Interferon- γ Reduces Melanosomal Antigen Expression and Recognition of Melanoma Cells by Cytotoxic T Cells

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In malignant melanoma, tumor-infiltrating lymphocytes are frequently reactive with melanosomal antigens. Achieving complete remissions by peptide therapy is frequently hampered by metastases evading immune recognition. The tumor microenvironment seems to favor reduced expression of target antigens by melanoma cells. Among candidate factors, interferon- γ (IFN- γ) (10^2 to 10^3 U/ml) suppressed expression of antigens MART-1, TRP-1, and gp100 by M14 melanoma cells as shown by immunohistology and fluorescence-activated cell sorting analysis, reducing MART-1 expression by >65%. Northern blot analysis revealed that reduced expression was regulated at the transcriptional level, demonstrating a 79% reduction in MART-1 transcript abundance after 32 hours of IFN- γ treatment. To evaluate consequences of IFN- γ exposure for immune recognition, MART-1-responsive T cells were reacted with pretreated HLA-matched melanoma cells. Cytotoxicity was reduced up to 78% by IFN- γ pretreatment, and was restored by addition of MART-1 peptide AAGIGILTV for 2 hours. Examination of melanoma lesions by quantitative reverse transcriptase-polymerase chain reaction revealed up to 188-fold more abundant IFN- γ transcripts when compared to control skin. Laser capture microdissection and immunohistology localized most IFN- γ -producing T cells to the tumor stroma. Reduced MART-1 expression was frequently observed in adjacent tumor cells. Consequently, IFN- γ may enhance inflammatory responses yet hamper effective recognition of melanoma cells. (*Am J Pathol* 2002, 160:521–528)

Most T cells infiltrating malignant melanoma tumors are reactive with differentiation antigens, expressed by ma-

lignant as well as normal melanocytes.¹ Such antigens include gp100, MART-1, tyrosinase, TRP-2, and TRP-1.² By comparison, fewer T cells respond to progression antigens expressed by malignantly transformed cells of diverse lineages such as MAGE-1 and MAGE-3.^{3,4}

Knowledge of target epitopes has provided opportunities for their clinical application to patients with metastatic melanoma. Patients can be immunized with antigenic peptides either directly or after previous incubation with autologous dendritic cells, frequently in combination with expression of immunostimulatory cytokines, ie, interleukin-2, IFN- γ , or granulocyte/macrophage colony-stimulating factor.^{5–8} Unfortunately, tumor cells develop immune escape mechanisms such as down-regulation of major histocompatibility complex (MHC) molecules at the cell surface, reduced activity of the transporter associated with antigen presentation, and modulation of the proteasome complex.^{9–11} Dedifferentiation associated with reduced target antigen expression can also lead to immune escape of tumor cells.¹²

The incentive for dedifferentiation among melanoma cells remains poorly understood to date. The microenvironment may contribute to dedifferentiation by providing circumstances that favor loss of expression of one or more melanosomal differentiation markers.^{13,14} In this regard, tumor cells at extracutaneous sites encounter different extracellular matrix molecules, cytokines, and growth factors compared to epidermal melanocytes.

To explore a mechanism for immune evasion, the potential of IFN- γ generated by activated tumor-infiltrating lymphocytes to modulate target antigen expression and subsequent immune recognition of melanoma cells was investigated. Consequences of IFN- γ exposure for target antigen expression were tested at the protein and RNA levels in sporadically metastatic M14 cells.¹⁵ Functional immune recognition of tumor cells pretreated in the presence or absence of IFN- γ was assessed using cytotoxic MART-1-reactive T cells and HLA-matched melanoma cells.¹⁶ Finally, the abundance of IFN- γ in primary and metastatic melanoma lesions was assessed by quantita-

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tive reverse transcriptase-polymerase chain reaction (RT-PCR), laser capture microscopy, and immunohistology. Such investigations can contribute to a further understanding of the dedifferentiation process, representing a potential immune escape mechanism for malignant melanoma cells.

