Comprehensive analysis of the frequency of recognition of melanoma-associated antigen (MAA) by CD8 melanoma infiltrating lymphocytes (TIL): implications for immunotherapy

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Fifty-nine tumor-infiltrating lymphocyte (TIL) cultures established from melanoma-invaded lymph nodes were screened for recognition of 28 melanoma-associated antigens (MAA) in association with 31 HLA molecules. Twenty-three (39%) TIL lines reacted to at least one melanoma antigen. Melanosomal proteins were recognized by 19 TIL populations and the most prominent responses against these proteins were directed against Melan-A/MART-1 (mainly in association with HLA-A*0201) and gp100 (in association with diverse HLA contexts). Ten TIL populations reacted against 10 tumor-specific antigens, in association with 8 different HLA molecules. HLA-A*0201 and B*3501-restricted responses were the most frequent with, respectively, 17 and 7 responses directed against 5 distinct antigens. Unexpectedly, the recognition by TIL of different MAA was frequently restricted by a single HLA in individual tumors, and there was no evidence for the existence of dominant MAA epitopes between tumors, except for Melan-A/MART-1 antigen. This analysis also led to the detection of 21 new HLA-peptide complexes recognized by melanoma TIL. This study, which is to our knowledge the most comprehensive analysis of TIL specificity to tumor antigens, has several implications for the design of immunotherapeutic strategies based on immunization against selected tumor epitopes.

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1 Introduction

More than 30 melanoma-associated antigens (MAA), targets of autologous $CD8^+$ cytotoxic T lymphocytes (CTL), have been identified during the last 10 years (for reviews see [1, 2]). These MAA can be roughly subdivided in two classes: proteins expressed exclusively by tumor cells, and proteins also expressed at a significant level by normal cells. Belonging to the first category, are: (i) the large family of cancer-testis antigens such as MAGE, GAGE, BAGE [1, 2], and LAGE [3], (ii) proteins expressed by unconventional gene expression, such as NA17-A [4], NA88-A [5], TRP-1 INT-2 [6], and (iii) mutated proteins [1, 2]. The main interest of these proteins is their tumor specificity, but they are expressed by a limited fraction of tumors. MAA belonging to the second category are the melanosomal proteins, such as Melan-A/MART-1, gp100, tyrosinase, gp75 or TRP-1 and TRP-2 [2] and the melanocyte receptor proteins, such as MC1R [7]. Interestingly, these proteins are expressed by a majority of melanoma tumors. However, as self proteins, they may present limitations for immunotherapies: either immunization against these antigens could fail, due to tolerance mechanisms, or, if efficient, it could result in autoimmune disorders.

The discovery of tumor antigen has resulted in the molecular identification of several major histocompatibility complex (MHC)-restricted tumor peptides, which has opened the way for antigen-targeted immunotherapies. Nonetheless, the relative interest of these many antigens remains unclear, and data documenting their level of expression by tumor cells as HLA-peptide complexes and their capacity to induce a T cell-dependent tumor rejection are lacking.

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Abbreviations: **MAA**: Melanoma-associated antigens **TIL**: Tumor-infiltrating lymphocytes

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The antigenicity/immunogenicity of MAA can only be evaluated indirectly through analysis of T cell reactivity to MHC-peptide complexes and relative antigenicity of known MAA can be assessed by comparing the frequency at which they are recognized *in vitro* by TIL.

This issue has recently been addressed in a preliminary way for some MAA (essentially for melanosomal proteins), using TAP-deficient cell lines presenting known epitopes from these antigens [8–10]. To circumvent the limitations of the above screening strategy, which requires prior knowledge of the recognized epitopes, Rosenberg and colleagues recently studied the reactivity of TIL populations to COS cells co-transfected with combinations of cDNA coding for a given MAA and HLA allele, and applied this technique for the analysis of 5 melanosomal proteins in association with 5 HLA-A contexts [9]. Here we extended this approach to the analysis of the reactivity of 59 TIL populations derived from melanoma-invaded lymph nodes, to 28 MAA presented by transfected COS cells in association with 31 HLA alleles.

The data obtained provide a relative frequency of recognition of these MAA by $CD8^+$ TIL in diverse HLA contexts. They also show the existence of many unknown epitopes derived from 12 of these MAA. By deriving TIL clones specific for several of these new MAA epitopes, we demonstrated that tumor cells express these epitopes on their surface, at levels sufficient to activate T cell effector functions. These new epitopes, once identified, may therefore represent new immunotherapeutic targets for cancer patients.

Altogether, these results suggest that the present approach may prove valuable for a systematic inventory of CTL tumor target antigens and epitopes in every type of cancer.

