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## Melanocyte Lineage-specific Antigen gp100 Is Recognized by Melanoma-derived Tumor-infiltrating Lymphocytes

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### Summary

We recently isolated a cDNA clone that encodes the melanocyte lineage-specific antigen glycoprotein (gp)100. Antibodies directed against gp100 are an important tool in the diagnosis of human melanoma. Since the gp100 antigen is highly expressed in melanocytic cells, we investigated whether this antigen might serve as a target for antimelanoma cytotoxic T lymphocytes (CTL). Here, we demonstrate that cytotoxic tumor-infiltrating lymphocytes (TIL) derived from a melanoma patient (TIL 1200) are directed against gp100. HLA-A2.1<sup>+</sup> melanoma cells are lysed by TIL from this patient. In addition, murine double transfectants, expressing both HLA-A2.1 and gp100, are lysed by TIL 1200, whereas transfectants expressing only HLA-A2.1 are not susceptible to lysis. Furthermore, the HLA-A2.1<sup>+</sup> melanoma cell line BLM, which lacks gp100 expression and is resistant to lysis, becomes susceptible after transfection of gp100 cDNA. Finally, HLA-A2.1<sup>+</sup> normal melanocytes are lysed by TIL 1200. These data demonstrate that the melanocyte differentiation antigen gp100 can be recognized in the context of HLA-A2.1 by CTL from a melanoma patient. Gp100 may therefore constitute a useful target for specific immunotherapy against melanoma, provided that no unacceptable cytotoxicity towards normal tissue is observed.

The melanocyte lineage-specific antigens recognized by mAb NKI-beteb are among the best diagnostic markers for human melanoma available to date (1). NKI-beteb reacts with melanoma cells throughout tumor development and does not cross-react with other tumor cell types or normal cells, except for melanocytes and pigmented cells in the retina. The antigen recognized by mAb NKI-beteb is an intracellular glycoprotein of approximately 100 kD (gp100) (1). Recently, we isolated a cDNA clone termed gp100-c1, which not only confers reactivity to mAb NKI-beteb, but also to two other mAbs used in diagnosis of malignant melanoma, HMB-50 and HMB-45 (2). Characterization of the gp100-c1 cDNA revealed that gp100 is a type I transmembrane glycoprotein highly homologous to another melanocyte-specific protein Pmel17 (Adema, G. J., A. J. de Boer, A. M. Vogel, W. A. M. Loenen, and C. G. Figdor, manuscript submitted for publication).

Although the mAbs recognizing gp100 are widely used for diagnostic purposes, they are less well applicable for therapeutic use since gp100 is predominantly expressed intracellularly. However, it has now been clearly demonstrated that

intracellular proteins can be processed and presented as peptides in association with MHC molecules to T lymphocytes (3, 4). Therefore, the gp100 antigen is a potential target for cellular immune responses against melanoma. CTLs recognizing melanoma tumor cells in a TCR-dependent and MHC-restricted manner have been isolated from tumor-bearing patients (for a review see reference 5). These CTLs often display cross-reactivity with allogeneic HLA-A2.1-matched melanomas, implying that shared melanoma antigens presented in the context of HLA-A2.1 are recognized (6, 7). Recently, it has been reported that some CTLs not only react with melanoma tumor cells, but also with normal melanocytes, suggesting recognition of differentiation antigens expressed by melanocytes (8).

Several approaches have been followed to unravel the nature of the antigens recognized by antimelanoma CTLs (9, 10). However, the identity of the antigens remains largely unknown. So far, only a genetic approach applied by Van der Bruggen et al. (9) and Brichard et al. (11) resulted in the identification of antigens recognized by CTL (9, 11). We have chosen to follow a reversed approach in that we focused on

a well-defined melanocytic antigen, the gp100 antigen, and investigated whether this antigen can serve as a target for antimelanoma CTLs.

Here, we demonstrate that the melanocyte differentiation antigen gp100 is specifically recognized by melanoma-derived tumor-infiltrating lymphocytes (TIL) in the context of HLA-A2.1.

