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Title
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Permalink
https://escholarship.org/uc/item/9cs89346

Journal
Cancer Discovery, 8(8)

ISSN
2159-8274

Authors
Mehta, Arnav
Kim, Yeon Joo
Robert, Lidia
et al.

Publication Date
2018-08-01

DOI
10.1158/2159-8290.cd-17-1178

Peer reviewed

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Author manuscript

Cancer Discov. Author manuscript; available in PMC 2019 August 01.

Published in final edited form as:

Cancer Discov. 2018 August ; 8(8): 935–943. doi:10.1158/2159-8290.CD-17-1178.

Immunotherapy resistance by inflammation-induced dedifferentiation

Arnav Mehta[1,2,*], **Yeon Joo Kim**[1,*], **Lidia Robert**[1], **Jennifer Tsoi**[1], **Begoña Comin-Anduix**[3,5], **Beata Berent-Maoz**[1], **Alistair J. Cochran**[4], **James S. Economou**[2], **Paul C. Tumeh**[1], **Cristina Puig-Saus**[1], and **Antoni Ribas**[1,5]

[1]Department of Medicine, David Geffen School of Medicine at UCLA, Los Angeles, CA

[2]Department of Medicine, Massachusetts General Hospital, Boston, MA

[3]Department of Surgery, David Geffen School of Medicine at UCLA, Los Angeles, CA

[4]Department of Pathology, David Geffen School of Medicine at UCLA, Los Angeles, CA

[5]Johnson Comprehensive Cancer Center, University of California, Los Angeles, CA

Abstract

A promising arsenal of targeted and immunotherapy treatments for metastatic melanoma has emerged over the last decade. With these therapies, we now face new mechanisms of tumor acquired resistance. We report here a patient whose metastatic melanoma underwent dedifferentiation as a resistance mechanism to adoptive T cell transfer therapy (ACT) to the MART-1 antigen, a phenomenon that had only been observed in mouse studies to date. After an initial period of tumor regression, the patient presented in relapse with tumors lacking melanocytic antigens (MART-1, gp100) and expressing an inflammation-induced neural crest marker (NGFR). We demonstrate using human melanoma cell lines that this resistance phenotype can be induced *in vitro* by treatment with MART-1 T-cell receptor expressing T cells or with TNFα, and that the phenotype is reversible with withdrawal of inflammatory stimuli. This supports the hypothesis that acquired resistance to cancer immunotherapy can be mediated by inflammation-induced cancer dedifferentiation.

Introduction

Despite the high initial response rates of patients to adoptive T cell transfer therapy (ACT), most patients relapse within a few months[1–3]. The prevailing hypotheses for how resistance to ACT develops in patients with melanoma are that either their T cells become ineffective, due to exhaustion or immune tolerance, or that a subset of tumor cells acquire a survival advantage, possibly by genetic alterations that increase proliferation or that result in the loss of presentation of the antigen targeted by ACT[4–9]. In recent years, an alternative mechanism, inflammation-induced dedifferentiation of tumor cells to precursor cells of

Correspondence: Antoni Ribas, M.D., Ph.D. Department of Medicine, Division of Hematology-Oncology, 11-934 Factor Building, Jonsson Comprehensive Cancer Center at UCLA, 10833 Le Conte Avenue, Los Angeles, CA 90095-1782, USA. Telephone: 310-206-3928. Fax: 310-825-2493. aribas@mednet.ucla.edu.

*These authors contributed equally to this work

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neural crest origin[10–12], has come to light from mouse studies and using human cell lines. Work by Landsberg and colleagues[11] in a mouse model demonstrated that ACT using T cells specific for the shared melanosomal antigen gp100 resulted in initial tumor responses followed by regrowth of cancer cells that had lost gp100 expression and dedifferentiated into a neural crest lineage. This tumor cell dedifferentiation was mediated by tumor necrosis factor alpha (TNFα) produced by the gp100 T cell receptor (TCR) transgenic T cells in response to gp100 antigen recognition.

The inflammatory cytokine-induced plasticity of tumor cells, with reversible loss of the tumor-targeting antigen[11, 13], may have widespread clinical implications on how best to target antigens in ACT. Here we show clinical and pathological findings from a patient who received MART-1-specific ACT and developed resistance to therapy in association with a dedifferentiated tumor phenotype that lacked conventional melanocytic antigens.

