MELOE-1 is a new antigen overexpressed in melanomas and involved in adoptive T cell transfer efficiency

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A cytotoxic T lymphocyte (CTL) clone was derived from a tumor-infiltrating lymphocyte (TIL) population infused to a melanoma patient who remained relapse free for 10 yr after this adoptive transfer. This clone recognized all melanoma cell lines tested and, to a lower extent, melanocytes, in the context of human histocompatibility leukocyte antigen A2 (HLA-A2), but it did not recognize other tumor cell types. The gene coding for the antigen recognized by this clone was identified by the screening of a melanoma complementary DNA expression library. This antigen is overexpressed in melanomas, compared with other cancer cell lines and healthy tissues, and was thus called *melanoma-overexpressed antigen* (*meloe*). Remarkably, the structure of *meloe* was unusual, with multiple short open reading frames (ORFs). The peptide recognized by the CTL clone was encoded by one of these ORFs, called MELOE-1. Using a specific HLA-A2/peptide tetramer, we showed a correlation between the infusion of TILs containing MELOE-1–specific T cells and relapse prevention in HLA-A2 patients. Indeed, 5 out of 9 patients who did not relapse were infused with TILs that contained MELOE-1–specific T cells, whereas 0 out of the 21 patients who relapsed was infused with such TIL-containing lymphocytes. Overall, our results suggest that this new antigen is involved in immunosurveillance and, thus, represents an attractive target for immunotherapy protocols of melanoma.

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Abbreviations used: HDAC, histone deacetylase; meloe, melanoma-overexpressed antigen; ORF, open reading frame; qPCR, quantitative PCR; SNP, single nucleotide polymorphism; TIL, tumor-infiltrating lymphocyte.

In the last 20 yr, many human melanoma antigens recognized by T cells have been identified using various methods such as cDNA cloning, MHC-bound peptide purification, or T cell induction against candidate peptides or proteins. These antigens have been classified into several groups: melanocytic differentiation antigens (such as Melan-A/MART-1) (1); cancer-germline antigens, shared by several tumors and male germline cells (such as MAGE antigens) (2, 3); mutated antigens generated by genetic alterations (such as CDK4) (4); antigens overexpressed in various tumor types (such as PRAME) (5); and antigens aberrantly expressed in tumors (such as NA17-A and NA88-A) (6, 7). However, despite their high number, the immunogenicity of these antigens has not been elucidated yet, with the exception of Melan-A/MART-1. Indeed, the immunogenicity of the Melan-A antigen in melanoma has been strongly suggested by the analysis of several active (8, 9) and passive (10–15) immunotherapy protocols targeting this antigen. The identification of such tumor antigens with a documented immunogenic potential remains a major issue to address for future immunotherapy protocols.

To this aim, we studied tumor-infiltrating lymphocyte (TIL) populations that had been infused to melanoma patients in an adjuvant setting between 1994 and 2006, and who are still relapse free (14, 16). We previously showed that the prevention of relapse was correlated with the infusion of tumor-specific T cells (17), and specifically for HLA-A*0201 patients, with the infusion of Melan-A–specific TILs (14).

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Nonetheless, in several TIL populations infused to relapse-free patients, a significant fraction of tumor-specific TILs remains of unknown specificity. To fully characterize these tumor-specific TILs and to look for new tumor antigens involved in relapse prevention, we used a TIL population infused to patient M170 in 1998, who is still relapse free today (18). This HLA-A2 TIL population contained a significant fraction of melanoma-reactive TILs, among which Melan-A/A2–specific lymphocytes and lymphocytes of unknown specificity were present. In this study, we show that this TIL population contained tumor-reactive lymphocytes specific for a new tumor antigen overexpressed in melanomas, melanoma-overexpressed antigen 1 (MELOE-1), and recognized by autologous TILs in the HLA-A2 context. Our study clearly shows a correlation between the infusion of T cells reactive against this new tumor epitope and relapse prevention of TIL-treated patients. Thus, this new antigen represents an attractive target for immunotherapy protocols of melanoma.

RESULTS

T cell clone selection and characterization

The M170 TIL population contained 16% of melanoma-reactive lymphocytes, among which 5% were specific for the Melan-A/A2 epitope and 11% were of unknown specificity (Fig. 1 A). This TIL population was then tested for recognition of a large panel of known antigens (Table I) transfected into COS cells with the class I HLA molecules of patient M170 (14, 19), and no response aside from the Melan-A/A2 response could be detected (unpublished data), suggesting that this population contained lymphocytes specific for new tumor antigens. To characterize them, we derived tumor-reactive $CD8^+$ T cell clones by limiting dilution. Eight of these CTL clones showed reactivity patterns consistent with the recognition of new antigens, and one of them, hereafter referred to as M170.48, was further characterized to determine the HLA context restricting its recognition. As illustrated by Fig. 1 B, the recognition of the autologous melanoma cell line occurs in the HLA-A2 context. To establish the distribution of the target antigen, we tested M170.48 reactivity toward various HLA-A2 tumor cell lines, including melanomas, ovarian carcinomas, lung carcinomas, breast carcinomas, renal carcinomas, and colon carcinomas, using a TNF release assay. As shown in Fig. 1 C, this T cell clone recognized all of the HLA-A2 melanoma cell lines tested but none of the other HLA-A2 tumor cell types. In addition, the M170.48 T cell clone weakly recognized HLA-A2 melanocytes (Fig. 1 D). However, this reactivity was much lower than that seen with a Melan-A/A2–specific T cell clone (Fig. 1 D, hatched bars).