## Materials and Methods

**Cell Culture.** TILs were generated by growth of single cell suspensions of metastatic melanomas with 1,000 U/ml IL-2 (Cetus Corp., Emeryville, CA) and were grown as was described previously (7). Melanoma lines Mel 397 and Mel 624 were obtained and grown as was reported previously (7). HLA-A2.1<sup>+</sup> melanoma lines MeWo (12) and BLM (13) and murine P815 transfectants were grown in DMEM (Gibco, Paisley, Scotland, UK) plus 7.5% heat-inactivated FCS (Gibco). JY, K562, and murine EL4 transfectants were cultured in IMDM (Gibco) plus 7.5% FCS. Murine cells were grown in the presence of  $5 \times 10^{-5}$  M  $\beta$ -ME, and all media contained antibiotics. Isolation of normal melanocytes from foreskin was performed by the method of Eisinger and Marko (14) with modifications as described previously (15). Melanocytes from passages two to three were used in chromium release assays.

**DNA Constructs and Transfection.** Plasmid pBJ1gp100neo was obtained by cloning the EcoRI fragment of the  $\lambda$  gp100-c1 cDNA clone (2) in the coding orientation in the polylinker of pBJ1-neo (16). Plasmid pBA2 containing a genomic fragment encoding HLA-A2.1 and human  $\beta_2$ -microglobulin was kindly provided by E. J. Baas (The Netherlands Cancer Institute, Division of Biochemistry, Amsterdam, the Netherlands). Plasmid pGK-hyg contains the hygromycin phosphotransferase gene (17). For the introduction of the HLA-A2.1 and  $\beta_2$ -microglobulin genes, EL4 cells were transfected with 18  $\mu$ g of pBA2 and 2  $\mu$ g of pGK-hyg DNA according to the calcium phosphate coprecipitation procedure (18) using Calciumphosphate Transfection Systems (GIBCO BRL, Gaithersburg, MD). 24 h after transfection, 500  $\mu$ g/ml hygromycin B (Calbiochem-Novabiochem Corp., La Jolla, CA) was added to the medium for selection of stable transfectants. HLA-A2.1<sup>+</sup> gp100<sup>+</sup> EL4 cells were obtained by transfection of stable HLA-A2.1<sup>+</sup> EL4 clones with 20  $\mu$ g of pBJ1-gp100 DNA and were selected with 500  $\mu$ g/ml hygromycin B and 1 mg/ml G418 (Gibco). BLM cells were transfected with 20  $\mu$ g of pBJ1-gp100neo DNA by calcium phosphate coprecipitation and were selected with 1 mg/ml G418. P815 A2.1 and P815 A2.1/gp100 cells were kindly provided by P. Coulie (Ludwig Institute, Brussels, Belgium).

**mAb and Flow Cytometry.** Phenotypic analysis of melanomas, transfectants, and normal melanocytes was performed by indirect immunofluorescence followed by flow cytometry using a FACScan<sup>®</sup> (Becton Dickinson & Co., Mountain View, CA). Purified anti-gp100 mAb NKI-beteb (1), and anti-HLA-A2 mAbs BB7.2 (culture supernatant) (19) and MA2.1 (ascites 1:500 dilution) (20) were used as primary reagents. FITC-conjugated GAM-IgG-F(ab')<sub>2</sub> (Zymed Laboratories, Inc., S. San Francisco, CA) was used for the second incubation. For the detection of the intracellular gp100 antigen cells were permeabilized in 0.01% digitonin and were subsequently fixed in 1% paraformaldehyde.

**Chromium Release Assay.** Chromium release assays were performed as described previously (7). Briefly,  $10^6$  target cells were incubated with 100  $\mu$ Ci Na<sup>51</sup>CrO<sub>4</sub> (Amersham International, Bucks, UK) for 1 h. Various amounts of effector cells were then added to  $2 \times 10^3$  target cells in triplicate wells of U-bottomed

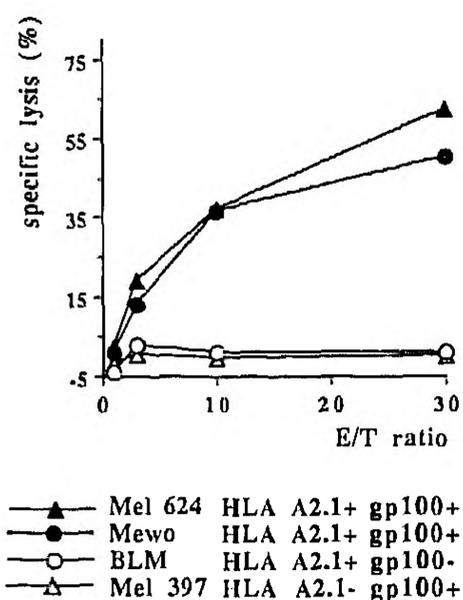
microtiter plates (Costar, Badhoevedorp, The Netherlands) in a final volume of 150  $\mu$ l. After 5 h of incubation, part of the supernatant was harvested and its radioactive content measured. Target cells were incubated for 48 h with 50 U/ml human (Boehringer, Ingelheim, Germany) or mouse recombinant IFN- $\gamma$  (TNO, Rijswijk, The Netherlands) before use in chromium release assays.

