytotoxicity induced by hMART-IT. Splenocytes from hMART-IT-treated mice were depleted of CD4⁺ or CD8⁺ T cells, followed by stimulation with hMART-IT *in vitro* and used as effector cells against B16F10 murine melanoma cells. Mock-depleted splenocytes

served as controls. As shown in Fig. 4a, mock-depleted effector cells from hMART-IT immunized mice lysed IFN γ -pretreated B16F10 melanoma cells, but the lytic activity was completely abolished by depletion of CD4⁺ T cells or CD8⁺ T cells prior to *in vitro* stimulation ($P < 0.005$ at an E:T ratio of 9:1), demonstrating that both CD4⁺ T cells and CD8⁺ T cells were critically involved in the induction of cytotoxicity by hMART-IT immunization.

Cytotoxicity against melanoma is primarily mediated by CD8⁺ T cells, but to a small degree also by CD4⁺ cells

We further tested if CD4⁺ or CD8⁺ T cells were directly responsible for the effector phase of cytotoxicity elicited by hMART-IT. Splenocytes from hMART-IT treated mice were stimulated *in vitro* with hMART-IT, depleted of CD4⁺ or CD8⁺ T cells just prior to the CTL assay and used as effector cells against B16F10 cells. As shown in Fig. 4b, mock-depleted effector cells from hMART-IT immunized mice lysed IFN γ -pretreated B16F10 melanoma cells, but the lytic activity was almost completely abolished by the depletion of CD8⁺ T cells ($P < 0.001$ at an E:T ratio of 27:1). Lytic activity was partially abolished by the depletion of CD4⁺ T cells ($P = 0.008$ at an E:T ratio of 27:1). These results demonstrated that the cytotoxic activity was dependent on CD8⁺ T cells primarily, but to some degree also on CD4⁺ T cells.

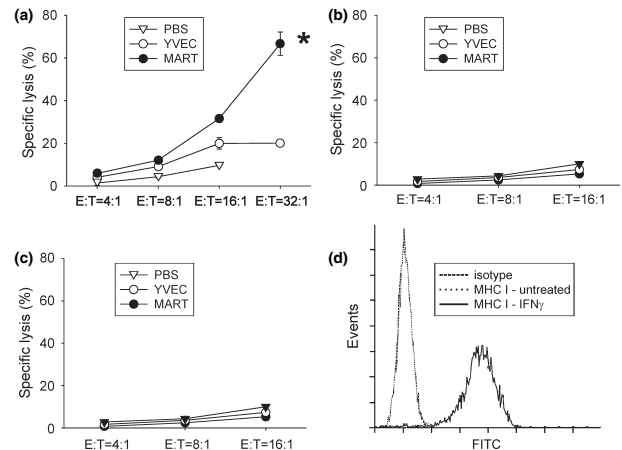


Figure 3. hMART-IT induces cytotoxic activity against B16F10. Splenocytes from mice treated with PBS, YVEC or hMART-IT were restimulated *in vitro* and used as effector cells in cytotoxicity assays against IFN γ -treated B16F10 melanoma cells (H-2^b) (a), untreated B16F10 cells (b) and EL-4 thymoma cells (H-2^b) (c) as targets. B16F10 treated with IFN γ , but not untreated B16F10 express MHC class I as determined by surface flow cytometry analysis (d). E:T effector:target ratio, *indicates significant difference ($P < 0.05$ for the hMART-IT-treated mice compared with PBS-treated mice or YVEC-treated mice).