Identification of the cDNA coding for the antigen

We screened a cDNA library derived from the M134 melanoma cell line (20) in COS-7 cells cotransfected with pHLA-A*0201 to characterize the antigen recognized by the M170.48 T cell clone. Among 800 pools of 100 pcDNA tested, one plasmid pool proved positive in this test, and the individual plasmid coding for the recognized antigen was recovered from it after a cloning step. This insert, namely *meloe*, spanning 2,128 bp, was sequenced and was found to contain a poly(A) tail and to be similar to the clone BC008026 isolated by the National Institutes of Health (NIH) Mammalian Gene Collection consortium (21). After expression vector cloning, cotransfection of BC008026 cDNA with HLA A*0201 into COS-7 cells also induced the recognition by the M170.48 T cell clone (Fig. 2 A), although these two sequences differed by four single nucleotide polymorphisms (SNPs; Fig. 2 B). To control the impact of these SNPs on the recognition of the M170.48 T cell clone, we sequenced the cDNA isolated from recognized (M134 and M117) and nonrecognized cell lines (A498 and SW480), and showed that this polymorphism did not affect tumor cell line recognition. The *meloe* sequence analysis showed a perfect colinearity with the genomic DNA, obtained by comparison with the sequence of the human genome released by Celera (22),

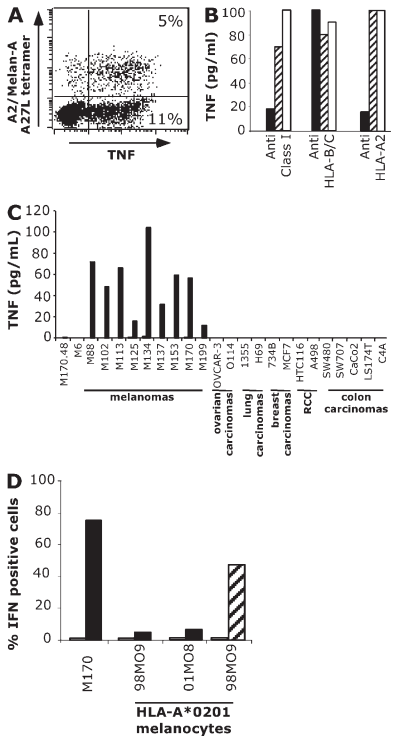

Figure 1. T cell clone selection and characterization. (A) Percentage of TNF-producing T cells and of HLA-A2/Melan-A_{A27L} tetramer–positive T cells in the M170 TIL population in response to the autologous melanoma cell line. 10^5 TILs and 2×10^5 melanoma cells were incubated for 5 h in the presence of Brefeldin A, stained with HLA-A2/Melan-A_{A27L} tetramer, fixed, and stained with anti-TNF antibody in a permeabilization buffer. 10^4 T cells were then analyzed by flow cytometry. (B) TNF secretion by the M170.48 T cell clone in response to the autologous melanoma cell line. 10^4 CTLs were added to 3×10^4 M170 melanoma cells in the presence of blocking antibodies directed against class I, A2, and B/C HLA, diluted to 1:50 (shaded bars), 1:500 (hatched bars), and 1:5,000 (open bars). CTL clone reactivity was assessed by a TNF release assay. (C) TNF response of the M170.48 CTL clone to HLA-A*0201 tumor cell lines. The M6 cell line (HLA-A2 negative) was used as a negative control. (D) IFN-γ response of the M170.48 CTL clone (shaded bars) and of a Melan-A/A2–specific CTL clone (hatched bars) to HLA-A*0201 melanocytes. The M170 cell line was added as a positive control.

which indicated absence of splicing. Finally, this sequence was found to be located in the third intron of the histone deacetylase (HDAC) 4 gene (Ensembl accession no. ENSG00000068024), on chromosome 2, in anti-sense orientation compared with the sequence of the HDAC-4 gene.

Identification of the peptide recognized by the M170.48 T cell clone

The *meloe* cDNA does not contain a long open reading frame (ORF) but multiple short ORFs. ORFs longer than 90 bp are illustrated in Fig. 3 A. The putative ORF described by the NIH Mammalian Gene Collection consortium was located between 486 and 689 bp (21). We tested this ORF and three additional ORFs (Fig. 3 A, black boxes) for recognition by M170.48, after transfection into COS-7 cells, with the HLA-A*0201 cDNA (Fig. 3 B). The ORFs tested (Table II) were chosen on the basis of preliminary results obtained on PCR fragments of *meloe* (unpublished data).

The ORF 1,230–1,370 bp encodes the protein bearing the peptide recognized by the specific T cell clone. This ORF encodes a 46–amino acid protein that contains two peptides (positions 16–24 and 36–44 on the protein sequence) able to bind to the HLA A*0201, with a high stability as predicted by BioInformatics and Molecular Analysis Section analysis (http://www-bimas.cit.nih.gov; Fig. 3 C). These two nonapeptides were tested for their recognition by the M170.48 T cell clone after loading onto T2 cells. Only the sequence 36–44 (TLNDECWPA) was recognized (Fig. 3 D and not depicted). We then tested the recognition of two additional peptides derived from this nonapeptide. We observed that addition of the serine at the C terminus (position 45) dramatically decreased the response of our CTL clone (Fig. 3 D, closed circles) and that deletion of the alanine at the C-terminal end (position 44) abrogated the CTL clone response (Fig. 3 D, open circles). In conclusion, the optimal peptide appeared to be the nonapeptide 36–44 (TLNDECWPA), with a half-maximal lysis of 10^{-8} M (Fig. 3 D, closed squares).

