# αvβ3-dependent cross-presentation of matrix metalloproteinase–2 by melanoma cells gives rise to a new tumor antigen

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**A large array of antigens that are recognized by tumor-specific T cells has been identified and shown to be generated through various processes. We describe a new mechanism underlying T cell recognition of melanoma cells, which involves the generation of a major histocompatibility complex class I–restricted epitope after tumor-mediated uptake and processing of an extracellular protein—a process referred to as cross-presentation—which is believed to be restricted to immune cells. We show that melanoma cells cross-present, in an αvβ3-dependent manner, an antigen derived from secreted matrix metalloproteinase–2 (MMP-2) to human leukocyte antigen A*0201-restricted T cells. Because MMP-2 activity is critical for melanoma progression, the MMP-2 peptide should be cross-presented by most progressing melanomas and represents a unique antigen for vaccine therapy of these tumors.**

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Abbreviations used: MMP; matrix metalloproteinase; MT1, membrane type 1; TIL, tumor-infiltrating lymphocyte; TPP, tripeptidyl peptidase.

Antigens recognized on tumor cells by CTLs consist of peptides presented by MHC class I molecules (1). These peptides originate in the tumor cell cytosol from the degradation of recently translated endogenous proteins, mainly by the proteasome. Most peptides are transported into the endoplasmic reticulum where they are loaded onto MHC class I molecules and then translocated to the cell surface as MHC–peptide complexes. It is assumed that those peptides also may be cross-presented in vivo by APCs (i.e., loaded onto MHC class I molecules from internalized tumor proteins), which could lead to the subsequent induction of tumor immunity (2, 3).

During the last 14 yr, a large array of human melanoma–associated antigens has been identified. Among these, several shared melanoma antigens have been targeted in immunization strategies. However, the therapeutic efficacy of this approach remains limited, despite significant induction of tumor-specific T cells (4, 5). One possible explanation is that targeted antigens are not suitable to induce efficient immune responses, which might be due, in part, to the generation of antigen-loss variants (6). A way to circumvent this limitation is to vaccinate against antigenic proteins whose expression is critical for tumor growth or invasiveness. In an attempt to identify such tumor cell proteins, we aimed at characterizing the antigens recognized by tumor-infiltrating lymphocytes (TILs) infused between 6 and 8 yr ago into patients who had melanoma who remain tumor-free (7, 8).

Here we show that the secreted matrix metalloproteinase–2 (MMP-2) is a novel shared melanoma antigen that is recognized by TILs in the HLA-A*0201 context. We also describe a new mechanism for the generation of this tumor epitope: cross-presentation, which is believed to be restricted to immune cells.

## RESULTS

### M134.12 T cell clone recognizes a shared melanoma antigen presented on HLA-A*0201

One $CD8^+$ TIL clone from the M134 patient (M134.12) killed the autologous M134 melanoma cell line and was able to secrete TNF, IFN-γ, and IL-2 when incubated with this cell line (Fig. 1 A and not depicted). To identify the restricting HLA allele, we used a panel of 29 allogenic melanoma cell lines that shared at least one HLA allele with the M134 cell line (HLA-A*0201, B*0801, Cw*0701). Half of HLA-A*$0201^+$ (12 out of 24), but none of HLA-A*$0201^-$, cell lines were recognized by M134.12 (Fig. 1 A and not de-

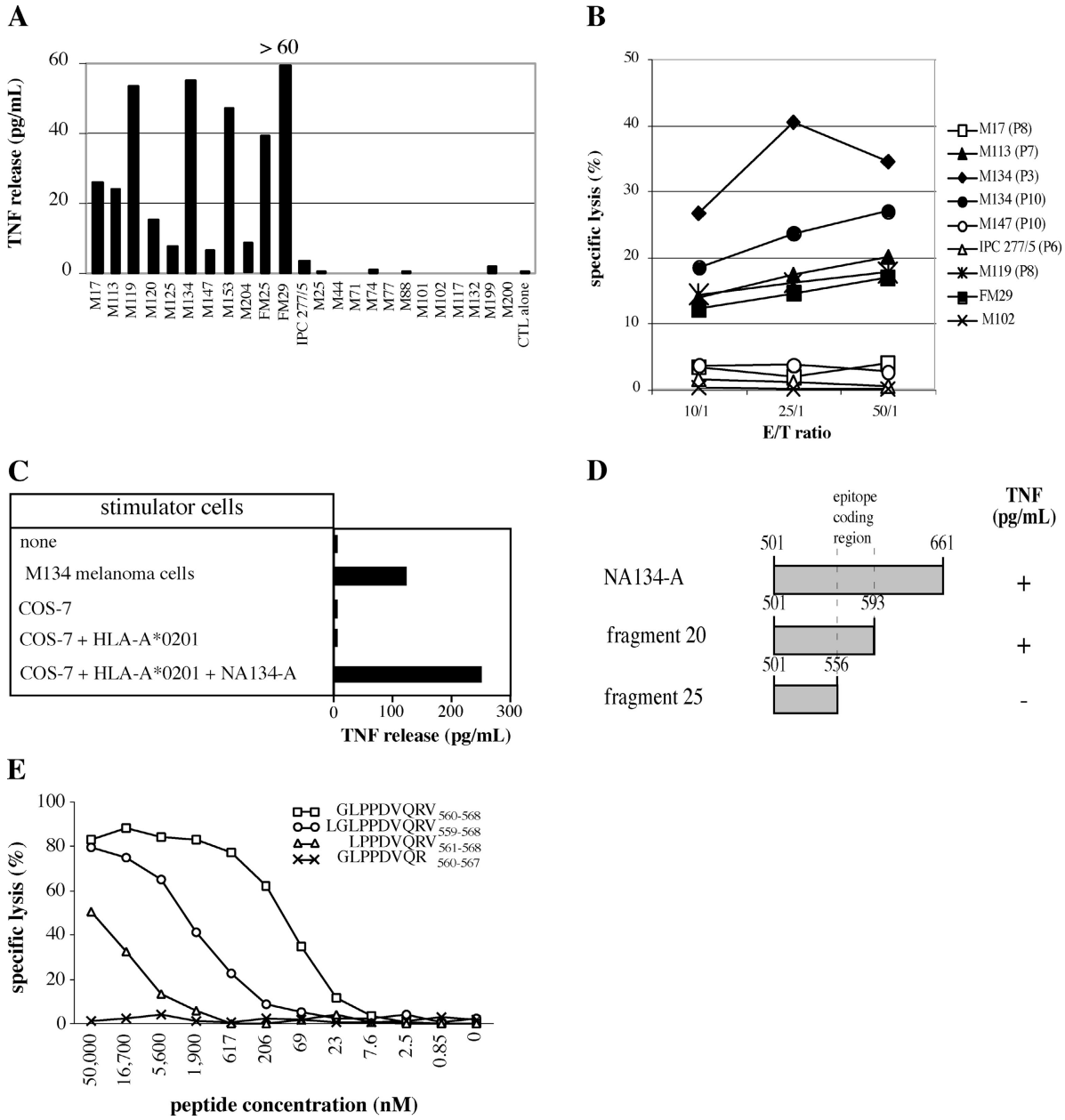


**Figure 1. The MMP-2 peptide$_{560-568}$ is the epitope recognized in the HLA-A*0201 context by the M134.12 CTL.** (A) TNF response of the M134.12 CTL clone to HLA-A*0201 melanoma cell lines. $10^4$ CTLs were added to $3.10^4$ melanoma cells, and the TNF content of the supernatant was tested after 6 h on WEHI-13 cells. (B) Lysis of freshly isolated HLA-A*0201 melanoma cells by the CTL clone (the number of passages after isolation is indicated in parentheses). M102 and FM29 melanoma cell lines were used as negative and positive controls, respectively. $^{51}$Cr-labeled tumor cells were cocultured with T cells at various effector/target ratios (E/T). Chromium release in the supernatants was measured after 4 h of incubation. (C, D) M134.12 CTL responses to COS-7 cells (E/T 1:3) transfected with indicated plasmids. CTLs were added 2 d after the transfection, and the TNF content of the supernatant was estimated 6 h later by testing the toxicity of WEHI-13 cells. (E) The MMP-2 epitope was determined by cytotoxicity to peptide-pulsed T2 cells. Target cells were chromium labeled and incubated for 30 min with the peptides at the indicated concentrations. The CTL clone was added at an E/T ratio of 10:1. Chromium release was measured after 4 h.