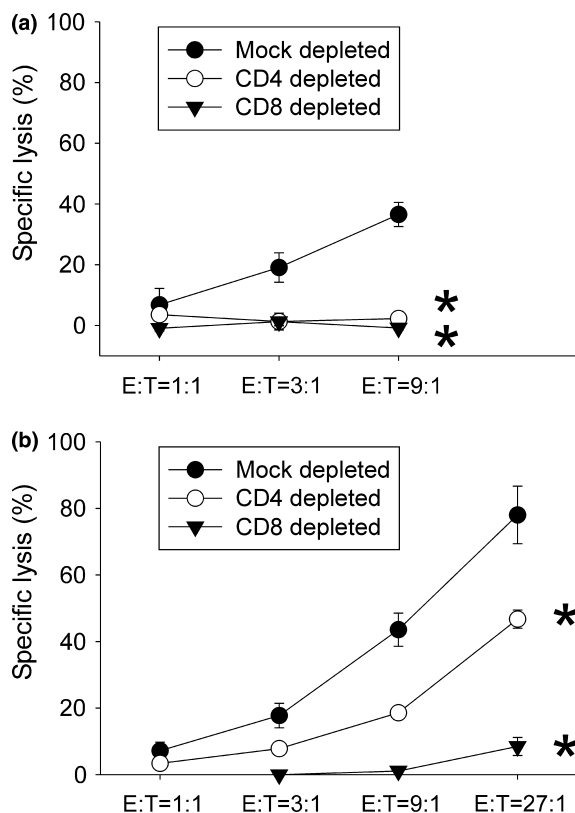


Figure 4. (a) During restimulation, both CD4⁺ and CD8⁺ T cells are required for efficient cytotoxicity. Splenocytes from hMART-IT-treated mice were depleted of CD4⁺ or CD8⁺ T cells, restimulated with hMART-IT and assayed for cytotoxicity against IFN γ -treated B16F10 melanoma cells. Mock-depleted effectors were used as controls. E:T effector:target ratio, *indicates significant difference ($P < 0.005$ for CD4-depleted effectors or CD8-depleted effectors compared with the mock-depleted effectors). (b) CD8⁺ T cells are primarily responsible for effector phase of cytotoxicity. Splenocytes from hMART-IT-treated mice were restimulated *in vitro* with hMART-IT for 5 or 6 days, depleted of CD4⁺ or CD8⁺ T cells prior to cytotoxicity assay and used as effector cells against IFN γ -treated B16F10 melanoma cells. Mock-depleted effectors were used as controls. E:T effector:target ratio, *indicates significant difference ($P < 0.05$ for CD4-depleted effectors or CD8-depleted effectors compared with the mock-depleted effectors).

hMART-IT induces granulocyte-macrophage colony stimulating factor (GM-CSF) and IFN γ production *in vitro*

Supernatants were obtained from splenocytes after *in vitro* stimulation with hMART-IT and analysed for cytokine production. As shown in Fig. 5a, splenocytes from mice treated with hMART-IT produced high levels of GM-CSF and IFN γ . In comparison, the production of GM-CSF and IFN γ by splenocytes from YVEC-treated, or PBS-treated mice was far less. Importantly, splenocytes from mice treated with hMART-IT produced more IFN γ and far more GM-CSF than splenocytes from mice treated with YVEC, demonstrating that production of GM-CSF and IFN γ was

to a large extent dependent on the expression of hMART-1 by yeast during *in vitro* stimulation.

We further tested if the cytokine production elicited by hMART-IT was dependent on the presence of CD4⁺ or CD8⁺ T cells. Splenocytes from hMART-IT-treated mice were depleted of CD4⁺ or CD8⁺ T cells, followed by stimulation with hMART-IT *in vitro* and analysed for cytokine production. As shown in Fig. 5b, the production of GM-CSF and IFN γ was abolished by the depletion of CD4⁺ T cells but not affected by the depletion of CD8⁺ T cells. These findings indicate that the production of GM-CSF and IFN γ *in vitro* was CD4⁺ T-cell dependent.

Tumors derived from hMART-IT administration show infiltration of CD3⁺ T cells and down-regulation of MART-1 expression

Because 27% of mice treated with hMART-IT developed tumors, we tested if immunoevasion contributed to tumor growth in vaccinated animals. Tumors from PBS-treated mice and those from hMART-IT-treated mice were analysed for the presence of infiltrating lymphocytes as well as the expression of MART-1 and MHC-class I. Tumor samples were stained with antibodies to CD3, MART-1 and MHC-class I by immunohistochemistry. Tumors from hMART-IT-treated mice showed dense infiltration of CD3⁺ T cells in and around tumors (Fig. 6a) whereas those from PBS-treated mice showed sparse infiltrates (Fig. 6b). MART-1 expression in tumors from hMART-IT-treated mice was weak and focal (Fig. 6c) compared with strong and diffuse expression in tumors from PBS-treated mice (Fig. 6d). The expression of MHC-class I was heterogeneous in tumors from both hMART-IT-treated and PBS-treated mice (data not shown) and was not correlated with infiltration of CD3⁺ T cells.