Table I. Antigen-coding cDNAs transfected into COS cells

	No.	Name
Shared tumor specific	1	MAGE-1
	2	MAGE-2
	3	MAGE-3
	4	MAGE-4
	5	MAGE-6
	6	MAGE-12
	7	GAGE-1
	8	GAGE-2
	9	GAGE-3
	10	GAGE-4
	11	GAGE-5
	12	GAGE-6
	13	Lage-1a
	14	Lage-1b
	15	Lage-2
	16	NA88-A
	17	NA17-A
	18	NA-134-A
	19	BAGE
	20	MAGE-C2
Differentiation	21	Melan-A
	22	gp100
	23	Tyrosinase
	24	MC1R
	25	TRP-2
	26	gp75
	27	NY-Mel-1
Mutated/other	28	Ras wild type
	29	Mutated Ras
	30	HSP70
	31	h-TERT
	32	survivin
Overexpressed	33	RAGE-1
	34	RAGE-2
	35	RAGE-3
	36	RAGE-4
	37	P53
	38	PRAME
	39	Her2-neu
	40	EphA2
	41	STEAP

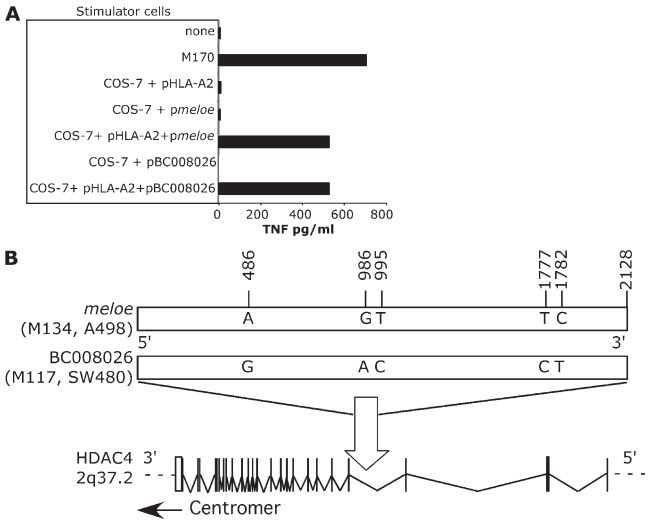

Figure 2. Characterization of the cDNA coding for the recognized antigen. (A) M170.48 TNF responses to COS-7 cells (E/T ratio = 1:3) transfected with the indicated plasmids. The T cell clone was added 2 d after the transfection, and the CTL clone reactivity was assessed by a TNF release assay. (B) Comparison of the nucleotide sequences of *meloe* and BC008026 cDNAs and the localization of this sequence on the HDAC-4 gene. The indicated nucleotides correspond to SNPs between the *meloe* sequence isolated from M134 and A498 tumor cell lines and the *meloe* sequence isolated from the M117 and SW480 cell lines, and the BC008026 cDNA sequence.

meloe is overexpressed in melanomas

To explain the absence of recognition of tumor cell lines other than melanomas, we examined the transcription level of *meloe* cDNA by quantitative PCR (qPCR; Table II) in a panel of HLA-A2 tumor cell lines and melanocytes. The mean level of *meloe* expression in two HLA-A2 melanocytes was used as a reference to establish its relative expression in other cell lines. This analysis showed that *meloe* expression in melanomas was higher than in melanocytes, with values ranging from 3- to 34-fold higher, whereas this expression was significantly lower in other tumor cell lines, with values ranging from 5- to 338-fold lower (Fig. 4 A). These results show that this antigen is overexpressed in melanomas, and thus, the protein encoded by the ORF 1,230–1,370 was called MELOE-1. Furthermore, transfection of *meloe* or *meloe-1* cDNA in HLA-A2 nonrecognized tumor cell lines induced their recognition by the M170.48 T cell clone (Fig. 4 B), showing that the absence of recognition of these tumor cell lines was caused by the underexpression of *meloe* cDNA. Finally, to address the question of the expression of this antigen in healthy tissues, we performed qPCR on a panel of 16 tissues. It appears that the expression of *meloe* in healthy tissues was always lower than in melanocytes. The highest *meloe* expression was found in the whole and fetal brain but remained, respectively, 1.5- and 1.8-fold below its expression in melanocytes (Fig. 4 C). Overall, these results suggest that this antigen could be safely targeted in immunotherapy protocols in melanoma, provided that its immunogenicity could be documented.