picted), which indicated that this CTL recognized a shared melanoma antigen presented by HLA-A*0201. Moreover, the CTL clone killed freshly isolated HLA-A*0201$^+$ melanoma cells (Fig. 1 B).

## MMP-2 is the antigen recognized by the CTL clone M134.12

Because M134.12 CTL did not recognize any of 54 previously identified tumor antigens, tested by expression in COS-7 cells (unpublished data), a cDNA library was made

from M134 melanoma mRNA. Plasmid DNA extracted from pools of bacterial colonies from this library was cotransfected with HLA-A*0201 into COS-7 cells. After 48 h, M134.12 T cells were added and TNF production was assayed. One plasmid pool proved positive in this test, and the individual plasmid coding for the recognized antigen was recovered from it after cloning (Fig. 1 C). It contained a 1.3-kb cDNA insert (hereafter referred to as NA134-A), which corresponded to the end of the MMP-2 gene transcript. To identify the MMP-2 epitope, HLA-A*0201 and truncated variants of the cDNA NA134-A were cotransfected into COS-7 cells. The stimulatory region mapped between amino acid 556 and 593 of the MMP-2 sequence (Fig. 1 D). By screening synthetic peptides spanning this region, the 9-mer peptide $MMP2_{560\text{-}568}$ (GLPPDVQRV) was found to be the epitope recognized by M134.12 T cells (Fig. 1 E).

### Presentation of the MMP-2 epitope is restricted to melanoma cell lines

MMP-2 is expressed frequently by normal and tumor cells (9, 10). Accordingly, MMP-2 expression was demonstrated first in a panel of 29 normal and malignant HLA-A*0201$^+$ cell lines through RT-PCR (Fig. 2), immunocytochemistry, and zymography analysis (Table I and not depicted). M134.12 T cells recognized 10 out of 15 HLA-A*0201$^+$/MMP-2$^+$ melanoma cell lines (Table I and Fig. 1 A). In contrast, 20 HLA-A*0201$^+$/MMP-2$^+$ nonmelanoma cell lines—tumoral and normal—were not recognized by this CTL (Table I). Therefore, the presentation of the 9-mer MMP-2 peptide on HLA-A*0201 seems to be a unique property of melanoma cell lines.

### The MMP-2 epitope is not presented by the endogenous pathway

Some HLA-A*0201$^+$ melanoma cells (M88, M101, and M117) and many nonmelanoma cells synthesized MMP-2 but failed to be recognized spontaneously by the M134.12 CTL clone (Table I), whereas they were recognized after peptide pulse (not depicted). Therefore, we wondered whether this could be due to a low level of MMP-2 expression in these cell lines. To address this question, we measured MMP-2 expression using semi-quantitative RT-PCR and protein release using an ELISA. Although several cell lines (M17, M113, M147, FM25, and FM29) that present the epitope showed a weak MMP-2 expression, some cell lines that failed to present this epitope had a relatively high MMP-2 transcription level (M101, M71, F257, and HAEC#8186; Fig. 2 and Table I). There is no correlation between mRNA transcription and peptide presentation. Therefore, we compared MMP-2 protein secretion with peptide presentation. Data show that at least two melanoma cell lines (M88 and M101) did not present the MMP-2 epitope although they secrete high amounts of the MMP-2 protein. Another cell line, M117, not recognized by the CTL clone was shown to express the MMP-2 protein by immunocytochemistry. Two other cell lines not recognized (M71 and M74) expressed MMP-2 mRNA but there is no evidence of protein synthesis because zymography and ELISA were negative (Table I). Therefore, at least three melanoma cell lines and many nonmelanoma cell lines (normal or tumoral) clearly synthesized the MMP-2 protein but failed to present the epitope (Table I). Therefore, we checked whether this could be due to the presence of mutations affecting the epitope coding region of MMP-2. To this end, we sequenced an 800-bp long cDNA epitope-coding region in three of these cells (M88, M117, and OVCAR). No mutation was found in these cDNA (unpublished data), which suggested that these cells express the wild-type MMP-2, and that the MMP-2 epitope could not be processed from it by the classic endogenous road. In support of this hypothesis, we observed that transfection of the full-length MMP-2 in HLA-A*0201$^+$ COS-7 cells or in tumor cell lines not recognized by the CTL clone failed to sensitize

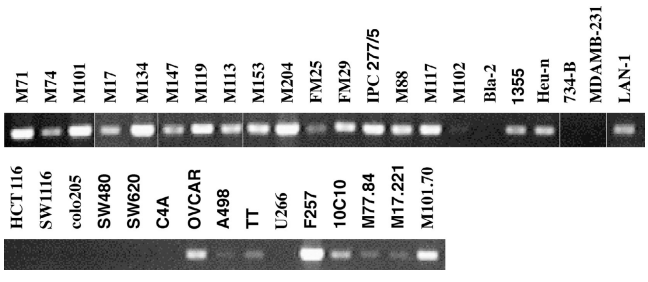


**Figure 2. Expression of the MMP-2 gene transcript.** mRNAs were extracted from tumor or normal cell lines before retro-transcription and MMP-2–specific amplification was performed for 27 cycles as described in Materials and methods.

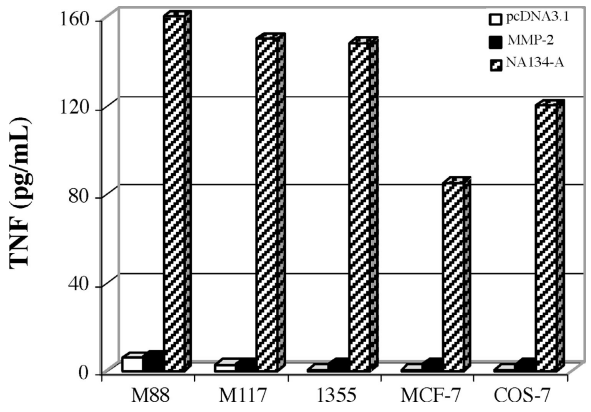


**Figure 3. The MMP-2 epitope is not presented from full-length MMP-2.** The M134.12 CTL was cocultured with HLA-A*0201$^+$ COS-7 cells or tumor cell lines transfected with plasmids coding for the full-length MMP-2 or the NA134-A fragment of MMP-2. $10^4$ CTLs were added to $3.10^4$ target cells, and the TNF content of the supernatant was tested after 6 h on WEHI-13 cells.