Discussion

MART-1 was originally identified by using cDNA expression cloning to functionally screen tumor infiltrating lymphocytes isolated from patients with metastatic melanoma (9). It is a melanosomal protein overexpressed in 80–90% of human melanomas (9,11). Human MART-1 is frequently recognized by CTLs and has been used as a target for immunotherapy of human melanoma (12,13). Most clinical trials employing MART-1 specific immunotherapy have utilized immunization with peptides and defined a restricted patient pool to be treated, because of the epitope specificity and MHC restriction of the T-cell receptor: For example, CD8 epitopes such as MART-1_{24–33}(34), MART-1_{26–35}, MART-1_{27–35}, and MART-1_{32–40} peptides are restricted to HLA-B45, HLA-B35, HLA-A2

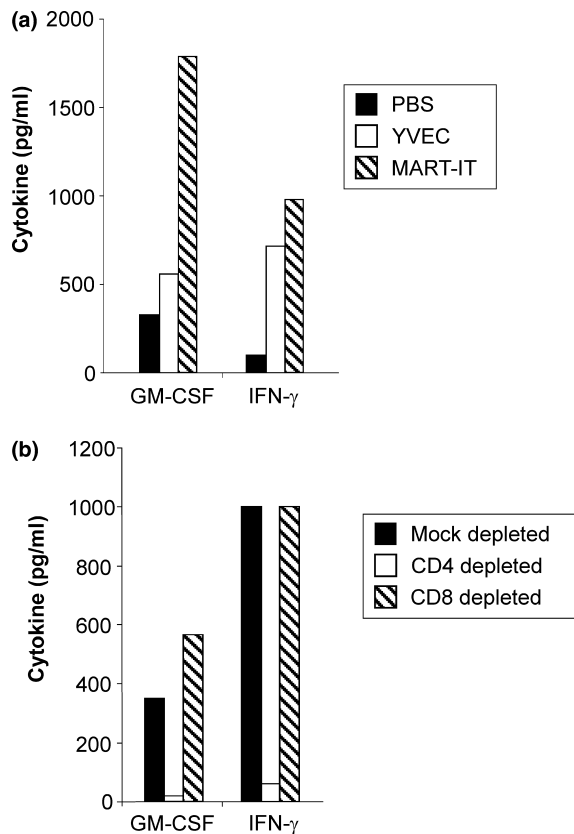


Figure 5. (a) hMART-IT induces GM-CSF and IFN γ production *in vitro*. Splenocytes from hMART-IT-treated, YVEC-treated, or PBS-treated mice were co-cultured with hMART-IT for 5–6 days. Supernatants were analysed for cytokines. Results represent a set of three mouse groups (MART, YVEC and PBS). Experiments were performed three times using different sets of mice and yielding similar results. (b) CD4⁺ T cells are the major subset required for the production of GM-CSF and IFN γ *in vitro*. Splenocytes from hMART-IT-treated mice were depleted of CD4⁺ or CD8⁺ T cells and restimulated *in vitro* with hMART-IT. Cytokine production was analysed using a microbead array assay. Mock-depleted splenocytes were used as controls. Results represent a set of three mouse groups (mock-depleted, CD4-depleted and CD8-depleted). Experiments were performed three times using different sets of mice and yielding similar results.

and HLA-A2 respectively (9,14–16) whereas a CD4 epitope, MART-1_{51–73} peptide, is restricted to HLA-DR4 (17). Dendritic cells (DCs) should naturally process and present peptides derived from full-length proteins in the context of their MHC class I and class II. Therefore, we hypothesized that specific T cells recognizing unidentified peptide-MHC complexes can be generated in any patient if full-length MART-1 containing epitopic sequences is provided in an appropriate setting.