Materials and Methods

Cultured Cells

The melanoma cell line M14 was cultured in 10% fetal bovine serum and standard antibiotics (100 IU/ml penicillin, 100 g/ml streptomycin, and 25 mg/ml amphotericin; Life Technologies, Inc., Gaithersburg, MD) in glutamine containing Dulbecco's modified Eagle's medium (Life Technologies, Inc.).¹⁵ The melanoma cell line 624.38 was cultured in glutamine containing Iscove's modified Dulbecco's medium (Mediatech Inc., Herndon, VA) with 10% normal human AB serum (Sigma Chemical Co., St. Louis, MO), 1% standard antibiotics and ciprofloxacin at 10 μ g/ml (Bayer Corporation, Kankakee, IL).¹⁶ Ciprofloxacin is routinely added to patient-derived cultures to prevent mycoplasma infestation. Similarly maintained melanoma cell cultures F002, F003, and F010 were derived from metastases by fine needle aspiration and used within 20 passages.¹⁶ The MART-1-reactive, HLA-A*0201-restricted A42 T-cell line was likewise maintained in medium described for 624.38 in the presence of 1000 IU/ml interleukin-2 (R&D Systems, Minneapolis, MN).¹⁷

Normal melanocytes were obtained from skin by proteolytic treatment as described previously and maintained in Medium 154 (Cascade Biologicals, Portland, OR) supplemented with 4% fetal bovine serum and standard antibiotics (Life Technologies, Inc.), 0.6 ng/ml basic fibroblast growth factor, 10 nmol/L endothelin-1, 10 nmol/L α -melanocyte stimulating hormone (α -MSH), 1 μ g/ml α -tocopherol, 5 μ g/ml insulin, and 1 μ g/ml transferrin (Sigma).¹⁸

Treatment by Inflammatory/Melanogenic Modulators

M14 cells were treated with 1000 IU/ml interferon- γ (IFN- γ), 100 ng/ml interleukin-13, or 10 μ g/ml transforming growth factor- β (all from R&D Systems) or with 0.6 ng/ml basic fibroblast growth factor, 1 nmol/L endothelin-1, 10 nmol/L α -MSH, 5 μ g/ml insulin (all from Sigma) for 72 hours. Cells were subsequently fixed in acetone for immunohistology or in 2% paraformaldehyde for fluorescence-activated cell sorting (FACS) analysis, or alternatively washed and harvested in Tri-Reagent (Sigma) for RNA isolation.

Immunohistology

Indirect immunostaining was performed on fixed adherent cells or tissue sections by preincubation in 10% normal human serum in phosphate-buffered saline (PBS),

followed by incubation in primary antibodies. Biotinylated antibodies to mouse immunoglobulins (Igs) were used as secondary antibodies to MEL5 (anti-TRP-1; Signet Laboratories, Dedham, MA), NKI-Beteb (anti-gp100; Sanbio, Uden, The Netherlands), M2-7 C10 (anti-MART-1; NeoMarkers, Fremont, CA), MEL-1 (anti-GD3; Signet Laboratories), and anti-CD3 (Pan-T cell marker; Becton Dickinson, San Jose, CA) in single-staining procedures, followed by peroxidase-conjugated streptavidin in the tertiary step. Alternatively, in double-staining procedures isotype-specific antibodies labeled with peroxidase or alkaline phosphatase were used in the second step (Southern Biotechnology Associates, Inc., Birmingham, AL).¹⁹ In the latter case, Fast Blue BB was first added as an alkaline-phosphatase substrate in the presence of 0.2 mg/ml naphthol As-Mx-phosphate and 1 mmol/L levanizole (Sigma) in 0.1 mol/L Tris-HCl buffer (pH 8.5), followed by 0.25 mg/ml amino ethyl carbazole (Sigma) in 0.1 mol/L NaAc (pH 5.2) as a peroxidase substrate. In single-staining procedures, only amino ethyl carbazole-staining was performed, followed by Mayer's hematoxylin counterstaining (Dakopatts, Carpinteria, CA) where indicated. Specimens were coverslipped in glycerol (Dakopatts).