**Table I.** MMP-2 expression and CTL recognition of tumor and normal cell lines

| | Target cells | | M134.12 recognition (TNF production) | MMP-2 expression | | | |
|---|---|---|---|---|---|---|---|
| | | | | RT-PCR | Immunocyto-chemistry | MMP-2 release (zymography) | ELISA (supernatants) |
| | | | | % | | | *ng/mL* |
| **Tumor cells** | melanoma | M17 | + | 1 | + | +/− | UND |
| | | M113 | + | 10 | + | + | 1 |
| | | M119 | + | 56 | ND | + | 2 |
| | | M134 | + | 100 (ref.) | + | + | 50 |
| | | M147 | + | 6,5 | + | + | 13 |
| | | M153 | + | 16 | + | + | 2 |
| | | M204 | + | >100 | + | + | 29 |
| | | FM25 | + | 3 | + | +/− | UND |
| | | FM29 | + | 13 | + | + | 1 |
| | | IPC 277/5 | + | 37 | + | + | 31 |
| | | M71 | − | 63 | ND | − | UND |
| | | M74 | − | 16 | ND | − | UND |
| | | M88 | − | 13 | + | + | 14 |
| | | M101 | − | 85 | ND | + | 64 |
| | | M117 | − | 29 | + | − | UND |
| | | M102 | − | 0 | − | − | UND |
| | lung | 1355 | − | 7 | + | +/− | 5 |
| | | Bla-2 | − | 0 | ND | ND | UND |
| | | Heu-n | − | 6 | ND | +/− | UND |
| | | 734-B | − | 0 | ND | − | UND |
| | | MDAMB-231 | − | 0 | ND | − | UND |
| | neuroblastoma | LAN-1 | − | 6 | − | + | UND |
| | | colo205 | − | 0 | ND | − | UND |
| | | C4A | − | 0 | ND | − | UND |
| | | HCT116 | − | 2 | ND | +/− | UND |
| | | Sw480 | − | 10 | + | − | UND |
| | | Sw620 | − | 0 | ND | − | UND |
| | | Sw707 | − | ND | ND | − | UND |
| | | Sw1116 | − | 0 | ND | − | UND |
| | renal cell carcinoma | A498 | − | 3 | + | − | UND |
| | | R28 | − | + | + | ND | ND |
| | ovary | OVCAR | − | 9 | + | +/− | 1 |
| | thyroid | TT | − | 3 | + | − | UND |
| | myeloma | U266 | − | 0 | − | − | UND |
| **Normal cells** | melanocyte | 98M09 (P6) | − | ND | + | + | ND |
| | | 01M08 (P4) | − | ND | + | + | ND |
| | | MHN 3 (P1) | − | ND | ND | − | UND |
| | | MHN 5 (P2) | − | ND | ND | − | UND |
| | keratinocyte | K1 | − | + | + | ND | ND |
| | fibroblast | MG | − | + | ND | ND | ND |
| | | HFFF2 | − | + | ND | ND | ND |
| | | F257 | − | >100 | ND | + | 89 |
| | endothelium | HAEC#8186 | − | >100 | ND | ND | ND |
| | CD8 T cell clones | 10C10 | − | 5 | ND | − | UND |
| | | M77.84 | − | 3 | ND | − | UND |
| | | M17.221 | − | 3 | ND | − | UND |
| | CD4 T cell clone | M101.70 | − | 20 | ND | − | UND |
| | EBV-B | ADAM | − | 0 | ND | − | UND |
| | | B17 | − | 0 | ND | − | UND |
| | | BM16 | − | 0 | ND | − | UND |
| | | Boleth | − | 0 | ND | − | UND |
| | | Dabin | − | 0 | ND | − | UND |

**Table I.** MMP-2 expression and CTL recognition of tumor and normal cell lines (continued)

| | M134.12 recognition (TNF production) | MMP-2 expression | | | |
|---|---|---|---|---|---|
| | | RT-PCR | Immunocyto-chemistry | MMP-2 release (zymography) | ELISA (supernatants) |
| Marie-Hélène | – | 0 | ND | – | UND |
| Marie-Luce | – | 0 | ND | – | UND |
| Grégoire | – | 0 | ND | – | UND |
| Le Pendu | – | 0 | ND | – | UND |

Tumor and normal human HLA-A*0201$^+$ cell lines were tested for MMP-2 expression by various approaches as indicated. PCR band intensities were measured individually from the original TIFF image (Fig. 2) with NIH image-J software after normalization with β-actin. Results are expressed as a percentage of the level of expression in M134 melanoma cell line. For R28, K1, MG, and HFFF2, MMP-2 products were detected, but not quantitated (+). ELISA and zymography assays from culture supernatants were used for evaluation of MMP-2 release. The TNF assay was performed by adding 10,000 lymphocytes to 30,000 target cells and testing the supernatants 6 h later on WEHI-13 cells. The number of passages is indicated in brackets for melanocytes.
UND, undetectable.

these cells to clone recognition. In contrast, the NA134-A fragment of MMP-2, transfected in the same conditions, did sensitize these cells to CTL clone recognition (Fig. 3). These data show that the absence of MMP-2 peptide presentation from endogenous or transfected full-length wild-type MMP-2 by tumor cells does not result from a defect of the antigen presentation capacity of transfected or spontaneously expressing cells, but results rather from a general inability of the classic endogenous processing pathway to generate the MMP-2 peptide from the full-length protein.

