mRNA-based dendritic cell immunization improves survival in ret transgenic mouse melanoma model

Adi Sharbi-Yungera,*, Mareike Greesb,*, Esther Tzehovala, Jochen Utikalb, Viktor Umanskyb, and Lea Eisenbacha

aDepartment of Immunology, Weizmann Institute of Science, Rehovot, Israel; bSkin Cancer Unit, German Cancer Research Center (DKFZ), Heidelberg, and Department of Dermatology, Venereology and Allergology, University Medical Center Mannheim, Ruprecht-Karl University of Heidelberg, Heidelberg, Germany

ABSTRACT
Malignant melanoma is characterized by a rapid progression, metastasis to distant organs and resistance to chemo and radiotherapy. Although melanoma is capable of eliciting an immune response, the disease progresses and the overall results of immunotherapeutic clinical studies are not satisfactory. Recently, we have developed a novel genetic platform for improving an induction of peptide-specific CD8+ T cells by dendritic cell (DC) based on membrane-anchored β2-microglobulin (β2m) linked to a selected antigenic peptide at the N-terminus and to the cytosolic domain of TLR4 at the C-terminus. In vitro transcribed mRNA transfection of antigen-presenting cells (APCs) resulted in an efficient coupling of peptide presentation and cell activation. In this research, we utilize the chimeric platform to induce an immune response in ret transgenic mice that spontaneously develop malignant skin melanoma and to examine its effect on the overall survival of tumor-bearing mice. Following immunization with chimeric construct system, we observe a significantly prolonged survival of tumor-bearing mice as compared to the control group. Moreover, we see elevations in the frequency of CD62L+CD44hi central and CD62L−CD44hi effector memory CD8+ T-cell subsets. Importantly, we do not observe any changes in frequencies of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in the vaccinated groups. Our data suggest that this novel vaccination approach could be efficiently applied for the immunotherapy of malignant melanoma.

Introduction
The incidence of melanoma cases is increasing worldwide and despite early detection, appropriate surgical resection and adjuvant therapy, the number of patients dying from metastatic disease continues to rise.1-3 The prognosis of advanced melanoma remains to be poor and the treatment options for metastatic disease are limited.1-3 After the standard therapy of stage III and IV melanoma, clinically disease-free patients have a 50–90% risk of recurrence. Despite recent therapeutic advances in metastatic melanoma dealing with the application of inhibitors targeting the mutated signaling molecule BRAF as well as antibodies blocking negative checkpoint CTLA-4 and PD-1,4,6 many treated patients do not respond to these treatments indicating that new approaches for melanoma immunotherapy are urgently needed.

It is well established that spontaneous anti-melanoma immune responses are directed in part against melanocyte differentiation antigens (e.g., gp100, tyrosinase (Tyr), tyrosinase-related protein-1 (TRP-1), TRP-2, MelanA).7 There are several therapeutic strategies aiming at enhancing the cellular antitumor immunity against these specific melanoma-associated antigens (MAAs). One such strategy is to use autologous DCs loaded with MAAs.8

DCs form a network of APCs that shape immune responses by linking innate and adaptive immunity.9 Proliferation, activation and differentiation of specific T-cell subsets are influenced by DCs. This makes DCs an attractive instrument for cancer immunotherapies.10,11 Antitumor therapeutic vaccines should be able to prime naive T cells, but most importantly, to induce the transition of existing memory T cells from non-protective to potent effector CD8+ T cells.11

However, the clinical impact of DC immunotherapy has been limited so far despite the induction of tumor-specific T-cell responses in many patients and occasional tumor regressions. Many reasons may explain this lack of success with DC vaccines. DC-based immunotherapies require optimization at several levels: (i) the maturation stimuli in use; (ii) the type and form of antigen to be loaded on DCs; (iii) the origin, subset and form of antigen to be loaded on DCs; (iv) the amount, frequency, route and the site of injections.12,13