Yeast has been shown to load antigens efficiently and stimulate immune responses (4,5). Yeast are avidly phago-

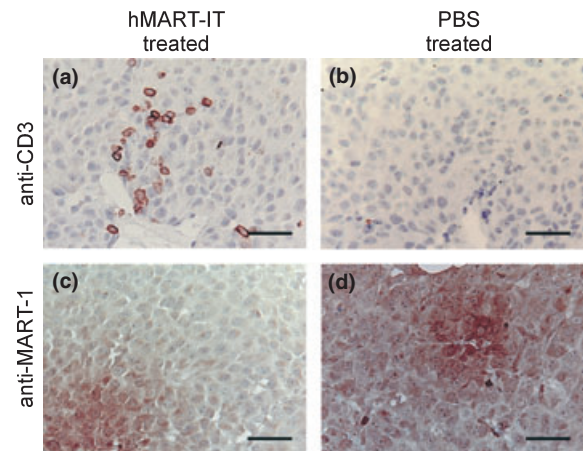


Figure 6. Tumors from hMART-IT show infiltration of CD3⁺ T cells and decreased expression of MART-1. C57BL/6 mice were treated with PBS or hMART-IT and challenged with B16F10 murine melanoma cells. Tumors from hMART-IT-treated mice (a, c) and those from PBS-treated mice (b, d) were immunostained with anti-CD3 (a, b) or anti-MART-1 antibody (c, d). Scale bars: 100 μ m.

cytized by DCs via interaction of mannan on yeast and mannose receptor (CD206) on DCs, thereby delivering tumor antigens directly and efficiently to DCs (4). β -1,3-D-glucan, a cell-wall component of *S. cerevisiae* yeast, possesses adjuvant potential as it leads to activation and maturation of DCs by binding to dectin 1 (18) and by stimulating toll-like receptors 2, 4 and 6 to activate MAPK and NF κ B (19,20). β -1,3-D-glucan also binds to complement receptor 3 to mediate phagocytosis by macrophages and enhance cytotoxicity of natural killer (NK) cells (21). In addition, yeast naturally mannosylate proteins. Protein antigens mannosylated by yeast have been shown to enhance MHC class I- and MHC class II-restricted antigen presentation and T-cell stimulation compared with non-mannosylated proteins (22–24). Thus, the use of yeast systems provides a potent immunostimulatory platform for the development of vaccines. Furthermore, immunization with xenogeneic proteins has been shown to enhance immunity to the native protein, resulting in greater immune responses and leading to improved anti-tumor immunity (25–27). Therefore, in this study, we investigated the utility of recombinant *S. cerevisiae* expressing a melanocyte/melanoma antigen as a prophylactic melanoma vaccine. Using a transplantable mouse melanoma model, we found that subcutaneous administration of recombinant yeast expressing a full-length melanocyte/melanoma protein provided protection against development of melanoma *in vivo* and elicited efficient immune responses involving both CD4⁺ and CD8⁺ T cells *in vitro*.

Generating specific CTLs is a crucial requirement for an effective anti-tumor immune response. The selective depletion of CD4⁺ or CD8⁺ T cells before *in vitro* stimulation

with hMART-IT demonstrated that the induction phase of cytotoxicity depends on both CD4⁺ T cells and CD8⁺ T cells. Stimulating both CD4⁺ and CD8⁺ T cells by separate epitopes from a full-length protein may be important in promoting strong CD8 T-cell responses and augmenting immune responses (28). Using a similar, yeast-based vaccine and knock-out animals, Stubbs et al. have previously demonstrated the requirement of CD8 T cells for induction of cytotoxicity (5). Our data obtained from depletion of CD4⁺ or CD8⁺ T cells before *in vitro* stimulation provide additional evidence for the requirement of both CD4⁺ and CD8⁺ T cells during the induction phase of cytotoxicity. In contrast, the selective depletion of CD4⁺ or CD8⁺ T cells after *in vitro* stimulation with hMART-IT demonstrated that the effector phase depends on CD8⁺ T cells primarily but to some degree on CD4⁺ T cells. As interpretation of these data, particularly CD4 depletion data, may be hampered by residual contaminant cells that were not completely depleted, we employed flow cytometry analysis to assess the efficacy of CD4 depletion. We found that more than 98% of the CD4 subset were reproducibly removed, indicating that the observed cytotoxicity was vastly from non-CD4 subsets. Although there is no doubt that CD8⁺ T cells are the major final effector cells in cytotoxicity and that CD4⁺ T cells have a 'helper' role for cytotoxicity, the finding of reduced cytotoxicity after CD4 depletion suggests that CD4⁺ T cells may also participate in the cytotoxicity directly. CD4⁺ CTLs have been observed in viral infections (29,30) and inflammatory diseases such as rheumatoid arthritis (31) and kill target cells either through perforin-dependent cytotoxic mechanism or Fas-dependent pathway (32,33). The reduced cytotoxicity after CD4 depletion prior to *in vitro* restimulation also suggests the presence of enriched suppressor T cells. However, we found a significant decline of CD4⁺ CD25⁺ foxp3⁺ T cells after *in vitro* stimulation (data not shown) and therefore this explanation is unlikely. Phenotypic and functional analysis of CD4⁺ T cells is yet to be conducted in our system to further elucidate the role of CD4⁺ T cells.