FACS Analysis

For membrane antigens, unfixed cells were stained in 10% normal human serum (NHS) in PBS and the primary antibody followed by fluorescein isothiocyanate-labeled anti-mouse Ig antibodies (BioSource, Camarillo, CA) or by biotinylated anti-mouse Ig followed by R-phycoerythrin-labeled streptavidin (Dakopatts). Antibodies used include MEL5, NKI-Beteb, and M2-7 C10, as well as antibodies L243 to HLA-DR antigens (Becton Dickinson) and B9.12.1 to HLA-A, HLA-B, and HLA-C antigens (Immunotech, Mar-seilles, France). For detection of intracellular antigens, cells were prefixed in 2% paraformaldehyde with 1% fetal bovine serum and 0.1% sodium azide (Sigma) in fluorescent antibody (FA) buffer (Difco, Detroit, MI) and permeabilized in 0.03% saponin in PBS, followed by immunostaining in the presence of 0.3% saponin (Sigma) by primary antibodies and secondary fluorescein isothiocyanate-labeled anti-mouse Ig antibodies (BioSource). A Coulter XL flow cytometer (Miami, FL) and Coulter Elite software were used to determine the mean fluorescence intensity of 5000 cells.

Northern Blot Analysis

RNA was isolated using Tri-Reagent according to the manufacturer's specifications (Sigma). Fifteen μ g per sample of formamide-denatured total RNA was loaded onto a 1% agarose gel in 6% formaldehyde and phosphate buffer and run at 90 V. RNA was transferred to a ZetaBind nylon membrane (Life Science Products Inc., Denver, CO) by capillary transfer overnight. RNA was crosslinked to the membrane by baking. The membrane was hybridized to 1.5×10^7 cpm of ³²P-labeled probe prepared using a random primer labeling kit (Life Technologies, Inc.). The probe for MART-1 mRNA was pre-

pared by RT-PCR amplification from total RNA derived from M14 cells with primers 5'-ATGCCAAGAGAAGATGCTCA-3' and 5'-TTAAGGTGAATAAGGTGGTGG-3', and verified by automated sequencing. Likewise, the gp100 probe was prepared with primers 5'-AGTCCCTGGATTGTGTTTC-3' and 5'-AGCAAGATGCCCGATCAG-3' and random primer labeled. Hybridization of the blot was performed in the presence of 40 μ g/ml of salmon sperm DNA at 65°C overnight. The washed blot was exposed to BioMax film (Kodak, Rochester, NY) at -80°C. After hybridization to the MART-1 probe was analyzed, the blot was stripped in 0.5% sodium dodecyl sulfate at 70°C for 1 hour. Effective stripping was confirmed by autoradiography before hybridization with the radiolabeled probe to gp100.

Cytotoxicity Assays

Cells characterized for HLA-A*0201 expression were plated at 10^4 cells/well in a 96-well plate and cultured in the presence or absence of 1000 IU/ml of IFN- γ for 72 hours, the same treatment used to compare the consequences of treatment by inflammatory/melanogenic modulators. Where noted, 10 μ g/ml of MART-1 peptide AAGIGILTV was subsequently added for 2 hours. Cells were then washed and cultured in the presence of 5 μ Ci/well of 51 Cr (Du Pont NEN Research Products, Boston, MA) at 37°C for 3 hours. The labeling medium was removed, and washed target cells were subsequently reacted with A42 T cells in the medium described for 624.38 cells at 37°C for 24 hours. Cytotoxicity was measured as cpm of 51 Cr release compared to spontaneous release (in absence of T cells) and total lysis (in the presence of 1% Triton X-100) according to the formula: percent killing equals cpm (in presence of T cells) - cpm (spontaneous)/cpm(total) - cpm(spontaneous).