## Results and Discussion

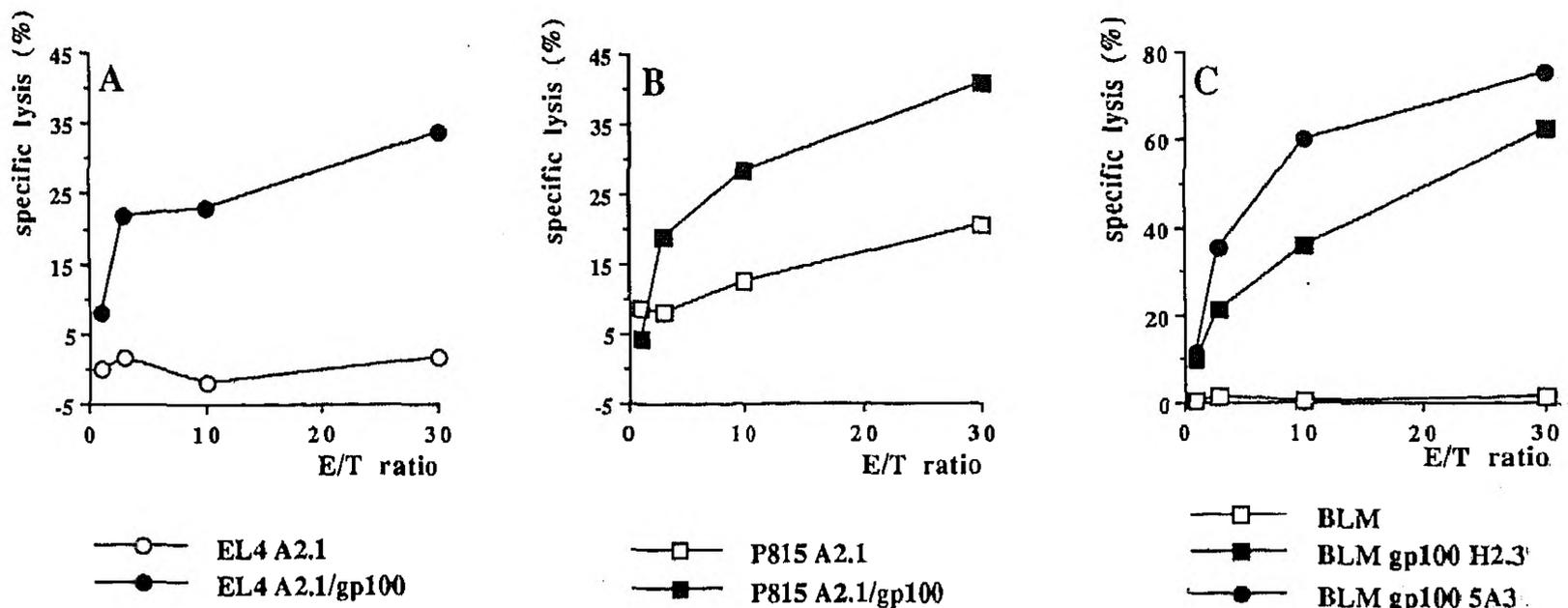
In search of gp100-specific CTLs we focused on HLA-A2.1 as a restriction element because of its widespread occurrence in caucasians and its presumptive dominant role in CTL reactivity against melanoma. A HLA-A2.1<sup>+</sup> TIL line, TIL 1200, was used for this study. This TIL line expresses TCR- $\alpha/\beta$ , CD3, and CD8 (data not shown).