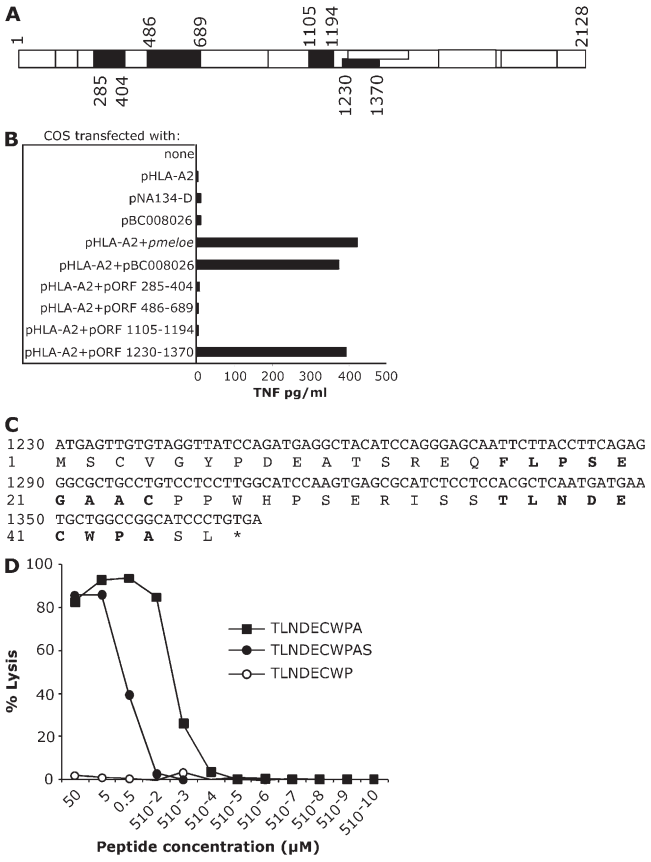

Figure 3. Characterization of *meloe*-derived peptide recognized by the M170.48 T cell clone. (A) Structure of *meloe* cDNA. Boxes illustrate ORFs >120 bp present along the *meloe* sequence, and black boxes correspond to ORFs tested for recognition by the CTL clone. (B) M170.48 TNF responses to COS-7 cells (E/T ratio = 1:3) transfected with the indicated plasmids. The T cell clone was added 2 d after the transfection, and the CTL clone reactivity was assessed by a TNF release assay. (C) Nucleotide and amino acid sequences of the ORF 1,230–1,370 of *meloe* isolated from the M134 cDNA library. The two candidate peptides are bolded. (D) Cytotoxicity of the M170.48 CTL clone against peptide-pulsed T2 cells. Target cells were ^{51}Cr-labeled for 60 min and incubated for 30 min with a range of the indicated peptides. The M170.48 T cell clone was added (E/T ratio = 10:1), and chromium release was then measured after a 4-h incubation period.

Presence of MELOE-1–specific lymphocytes in TIL populations infused to relapse-free patients

To address the question of the immunogenicity of this new epitope, we used a specific HLA-A2/peptide tetramer to look for the presence of specific lymphocytes among 30 HLA-A2 TIL populations derived from melanoma-invaded lymph nodes. All of those TIL populations had been infused to melanoma patients in an adjuvant setting between the years 1998 and 2002. After this treatment, 21 of these patients relapsed and 9 remained relapse free. Using a specific HLA/peptide tetramer, we detected the presence of MELOE-1/A2–specific T cells in five out of nine TIL populations that had been infused to relapse-free patients, with frequencies ranging from 0.07 to 3.8% among $CD8^+$ TILs (Fig. 5 A, top). In contrast, we did not observe the presence of such T cells among the TILs infused to the 21 HLA-A2 patients who relapsed. An example of 5 out of these 21 negative TIL populations is shown in Fig. 5 A (bottom). These results document the existence of a correlation between the presence of MELOE-1–specific lymphocytes among infused TILs and relapse prevention ($P < 0.001$), and thus, suggest the potential immunogenicity of this new HLA-A2 melanoma epitope.

Finally, to address the question of the diversity and tumor reactivity of the MELOE-1/A2–specific repertoire, specific lymphocytes were sorted by monomer-based immunomagnetic sorting (23) from the five positive TIL populations. Fig. 5 B (insets) illustrates the purity of sorted TILs assessed by specific tetramer labeling. We also attempted to sort five negative TIL populations with monomer-coated beads, but no MELOE-1–specific cells were obtained (unpublished data). This last result formally documented the absence of such cells in those populations, or at least showed that the frequencies of MELOE-1–specific T cells were too low to allow their purification by multimer sorting. The diversity of TCR Vβ usage of sorted populations was assessed with a panel of 25 anti-Vβ antibodies representing the most frequently expressed Vβ chains within a normal repertoire. In M117, M170, and M278 sorted populations, eight or six different Vβ chains were significantly expressed (>1%) by MELOE-1/A2–specific TILs, indicating the presence of a rather polyclonal-specific TCR repertoire (Fig. 5 B). TCR diversity of M134 sorted TILs

was much lower, with a strong dominance of lymphocytes expressing the Vβ13.1 chain (Fig. 5 B). This may be related to the low fraction of MELOE-1–specific T cells present in this population before sorting (0.3% of $CD8^+$ TILs; Fig. 5 A), which was probably poorly diverse. Finally, we could not determine the dominant Vβ chain expressed by TILs sorted from M180 with our panel of antibodies. Therefore, no dominant Vβ usage could be observed within these three sorted TIL populations. Finally, to support the potential role of MELOE-1/A2–specific TIL transfer in relapse prevention, we studied the reactivity of sorted TIL populations on HLA-A*0201 melanoma cell lines that spontaneously express the MELOE-1 antigen. All sorted T cell lines were lytic against melanoma cell lines (Fig. 6 A and not depicted) and produced IFN-γ and TNF upon stimulation by these cells, with levels similar to the M170.48 CTL clone, and to a lower extent IL-2 (Fig. 6 B and not depicted).

DISCUSSION

The *meloe* gene is located on the third intron of the HDAC-4 gene and translated in anti-sense orientation in comparison with the HDAC-4 gene (24). There is a perfect colinearity between the *meloe* gene and its corresponding cDNA, showing an absence of splicing of this gene. Furthermore, the structure of this 2.1-kb cDNA is rather unusual, with multiple short ORFs instead of a unique long ORF. The protein encoded by the $ORF_{1230\text{-}1370}$ (MELOE-1) contains a peptide that was recognized by a CTL clone derived from melanoma-specific TILs, in the HLA-A*0201 context. This CTL clone recognized all of the HLA-A*0201 melanoma cell lines tested, and to a lower extent HLA-A*0201 melanocytes. On the other hand, this T cell clone failed to recognize any of the other tumor cell lines tested (Fig. 1 C). Moreover, another epitope derived from the protein encoded by an alternative ORF, also recognized in the HLA-A*0201 context by a CTL clone deriving from the same TIL population, is under characterization. This suggests that *meloe* could be an interesting source of tumor epitopes recognized by melanoma TILs.