Results

Clinical case report

A 60-year-old man with multiple pigmented nevi over his body presented with metastatic melanoma and was enrolled in a clinical trial of MART-1 TCR engineered ACT immunotherapy in combination with MART-1 peptide pulsed dendritic cells (DC) and high dose interleukin-2 (IL-2), administered after a lymphodepleting conditioning regimen (Figure 1A) (NCT00910650). Two years prior he had noted a mole on his back that had grown and bled occasionally, however it spontaneously regressed over time. Three months prior to presentation, he noted a right posterior neck mass and one month prior to presentation he was admitted for abdominal pain and jaundice. Endoscopic retrograde cholangio-pancreatography (ERCP) revealed peripancreatic masses and a fine-needle biopsy raised the possibility of melanoma. A whole-body PET/CT scan revealed multiple metastases in the lungs, head and tail of the pancreas, stomach wall, right lobe of the liver, and peritoneal and mesenteric regions (Figure 1B). An incisional biopsy of the right posterior neck mass confirmed a MART-1-positive metastatic melanoma. HLA typing of the patient revealed HLA-A*0201 positivity, making him a candidate for this clinical trial. This patient was treated before the approvals of immunotherapies that have shown improvement of overall survival in patients with advanced melanoma, such as ipilimumab, nivolumab and pembrolizumab[14–16].

Per study protocol (Figure 1A), the patient underwent a non-myeloablative conditioning regimen of cyclophosphamide (60 mg/kg/day, day −7 and −6) given with mesna (60 mg/kg/day) followed by fludaribine (25 mg/m^2, days −5 to −1). On day 0, the patient received adoptive transfer of 1×10^9 lymphocytes that had been retrovirally transduced with the F5 MART-1-specific TCR. On day +1, +14 and +30, the patient received a MART-$1_{26–35}$ peptide pulsed DC vaccines (1.8×10^7, 1.8×10^7 and 1.7×10^7 cells, respectively). Between days +1 to +7 he received high-dose IL-2 administered every 8 hours to tolerance. Over the course of the study, peripheral blood was collected at regular intervals for basic laboratory tests and immune monitoring, and he underwent baseline and on-therapy biopsies and PET/CT scans.

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At the time of treatment, the patient presented with diffusely metastatic disease confirmed by incisional biopsy of his left neck lesion to be stage IVc melanoma with homogenously positive MART-1 (Melan-A) expression by immunohistochemistry (IHC) (Figure 1B). The patient tolerated infusion of F5 TCR T cells, followed by IL-2 treatments, and MART-$1_{26–35}$ peptide pulsed DC vaccines with the main adverse effect being a whole body skin rash. The patient was able to return to work one month post-infusion. An early follow-up PET/CT scan at 35 days post-infusion demonstrated a dramatic decrease in FDG uptake in the vast majority of the patient's baseline lesions, with increased uptake in seven lesions and the presence of two new lesions with mild FDG uptake (Figure 1C, new lesions marked with black arrows and biopsied lesion marked with a red arrow). A subsequent PET/CT scan at 84 days post-infusion demonstrated objective responses on most baseline lesions by Response Evaluation Criteria in Solid Tumors (RECIST). However, there were also several new and increasingly FDG-avid lesions (Figure 1D, biopsied lesion marked with a red arrow) concerning for progression of disease including on his right anterior chest, liver, and paraspinal, paraaortic and mesenteric regions among others. The right anterior chest wall lesion was biopsied at that time for further investigation of his progressing disease. The patient's progression free survival was 3 months and his overall survival was 5 months.

Loss of MART-1 antigen in regressing and relapsed tumors

The initial biopsy of the patient's left neck lesion (Figure 1B, red arrow) showed an atypical malignant epithelioid neoplasm that was consistent with melanoma. Immunohistochemistry staining was performed and showed strong, diffuse S100 and melanocytic antigen expression, including MART-1, gp100 and tyrosinase (Figure 1E). One month after infusion of the F5 TCR T cells, the same left neck lesion was biopsied but showed a heterogeneous pattern of MART-1 staining with multifocal areas of low or absent MART-1 expression (Figure 1F). S100 staining showed no difference in intensity and distribution of expression when compared to the initial biopsy, however, gp100 and tyrosinase staining patterns mimicked the multifocal MART-1 pattern (Figure 1F). At the time of progression, an anterior chest lesion biopsy revealed the complete absence of MART-1 expression, along with absence of gp100 and tyrosinase, but again a normal S100 staining pattern (Figure 1G). The global loss of melanocytic antigens, as opposed to just MART-1, suggested a phenotype switch[13] within the progressing tumor.