In addition to the limitations of DCs immunotherapy, numerous immunosuppressive cells and factors strongly contribute to the impairment of the antitumor immune response leading to the tumor progression. Among them are MDSCs, which represent a heterogeneous population of immature myeloid cells that could severely impair antitumor activities of T cells.14-16 Tregs are considered to be pivotal mediators of peripheral tolerance and immune suppression. However, they are highly enriched in the tumor microenvironment and are...
considered to be important for limiting antitumor T-cell-mediated immune responses.\textsuperscript{17,18}

We have earlier developed a novel genetic platform for the induction of antigen-specific effector CD8\textsuperscript{+} T cells by DCs vaccination using the MHC-I light chain, \textbeta{2}m.\textsuperscript{9} To this end, we have converted \textbeta{2}m into an integral membrane protein by linking antigenic peptides to its N-terminus and the intracellular toll-like receptor (TLR4) signaling domain to its C-terminus. We showed that efficient peptide presentation can be coupled to constitutive TLR4 signaling through the polypeptide product of a single gene, and that this dual effect can be achieved by virtue of mRNA electroporation.\textsuperscript{18} We also showed that these constructs can mediate an effective antitumor activity in the transplantable B16 melanoma model.\textsuperscript{20}

In this study, we utilize this chimeric mRNA construct system to examine whether DCs immunization will affect melanoma progression in a spontaneous \textit{ret} transgenic mouse melanoma model that closely resembles human melanoma regarding histopathology and clinical development.\textsuperscript{21,22} In addition, primary skin melanomas and metastases in lymph nodes (LN) and distant organs of transgenic mice express similar MAAs to those expressed in human melanoma and B16 tumors (such as gp100, Tyr, TRP-1 and TRP-2).\textsuperscript{22,23}

Our data suggest that immunotherapy with DCs-based antitumor vaccine can significantly improve the survival of tumor-bearing \textit{ret} transgenic mice. Analyzing the mechanism of such effect, we observed elevated frequencies of central and effector memory T-cell subsets in vaccinated mice. Interestingly, we found a significant survival improvement only in the group of mice, which was immunized with a vaccine composed of mRNA mixture of the two MAAs, gp100 and TRP-2, indicating that an aggressive disease may require more vaccine combinations. Moreover, in order to enhance the vaccine efficacy, we should consider applying Tregs and MDSCs inhibitors in the treatment protocol.

**Materials and methods**

**Mice**

C57BL/6 mice expressing human \textit{ret} transgene in melanocytes under the control of mouse metallothionein-I promoter–enhancer\textsuperscript{24} were provided by Dr I. Nakashima (Chubu University, Aichi, Japan). Animals were crossed and kept under specific pathogen-free conditions in the animal facility of German Cancer Research Center (Heidelberg, Germany). Experiments were performed in accordance with government and institutional guidelines and regulations.

**Antibodies**

The anti-mouse APC-CD11b, APC-CD62L, APC-CD25, PE-CD44, FITC-CD45RB, FITC-CD3, Pe-Cy7-Gr1, Pe-Cy7-CD4, PerCp-Cy5.5-CD45.2 and PerCp-Cy5.5-CD3 were purchased from BD Biosciences (San Jose, CA). The anti-mouse PE-F4/80, PE-FoxP3 and FITC-MHC-II were purchased from eBio-science (San Diego, CA). The anti-mouse APC-Cy7-CD11c and APC-Cy7-CD8 and the PE-conjugated anti-human \textbeta{2}m antibody were purchased from Biolegend (San Diego, CA).