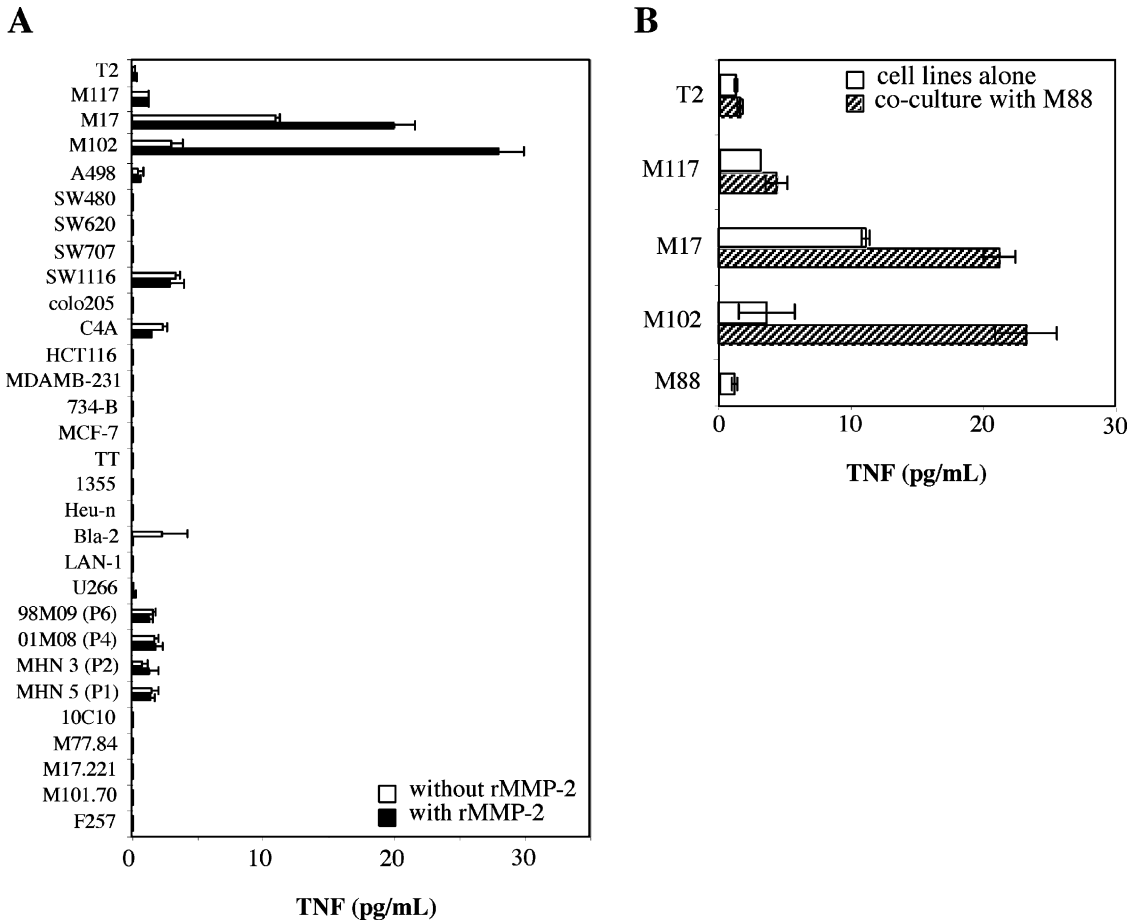


**Figure 4. Uptake of soluble MMP-2 leads to peptide presentation by melanoma cells.** Tumor or normal cell lines and T2 cells were cultured with (A) rMMP-2 (100 ng/ml) or (B) an MMP-2–secreting cell line (M88) not recognized by the CTL M134.12 (ratio 1:1). The CTL was added the next day and corresponding supernatants were tested for TNF content 6 h later on WEHI-13 cells. Histologic origins of the cell lines are indicated in Table I. The number of passages is indicated in parentheses for melanocytes. Standard deviations were obtained from duplicates.

### The MMP-2 epitope presented by melanoma cells comes from exogenously acquired MMP-2 protein

Because MMP-2 normally is secreted as a proenzyme, we addressed a possible requirement for MMP-2 secretion for its presentation by melanoma cell lines. The data obtained from zymography assay show that 10 melanoma cell lines recognized by the M134.12 CTL had a gelatinolytic activity in their supernatant, showing that they secrete proMMP-2, whereas 4 of the 6 melanoma cell lines which were not (M71, M71 and M117) or poorly recognized (M17), lacked or poorly displayed any such activity (Table I). To assess directly whether secreted MMP-2 could be uptaken, processed, and presented to T lymphocytes, we incubated several HLA-A*0201$^+$ tumor (melanoma and nonmelanoma) and normal cell lines (freshly isolated melanocytes, fibroblasts, and T cell clones), which were not or poorly recognized by the CTL clone, with exogenous MMP-2. These cell lines were cultured with 100 ng/ml rMMP-2 (Fig. 4 A) or with the M88 melanoma line that secretes MMP-2 but does not stimulate the M134.12 CTL (Fig. 4 B). This allowed M17 and M102, but not M117 and nonmelanoma cell lines, to be sensitized to M134.12 CTL recognition. Moreover, 10 EBV-B cell lines, incubated with rMMP-2, did not stimulate the CTL clone (unpublished data). T2 cells cultured in the same conditions failed to be sensitized to clone recognition; this showed that melanoma cell sensitization did not result from passive binding to HLA-A*0201 of contaminating peptide fragments in the recombinant protein preparation (Fig. 4, A and B). Therefore, some melanoma cell lines are able to process the MMP-2 peptide from an exogenous supply of the protein and so can perform cross-presentation. Moreover, this cross-presentation of secreted MMP-2 seems to be very efficient because it was observed using the supernatant of a melanoma cell line (M88) that contained 14 ng/ml of the protein (Fig. 4 B).

### MMP-2 epitope presentation is proteasome- and clathrin-coated vesicle–dependent

To understand how naturally secreted and exogenous MMP-2 could be processed and associated to HLA-class I in melanoma cells, we studied the effect of various inhibitors of peptide presentation pathways on MMP-2 peptide presentation by two spontaneously presenting cell line (M134 and FM29) and by the M102 cell line induced to present the epitope from an exogenous supply.

Monodansylcadaverine, an inhibitor of clathrin-mediated endocytosis (11), significantly blocked natural presentation of the MMP-2 peptide by melanoma cell lines, M134 and FM29, as well as presentation of added rMMP-2 by the MMP-2$^-$ M102 cell line; however it had no effect on the presentation of added synthetic 9-mer MMP-2 peptide (except at the 100 μM concentration, which seemed to be toxic for the M134 cell line; Fig. 5 A). In contrast, amiloride, an inhibitor of macropinocytosis; cytochalasin B and D, inhibitors of actin polymerization; ammonium chloride, which prevents lysosome and endosome acidification; pepstatin A, which inhibits the cathepsin D enzyme; and leupeptin, an inhibitor of serine and cysteine proteases, did not alter the MMP-2 peptide presentation by these melanoma cells (unpublished data). A specific inhibitor of proteasomal activities, lactacystin (which inhibits tryptic, chymotryptic, peptidylglutamyl peptide hydrolase, and branched chain amino acid–preferring protease activities) strongly inhibited MMP-2 epitope presentation (Fig. 5 B). Finally, a role of cytosolic peptidases was addressed: E64, an inhibitor of cysteine proteases (12); alanine-alanine-phenylalanine-chloromethylke-

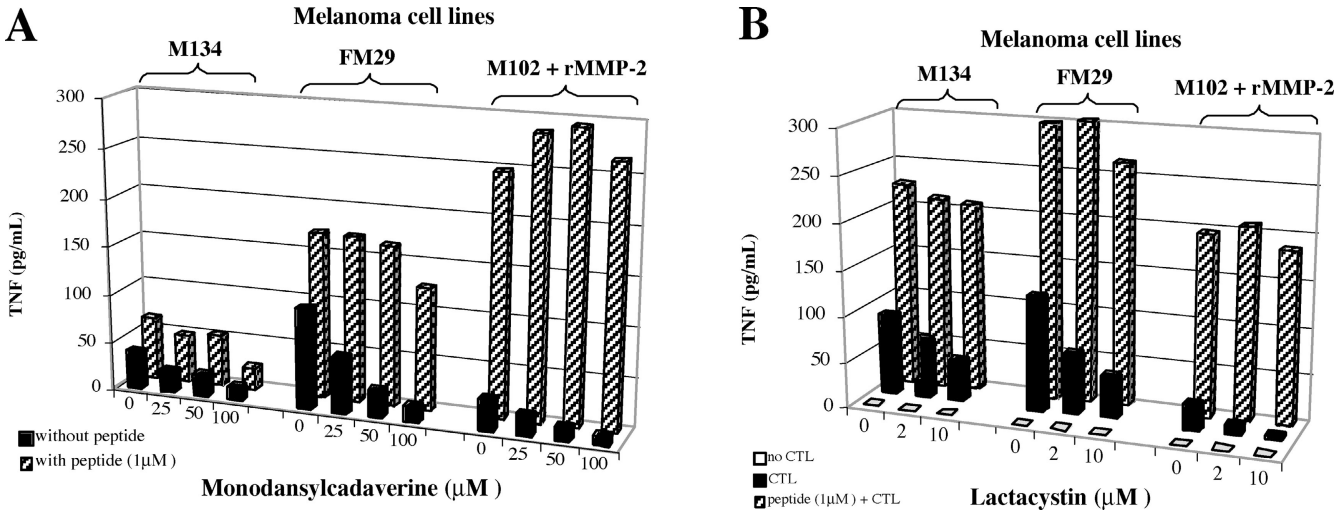


**Figure 5. MMP-2 is processed by a clathrin-coated pits- and proteasome-dependent pathway.** Melanoma cell lines (M134, FM29, and M102) were treated overnight with (A) monodansylcadaverine or (B) lactacystin. The M102 cell line also was cultured overnight in presence of rMMP-2 (100 ng/ml) before the CTL stimulation assay. Cells were washed and cocultured with the M134.12 CTL (E/T 1:3) before TNF estimation 6 h later on WEHI-13 cells.