Cytokines play an important role in tumor formation and sharpening host immunity and therapeutic manipulation of the cytokine environment constitutes one strategy to stimulate protective responses. Our current study showed CD4⁺ T-cell dependent GM-CSF and IFN γ production from hMART-IT-treated mice. GM-CSF activates DCs, macrophages, granulocytes and NKT cells, thereby improving tumor antigen presentation (34). Immunotherapy with cytokines or with tumor cells, dendritic cells or stroma cells genetically modified to express various cytokines showed that GM-CSF was the most potent molecule for augmenting tumor immunity among various cytokines including IL-2, IL-6, IL-12, IFN γ and TNF α (35–38). GM-CSF has been used as a systemic or local cytokine treatment for

melanoma in clinical trials with some clinical benefits (35,39,40). IFN γ is a Th-1 cytokine with multiple biological functions. It is produced by various cells including T cells, B cells, macrophages, DCs, NK cells and NKT cells, and plays a major role in the preventive and defensive immune responses against infectious pathogens (41). It also works as a proinflammatory cytokine to induce other cytokines such as TNF α . In addition to its well-established role in balancing Th1 versus Th2 type immune responses, IFN γ was recently demonstrated to regulate the generation and activation of CD4⁺ CD25⁺ T-regulatory cells (42). Taken together, the cytokine profiles elicited by hMART-IT suggest favourable environment for immunological help by CD4⁺ T cells to augment effective anti-tumor immune responses.

In spite of the strong induction of CTLs and cytokine production *in vitro*, not all mice pretreated with hMART-IT were protected from tumor challenge *in vivo*. The immunohistochemical analysis of tumors derived from hMART-IT-treated mice demonstrated down-regulation of MART-1 expression despite the recruitment of tumor infiltrating T lymphocytes. This suggests an immunoevasion mechanism by B16F10 melanoma cells that undergo a process of immunoeediting through interaction with the immune systems to evade immune-mediated control by hMART-IT treatment. Several reports have shown that antigen-specific vaccine therapy is associated with the *in vivo* development of antigen-loss variants, especially with antigens whose expression is not required by tumor cells for the maintenance of transformed phenotype (43,44). Promoter methylation and the release of a soluble protein inhibiting promoter activity have been recently described to decrease expression of tumor antigens in human melanomas (45,46). The combination of multiple vaccines to target diverse antigens or targeting antigens that are essential for tumor survival and progression as well as modifying local inhibitory environment could improve the efficacy of this treatment.

While prevention of melanoma is a favourable approach, the use of hMART-IT in a therapeutic setting will be interesting to study. However, the therapeutic efficacy of a vaccine is difficult to evaluate in the B16F10 melanoma model because of its rapid tumor growth. Other mouse models with slowly growing tumors may serve as better therapeutic models because they provide sufficient time for mice to generate cellular-mediated immune responses against tumors. Adoptive transfer of *in vitro* stimulated lymphocytes using the vaccine or combination of the vaccine with chemotherapy or inhibitors may also overcome the experimental problem in the therapeutic setting. We are currently investigating the therapeutic potential of hMART-IT in a genetically engineered mouse model that spontaneously develops

melanoma with a protracted course more similar to melanomas in humans (47).

The hMART-IT vaccine presented here has several obvious advantages as a prophylactic melanoma vaccine. First, hMART-IT does not require expensive, time-consuming preparation and expansion of cells. Secondly, it is safely and easily administered with subcutaneous injection. Thirdly, this methodology does not require precise knowledge of epitopic sequences and their matched HLA types, making the vaccine applicable to broad populations with diverse HLA types. Fourthly, hMART-IT stimulates both CD4⁺ T cells and CD8⁺ T cells simultaneously in mice. Therefore, hMART-IT could be utilized as an effective prophylactic vaccine to target high-risk melanoma patients, including patients with personal or family history of melanoma and patients with large congenital or dysplastic nevi.

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