CD8 cytotoxic T-lymphocyte infiltration in MART-1-deficient tumor

Prior to ACT, the majority of CD8 CTLs were observed at the periphery of the tumor with few CD8 CTLs present in the central regions of the tumor parenchyma (Figure 1E and 2A). At 35 days post-infusion of F5 TCR T cells, a prominent but multifocal lymphocytic infiltrate was observed within the tumor, largely at the periphery with moderate infiltration of the center of the tumor (Figure 1F and 2B). At the time of relapse, the anterior chest biopsy revealed a predominance of CD8 CTLs diffusely in all areas of the tumor (Figure 1F and 2C), in direct contrast to the pattern observed on initial biopsy of pre-treated tumors. Evidence supports that the presence of CD8 CTLs is indicative of an increased inflammatory milieu within the tumor microenvironment[11].

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Expression of an inflammation-induced dedifferentiation marker in relapsed tumor

The nerve growth factor receptor (NGFR or CD271) is a member of the tumor necrosis factor (TNF) superfamily group of receptors, and has been shown to be a marker of cancer stem cells in melanoma[10]. The expression of melanocytic antigens, such as MART-1 and gp100, is negatively correlated with the expression of NGFR [10]. Importantly, inflammatory signals, largely due to TNFα, result in increased expression of NGFR in mouse models of melanoma and human cell lines *in vitro*[11]. In this patient, the expression of NGFR was low in pre-treated tumor biopsies (Figure 1E and 2A). However, increased multifocal expression of NGFR was observed in the tumor 35 days post-infusion of F5 TCR T cells (Figure 1F and 2B). Importantly, the expression of NGFR was negatively correlated with expression of MART-1 and gp100 within the tumor, and was observed predominantly in regions of increased CD8 CTL infiltration, thus suggesting that its expression was linked to regions of the tumor with increased inflammatory mediators (Figure 1F and 2B). In the progressing tumor, this phenomenon was even more apparent as the vast majority of the tumor cells did not express MART-1, and had strong NGFR expression with heavy infiltration of CD8 CTLs (Figure 1G and 2C).

F5 TCR T cells induce dedifferentiation in human melanoma cell lines

To determine if F5 TCR transduced T cells are sufficient to induce dedifferentiation of human melanoma cells *in vitro*, we cultured the human melanoma cell line M397 with conditioned media from the co-culture of F5 TCR T cells with M397 cells. We were unable to derive cell lines from the patient's biopsy samples, thus the M397 line was chosen due to its HLA-A2 positivity and expression of MART-1. M397 cells cultured with conditioned media generated from co-culture with F5 TCR transduced T cells demonstrated decreased surface expression of MART-1 and upregulation of NGFR (Figure 3A). No effect was seen on M397 cells that remained untreated or that were treated with conditioned media from untransduced T cells (Figure 3A), thus suggesting that conditioned media from F5 TCR T cells is sufficient to induce dedifferentiation of human melanoma cells.

A report in preclinical models had suggested that inflammation-induced dedifferentiation in mouse models of melanoma and in human cell lines is mediated by TNFα[11]. To test if F5 TCR T cells produce TNFα upon antigen encounter, we performed intracellular staining of F5 TCR T cells co-cultured with M397 cells and found significantly increased TNFα expression in these cells compared to untransduced cells (Figure 3B).

TNFα induces reversible dedifferentiation and markers of ACT resistance in human melanoma

To verify TNFα-induced dedifferentiation in human melanoma cell lines, we treated eight previously described patient-derived MART-1-expressing melanoma cell lines[17] with either TNFα or DMSO for three days, and analyzed them for surface expression of MART-1 and NGFR (Figure 4A,B). Consistent with previous findings, we found that TNFα treatment lead to dedifferentiation characterized by decreased surface MART-1 expression, and upregulation of NGFR (Figure 4A,B). To better understand the molecular mechanism underlying this phenotypic transformation, we performed RNA-sequencing of three different melanoma cell lines, M229, M263 and M297, treated with either TNFα or DMSO for three

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days. Gene-set enrichment analysis (GSEA) of differentially expressed genes between TNFα and DMSO treated cells (Table S1 and Table S2) revealed that TNFα treated cells were enriched for genes characteristic of the TNFα inflammatory response (normalized enrichment score or NES = 2.21, FDR < 0.001), epithelial to mesenchymal transition (NES = 1.92, FDR < 0.001) and neural crest stem cells (NES = 1.49, FDR = 0.016) (Figure 4C). Moreover, genes involved in the microphthalmia-associated transcription factor (MITF) pathway[18], including melanocytic antigens such as MART-1, tyrosinase and pmel (same as gp100), were enriched in DMSO samples, and down-regulated in TNFα treated cells (NES = −2.38, FDR < 0.001) (Figure 4C and Table S1). Interestingly, TNFα treatment also led to the enrichment of several pathways characteristic of the innate anti-PD-1 resistance (IPRES) gene signature[19] (Table S2). This included enrichment of genes involved in EMT, angiogenesis and hypoxia, among others (Table S2), which is reflective of a more invasive melanoma phenotype and that has been associated with increased NGFR expression[20, 21].