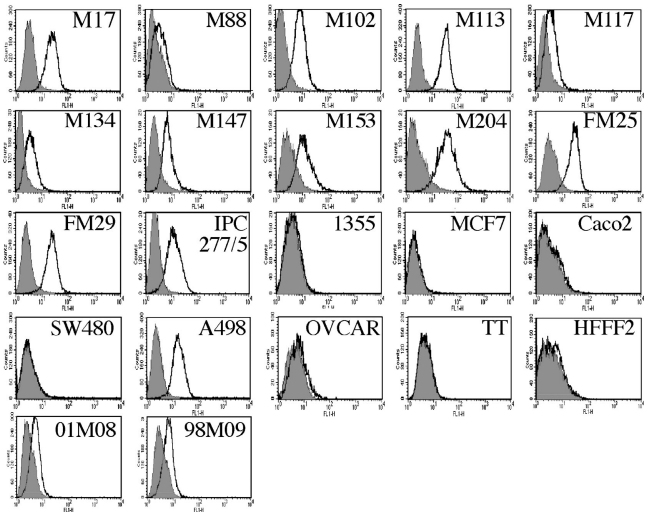


**Figure 6. αvβ3 expression.** Cell lines were stained for 30 min at 4°C with an anti-αvβ3-FITC mAb (23C6, BD Biosciences). Negative controls were performed by incubating the cells with isotype-matched FITC control antibody. Labeling was analyzed on a FACScan flow cytometer using Cellquest software (Becton Dickinson).

ton, an inhibitor of tripeptidyl peptidase (TPP) I; TPP II; bleomycin hydrolase and puromycin-sensitive aminopeptidase enzymes (13, 14); and JA-2, an inhibitor of the Thymet oligopeptidase (15), had no effect on the presentation of the MMP-2 epitope by melanoma cell lines (unpublished data). Therefore, endocytosis of exogenous MMP-2 through clathrin-coated pits and proteasome activity are required for MMP-2 peptide presentation.

### MMP-2 epitope presentation depends on αvβ3 expression by melanoma cells

For two melanoma cell lines, M88 and M117, as well as for several nonmelanoma cell lines, spontaneous secretion or addition of rMMP-2 did not allow their recognition by M134.12 T cells (Fig. 4, A and B); this suggested the existence of additional factors which underlie MMP-2 epitope cross-presentation. Therefore, we asked whether MMP-2 uptake was mediated by a receptor. Because secreted MMP-2 is known to interact with various membrane-bound molecules, such as CD91 (16), membrane type-1 (MT1)-MMP (17), and αvβ3 (18), on tumor cell lines, the role of these molecules as receptors or cofactors for MMP-2 peptide presentation was addressed, first, by analyzing the expression of these molecules in a panel of HLA-A*0201$^+$/MMP-2$^+$ cell lines recognized by the CTL clone. Only the expression of αvβ3 correlated with T cell clone recognition (Table II and Fig. 6). This suggested that the expression of αvβ3, but not that of CD91 or MT1-MMP, is required for MMP-2 epitope presentation. Therefore, we analyzed the effect of blocking the αvβ3 receptor using a mAb. An anti-CD91

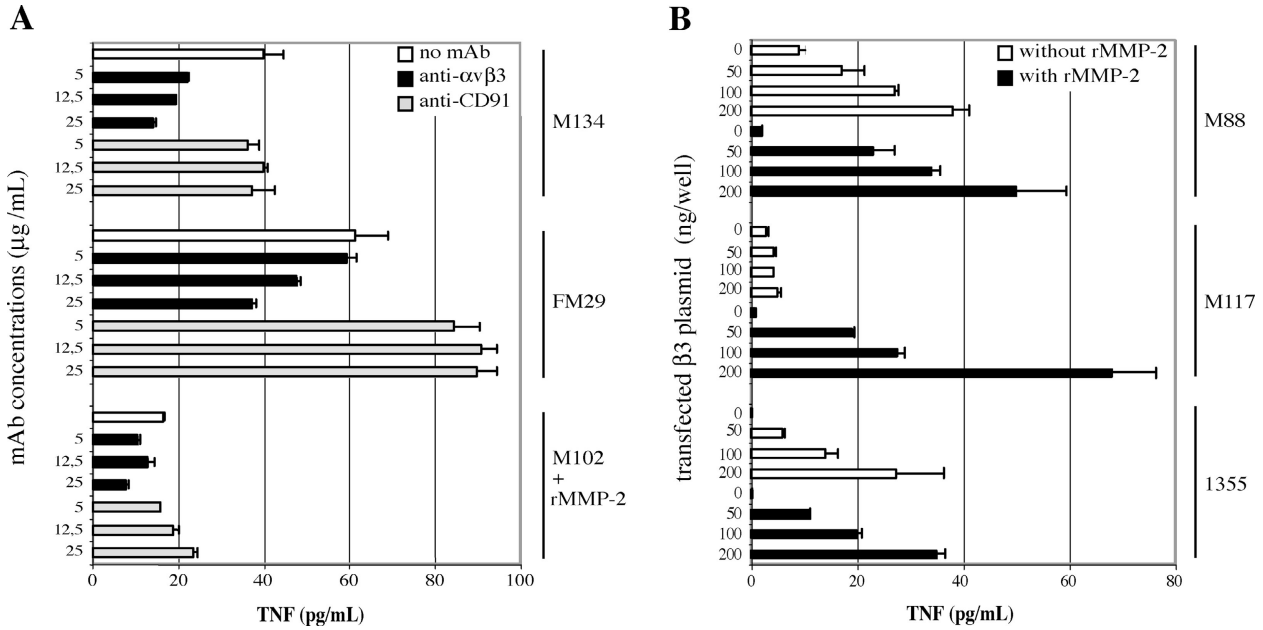


**Figure 7. MMP-2 peptide presentation depends on αvβ3 expression.** (A) Melanoma cell lines were incubated with anti-αvβ3 or anti-CD91 mAb (at indicated concentrations in μg/ml) for 1 h at room temperature. The CTL M134.12 was added (E/T ratio 1:3) before estimation of the TNF content of the supernatants 6 h later on WEHI-13 cells. The graph is representative of four different experiments and standard deviations were obtained from duplicates. (B) A plasmid coding for the β3 chain transfected in αvβ3$^-$/MMP-2$^+$ tumor cell lines with a lipofectamine reagent kit. 24 h later, these cell lines were or were not cultured overnight with rMMP-2 (100 ng/ml) before CTL stimulation assay. The TNF content of the supernatants was tested after 6 h on WEHI-13 cells. Standard deviations were obtained from duplicates.