**Generation of DCs from murine bone-marrow cells**

Murine bone marrow derived DCs (BMDCs) were performed as described by Lutz et al.\textsuperscript{24} with minor modifications. Briefly, bone-marrow (BM) cells from femurs and tibiae of 4–6 weeks old C57BL/6, female mice were cultured in DC medium containing RPMI (GibcoBRL, Grand Island, NY), supplemented with 10\% FBS (Hyclone, Logan, UT), 10 mM HEPES, 2 mM L-glutamine, 50 mM 2-mercaptoethanol (2-ME), gentamicin and 200 U/mL recombinant mouse (rm)GM-CSF (Prospec, Rehovot, Israel). On day 8, non-adherent cells were harvested and further cultured in fresh medium containing 100 U/mL rmGM-CSF for 24 h. Non-adherent cells were analyzed by flow cytometry for the expression of CD11c, CD80, CD86, Gr1, F4/80, CD11b and MHC class II, followed by mRNA electroporation procedure.

**mRNA constructs assembly and expression**

The \textbeta{2}m-\textalpha{2}m and \textbeta{2}m-\textalpha{2}m\textbeta{b} backbones were covalently linked to the tumor-associated peptides as previously described.\textsuperscript{19} Human gp100\textsubscript{25–33} or mouse TRP-2\textsubscript{180–188} peptides were used.

**In vitro mRNA transcription**

Template DNA cloned in the pGEM4Z-A64 vector was prepared with the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA) and linearized via the SpeI restriction site positioned at the 3’ end of the poly (A) tract of the vector. 1 \mu g of linear plasmid was used for \textit{in vitro} mRNA transcription with AmpliCap-Max T7 High Yield Message Maker Kit (CellScript, Biotechnologies, Madison, WI). The concentration and quality of the mRNA was assessed by spectrophotometry.

**mRNA electroporation**

Procedure was performed as previously described by Cafri et al.\textsuperscript{26} Shortly, BMDCs cultured for 8 d, were washed twice with OptiMEM medium (GibcoBRL) and resuspended in 150 \mu L OptiMEM medium containing 20 \mu g transcribed mRNA and electroporated using BTX ECM 830 electroporator (BTX, Holliston, MA) 400 V, 0.9 ms, one pulse, using a 2 mm cuvette. Cells were resuspended in 5 mL growth medium and transferred into tissue culture plates for further incubation. Constructs expression was assessed by flow cytometry 6 h after electroporation.

**Immunizations**

BMDCs were electroporated with 10–20 \mu g of transcribed mRNA (5 \mu g of each construct according to the vaccinated group) followed by 6 h incubations at 37°C. Cells were washed three times and resuspended to 2.5 \times 10^6/mL in PBS. In all experiments, mice were vaccinated i.p. with 200 \mu L cell suspension (0.5 \times 10^6 cells per mouse) three times weekly.
Flow cytometry analysis

Mice were sacrificed 10 d after the last vaccination. Cell suspensions from spleens, BM, LNs and skin tumor were prepared and adjusted to $1 \times 10^6$ cells/100 μL in staining buffer (PBS with 0.5% BSA and 0.1% sodium azide). Fluorochrome-conjugated antibodies were added for 30 min at 4°C in the dark. Acquisition was performed by six-color flow cytometry using FACS Canto II with FACSDiva software (both from BD Biosciences). The compensation control was performed with BD CompBeads set (BD Biosciences) using the manufacturer’s instruction. FlowJo software (Tree Star, Ashland, OR) was used to analyze at least 100,000 events. Data were expressed as dot plots.

Statistical analysis

Statistical analysis of cell surface markers and Tregs was conducted with one-way ANOVA. Significance of the differences was assessed by Dunnett’s multiple comparison posttest. Survival curves were generated using the product limit (Kaplan–Meier) method and comparisons were conducted using the log-rank (Mentel–Cox) test. A value of $p < 0.05$ was considered statistically significant.