Amongst the differentially expressed genes with TNFα treatment of our melanoma cell lines were LIF and IL-8, both of which are known to promote EMT and tumor resistance[22, 23], thus suggesting autocrine signaling through these proteins may play a role in tumor dedifferentiation. We thus measured the secretion of IL8 and LIF in the supernatant of M397 cells treated with TNFα, or co-cultured with untransduced or F5 TCR T cells. Both TNFα and F5 TCR T cell treatment of M397 cells resulted in increased IL8 and LIF secretion (Figure S1A). However, neither IL8 nor LIF treatment of M397 cells was sufficient to induce dedifferentiation (Figure S1B), thus suggesting the melanoma resistance phenotype observed in this study is a direct effect of TNFα or may be mediated by factors other thank IL8 and LIF.

To test the reversibility of inflammation-induced dedifferentiation in our melanoma cell lines, we treated M397 cells for three days with TNFα and subsequently removed the inflammatory media for seven days (Figure 4D). Withdrawal of inflammatory media resulted in increased surface MART-1 expression and loss of NGFR expression (Figure 4D), thus suggesting that inflammation-induced dedifferentiation is reversible and that persistent TNFα exposure is required to maintain this phenotypic state.

Discussion

We report a case of melanoma dedifferentiation as a mechanism of immune escape in a patient treated with cancer immunotherapy, a phenomenon previously only described in mouse models and human cell lines[11, 13, 20]. The notion of phenotypic switching of melanoma is consistent with our current model of melanoma resistance; that is, we see the loss of the target antigen, in this case MART-1, in progressing tumors, which is a phenomenon frequently encountered by pathologists. The global loss of melanocytic markers, including gp100 and tyrosinase, in conjunction with the expression of the inflammation-induced neural crest marker NGFR, suggest that the tumor cells acquired a dedifferentiated state that is reflective of earlier stages of embryological development of melanocytes, cells that originally arise from within the neural crest. While it has been suggested that this plasticity of melanoma cells is reversible in mouse models[11], this cannot readily be tested in human patients. Our *in vitro* data using human melanoma cell

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lines suggests, however, that the same phenomenon may apply to humans, with cells dedifferentiating in the setting of inflammation with the possibility of re-differentiating after inflammation resolves.

Our RNA-sequencing analysis of melanoma cell lines treated with TNFα suggests that the pathways of inflammation-induced dedifferentiation may overlap with those of innate anti-PD-1 resistance (Table S2). In particular, the enrichment of genes involved in EMT suggest that dedifferentiation may reflect a more invasive phenotype. This has parallels to resistance patterns observed with BRAF inhibition, and may suggest that the concept of phenotypic switching applies more broadly as a resistance mechanism to other modes of melanoma therapy [13, 18, 20, 21, 24, 25]. In particular, several hypotheses suggest that the expression of MITF serves as a rheostat for melanoma cell phenotypes, with high levels of MITF promoting differentiation, moderate levels promoting proliferation, and low levels promoting invasiveness[26]. MITF is a critical factor for expression of melanocyte pigment genes, and loss of MITF and MART-1[18] with elevated expression of AXL receptor tyrosine kinase and NGFR[20, 21, 27] has been implicated in switching of melanoma cells from a proliferative to an invasive phenotype. Given that invasive cells are more resistant to BRAF inhibition[28], this phenotypic switching may serve as a mechanism to develop resistance to such inhibitors. Single-cell studies of human melanomas at different stages of treatment have revealed that most tumors contain heterogeneous subsets of low and high MITF expressing cells, with the relative composition of such cells shifting towards low MITF expressing cells in BRAF or MEK inhibitor treated patients[27].

This case report suggests that inflammation-induced dedifferentiation of melanoma cells may play a role in evading ACT in a subgroup of patients. In the clinical trial, 13 patients were evaluable for tumor response and nine patients (69%) showed evidence of tumor regression; however, they all had disease progression within six months[29], similar to other TCR engineered ACT trials[30]. There were four patients with biopsies at progression and only this case had evidence of dedifferentiation. This highlights the critical need for more studies investigating the underlying mechanisms of ACT resistance in human patients. These data are important in suggesting that future ACT protocols may benefit from the simultaneous targeting of multiple tumor antigens using mixed T cell populations and from broadening of the response by concurrent administration of checkpoint inhibitors, so that dedifferentiation, and loss of critical melanocytic antigens such as MART-1 and gp100, do not contribute to treatment resistance. In addition, concurrent reduction of the T cell-induced inflammatory milieu that is associated with elevated NGFR expression may also enhance therapeutic efficacy.