**Table II.** MMP-2 interacting molecules expression by target cells

| | | | Expression of | | | |
|---|---|---|---|---|---|---|
| | | | | | αvβ3 | |
| | Target cells | M134.12 recognition (TNF production) | CD91 (Ab staining) | MT1-MMP (RT-PCR) | β3 chain (RT-PCR) | Ab staining (RFI) |
| | | | | | % | |
| **Melanoma cells** | M17 | + | ND | +/− | 84 | 6.6 |
| | M113 | + | ND | + | 182 | 4.6 |
| | M119 | + | ND | ND | 34 | 3.7 |
| | M134 | + | +/− | + | 100 (ref.) | 3.5 |
| | M147 | + | +/− | + | 57 | 2.5 |
| | M153 | + | +/− | +/− | 113 | 4.7 |
| | M204 | + | − | + | 67 | 7.6 |
| | FM25 | + | ND | + | 126 | 1.8 |
| | FM29 | + | + | + | 37 | 6.3 |
| | IPC 277/5 | + | ND | +/− | 143 | 3.6 |
| | M71 | − | ND | ND | 54 | 3.3 |
| | M74 | − | ND | ND | 14 | 3.5 |
| | M88 | − | +/− | + | 6 | 1.1 |
| | M101 | − | ND | ND | 7 | 1.3 |
| | M117 | − | +/− | + | 249 | 1.3 |
| | M102 | − | − | + | 96 | 3.2 |
| **Nonmelanoma tumor cells** | 1355 | − | +/− | − | 4 | 1 |
| | Bla-2 | − | ND | ND | 4 | 2 |
| | Heu-n | − | ND | ND | 0 | 1 |
| | LAN-1 | − | ND | ND | 0 | 1 |
| | HCT116 | − | ND | ND | 0 | 1 |
| | Sw480 | − | − | − | 0 | 1 |
| | Sw707 | − | ND | ND | 0 | 1 |
| | A498 | − | + | + | 58 | 5.5 |
| | R28 | − | ND | + | ND | ND |
| | OVCAR | − | ND | − | 5 | 1 |
| | TT | − | ND | − | 0 | 1 |
| **Normal cells** | 98M09 (P6) | − | ND | ND | ND | 1.3 |
| | 01M08 (P4) | − | ND | ND | ND | 1.2 |
| | MHN 3 (P1) | − | ND | ND | ND | 1.1 |
| | MHN 5 (P2) | − | ND | ND | ND | 1 |
| | K1 | − | ND | ND | ND | ND |
| | MG | − | ND | ND | ND | ND |
| | HFFF2 | − | ND | ND | ND | 1 |
| | F257 | − | ND | ND | 5 | 1.2 |
| | HAEC#8186 | − | ND | ND | ND | ND |
| | 10C10 | − | ND | ND | 0 | 1 |
| | M77.84 | − | ND | ND | 0 | 1 |
| | M17.221 | − | ND | ND | 0 | 1 |
| | M101.70 | − | ND | ND | 0 | 1 |

Tumor and normal human HLA-A*0201$^+$/MMP-2$^+$cell lines and the M102 melanoma cell line (HLA-A*0201$^+$/MMP-2$^-$) were tested for MMP-2 interacting molecule expression by RT-PCR (MT1-MMP and β3) or by antibody staining (anti-αvβ3 [23C6] and anti-CD91 [A2MR-α2]). For β3, PCR band intensities were measured individually from the original TIFF image with NIH image-J software after normalization with β-actin. Results are expressed as a percentage of the level of expression in M134 melanoma cell line. To evaluate the level of αvβ3 expression, we determined RFI values as described in Material and methods. See Table I for the cell nature and origin.

was used as a control. The anti-αvβ3, but not the anti-CD91, antibody inhibited MMP-2 peptide recognition of αvβ3$^+$ melanoma cell lines, M134 and FM29, and of the M102 cell line cultured with rMMP-2 (Fig. 7 A). Conversely, transfection of a plasmid coding for the β3 chain in melanoma cell lines, M88 and M117, and also in the non-small cell lung carcinoma 1355 cell line, sensitized these cells to M134.12 CTL recognition, directly for M88 and 1355 or in the presence of rMMP-2 for M117 that did not secrete this protein (Fig. 7 B). Therefore, the presentation of MMP-2 epitope on HLA-A*0201$^+$ tumor cells seems to depend on the expression of αvβ3 by these cell lines.

To document better the expression of this molecule, we performed a systematic measurement of β3 and αvβ3 expression in cell lines expressing the MMP-2 transcript, by semi-quantitative RT-PCR of the β3 chain and immunofluorescence of the entire integrin, respectively (Table II). Data show that high amounts of β3 transcript were found in melanoma cells but also in one renal cell carcinoma line, A498 (Table II). No correlation was observed between β3 mRNA levels and cell surface expression of αvβ3 (Table II). This could be due to posttranscriptional regulation of the β3 chain expression or to the formation of other heterodimers with one of these two chains, such as αvβ5 or αIIbβ3 (19). Most cell lines expressing the MMP-2 epitope (with the exception of FM25) seem to express the αvβ3 integrin at higher levels than cell lines that do not present it (Table II). Therefore, the restricted expression of MMP-2 epitope by melanoma cells should be related, in part, to the unique capacity of these cells to express significant levels of αvβ3 on their surface.

## DISCUSSION

Here we showed that an HLA-A*0201–restricted peptide from the MMP-2 may be cross-presented by αvβ3$^+$-expressing tumor cells following clathrin-coated pits–mediated uptake of extracellular MMP-2 and proteasome activity. The endogenous classic cytosolic pathway did not present this peptide. The HLA-A*0201–cross-presented MMP-2 peptide was identified as the epitope recognized by a CTL clone present among the TILs of a patient who had melanoma and received passive transfer of autologous tumor-reactive TILs 8 yr ago and who remains tumor-free (7, 8). Whether this clone had taken part in a protective antitumor response is unknown, but this lytic activity should occur in vivo because the CTL clone killed freshly isolated melanoma cells from this patient. Using HLA-A*0201/MMP-2$_{560\text{-}568}$ tetramers, we recently found MMP-2–specific lymphocytes among the TILs from 5 out of 32 patients who were treated in the same clinical assay (Godefroy et al., unpublished data).

Our results demonstrate for the first time that cross-presentation is used by melanoma cells for the generation of class I–restricted antigens, a mechanism that is believed to be essentially restricted to immune cells. Moreover, cross-presentation of secreted MMP-2 seems to be very efficient because it was observed using the supernatant of a melanoma cell line (M88) that contained 14 ng/ml of the protein, whereas OVA peptide cross-presentation through FcR-dependent internalization required OVA concentrations that ranged from 0.1 to 1 μg/ml (20).

MMP-2, also known as gelatinase-A or type IV collagenase, is a proteolytic enzyme that is secreted by normal and tumor cells; essentially, it degrades components of the extracellular matrix, basal membranes, and specific cell modulators (9). It is secreted as a latent proenzyme comprising four domains: a peptide secretion domain, a prodomain, a catalytic domain, and a COOH-terminal hemopexin-like domain. MMP-2 becomes active extracellularly by cleavage of its prodomain after interactions with membrane-bound MT1-MMP and soluble tissue inhibitors of metalloproteinase-2; reference 17). Secreted MMP-2 also interacts with other membrane proteins, such as the CD91/thrombospondin-2 complex (16), and with integrins, including αvβ3 (18, 21, 22); this restricts the area of MMP-2 activity and regulates extracellular MMP-2 levels through receptor-mediated uptake.