Laser Capture Microscopy

Eight- μ m frozen sections were cut; followed by treatment with 70% ethanol, acetone, filtered Mayer's hematoxylin, water, eosin Y, graded ethanol, and xylene; and 30 minutes of drying in a dessicator. Cells within tumor lobes were captured with a PixCell II Laser Capture Microdissection System (Arcturus Engineering Inc., Mountain View, CA) and captured cells were immediately transferred to Tri-Reagent for RNA isolation, performed according to the manufacturer's specifications.

Quantitative RT-PCR

RNA (250 ng) was reverse-transcribed in the presence of 62.5 U of Multiscribe reverse transcriptase (RT), 5.5 mmol/L MgCl₂, 0.5 mmol/L (per nucleotide) dNTPs, 2.5 μ mol/L random hexamers, 20 U RNase inhibitor, and RT buffer (Perkin Elmer, Norwalk, CT) at 48°C for 30 minutes, followed by a 5-minute inactivation of RT activity at 95°C. For quantitative PCR, 10% of the RT reaction was combined (in triplicate) with 62.5% TaqMan PCR Master mix (Perkin Elmer), 1 μ mol/L of primers 5'-AGCTCTG-

Figure 1. IFN- γ -induced repression of melanosomal antigen expression by M14 cells. M14 cells were exposed to 1000 U/ml of IFN- γ or 10 nmol/L of α -MSH for 72 hours and stained with antibodies to TRP-1, MART-1, and GD3. Note reduced expression of both melanosomal antigens TRP-1 and MART-1 after exposure to IFN- γ . By contrast, expression of membrane antigen GD3 is maintained in the presence of both IFN- γ and α -MSH. Original magnification, $\times 36$.

CATCGTTTTGGGTT-3' and 5'-GTTCCATTATCCGCTACATCTGAA-3' for IFN- γ or 5'-GGCACCCAGCACAAATGAAG-3' and 5'-GCCGATCCACACGGAGTACT-3' for β -actin, as well as 1 μ mol/L of probes FAM-TCTTGCTGTTACTGCCAGGACCCA-TAMRA or FAM-TCAAGATCATTGCTCCTCCTGAGCGC-TAMRA, respectively.²⁰ Samples were heated 2 minutes at 50°C and 10 minutes at 95°C, followed by 42 cycles of 15 seconds at 95°C, 1 minute at 60°C in an Abi-Prism 7700 Sequence Detection System (Perkin Elmer), and the threshold cycle C_T for significant fluorescence was noted.

Relative IFN- γ mRNA levels were calculated following Perkin-Elmer guidelines. Briefly, C_T values for actin mRNA were subtracted from IFN mRNA C_T values. The difference found for control skin was subtracted from values obtained for melanoma samples, providing a value for $\Delta\Delta C_T$. The relative concentration of IFN- γ mRNA is represented by $2^{-\Delta\Delta C_T}$ (shown $\pm \sigma_{n-1}$).

Results

Immunohistology of M14 Cells Before and After Exposure to Various Treatments

Figure 1 illustrates MART-1 and TRP-1 down-regulation after exposure to IFN- γ for 72 hours, as well as sustained expression of membrane antigen ganglioside D3 (GD3). This is supportive of specific repression of melanosomal antigens. Exposure to other compounds tested did not markedly affect expression as exemplified by MART-1, TRP-1, and GD3 staining after exposure to α -MSH in Figure 1. Thus, IFN- γ was chosen as a factor potentially affecting target antigen expression by melanoma cells.

Table 1. FACS Analysis of M14 Cells after Exposure to IFN- γ

Marker	MFI (control)	MFI (with IFN- γ)
MART-1	3.38	0.66
gp100	3.83	1.73
TRP-1	4.72	2.39
HLA-DR	2.91	15.1
HLA-ABC	6.91	15.1

MFI, mean fluorescence intensity.