Results

We have recently described the development and assessment of recombinant bi-functional β2m-based polypeptides, which couple MHC-I presentation to constitutive TLR4 activation. To test whether these polypeptides confer antitumor activity in a spontaneous melanoma model, we have used ret transgenic mice. We have applied both the Kb and TLR4 anchors covalently linked to either hgp10025–33 or TRP-2180–188 antigens. The use of both anchors, and not only one, was due to our previous data showing that although the Kb anchored peptides are more stable on the cell surface, the TLR4 anchor is important for transmitting DC maturation signals. We utilize the recombinant bi-functional β2m-based polypeptides system for the immunization of melanoma-bearing mice. The vaccination by a single peptide (derived from gp100 or TRP-2) with both anchors (Kb and TLR4) is designated as a double construct. Composition of the different combinations used in the experiment and their designations are shown in Fig. 1.

For every type of construct, 5 μg of mRNA were electroporated into BMDCs, resulting in total of 10 μg mRNA in the gp100 and TRP-2 groups and 20 μg in the quadrate constructs (Mix) group. Although a trend for improved survival was observed in the group treated with gp100 containing double constructs, immunization with a double construct of a single peptide did not significantly increase the survival of mice (Fig. 2). However, the use of the constructs mixture containing gp100-Kb, gp100-TLR4, TRP-2-Kb and TRP-2-TLR4 (quadrate...
constructs) in the Mix group significantly improved survival and prolonged life expectancy of mice up to day 55 (p < 0.05; Fig. 2).

Next, we investigated the changes in the immune cells that could be involved in the survival benefit of melanoma-bearing mice upon the vaccination. We have collected tissues of BM, metastatic LNs, spleen and skin tumors of treated groups 10 d after the last vaccination and performed a phenotypic analysis of T lymphocytes. As shown in Fig. 3A, the frequency of CD8$^+$ was observed in metastatic LN from the TRP-2 group (p < 0.05), whereas skin tumors of these mice displayed no statistically significant changes in Tregs as compared to the control group (Fig. 5A). Furthermore, the vaccination did not induce any changes in MDSC in melanoma lesions and lymphoid organs as compared to non-treated mice (Fig. 5B).

Figure 3. Phenotypic analysis of CD8 memory T cell subsets following vaccination with mRNA electroporated DCs. Melanoma-bearing ret transgenic mice were vaccinated three times at weekly intervals with BMDCs electroporated with 10–20 μg transcribed mRNA of gp100 (n = 5), TRP-2 (n = 6), Mix construct combinations (n = 12) or empty construct (Control group, n = 12). 10 d after the last vaccination melanoma lesions (skin tumors and metastatic LN), BM, and spleens were analyzed by flow cytometry. CD8$^+$ CD62L$^+$CD44$^+$ central memory (A) and CD8$^+$ CD62L CD44$^+$ effector memory (B) T cells were shown as the percentage of respective cells among total CD8$^+$ T cells. *p < 0.05, **p < 0.01, ***p < 0.001.

To characterize central and effector memory CD4$^+$ T-cell subsets, we have used CD44 and CD62L markers similar to CD8$^+$ T cells.25 We have found significantly elevated frequencies of CD4$^+$ CD62L$^+$CD44$^+$ central memory T cells in the LN of the control compared to the gp100 group (p < 0.05; Fig. 4A). In the spleen, we detected significantly elevated levels of CD4$^+$ memory cells in the control group as compared to all other groups. Analyzing CD4$^+$ CD62L$^+$CD44$^+$ effector memory T cells, we observed their enhanced frequencies in the tumor from mice treated with gp100 construct as well as in the BM from mice of Mix group as compared to the control group (p < 0.05; Fig. 4B).

Next, we investigated the effect of vaccination with different constructs on the frequency of two major immunosuppressive cell populations, i.e., CD4$^+$ CD25$^+$FoxP3$^+$ Tregs and CD11b$^+$Gr1$^+$ MDSCs. Studying Tregs, we demonstrated a tendency to the decrease in the BM from Mix group as compared to that in control group (Fig. 5A). A significant downregulation of Treg frequencies was observed in metastatic LN from the TRP-2 group (p < 0.05), whereas skin tumors of these mice displayed no statistically significant changes in Tregs as compared to the control group (Fig. 5A).