The *meloe* antigen could be classified into the family of "melanocytic differentiation antigens" because of its expression in melanocytes and melanoma cell lines (25). Nonetheless, it could be also classified into the family of aberrantly expressed antigens because of the particular location of *meloe* gene in the third intron of the HDAC-4 gene, according to the example of NA17-A antigen, located in an intron of the GnT-V gene (6). Unlike classical differentiation antigens such as Melan-A or tyrosinase (18, 26, 27), we detected by qPCR a residual expression of the *meloe* gene in other cancer cell lines, even if this expression level was too low to induce their recognition by our specific CTL clone, as recently shown for a mouse prostate tumor epitope derived from histone H4 (28). *meloe* also differs from a classical differentiation antigen by its overexpression in melanomas, compared with normal melanocytes. Thus, *meloe* seems to be specifically overexpressed in melanomas, and therefore, this new antigen presents both the properties of tissue specificity and overexpression in cancer. The reasons for its overexpression in melanomas could be because of the regulation of *meloe* promoter. As described for the MAGE-1 gene, a transient and general process of demethylation in tumors could be followed by a persistent inhibition of remethylation caused by the presence of melanoma-specific transcription factors (29). In this way, *meloe* overexpression in melanomas could be caused by the hypomethylation of its promoter in melanoma cell lines. In another way, melanoma-specific transcription factors, such as microphthalmia-associated transcription factor, which controls the expression of the three main melanocytic differentiation antigens (30, 31), could also control the overexpression of *meloe* in melanomas.

Table II. Sequences of primers used for qPCR and ORF subcloning

Primers	5′–3′ sequences
qPCR	
RPLPO forward	GTGATGTGCAGCTGATCAAGA
RPLPO reverse	GATGACCAGCCCAAAGGAGA
β2-microglobulin forward	TTCACCCCCACTGAAAAAGAT
β2-microglobulin reverse	GGCATCTTCAAACCTCCATGAT
meloe forward	GTCCTCCCCAGCACCAGAGT
meloe reverse	AGCCTGCCATCTGCAATCCT
ORF subcloning	
(285–404) forward	aaa*GAATTC*gccaccATGAGTGAAAATGCAGGAGG
(285–404) reverse	aaa*CTCGAG*TCACTGGCACAGTGCAG
(486–689) forward	aaa*GAATTC*gccaccATGTCCGTAGGGAGACGC
(486–689) reverse	aaa*CTCGAG*TCACCCTAGAGCTGCCAAG
(1,105–1,194) forward	aaa*GAATTC*gccaccATGAAATTTGAATTAATTTGCAGAAC
(1,105–1,194) reverse	aaa*CTCGAG*TTAATTCCTAAGGCATTCATTC
(1,230–1,370) forward	aaa*GAATTC*gccaccATGAGTTGTGTAGGTTATCC
(1,230–1,370) reverse	aaa*CTCGAG*TCACAGGGATGCCGGCC

The italicized letters correspond to the enzyme restriction sites. The lowercased letters correspond to the Kozak sequence, which is described in the Materials and methods.

Because of its expression widely shared by melanoma cell lines, and to the existence of an epitope recognized by a melanoma-reactive CTL in the HLA-A*0201 context, the MELOE-1 antigen could be a promising target for future immunotherapy protocols of melanoma, provided that its usage remains safe in patients and that its immunogenicity could be documented.

Our data provide some arguments concerning the safety of immunization of patients with this antigen or of adoptive transfer with specific CTLs. Indeed, the expression level of *meloe* in healthy tissues is always lower than in melanocytes, which are weakly recognized by the MELOE-1–specific CTL clone to a lower extent than by Melan-A–specific CTLs (Figs. 1 D and 4 C). Furthermore, none of the other HLA-A*0201 cancer cell lines was able to spontaneously induce any reactivity of the MELOE-1–specific CTLs (Fig. 1 C), even after a 48-h treatment with IFN-γ (not depicted). MELOE-1–specific CTL clones could only be activated by such tumor cell lines when they were previously transfected by the *meloe* cDNA or the cDNA coding for $ORF_{1230-1370}$ (Fig. 4 B), showing that the expression level of *meloe* in other cancer cell lines was too low to induce the activation of specific lymphocytes. This expression level is similar to that measured in healthy tissues by qPCR (Fig. 4 C), suggesting that an immunization with this antigen should not induce deleterious reactions in healthy tissues, although we could expect the induction of some vitiligo because of the expression of *meloe* in melanocytes.

The second point concerns the immunogenicity of this new antigen and especially the immunogenicity of the HLA-A*0201–restricted epitope. To answer this main question, we checked the presence of MELOE-1–specific lymphocytes among TIL populations, which had been infused to stage III melanoma patients after lymph node excision, in an adjuvant setting. In retrospective studies of this adoptive transfer protocol, we already showed that the infusion of melanoma-specific TILs had a significant impact on the relapse prevention of treated patients (17). More recently, we found that prolonged relapse-free survival of TIL-treated patients correlated with the infusion of Melan-A–specific lymphocytes (14), although a significant fraction of tumor-specific TILs remains of unknown specificity in several TIL populations infused to relapse-free patients. We detected a significant fraction of MELOE-1–specific lymphocytes, by tetramer labeling, in