Effect of IFN- γ on Expression of Melanosomal Antigens at the Protein Level

The effect of IFN- γ on expression of TRP-1, MART-1, gp100, HLA-DR antigens, and HLA-A, HLA-B, and HLA-C antigens was quantified by FACS analysis as shown in Table 1. Up-regulated expression of HLA-DR and HLA-A, HLA-B, and HLA-C antigens confirms the efficacy of IFN- γ treatment. Simultaneously, it can be observed that expression of MART-1 was reduced by 80%, of TRP-1 by 49%, and of gp100 by 54%. Conversely, expression of HLA-DR and HLA-A, HLA-B, and HLA-C antigens was increased 5.2-fold and 2.2-fold, respectively, by the same treatment.

As the standard concentration of IFN- γ of 1000 U/ml used is higher than considered physiologically relevant, expression of MART-1 as the model antigen was tested after exposure to different IFN- γ concentrations. Mean fluorescence intensities for MART-1 shown in Figure 2

Figure 2. Loss of MART-1 protein expression in the presence of varying IFN- γ concentrations and its reversibility. FACS analysis of MART-1 expression in M14 cells treated with the indicated concentrations of IFN- γ for 72 hours. Where indicated by /R and for the concentrations shown, the cytokine was withdrawn and cells were cultured another 72 hours before harvesting cells for FACS analysis.

Figure 3. Northern analysis of MART-1 and gp100 expression in the presence of IFN- γ . Expression of MART-1 and gp100 after exposure to 1000 U/ml of IFN- γ for 0, 8, 16, or 32 hours was demonstrated by sequential hybridization of a Northern blot to appropriate probes. Note reduced expression after prolonged exposure to IFN- γ .

supported the notion that expression is also affected at lower concentrations of the cytokine. Mean fluorescence intensity decreased from 12.8 before treatment by 64.9% to 4.5 at 62.5 IU/ml. The decrease was similar for all concentrations assessed, decreasing by 68.2% to 4.1 at 1000 IU/ml. Thus, the majority of the effect occurs at less than the lowest concentration assessed. In a follow-up experiment, it was determined that suppressed expression was completely maintained at concentrations of 62.5 IU/ml, whereas fluorescence intensities rose to 5.9 at 31.25 U/ml and returned to the untreated value at concentrations of 15.1 IU/ml IFN- γ and below.

To determine whether loss of expression was permanent, cells treated with either 500 IU/ml or 1000 IU/ml of IFN- γ for 3 days were washed and cultured for an additional 3 days in absence of IFN- γ . Subsequent evaluation of MART-1 expression by FACS analysis revealed mean fluorescence intensities of 9.4 and 7.6 (Figure 2). Such a 63.5% and a 40.9% reversion, respectively, toward the level of MART-1 expression observed before treatment, demonstrated that repression of MART-1 expression by IFN- γ is dependent on the continued presence of IFN- γ .

Consequences of Exposure to IFN- γ for Transcription of Melanosomal Antigens

Figure 3 illustrates reduced expression of MART-1 as well as gp100 at the RNA level in melanoma cells exposed to IFN- γ . The intensity of bands hybridizing to the MART-1 cDNA probe as measured by NIH Image 1.62 software did not decrease in the lane representing 8 hours of treatment, and decreased thereafter by 44.3% and by 78.9% at 16 hours and 32 hours of treatment, respectively, when compared to untreated cells. The stripped blot, exposed similarly to a gp100 cDNA probe exhibited the same tendency, with a decrease in transcript abundance of 51.6% during the last 16 hours of treatment. It

Figure 4. Reduced cytotoxicity of A42 cells toward multiple melanoma cell cultures in the presence of IFN- γ and its restoration by MART-1 peptide. **A:** ^{51}Cr release by multiple melanocytic cell cultures in the presence or absence of 1000 U/ml of IFN- γ is shown. Note reduced recognition of patient-derived melanoma cells, but not of melanocytes after cytokine treatment. HLA-A2 $^{+}$ melanocytes derived from a vitiligo patient similarly showed a lack of reduced recognition in the presence of IFN- γ (results not shown). Mf9807 normal melanocytes are HLA-A*0201-negative. Effector:target ratios were 10:1. SE of chromium release measurements did not exceed 15%. **B:** Recognition of the IFN- γ -treated 624.38 cell line is increased after the addition of synthetic MART-1 peptide at 10 $\mu\text{g}/\text{ml}$. Such an increase in the cytotoxic response was absent if target cells were not pretreated with IFN- γ (results not shown). SE of chromium release measurements did not exceed 15%.

thus seems that IFN- γ affects melanosomal antigen expression by reducing mRNA levels.