Methods

Study design

This was a pilot clinical trial for patients with metastatic melanoma (Clinicaltrials.gov number NCT00910650)[31–33]. The study protocol and its amendments were approved by the University of California, Los Angeles Institutional Review Board. The patient provided written informed consent.

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Manufacture of cell products

The patient underwent leukapharesis to collect peripheral blood mononuclear cells (PBMCs). T cells and DCs were manufactured as previously described[31–33]. Briefly, PBMCs were activated using anti-CD3 antibodies (OKT3) and IL-2 to enrich for T cells, and retrovirally infected with the MSGV1-F5AfT2AB vector. Cells were infused fresh. DCs were produced by *ex vivo* differentiation of PBMCs using granulocyte/monocyte colony stimulating factor (GM-CSF) and interleukin-4 (IL-4), and pulsed with the MART-$1_{26–35}$ anchor-modified immunodominant peptide in the HLA-A2.1 allele[29]. Full details are provided in the supplemental appendix online.

Tumor pathology and immunohistochemistry (IHC)

Formalin-fixed paraffin embedded baseline and on-therapy biopsies were cut and stained with hematoxylin and eosin, anti-S100, anti-Melan-A, anti-CD8, and anti-NGFR antibodies at the UCLA Anatomic Pathology Immunochemistry and Histology Laboratory as previously described[34].

Cell culture, in vitro stimulation and flow cytometry

Human melanoma cell lines were cultured as previously described[17]. For *in vitro* dedifferentiation experiments, MART-1-specific T cells and untransduced T cells were co-cultured with M397 cells and supernatant was obtained after 24 hours. This conditioned media was diluted 1:1 in fresh culture media for use in subsequent cultures. One million M397 cells were then seeded and treated with TNFα (1000 IU/ml), conditioned media from MART-1 F5 TCR transduced T cells, conditioned media from mock untransduced T cells, or no treatment for three days. To test reversibility of dedifferentiation, the treatment media was removed from some replicates on day three and replaced with fresh culture media for seven days. All cells were stained with anti-NGFR and anti-MART-1 fluorescently conjugated antibodies for analysis by flow cytometry. Intracecllular staining was performed using anti-TNFα antibodies after fixation using the Fixation/Permeabilization Solution kit (BD Biosciences). Samples were analyzed using an LSR-II flow cytometer (BD Biosciences) and the data was analyzed using FlowJo software (Tree Star, Inc.). Full details of all *in vitro assays* are provided in the supplemental appendix online.

Bulk RNA-sequencing and analysis

RNA was extracted for three melanoma cell lines treated with either DMSO or TNFα, and processed for library preparation. Samples were sequenced by 50bp single-end sequencing on an Illumina HiSeq 2500 sequencer. Reads were mapped using TopHat2 v2.0.9[35], and gene expression was quantified using Cufflinks v2.2.1 and Cuffnorm[36]. Gene Set Enrichment Analysis (GSEA) was used to assess enrichment across several gene sets on the Molecular Signatures Database (MSigDB) and the pathways for IPRES[19]. Full details are provided in the supplemental appendix online.

Supplementary Material

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Acknowledgments

This study was funded in part by the UCLA/Caltech Medical Scientist Training Program (to A.M and Y. J. K) and a NIH National Research Service Award CA183220 (to A.M.), NIH grants R35 CA197633 and P01 CA168585, the Parker Institute for Cancer Immunotherapy, the Ressler Family Fund, the Samuels Family Fund, the Grimaldi Family Fund, and the Garcia-Corsini Family Fund (to A.R.). We would like to thank Nathan Phillips and the UCLA Technology Center for Genomics and Bioinformatics Core Facility for their assistance in processing samples for genomic analysis. We would also like to thank Daniel E. Speiser for his helpful discussions about this work and critical review of our manuscript.

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Statement of significance

We report a patient whose metastatic melanoma underwent inflammation-induced dedifferentiation as a resistance mechanism to ACT to the MART-1 antigen. Our results suggest that future melanoma ACT protocols may benefit from the simultaneous targeting of multiple tumor antigens, modulating the inflammatory response, and inhibition of inflammatory dedifferentiation-inducing signals.