The epitope identified here is localized in the hemopexin-like domain of MMP-2 that is shown to interact with αvβ3 (22). Previous studies of the same group and others showed that αvβ3 is a receptor of melanoma cells for active MMP-2 (18), whose expression level in these tumors correlates with progression and metastasis (10, 23, 24). We provide experimental evidence that this integrin is involved in MMP-2 peptide cross-presentation, possibly by mediating MMP-2 uptake.

We showed also that MMP-2 peptide presentation depends on clathrin-coated vesicles and proteasome. This raised the question of how MMP-2 could reach the proteasome following endocytosis. Available knowledge on antigen processing suggests that this should involve a retrotranslocation from the endosome to the cytosol, possibly by the ER membrane Sec61 pore complex or the Derlin complex (25, 26). This implies that internalized MMP-2 gains access to the ER by transiently available continuities with the lumen of the endosome (in this case, cross-presentation could occur in the ER proper; 27–29), or that the MMP-2–containing endosome is self-sufficient for mediating cross-presentation, incorporating Sec61, associated proteasomes, and peptide-loading complexes (27, 30).

Tumor cells that expressed the MMP-2 protein but failed to secrete it (M117, Sw480, A498, OVCAR, TT), or those that secreted MMP-2 but lacked the αvβ3 integrin (M71, M74, M88, 1355), did not present the MMP-2 epitope. Nevertheless, some of these tumor cells could be sensitized by the addition of exogenous MMP-2 or by transfection of a plasmid coding for the β3 chain. Therefore, in contrast with other HLA class I–restricted tumor epitopes, the MMP-2 peptide cannot be processed from the native intracellular MMP-2 through the classic endogenous pathway. One likely explanation is that native MMP-2 escapes from protease degradation or is cleaved differently from NA134-A because of

its sequence, conformation, or localization. proMMP-2 contains a secretion peptide and a prodomain that are absent in the NA134-A peptide. Disulphide bonds and α-helical stretches, located in the prodomain (31), could be responsible for the absence of MMP-2 epitope generation by the endogenous pathway (32, 33). Alternatively, the secretion peptide could segregate native MMP-2 from the cytosol, and prevent its access to the proteasome (34). Whatever mechanism underlies defective presentation of the MMP-2 epitope by the endogenous road, MMP-2 needs to be secreted, internalized, and processed for peptide presentation.

Cutaneous melanoma is a highly invasive and metastatic tumor. Degradation and remodeling of the extracellular matrix and basement membranes by MMP-2 are essential steps in these processes that depend essentially on the coordinated production of active MMP-2 by tumor or stromal cells, and expression of αvβ3 by melanoma cells (24, 35–37). Therefore, most progressive HLA-A*0201 melanoma cells should cross-present the MMP-2 peptide both from the MMP-2 they secrete, as melanoma cell lines do in vitro, and from the one secreted by the surrounding stromal cells. This should allow higher in vivo MMP-2 epitope presentation by these melanoma cells than in vitro by tumor cell lines. Although many normal and tumor tissues express MMP-2, we observed that melanoma cell lines only did present the MMP-2 epitope. This might be due to a restricted coexpression of MMP-2 and αvβ3 by these cells. Therefore, MMP-2 seems to be a melanoma-specific antigen. Hence, this antigen could be a relevant target for immunotherapy of HLA-A*0201 melanoma patients. Nonetheless, an appropriate animal model will be necessary to confirm the restricted expression of the MMP-2 peptide to melanoma cells and to assess the potential autoimmune risk associated with MMP-2 vaccination.

Because MMP-2 plays a key role in tumor angiogenesis, growth, and metastasis (22, 38–40), synthetic and natural MMP inhibitors have been used for anti-tumor therapies. Despite disappointing clinical results it is hoped that MMP inhibitors might be efficient anti-invasive agents at early disease stages or as adjuvant treatments (10, 41). Recent studies supported an anti-invasive effect of the PEX peptide (42). Because the MMP-$2_{560-568}$ epitope is contained within the PEX αvβ3 interacting sequence of MMP-2, vaccines containing such a sequence, or a recombinant DNA which encodes it, could, induce an antimelanoma invasive effect and T cell–mediated responses against the MMP-2 epitope by competing with MMP-2 binding. Such vaccines also should have the unique property of simultaneously inducing, or increasing, epitope presentation on the tumor target cells and stimulating T cell responses.

Finally, because MMP-2 also was reported to play a role in tumor-mediated immunosuppression through the proteolytic cleavage of the IL-2Rα that is expressed by tumor reactive T cells (43), MMP-2 vaccines that are able to inhibit MMP-2 activity also should potentiate the vaccine effect by avoiding growth factor starvation of activated T cells.

In conclusion, the present data suggest that MMP-2 is a unique tumor antigen for vaccine therapy of invasive melanoma tumors because it opens the way for innovative strategies that combine vaccine and anti-invasive therapeutic approaches for the treatment of patients who have HLA-A*0201 melanoma. More generally, the demonstration that melanoma cells can perform cross-presentation—a process which is believed to be restricted to immune cells—raises the possibility for redirecting preexisting T cells responses that are specific for common environmental antigens against melanoma cells, through immunization against proteins fused to αvβ3 noncatalytic binding fragments of MMP-2.

## MATERIALS AND METHODS

**Cell lines and TIL cultures.** The CTL clone, M134.12, was isolated from a tumor-invaded lymph node of patient M134 who had melanoma. This patient had been included in a clinical trial consisting of TILs plus IL-2 infusions (7). This protocol was approved by the Institutional Ethics Committee and registered with regulatory state authority. Melanoma (M), colorectal cancer (C) and renal cell carcinoma cell lines (R) were established from metastatic tumors or tumor invaded–lymph node fragments that were obtained after patient's consent. The various cell lines that were used in this study have been published (44–46). Melanoma cell lines, FM25 and FM29, were gifts from J. Zeuthen (Danish Cancer Society Research Center, Copenhagen, Denmark). Ovary, neuroblastoma, renal carcinoma, and thyroid cell lines (OVCAR, LAN-1, A498, and TT, respectively) were gifts from C. Saï (INSERM U601, Nantes, France). Colorectal carcinoma (Sw480, Sw620, Sw707, Sw1116, HCT116, colo205), lung carcinoma (Heu-n and Bla-2), and breast carcinoma cell lines (MDAMB-231, 734-B) were gifts from M. Grégoire (INSERM U601, Nantes, France), S. Chouaib (INSERM U487, Villejuif, France), and D. Jäger (Klinik und Poliklinik für Onkologie, Zürich, Germany), respectively. K1 keratinocyte cell line was prepared from foreskin fragments of healthy donors that were incubated overnight in trypsin at 4°C. Normal melanocytes, MHN 3 and MHN 5, were gifts from S. Tartare-Deckert (INSERM U597, Nice, France). Normal melanocytes (98M09 and 01M08), fibroblasts (MG and HFFF2), EBV-B cell lines, and endothelial cell lines (HAEC#8186), were gifts from M. Regnier (L'Oréal Laboratory, Paris, France), M. Grégoire (INSERM U601, Nantes, France), H. Vié (INSERM U601, Nantes, France) and B. Charreau (INSERM U643, Nantes, France), respectively. Tumor cells were treated with lactacystin, epoxomicin, E-64, amiloride, monodansylcadaverine, cytochalasins B and D, ammonium chloride, pepstatin A, leupeptin (Sigma-Aldrich), AAF-CMK (Tebu), and JA-2 (I. Smith, Baker Medical Research Institute, Melbourne, Australia), overnight at 37°C.