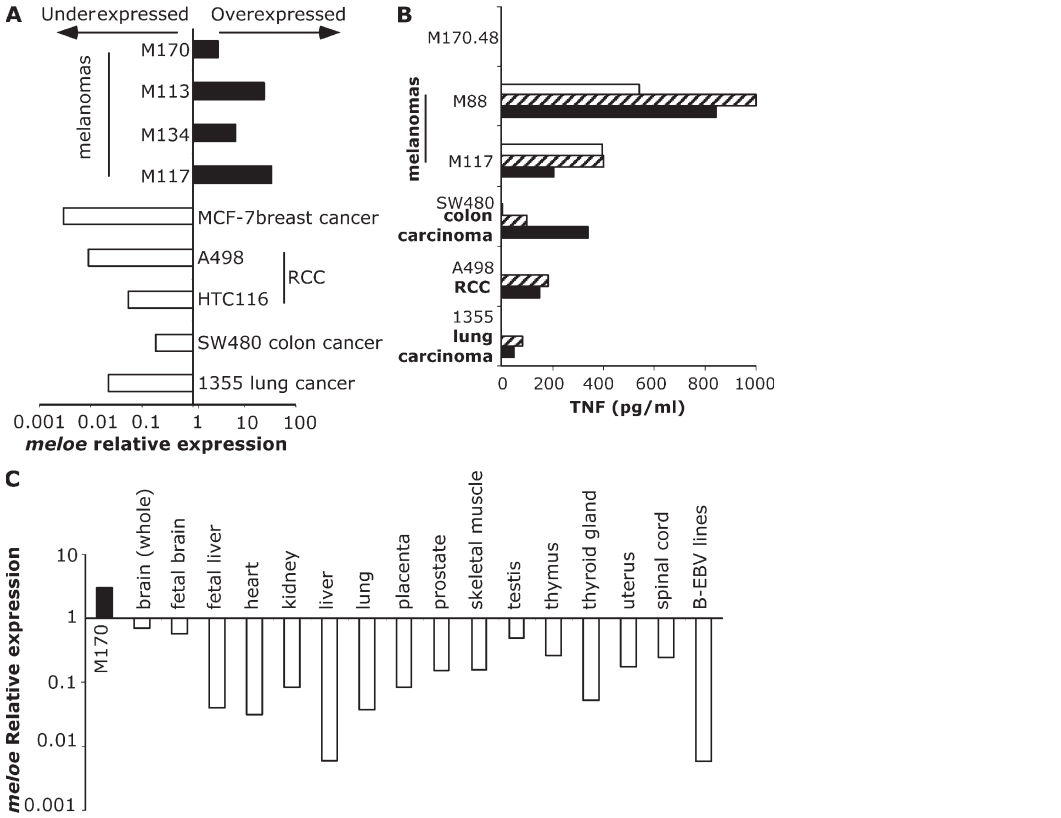

Figure 4. Preferential expression of *meloe* cDNA in melanoma cell lines measured by qPCR, and impact of *meloe* expression on specific CTL clone activation. (A) Four melanoma, one breast cancer, two renal carcinoma, and one lung cancer cell lines were tested by qPCR for the expression of *meloe*. RPLP0 and β2-microglobulin gene expression were used as internal controls. The relative expression of *meloe* was calculated after normalization on the efficiency of the PCR reaction and the mean expression of these two housekeeping genes, reported to its normalized expression in melanocytes. (B) TNF secretion by the M170.48 CTL clone in response to HLA-A2 tumor cell lines nontransfected (open bars) or transfected with *meloe* (hatched bars) or *meloe*-1 (shaded bars) expression plasmids. Tumor cells were transiently transfected with 100 ng of each plasmid with a lipofectamine reagent kit. 10^4 CTLs were added to 3×10^4 target cells, and the CTL clone reactivity was assessed by a TNF release assay. (C) *meloe* relative expression measured by qPCR in 16 human healthy tissues.

5 out of 9 TIL populations infused to HLA-A2 patients who remained relapse free from 7 to 13 yr, whereas no MELOE-1–specific lymphocytes could be observed in 21 TIL populations infused to patients who relapsed. The statistical analysis of these results performed by a χ^2 test documents a correlation between the prevention of the relapse of TIL-treated HLA-A2 patients and the presence of MELOE-1–specific CTLs among those TILs ($P < 0.001$). This main result provides a strong argument in favor of the implication of MELOE-1 antigen in the immunosurveillance of patients treated by adoptive transfer of TILs. This role in immunosurveillance has not been formally elucidated for the majority of melanoma antigens identified, except for Melan-A/MART-1, which seems clearly involved in clinical responses of melanoma patients treated by immunotherapy (10–12, 14, 15).

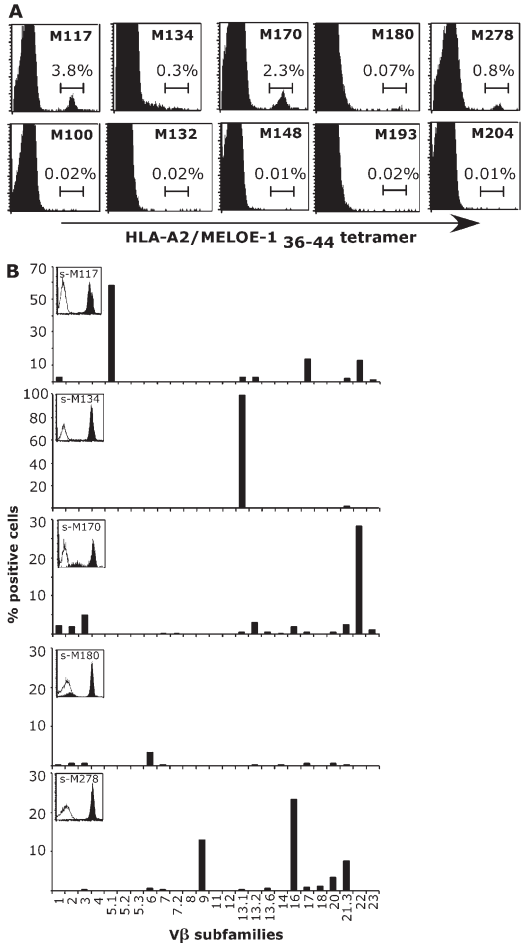

Figure 5. Detection of MELOE-1/A2–specific CTLs in TILs infused to relapse-free melanoma patients and analysis of their repertoire diversity. (A) HLA-A2 TIL populations labeled with the A2/MELOE-1_{36-44} tetramer. (top) TILs infused to relapse-free patients. (bottom) TILs infused to patients who relapsed. TILs were coincubated with MELOE-1 tetramer and anti-CD8 mAb. Values indicate the percentage of tetramer-positive cells among $CD8^+$ TILs. (B) Repertoire diversity of multimer-sorted populations was evaluated by labeling with 25 anti-Vβ mAbs. Insets illustrate the purity of each sorted TIL population, assessed by MELOE-1–specific tetramer labeling.