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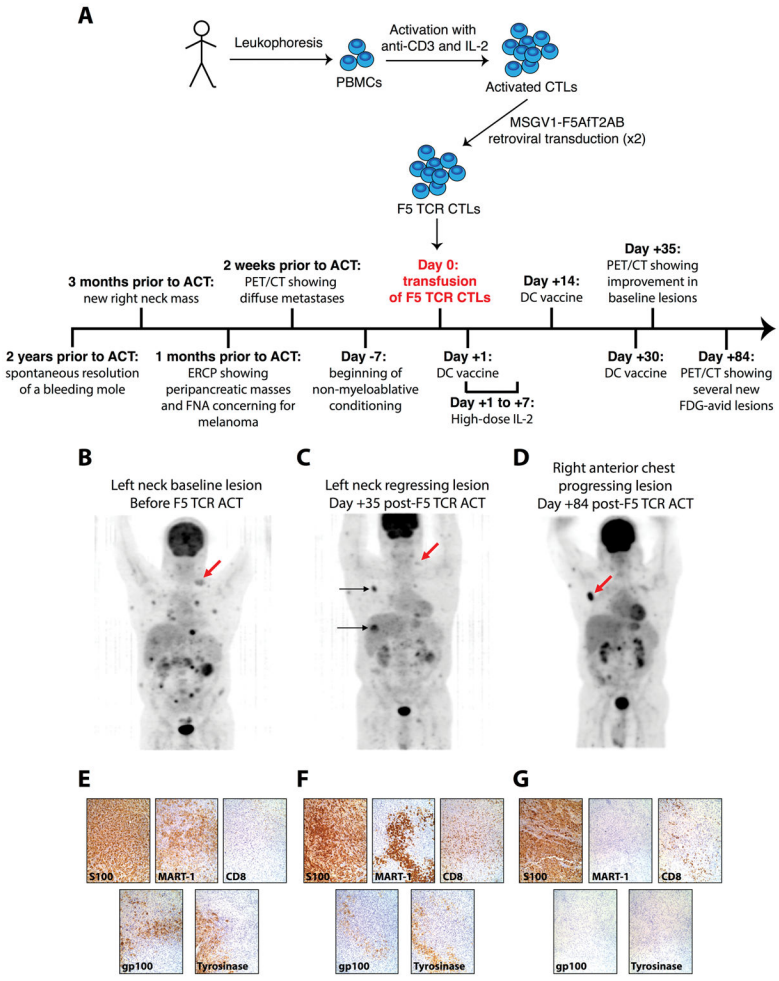

Figure 1.

Progressive tumors after ACT demonstrate loss of melanocytic antigens. (A) Overall treatment course for this patient. The patient was treatment naïve at the time of initiation of the ACT protocol. The patient underwent leukophoresis, and PBMCs were used to manufacture T cells for ACT and DCs for use in a MART-$1_{26–35}$ pulsed DC vaccine. The patient underwent non-myeloablative conditioning with cyclophosphamide and fludarabine started at day −7 prior to ACT. He received the DC vaccine on days +1, +14 and +30 after ACT. Several follow-up PET/CT scans were performed, initially showing disease regression at day +35 and eventual progression by day +84. (B)–(D) PET/CT scans of the patient are shown at (B) baseline before ACT, (C) during tumor regression on day +35 of ACT and (D) during tumor progression on day +84 of ACT. Red arrows indicate biopsied tumors and black arrows indicate progressing lesions at day +35. (E)–(G) Immunohistochemistry of the patient's tumor for expression of S100, MART-1, CD8, gp100 and tyrosinase at (E) baseline before ACT, (F) during tumor regression and (G) during tumor progression. A heterogeneous, multifocal loss of MART-1 and gp100 is seen during tumor regression, with complete loss of these melanocytic antigens at the time of tumor progression.