**HLA-A2.1-restricted Killing of Melanoma Tumor Cells by TIL 1200 Corresponds to gp100 Expression.** Cytolytic activity of TIL 1200 was analyzed using a panel of human melanoma cell lines. As shown in Fig. 1, TIL 1200 efficiently lysed HLA-A2.1<sup>+</sup> Mel 624 and MeWo melanoma tumor cells, which both express gp100, whereas no reactivity towards HLA-A2.1<sup>-</sup> gp100<sup>+</sup> Mel 397 cells was seen. It is interesting to note that we observed that HLA-A2.1<sup>+</sup> BLM melanoma cells also are resistant to lysis by TIL 1200 (Fig. 1). Previously, we have shown that BLM cells lack the expression of the gp100 antigen, both at the protein and at the mRNA level (2). Furthermore, HLA-A2.1<sup>+</sup> EBV-transformed B cells (JY), which also lack gp100 expression, and K562 cells, were not lysed by TIL 1200 (data not shown). Together, these data demonstrate that TIL 1200 displays HLA-A2.1-restricted killing which correlates with gp100 expression.

**TIL 1200 Recognizes HLA-A2.1<sup>+</sup> gp100<sup>+</sup> Transfectants.** To explore whether TIL 1200 recognized a gp100-derived peptide in the context of HLA-A2.1, we cotransfected murine EL4 cells with a genomic fragment encoding HLA-A2.1 together with a plasmid conferring hygromycin resistance. Stable hygromycin-resistant transfectants were selected and analyzed for HLA-A2.1 surface expression by flow cytometry. EL4 cells expressing HLA-A2.1 (EL4 A2.1) were subsequently transfected with pBJ1-gp100neo, which encodes gp100 and confers resistance to G418. Stable transfectants were selected and were screened for gp100 expression using mAb



**Figure 1.** HLA-A2.1-restricted killing of gp100<sup>+</sup> melanoma tumor cells by TIL 1200. Lysis of chromium-labeled cells was measured after 5 h. All melanoma cell lines except BLM express the gp100 antigen. One representative experiment out of three.



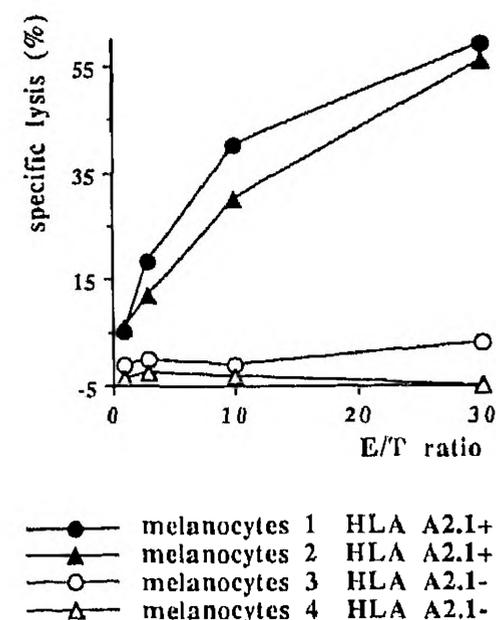
**Figure 2.** Lysis of HLA-A2.1<sup>+</sup> gp100<sup>+</sup> transfectants by TIL 1200. Murine EL4 and P815 cells were transfected with both gp100 and HLA-A2.1 DNA, and human BLM cells were transfected gp100 DNA as described in Materials and Methods. Isolated clones were tested for sensitivity to lysis by TIL 1200. (A and B) Representative examples of experiments performed with four individual EL4 A2.1/gp100 and P815 A2.1/gp100 clones. (C) One experiment out of three, using BLM gp100 clones H2.3 and 5A3.