According to the example of Melan-A antigen, a diverse and tumor-reactive T cell repertoire is necessary to develop protocols of adoptive transfer of specific T cells in melanoma patients, as well as for vaccination trials. We addressed this last issue by analyzing the diversity and reactivity of an MELOE-1–specific repertoire in the five TIL populations that contained MELOE-1–specific T cells. After the selection of MELOE-1–specific T cells from TILs by immunomagnetic sorting with multimer-coated beads (23), we showed that the MELOE-1–specific repertoire was diverse in three out of five TIL populations and much more limited for the M134 TIL population, which contained a low fraction of MELOE-1–specific T cells (Fig. 5, A and B), which could explain this poorer diversity. We could not determined the dominant Vβ chains expressed by M180 sorted TILs with our panel of antibodies (Fig. 5 B). The repertoire analysis of these TIL populations did not reveal any recurrence of a particular Vβ usage, as previously described for the Melan-A–specific repertoire (23).

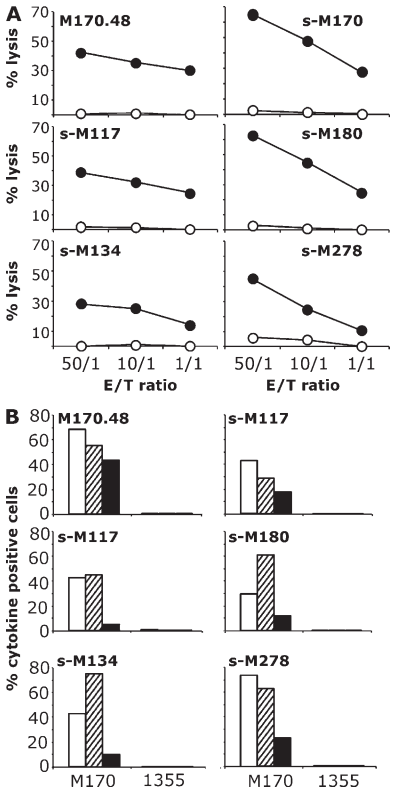

Figure 6. Reactivity of MELOE-1/A2–specific TILs against HLA-A2 tumor cell lines. (A) Lysis of the M170 melanoma cell line (closed circles) and of the 1355 lung carcinoma cell line (open circles) by the M170.48 CTL clone and MELOE-1–specific TIL populations. ^{51}Cr-labeled tumor cells were co-cultured with T cells at various E/T ratios. Chromium release in the supernatants was measured after a 4-h incubation period. (B) Cytokine production by the M170.48 CTL clone and MELOE-1–specific TIL populations in response to M170 melanoma cells. Effector and target cells were incubated at a 1:2 ratio in the presence of Brefeldin A and stained with anti-TNF antibody (open bars), anti- IFN-γ antibody (hatched bars), or anti–IL-2 antibody (closed bars), and 10^4 T cells were analyzed by flow cytometry.

These five MELOE-1–specific TIL populations were as reactive as the M170.48-specific T cell clone against HLA-A2 melanoma cell lines, as shown by lysis (Fig. 6 A) and IFN-γ and TNF-α production in response to melanoma cells, and, to a lower extent, IL-2 production. Furthermore, we recently demonstrated that MELOE-1–specific T cells could be also selected and amplified from the PBMCs of melanoma patients (unpublished data), which remain the most convenient source of tumor-specific T cells usable for all HLA-A2 patients enrolled in adoptive transfer protocols.

In conclusion, this new melanoma antigen, *meloe*, appears to be a very relevant target for immunotherapy protocols. Its overexpression by all melanoma cell lines tested, the particular structure of the transcript with multiple ORFs, its potential as a source of class I and II epitopes, the characterization of an epitope derived from one of these ORFs and recognized in the frequently expressed HLA-A2 context, and, finally, its implication in the immunosurveillance of TIL-treated HLA-A2 melanoma patients are convincing arguments to use this antigen in vaccination trials or to target it by the adoptive transfer of specific T cells.

MATERIALS AND METHODS

Cell lines and TIL cultures. 20 T cell populations were expanded from cryopreserved samples of TILs (derived from tumor-invaded lymph nodes) infused to melanoma patients included in a phase I/II protocol between the years 1994 and 1998. This clinical trial aimed at examining the survival of stage III melanoma patients randomly treated by IL-2 alone, or TIL plus IL-2, in an adjuvant setting (16). 10 additional HLA-A2 stage III melanoma patients, treated with TIL and IL-2 in an adjuvant setting, were also analyzed in the present study. They have been enrolled between December 2001 and June 2006 either in a clinical open study or in a multicentric trial. All of these clinical trials were approved by the local ethics committee Comité de Protection des Personnes Ouest IV–Nantes and the Agence française de sécurité sanitaire des produits de santé. TIL samples were expanded according to a previously described procedure (32, 33). TILs containing tumor-specific T cells were cloned by limiting dilution (34), and tumor-specific T cell clones were amplified as previously described (33). Melanoma cells lines and colorectal carcinoma cell line C4-A were established, respectively, in the Unit of Cellular Therapy and in our laboratory. Mouse fibrosarcoma WEHI 164 clone 13 and COS-7 cells were obtained from T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). Ovary carcinoma cell lines (OVCAR-3 and O114) and renal carcinoma cell line A498 were gifts from C. Saï (Institut National de la Santé et de la Recherche Médicale [INSERM] U892, Nantes, France). Colorectal carcinoma cell lines (CaCo-2, Sw480, Sw707, and LS174T), renal carcinoma cell line HTC116, and breast carcinoma cell line 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). Breast cancer cell line MCF-7 was obtained from the American Type Culture Collection. Normal melanocytes (98M09 and 01M08) were gifts from M. Regnier (L'Oréal Laboratory, Paris, France). EBV-B cell lines were gifts from H. Vié (INSERM U601, Nantes, France).