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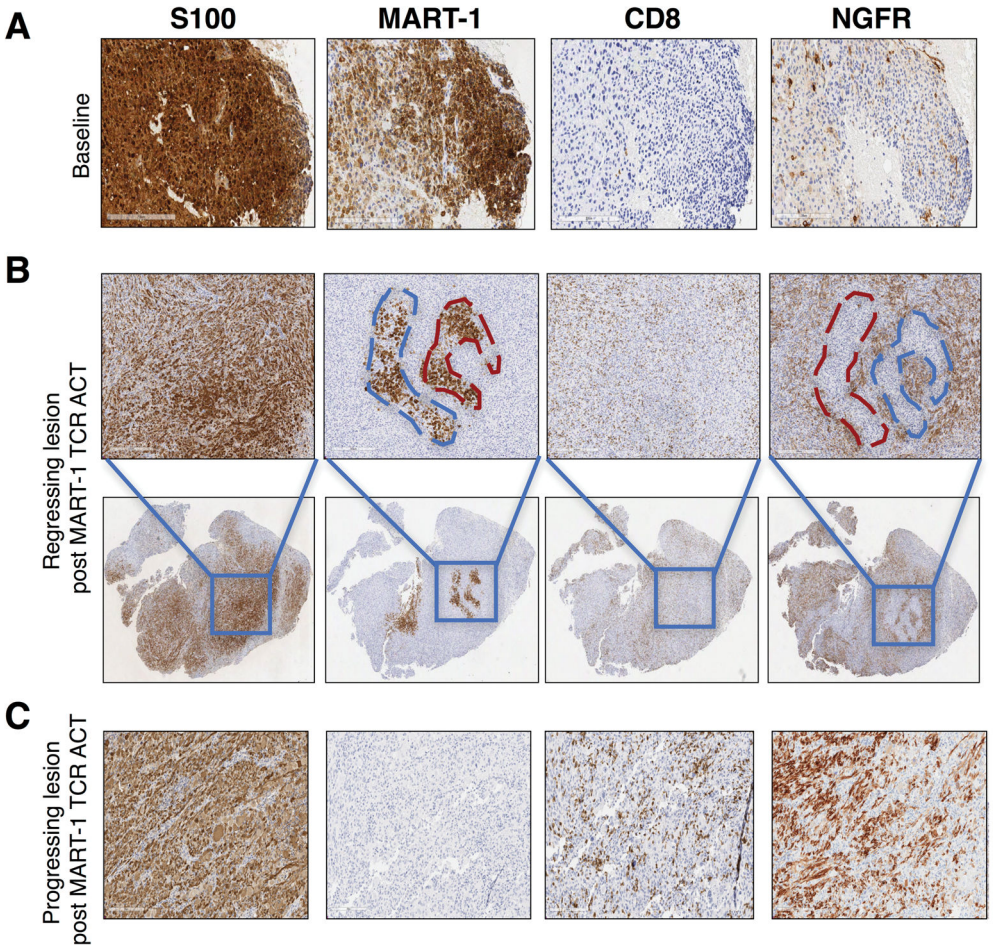

Figure 2.

Loss of melanocytic antigens is associated with increased NGFR expression and T cell infiltration. (A)–(C) Immunohistochemistry of the patient's tumor for S100, MART-1, CD8 and NGFR (CD271) at (A) baseline before ACT, (B) during tumor regression and (C) during tumor progression. The loss of MART-1 is correlated with higher NGFR expression and CD8 T cell infiltration in both regressing and progressing tumors, thus suggesting inflammation-induced tumor dedifferentiation of these tumors.

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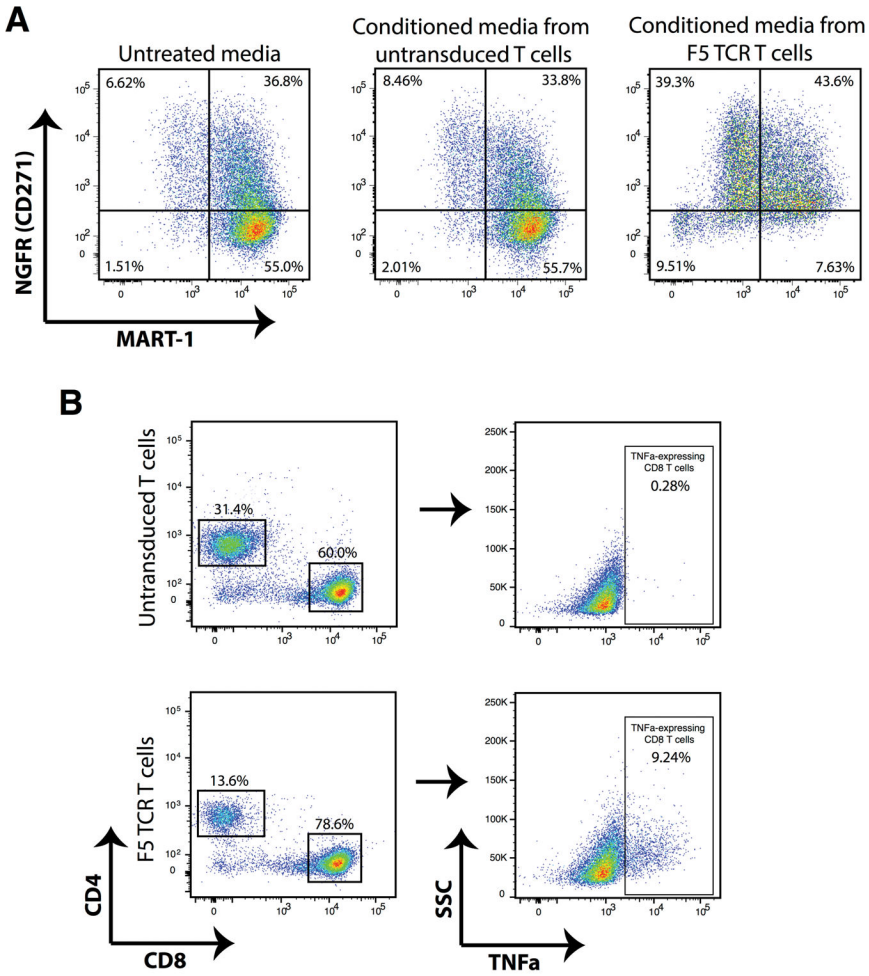

Figure 3.