Discussion

We have previously shown in the transplantable B16 melanoma model that the peptide-hβ2m-TLR4 constructs were expressed at the cell surface of BMDCs and prolonged effective peptide presentation up to at least 3 d post-mRNA electroporation.20 Moreover, we observed an increased antigen-specific reactivity indicated by an elevated cytokine production by effector CD8$^+$ T cells and their enhanced cytotoxic activity in in vivo killing assay as compared to peptide-loaded mature BMDCs.20 This system allows continuous peptide presentation and improves the prospects of peptide-bearing DCs to enter the LNs and encounter peptide-specific naive T cells. Moreover, by using mRNA rather than cDNA as gene carrier, we can prevent the development of long-term toxic effects arising from continuous TLR4 activation.19,20

However, the ability of a transplantable model system to adequately predict therapeutic responses in human patients is limited. In this study, we attempt to utilize our mRNA constructs system in ret transgenic mice that develop spontaneously skin tumors and metastases in the BM, lungs, liver and brain.21,22 Moreover, melanoma lesions expressed several MAs such as tyrosinase, gp100, TRP-1 and TRP-2. It has been also reported that ret transgenic mice could develop specific T-cell responses against TRP-2 upon vaccination.21,22

Here, ret transgenic mice with clinically visible tumors were vaccinated with DCs electroporated with mRNAs constructs for gp100 and TRP-2. We demonstrated that an
immunotherapy with a double mix anchor (Kb and TLR4), for a single antigen (gp100 or TRP-2) induced some increase in mouse survival. However, the application of mRNA containing a double mix anchor and both antigens, quadrate constructs (Mix), significantly improved survival of tumor-bearing mice.

We have previously characterized the different constructs used in this study and performed phenotypic analysis of healthy immunized naive C57BL/6 mice. We tested the activation status of T lymphocytes, by the expression of CD44 and CD62L on cell surface. We have shown that vaccination with quadrate constructs Mix induced elevation in the effector-memory CD8^+ T-cell population and decrease in the total number of memory cells. We have also analyzed Tregs frequency cells where no statistically significant differences were seen among all treatments.

Here, we analyzed the effect of vaccination on different immune cell subsets in melanoma lesions (skin tumors and metastatic LN) as well as lymphatic organs (spleen and BM) from ret transgenic transgenic mice.

**Figure 4.** Phenotypic analysis of CD4^+ memory T cell subsets following vaccination with mRNA electroporated DCs. Melanoma-bearing ret transgenic mice were vaccinated three times at weekly intervals with BMDCs electroporated with 10–20 μg transcribed mRNA of gp100 (n = 5), TRP-2 (n = 6), Mix construct combinations (n = 12) or empty construct (Control group, n = 12). 10 d after the last vaccination melanoma lesions (skin tumors and metastatic LN), BM, and spleens were analyzed by flow cytometry. CD4^+ CD62L^− CD44^hi central memory (A) and CD4^+ CD62L^+ CD44^hi effector memory (B) T cells were shown as the percentage of respective cells among total CD4^+ T cells. *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure 5.** Phenotypic analysis of Tregs and MDSCs populations following vaccination with mRNA electroporated DCs. Melanoma-bearing ret transgenic mice were vaccinated three times at weekly intervals with BMDCs electroporated with 10–20 μg transcribed mRNA of gp100 (n = 5), TRP-2 (n = 6), Mix construct combinations (n = 12) or empty construct (control group, n = 12). 10 d after the last vaccination melanoma lesions (skin tumors and metastatic LN), BM, and spleens were analyzed by flow cytometry. CD4^+ CD25^+ FoxP3^+ Tregs (A) and CD11b^+ Gr1^+ MDSCs (B) were shown as the percentage of CD4^+ and total monocytes respectively. *p < 0.05, **p < 0.01, ***p < 0.001.
mice. We observed an elevated frequency of CD8+CD62LhiCD44hi central memory and CD8+CD62LloCD44hi effector memory T cells in the Mix group as compared to the control group. CD8+ effector memory T cells were also accumulated in mice vaccinated with gp100 construct as compared to the control. These data are consistent with our previous results, showing that the Mix treatment, expressing the two antigens, induced the most effective CD8+ response in a B16 transplantable melanoma model owing to its dual functionality, which allowed long-lasting peptide presentation through K0 in conjunction with DCs maturation driven by TLR4.20