NKI-beteb (EL4 A2.1/gp100). In collaboration with P. Coulie a similar panel of transfectants was generated in murine P815 cells (P815 A2.1 and P815 A2.1/gp100). Using these murine transfectants as target cells in chromium release assays, we clearly observed gp100 specific lysis by TIL 1200 (Fig. 2, A and B). The percent specific lysis (25–35%, E/T 30:1) of murine EL4 A2.1/gp100 and P815 A2.1/gp100 transfectants by TIL 1200 was somewhat lower compared with that observed with the HLA-A2.1<sup>+</sup> gp100<sup>+</sup> human melanoma cells (45–60%, E/T 30:1). This difference may be explained by nonmatched accessory molecules between human TILs and murine transfectants. To overcome this we introduced the gp100 antigen into human HLA-A2.1<sup>+</sup> gp100<sup>-</sup> BLM melanoma cells by transfection of pBJ1-gp100neo. Stable BLM gp100 clones were tested in chromium release assays using TIL 1200. As shown in Fig. 2 C, BLM gp100 clones H2.3 and 5A3 were as sensitive to lysis by TIL 1200 as Mel 624 and MeWo cells which express the gp100 antigen endogenously. The gp100 specificity of TIL 1200 was further demonstrated by the absence of lysis of G418-resistant BLM cells not expressing gp100, excluding the possibility that neomycin-derived peptides are recognized (data not shown). We conclude from these data that TIL 1200 recognizes a gp100-derived peptide presented in HLA-A2.1.

*HLA-A2.1<sup>+</sup> Normal Melanocytes Are Lysed by TIL 1200.* Since gp100 is expressed in all cells of the melanocytic lineage, one would predict that normal melanocytes are also recognized by TIL 1200. To investigate this, short-term cultures of HLA-A2.1<sup>+</sup> and HLA-A2.1<sup>-</sup> normal melanocytes were tested for lysis by TIL 1200. Melanocytes used in this study all expressed the gp100 antigen to a similar extent (data not shown). HLA-A2.1<sup>+</sup> melanocytes were efficiently lysed, whereas HLA-A2.1<sup>-</sup> melanocytes were resistant to lysis by TIL 1200 (Fig. 3). These results demonstrate that TIL 1200 is also reactive with gp100-derived peptides presented in HLA-A2.1 by normal melanocytes.

Lysis of HLA-A2.1<sup>+</sup> gp100<sup>+</sup> melanocytes rules out the possibility that TIL 1200 recognizes a peptide epitope of gp100 mutated in melanoma tumor cells. This is further demonstrated by the fact that TIL 1200 is reactive with several allogeneic HLA-A2.1 matched, gp100<sup>+</sup> melanoma tumor lines. We therefore conclude that TIL 1200 recognizes a non-mutated peptide derived from the melanocyte differentiation antigen gp100 expressed by melanoma tumor cells, as well as by normal melanocytes.

Furthermore, our data show that gp100 can be immunogenic, suggesting that melanocyte differentiation antigens may serve as tumor rejection antigens. This hypothesis is strengthened by: (a) the finding that antibodies directed against this type of antigens (e.g., gp75) are present in the serum of melanoma patients (21, 22); (b) the recent observation that several antimelanoma CTLs display cross-reactivity with normal melanocytes (8); and (c) the identification of the melanocyte lineage-specific tyrosinase protein as a target for antimelanoma CTLs (11). Immunogenicity of melanocyte differenti-



**Figure 3.** Lysis of HLA-A2.1<sup>+</sup> normal melanocytes by TIL 1200. Short-term cultures of foreskin-derived melanocytes were tested for sensitivity to lysis by TIL 1200. Expression of HLA-A2.1 was analyzed by flow cytometry using mAbs BB7.2 (19) and MA2.1 (20).

ation antigens may also explain the clinical observation that melanoma patients often display local depigmentation of their skin (23). The occurrence of local depigmentation in melanoma patients has been reported to correlate with prolonged survival (23) and may result from the destruction of melanocytes as a consequence of an immune response directed against melanocyte differentiation antigens (24). Other clinical data, indicating that vitiligo (local depigmentation of the skin) is associated with improved survival of melanoma patients (25), are in line with this hypothesis. TILs have been shown to mediate regression of metastatic melanoma in patients treated with TILs plus IL-2 (26). Of particular interest is the fact that therapy with TIL 1200 was associated with objective tumor regression in the autologous patient (Rosenberg, S. A.,

unpublished data), suggesting that CTLs directed against melanocyte differentiation antigens can cause tumor regression in vivo.

Since melanocyte differentiation antigens such as gp100 are widely expressed in melanoma tumor cells, they may constitute a useful target for specific immunotherapy against melanoma. Although no unwanted toxicity was observed in the autologous patient upon TIL 1200 treatment, adverse side effects upon gp100 immunization, such as the destruction of normal melanocytic cells and retinal pigment epithelium cells, remain a possibility and require further study in animal models. Furthermore, it will be important to determine how widely T cell-mediated gp100 reactivity is observed in melanoma patients.

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