**cDNA library.** Poly-$A^+$ RNA was extracted from M134 cells using the Fast Track 2.0 mRNA extraction kit (Invitrogen) and converted to cDNA using a cDNA synthesis kit (Stratagene). cDNA were ligated to EcoRI adaptors, digested with XhoI, and inserted at the EcoRI and XhoI sites of expression vector pcDNA3.1 (Invitrogen). Recombinant plasmids were electroporated into *Escherichia coli* XL1 (Stratagene). For screening, 574 pools of 100 ampicillin-resistant bacteria were constituted. Plasmid DNA was extracted from each pool with the QIAprep Spin Miniprep kit (QIAGEN).

The positive plasmid was opened with XbaI and XhoI and digested with exonuclease III with the Erase-a-base System before ligation (Promega) to define the region coding for the epitope.

**cDNA sequencing.** cDNA were sequenced by the dideoxy chain termination method (USB). NA134-A corresponded to the end of the MMP-2 sequence (PubMed/blast accession no. NM_004530).

**Transient transfection of COS-7 cells and tumor cell lines.** Transfection of COS-7 cells was performed by the DEAE-dextran-chloroquine

method, as described (47). Tumor cells were transfected with a lipofectamine reagent kit (Invitrogen), according to the manufacturer's instructions. The β3-GFP plasmid was provided by B. Ihmof (Pathology Department, Geneva, Switzerland).

**Synthetic peptides.** The MMP-2 peptides were purchased from Synt:em and were >85% pure by reversed-phase HPLC. Lyophilized peptides were dissolved in water containing 0.1% trifluoroacetic acid at 10 mM and stored at −80°C. Cell lines were pulsed for 1 h at 37°C with peptides and washed.

**T cell stimulation assays.** For the TNF assay, T cells ($10^4$) were added to $3.10^4$ stimulator cells (cells transfected for 48 h or tumor and normal cell lines). Culture supernatants, harvested 6 h later, were tested for TNF content by measuring lysis of WEHI 164 clone 13 in an MTT colorimetric assay (48).

The chromium release assay was performed as described elsewhere (46). In brief, $10^3$ or $3.10^3$ $^{51}$Cr-labeled target cells (peptide-pulsed T2 cells or tumor cells, respectively) were cocultured with T cells at various effector/target ratios. Chromium release in the supernatants was measured after 4 h of incubation.

For blocking assays, target cell lines were incubated 1 h at room temperature with anti-αvβ3 or anti-CD91 (respectively 23C6 and A2MR-α2, BD Biosciences) at indicated concentrations before CTL addition and TNF assays.

Sensitization of melanoma cell lines to CTL clone recognition was performed by overnight incubation in 96-well rounded bottom plates at 37°C with recombinant MMP-2 (rMMP-2; 100 ng/ml; BIOMOL Research Laboratories, Inc.) or the MMP-2 secreting melanoma cell line, M88 (ratio 1:1), not recognized by the CTL clone.

**RT-PCR assays.** Total RNA was extracted using the Trizol reagent (GIBCO BRL), according to the manufacturer's instructions, and was used for cDNA synthesis (49).

For amplification of MMP-2 cDNA, PCR assays were performed with primers 5′-TGGGCAACAAATATGAGAGC-3′ and 5′-CGGCATCCAGGTTATCGGGG-3′ (792 bp), for 27 cycles (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C). For amplification of β3 cDNA, PCR assays were performed with primers 5′-TGAGAAGTGCCCCACCTG-3′ and 5′-TGGCTGTGTCCCATTTTGC-3′ for 27 cycles (1 min at 94°C, 1 min at 60°C, and 1 min at 72°C). Samples were normalized for RNA and cDNA integrity and quantity by PCR amplification of human β-actin cDNA (49).

For semi-quantitative expression measurements, cDNA was synthesized as above. RNA obtained from M134 cells was used undiluted or was diluted serially in *E. coli* tRNA for each series of quantitative PCR.

**Immunocytochemistry.** Cells were spread on slides (500 revolutions/min for 5 min), fixed with acetone (10 min), and incubated successively with a MMP-2–specific antibody (5 μg/ml; CA4001; Serotec), before using the Dako-ChemMate kit (DakoCytomation) according to the manufacturer's instructions. Counter-staining was performed with hematoxylin.

**ELISA assays.** Release of MMP-2 by cell lines was quantitated by an ELISA kit (Calbiochem) according to the manufacturer's instructions. This "sandwich" enzyme immunoassay was performed on conditioned media obtained from cell lines ($3.10^5$ cells) cultured with RPMI 1640 medium (300 μl) without stromal-vascular fraction in 24-well plates for 48 h.

**Gelatin substrate gel zymography.** Secretion of MMP-2 was evaluated by gelatin zymography, as described previously (50). Conditioned media, obtained from cell lines ($3.10^5$ cells) cultured with RPMI 1640 medium (300 μL) without stromal-vascular fraction in 24-well plates for 48 h, were mixed with SDS buffer without reducing agent, and proteins were subjected to SDS-PAGE in 7.5% polyacrylamide gels containing gelatin at 1 mg/ml. After electrophoresis, SDS was removed from the gel by incubation in 2.5% triton X-100 for 1 h at room temperature. The gels were incubated in a buffer containing 50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$, pH 7.6, for 24 h at 37°C. The gels were stained with coomassie blue R250. Proteolytic activities were evidenced as clear bands against the blue background of stained gelatin.

**Immunostaining of cell lines.** Tumor and normal cell lines were analyzed for surface molecule expression by direct or indirect immunofluorescence and flow cytometric analysis. In brief, $10^5$ cells were stained with the corresponding mAb (anti-αvβ3 [23C6, BD Biosciences]) and anti-CD91 (A2MR-α2, BD Biosciences) for 30 min at 4°C. Negative controls were performed by incubating the cells with isotype-matched control antibody. For indirect staining, cells were incubated for 30 min with the secondary PE-labeled antibody. Labeling was analyzed on a FACScan flow cytometer using Cellquest software (Becton Dickinson). 10,000 cells were gated with FSC/SSC parameters and analyzed. The ratio of fluorescence intensity was calculated for αvβ3 expression as follows: mean fluorescence intensity obtained with the test/mean fluorescence intensity obtained with the negative control.

We thank M. Marzin for immunocytochemical staining of tumor and normal cell lines. We thank S. Tartare-Deckert and M. Regnier for providing us with melanocytes. We thank M. Bonneville, T. Boon, S. Amigorena, L. Old, and J.F. Fonteneau for critically reviewing the manuscript.

This work was supported by the Ligue Nationale Contre le Cancer (Labellisation 2001 and 2005). E. Godefroy was supported by a doctoral fellowship from the Association pour la Recherche contre le Cancer.

The authors have no conflicting financial interests.

**Submitted: 15 October 2004**
**Accepted: 27 May 2005**

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