Studying CD4+ memory T cells, we observed elevated levels of CD4+CD62LhiCD44hi central memory cells in the control group as compared to the gp100 group in the LNs and elevated levels of these cells in the control group as compared to all other groups in the spleen. This might be explained by the fact that the contribution of CD4+ T cells to antitumor immunity is contradictory due to their heterogeneity.26 For instance, it was described that IL-4 cytokine, secreted by CD4+ T cells, may exert antitumor effect;27 however, the frequency of antigen-specific CD4+ cells that produce IL-5 has been correlated with progressive growth of melanoma.28 Further investigation of different CD4+ T-cell subsets are required to determine their function and contribution to the tumor progression.

Studying CD4+ effector memory T cells, we found an increase in its frequency in the tumor tissue from mice treated with gp100 construct as well as in the BM of Mix group as compared to the control group. This CD4+ effector memory T-cell subpopulation should be also further analyzed to determine its contribution to the tumor regression.

Tregs have been accepted to play a major role in maintenance of self-tolerance in healthy hosts.29 Moreover, these cells may hamper an effective antitumor immune response in cancer patients and tumor-bearing mice.30,31 Therefore, we monitored the Treg frequency in melanoma lesions and lymphatic organs. Importantly, we found no significant alterations of these immunosuppressive cells, which was in agreement with our previous findings obtained with the same construct in transplantable B16 melanoma model.20

Another crucial immunosuppressive cell population is represented by MDSCs that inhibit antitumor T-cell responses by multiple mechanisms and can be considered as one of the most important components of tumor-induced immunosuppression melanoma.14,15,32,33 It has been previously described that MDSCs accumulate in peripheral lymphoid organs and migrate to tumor sites, where they contribute to immunosuppression.15,16,33,34 Furthermore, some evidence suggests that MDSCs can induce an expansion of Tregs.35 In addition, a transient elevation in MDSCs in mice following immunization was demonstrated.36,37 Previous studies performed on ret transgenic tumor-bearing mice demonstrated that targeting of MDSC by the phosphodiesterase-5 inhibitor sildenafil or ultra-low dose paclitaxel significantly increased animal survival associated with the restoration of antitumor T-cell functions.23,38 On the other side, it has been reported that, T cells may promote melanoma progression in this mouse model by favoring protumoral properties of tumor-infiltrating myeloid cells that further inhibit functions of immune effector cells.39 In this study, we found no statistically significant changes in MDSC frequencies in melanoma lesions and lymphoid organ upon vaccination with modified DCs, indicating that this immunization does not display side effects dealing with the MDSC accumulation. However, the immunosuppressive activity of these cells has to be monitored in further experiments.

In summary, we have demonstrated that DC vaccination can improve the survival of melanoma-bearing ret transgenic mice and induce an increase in frequencies of CD8+ effector and central memory T cells without any stimulatory effect on immunosuppressive Tregs and MDSCs. Together with our previous data on the stimulation of antitumor activities based on mRNA electroporated DCs,20 our findings demonstrate that this vaccine approach can be efficiently applied for the melanoma immunotherapy. We suggest that the efficiency of DC vaccination could be significantly improved by combining it with targeting of MDSCs and Tregs to neutralize immunosuppressive tumor microenvironment.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

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