IFN- γ -Affecting Immune Recognition of Target Cells

Apart from reducing melanosomal antigen expression, IFN- γ induces expression of MHC molecules as confirmed in Table 1. Consequently, the recognition of target cells exposed to IFN- γ will depend on the relative importance of HLA up-regulation and target antigen suppression. The prevailing effect was analyzed by reacting target cells with MART-1-responsive cytotoxic T cells as shown in Figure 4A. The percent killing is reduced by up to 78% for melanoma cells pretreated with 1000 U/ml IFN- γ compared to untreated cells. By contrast, it can be observed that recognition of Mf9931 P2 normal melanocytes was not affected by IFN- γ pretreatment. As ex-

pected, Mf9807 P11 HLA-mismatched melanocytes were not recognized by A42 MART-1-reactive T cells.

In Figure 4B, reduced recognition of IFN- γ -treated 624.38 melanoma cells was restored in the presence of the synthetic MART-1 peptide AAGIGILTV added at 10 $\mu\text{g}/\text{ml}$ 2 hours before incubation with A42 T cells. Thus, reduced recognition of target cells in the presence of IFN- γ is because of loss of MART-1 expression.

IFN- γ Expression in Melanocytic Lesions

Local expression of IFN- γ in primary and metastatic melanoma lesions was analyzed by quantitative RT-PCR. Of three specimens investigated, one contained 10-fold elevated concentrations of IFN- γ transcripts whereas others contained levels similar to control skin (Table 2). By contrast, metastatic melanoma tissue contained an elevated IFN- γ concentration of 188 times that detected in control skin.

Tumor cells are clustered in tumor lobes separated by tumor stroma. To analyze whether IFN- γ concentrations are elevated within tumor lobes or solely within the tumor stroma, the former was isolated by laser capture microscopy as shown in Figure 5 and RNA from the captured tissue was again used for quantitative RT-PCR. Comparing IFN- γ mRNA in captured tissue to control skin, it appeared that the majority of IFN- γ is encoded by cells present in the stroma, as levels were elevated 4.5-fold in the captured tissue compared to the control skin values. Focally however, IFN- γ -producing T cells will generate high levels of the cytokine.

A Potential Source for Elevated IFN- γ Expression

An explanation for the differences in IFN- γ transcript levels presented in Table 2 may be found in differential abundance of T cells as the source of this cytokine. T-cell infiltration of the tissue samples represented in Table 2 is shown by immunohistochemistry in Figure 6, A to E. It can be observed, that infiltrating T cells were indeed relatively abundant in metastatic melanoma, with fewer T cells in melanoma *in situ* specimens. Among the latter, sample C displayed the highest T-cell frequencies, in concordance with the observed increase in IFN- γ transcripts observed in this sample.

In the immuno-double staining performed to simultaneously observe MART-1 expression by melanoma cells and their proximity to T cells shown in Figure 6F, a halo of

Table 2. Quantitative RT-PCR Analysis of IFN- γ Expression in Melanocytic Lesions

Tissue source	Comparative (IFN- γ mRNA)
Control skin	1.0 (range, 0.6–1.7)
Melanoma <i>in situ</i> A (scalp)	0.5 (range, 0.3–0.9)
Melanoma <i>in situ</i> B (back)	1.2 (range, 0.2–3.4)
Melanoma <i>in situ</i> C (back)	9.8 (range, 7.0–13.6)
Melanoma metastasis (spine)	188.7 (range, 153.3–232.3)

Figure 5. Laser capture of melanoma cells. A hematoxylin-stained, 10- μ m frozen section of metastatic melanoma tissue is shown, together with captured tissue showing selective capture of tissue from a tumor lobe and exclusion of the tumor stroma. The resulting section lacking captured cells is also represented. Original magnification, $\times 50$.

reduced MART-1 expression was observed in tumor cells in close proximity to tumor-infiltrating T cells, suggesting that IFN- γ -generating T cells may be actively suppressing MART-1 expression in adjacent tumor cells.