Functional analysis of T cells. Cytotoxic activity of T cells was measured in a standard 4-h assay against ^{51}Cr-labeled cells (peptide-loaded T2 cells or tumor cell lines) (23). Measurement of TNF produced by T cells in response to tumor cells or transfected COS-7 cells (19) was performed as previously described, using WEHI 164 clone 13 cells (35). mAb against HLA class I (clone W6.32), HLA-B/C (clone B1.23.2), and HLA-A2 (clone BB7.2), added to cultures in some experiments, were produced in our laboratory from hybridomas obtained from the American Type Culture Collection for W6.32 and BB7.2 antibodies and from F. Lemonier (Pasteur Institute, Paris, France) for B1.23.2 antibody. Intracellular staining of cytokines was performed as previously described on stimulated T cells (36).

cDNA library and ORF constructs. The M134 cDNA library has been inserted in pcDNA3.1, as described previously (20), and recombinant plasmids were electroporated into *Escherichia coli* XL1 (Stratagene). For screening, 800 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 sequenced by the DNA Sequencing Facility of the Institut Fédératif de Recherche 26. The various ORFs from the *meloe* sequence were generated by PCR (primers are available in Table II). Oligonucleotides were designed with EcoRI and XhoI adaptators for subcloning in pcDNA3, and with a Kozak sequence (gccaccATG) for the upper primer and a stop codon for the lower primer.

Synthetic peptides. Peptides were purchased from Eurogentec. Purity (>70 or >90% for tetramer production) was controlled by reversed-phase HPLC. Peptides were lyophilized, dissolved in DMSO at 10 mg/ml, and stored at −20°C.

Real-time PCR. Total RNA was extracted from tumor cell lines, melanocytes, and B-EBV cell lines by TRIzol reagent (Invitrogen). RNA from healthy tissues were purchased from Clontech Laboratories, Inc. The quality of RNA samples was controlled using a bioanalyzer (Agilent 2100 Bioanalyzer; Agilent Technologies), and all of the samples exhibited an RNA integrity number >7. Retrotranscription was performed using 1 μg of total RNA, random hexamers, and SuperScript II reverse transcriptase (Invitrogen). Relative quantification of *meloe*, RPLPO, and β2-microglobulin expression was performed using Brilliant SYBR Green QPCR in an Mx4000 machine (Stratagene). 10 ng of cDNA from samples was added to SYBR Green Master Mix in an Mx4000 machine with forward and reverse primers (Table II) at 200 nM in a final volume of 25 μl. Thermal cycling was one step at 95°C for 10 min, followed by 40 cycles at 95°C for 30 s, 63°C for 1 min, and 72°C for 1 min. The efficiency of PCR reaction was determined with a series of 10-fold–diluted cDNA from patient M170, performed in parallel to plot the standard curves for *meloe*, RPLPO, and β2-microglobulin. Duplicate dilution series were included as standards in each run. Mean threshold cycle (*CT*) values from duplicate PCR reactions were normalized to mean *CT* values for two housekeeping genes (β2-microglobulin and RPLPO) from the same cDNA preparations. The relative expression ratio of a target gene was calculated based on the PCR efficiency (*E*) and the *CT* deviation between a given cell line (*x*) and a reference cell line (*calibrator*), expressed in comparison with the mean of the housekeeping genes (37): ratio = $(E\text{target})^{\Delta CT\text{target(calibrator}-x)}$/ mean$((E\text{housekeeping})^{\Delta CT\text{housekeeping(calibrator}-x)})$.

Tetramer and Vβ labeling. HLA-A*0201/MELOE-1 and HLA-A*0201/Melan-A α3-mutated monomers were generated by the recombinant protein facility (INSERM U892), as previously described (38). TIL populations and the M170.48 T cell clone were coincubated for 1 h at 4°C in the dark with 10 μg/ml of MELOE-1 tetramer and 5 μg/ml CD8 mAb, and 10^4 events were analyzed on a FACSCalibur (BD Biosciences). A panel of 25 anti-Vβ mAbs (Vβ1, -2, -3, -4, -5.1, -5.2, -5.3, -6, -7, -7.2, -8, -9, -11, -12, -13.1, -13.2, -13.6, -14, -16, -17, -18, -20, -21.3, -22, and -23) was used to analyze the diversity of sorted TIL populations (Beckman Coulter).

Immunomagnetic cell sorting and expansion of T cell–sorted populations. 20 μg/ml of HLA-A*0201/MELOE-1 monomers were incubated for 1 h at room temperature with 6.7×10^6 streptavidin-coated beads (Dynabeads M-280 streptavidin; Invitrogen) and washed in PBS/0.1% BSA. 5×10^6 TILs were rotated for 4 h at 4°C with monomer-coated beads (23, 38). After 10 washes, bead-coated cells were expanded using a polyclonal T cell stimulation protocol (33).

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