F5 TCR T cells induce dedifferentiation of human melanoma cell lines. (A) M397 cells were left untreated, or cultured for three days with conditioned media obtained from co-culture of either untransduced or F5 TCR T cells with M397 cells. Flow cytometry was subsequently performed for surface expression of MART-1 and NGFR. (B) Untransduced or F5 TCR T cells were co-cultured with M397 cells for six hours. Flow cytometry was subsequently performed with intracellular staining for TNFα.

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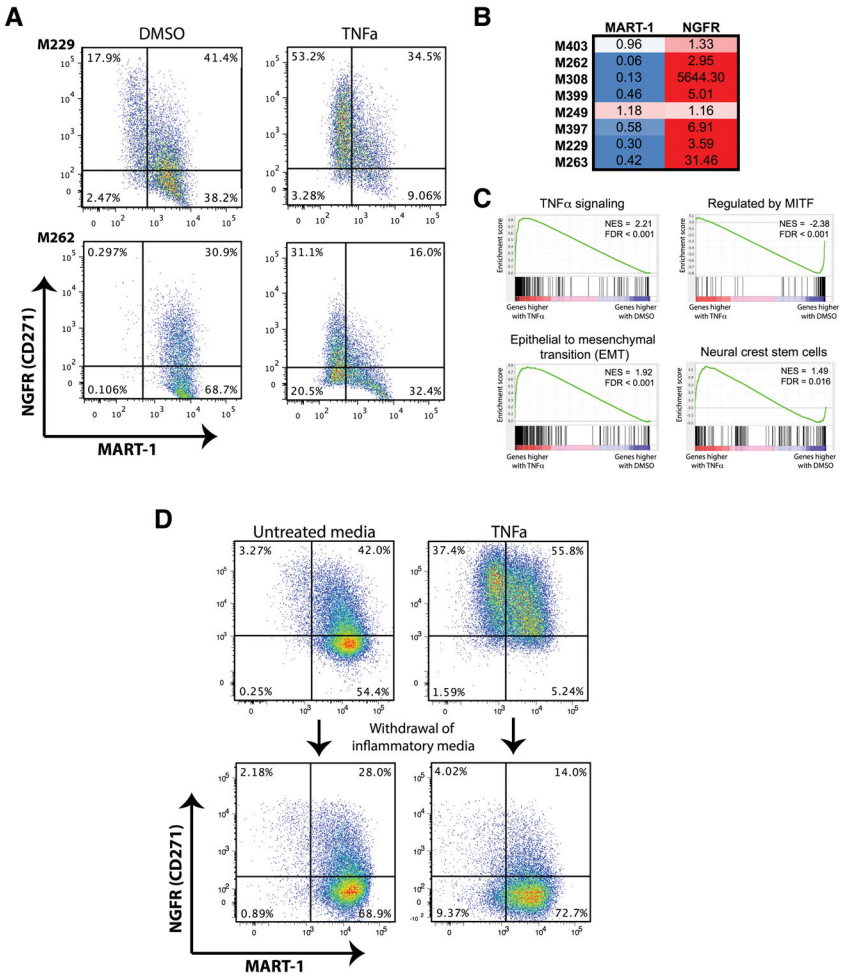

Figure 4.

TNFα treatment induces a reversible dedifferentiation of several *BRAF*^{V600} mutant melanoma cell lines. (A) M229 and M262 *BRAF*^{V600} mutant cell lines were treated with DMSO or TNFα (1000U/mL) for 3 days. The surface expression of MART-1 and NGFR at the end of this period was quantified using flow cytometry. (B) Normalized median fluorescence intensities of MART-1 and NGFR expression by flow cytometry in several *BRAF*^{V600} mutant melanoma cell lines treated with DMSO or TNFα for 3 days. Red and blue boxes highlight increased and decreased expression with TNFα treatment, respectively. (C) GSEA analysis of differentially expressed genes between melanoma cell lines treated with TNFα or DMSO for 3 days. An enrichment of genes characteristic of TNFα signaling, EMT and neural crest stem cells was seen in TNFα treated cells, and an enrichment of genes involved in the MITF pathway was seen in DMSO treated cells. (D) M397 cells were left untreated or treated for 3 days with TNFα. Inflammatory media was then replaced with fresh culture media for 7 days, and cells were subsequently analyzed by flow cytometry for surface expression of MART-1 and NGFR.