Discussion

Reduced expression of melanosomal antigens poses a risk to melanoma patients, as dedifferentiation leaves melanoma cells less recognizable to the immune system.² The microanatomy of melanocytic lesions seems to affect the level of expression of differentiation antigens. This was suggested by expression analysis performed on frozen tissue and cultured cells as previously reported.¹³ As melanocytic cells encounter an altered microenvironment on entering the dermal compartment, it was likely that factors encountered within this environment affect the expression pattern of differentiation antigens. Candidate compounds used for the present study were chosen for their known involvement in melanogenesis or inflammation.

Among factors tested, IFN- γ most markedly altered expression of differentiation markers expressed in the melanosomal compartment of the cell. Comparable expression of lysosome-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2) in cells treated with and without IFN- γ has since indicated that down-modulation is not associated with reduced melanosome content (results not shown). Because of its frequent administration to

Figure 6. T-cell infiltration of primary and metastatic tumors. Six- μ m frozen sections of the same control and tumor tissues used for quantitative RT-PCR analysis as shown in Table 2 were immunostained with antibodies to CD3 to compare pan-T cell infiltration. **A–C:** Melanoma *in situ* samples presented in the order shown in Table 2. **D:** Control skin. **E:** Melanoma metastasis. **F:** MART-1 (blue) and CD3 (red) immuno-double staining of metastatic tissue. It can be observed that infiltrates are present in all tumor samples, yet T cells are more abundant in (sections of) metastatic tumor tissue. **F:** A halo of apparently viable cells with reduced MART-1 expression can be observed surrounding tumor-infiltrating T cells, possibly as a consequence of IFN- γ secretion by these T cells. Control stainings performed in the absence of one and both of the primary antibodies confirmed these staining patterns (results not shown). Original magnifications: $\times 100$ (**A–E**); $\times 132$ (**F**).

tumor patients, it should also be noted that exposure to 1000 U/ml of IFN- α did not affect melanosomal antigen expression (results not shown).

In a separate study, it was shown by quantitative RT-PCR that exposure to 20 μ mol/L of α -MSH can elevate TRP-1 and TRP-2 transcript abundance ~4.5-fold and 1.5-fold, respectively, in F002 melanoma cells (not shown). M14 cells have a much higher baseline expression of TRP-1 than F002 cells, so that such induction cannot be well appreciated in Figure 1. The apparent antagonism between α -MSH and IFN- γ is of interest in the light of the opposing role of these compounds in the immune response, α -MSH being anti-inflammatory whereas IFN- γ supports inflammation.^{21,22}

Dedifferentiation is poorly understood at present. Expression of melanosomal antigens is clearly not essential to the viability of melanoma cells. It has been suggested that depigmentation observed in malignant melanoma cells is the consequence of aberrant intracellular trafficking of melanosomal tyrosinase.²³ It was thus of interest to define the gene expression level affected by IFN- γ treatment. The cytokine clearly affected expression at the RNA level. The effect of IFN- γ was not permanent, as suppression of MART-1 expression could be reversed as shown in Figure 3. This suggests that additional mechanisms contribute to the formation of tumors that persistently lack expression of one or more differentiation antigens, even in absence of modulating factors. It is possible that epigenetic modulation eventually consolidates antigen loss. Yet importantly, reduced expression of MART-1 after exposure of melanoma cells to IFN- γ was sufficient to reduce the efficacy of MART-1-reactive T cells by up to 78%. This is of particular interest in light of elevated MHC class I and II expression known to occur after exposure to IFN- γ .^{24,25} Preliminary experiments suggested that reduced MART-1 expression can similarly suppress T-cell activation, as HLA-matched dendritic cells pulsed with antigenic lysates prepared from melanocytic cells induced T-cell activation to an extent correlating with MART-1 content of the lysate (not shown). Such results are compatible with high antigen-expression level requirements for effective pulsing of dendritic cells.²⁶ It should be noted that IFN- γ exposure can modulate expression of hundreds of genes in a cell-type-dependent manner.²⁵ Among these, some may contribute to immune escape of tumors through pathways other than through reduced target antigen expression. For example, IFN- γ exposure modulates expression of cellular adhesion molecules, thereby augmenting tumor dissemination and metastasis, and potentially providing a safe haven for tumor cells entering immune-privileged sites.^{25,27} Moreover, IFN- γ may contribute to reduced tumor recognition by inducing expression of the immunoproteasome at the expense of the standard proteasome, as only the latter seems capable of cleaving MART-1 to render immunogenic peptides.²⁸

Although a suppressive effect of IFN- γ on melanoma recognition was previously noted, such effect was not assigned to reduced antigen expression by tumor cells.²⁹ In fact, reduced expression of gp100 was not substantiated by a study reported by Takechi and col-

leagues,³⁰ demonstrating increased expression of this antigen after IFN- γ exposure. As we have presently expanded our studies for the most dramatically affected antigen MART-1 at the expense of studies related to other melanosomal antigens, we cannot exclude the possibility that gp100 is differentially affected in melanoma cells of different origins. It will be important to further define the consequences of cytokine exposure for tumor cell recognition with T-cell clones to additional melanosomal antigens. Also, it should be taken into account that others have reported an augmented humoral response to melanosomal antigens after overexpression of IFN- γ by melanoma cells.³¹ Augmenting cell-mediated immunity has been the primary focus in tumor immunology as it can be directed to a greater arsenal of antigens than humoral immunity, the latter being effective only for antigens expressed at the cell surface.³² Nevertheless, the humoral response may well compensate, at least in part, for reduced cellular cytotoxicity. In fact, our results demonstrating sustained expression of membrane antigen GD3 in the presence of α -MSH as well as IFN- γ is supportive of this concept, as antibodies to GD3 have been proposed for the treatment of melanoma patients.³³

Interestingly, a suppressive effect of IFN- γ on tumor antigen gp70 expression and subsequent tumor killing was also recently reported for murine colon carcinoma.³⁴ Taking into account that T cells are the most likely source for IFN- γ , it is likely that within a MART-1-expressing tumor, infiltrating activated T cells can both eliminate part of the tumor cells, and provide an effective immune escape mechanism to others. Even a few remaining tumor cells can endanger the patient, particularly if such tumor cells lose expression of target antigens and can thus no longer be recognized by tumor-infiltrating lymphocytes. Such extrapolation to the *in vivo* situation is of particular interest considering the high concentrations of IFN- γ , as well as the abundance of T cells detectable in metastatic melanoma lesions. Despite a more elaborate immune response, T cells do not effectively eliminate the tumor. By contrast, in vitiligo minute infiltrates effectively eliminate all pigment-producing cells from the skin, leading to progressive depigmentation.^{35,36} This is understandable, as levels of IFN- γ generated in the course of this immune response will not affect recognition of normal melanocytes (see Figure 5). Given the threshold level of antigen expression required for effective recognition by T cells, cytotoxicity toward normal melanocytes after IFN- γ exposure is likely explained by relatively high levels of remaining target antigen expression in these cells.³⁷ The apparent sustained recognition of IFN- γ -exposed melanocytes by CTL may explain how some patients develop extensive vitiligo with concomitant growth of metastatic tumor sites apparently escaping T-cell recognition.

Taken together, these novel findings indicate that despite its stimulatory effect on MHC molecule expression, IFN- γ can reduce target antigen expression and recognition of melanoma cells by CTL. Consequently, this aspect of IFN- γ action should be considered in the development of melanoma therapy.

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