2 Results

2.1 Responses against differentiation melanoma antigens

As shown in Fig. 1, 19 out of 59 tested TIL cell lines recognized at least one out of the four differentiation antigens Melan-A/MART-1, tyrosinase, gp100 or gp75. Melan-A/MART-1 and gp100 were the most frequently recognized, by 12 and 9 TIL, respectively (Fig. 1A, B; and 5A). Most Melan-A/MART-1-specific responses (11 out of 12) were restricted by the HLA-A*0201 allele, and these 11 TIL populations recognized T2 cells pulsed with the immunodominant 27–35 and 26–35 epitopes (data not shown). The other Melan-A/MART-1-specific TIL population, M28, was restricted by B*3501.

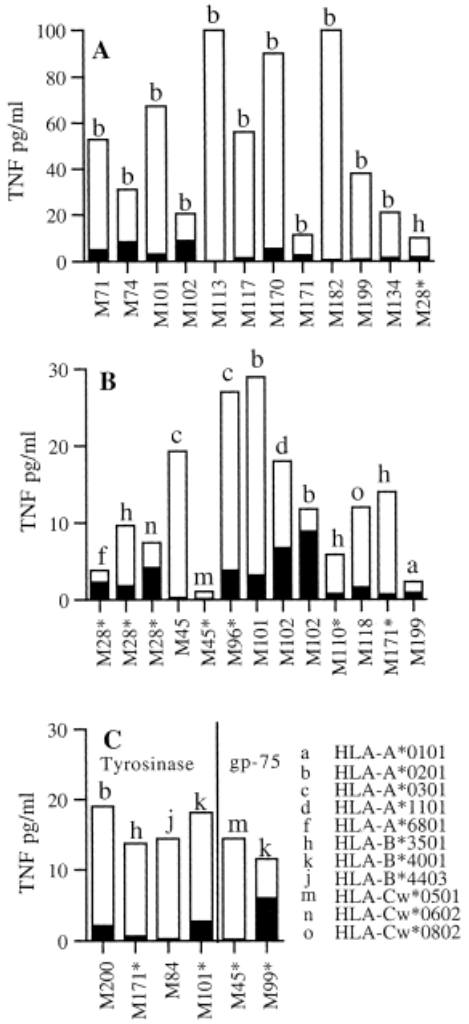

Fig. 1. Responses of TIL populations against melanocytic differentiation antigens. COS cells were transfected with pcDNA3 vector coding for an HLA molecule and with pcDNA3 vector coding for an antigen. After 48-h incubation, COS7 cells are incubated 6 h at 37 °C with TIL populations. TNF secreted in the supernatant was then measured. (■) TNF secretion of TIL populations in response to COS7 cells transfected with the cDNA encoding the HLA molecule alone. (□) TNF secretion of TIL populations in response to COS7 cells transfected with cDNA encoding the HLA and the antigen. (A) Responses against Melan-A/MART-1, (B) responses against gp100, (C) responses against tyrosinase and gp75 antigens. * Indicates TIL populations that recognized not described MHC/peptide complexes.

gp100 recognition by TIL was observed in 9 different HLA contexts: HLA-A*0101, *0201, *0301, *1101, *6801; HLA-B*3501; HLA-Cw*0501, *0602 and *0802 (Fig. 1B, and 5A). Six of these HLA alleles restricted the recogni-

tion of gp100 by only one TIL population. However, B*3501 restriction of gp100 recognition was observed for three TIL populations, and A*0201 and A*0301 restrictions were observed for two populations. gp100 was recognized by three TIL populations (M45, M28 and M102, see Fig. 1B) in several HLA contexts.

Tyrosinase was recognized by four and gp75 by two TIL populations (Fig. 1C). Tyrosinase was recognized in four different HLA contexts (A*0201, B*4403, B*3501 and B*4001) and gp75 in two HLA contexts (B*4001 and Cw*0501).

2.2 Responses against tumor-specific MAA

As shown in Fig. 2, only 10 of these antigens, out of 21 tested, were recognized by 10 TIL populations out of 59. MAGE-1 and MAGE-3 were recognized by 1 TIL population (MAGE-1 by TIL M99 in the HLA-B*4001 context, and MAGE-3 by TIL M171 in the HLA-B*3501 context). MAGE-6 was recognized by 2 TIL populations (TIL M171 that also recognized MAGE-3 in the same HLA-B*3501 context, and TIL M91 in the HLA-Cw*0401 context).

LAGE-1b was recognized by 2 TIL populations (M91 TIL population in the HLA-B*3501 context, and TIL M117 in the HLA-A*0201 context) and LAGE-2/.NY-ESO-1 was recognized by only 1 TIL (M118 in the HLA-B*3503 context). GAGE-5 was recognized by 2 TIL (M45 in the HLA-A*0301 context, and M167 in the A*2402 context) and both TIL cross-reacted, in the same context, with GAGE-3 and GAGE-2, respectively .

Two TIL populations (M17 and M74) recognized NA17-A in the A*0201 context and 1 TIL population (M88) recognized the recently cloned MAA NA88-A [5].

2.3 Specificity of TIL responses was confirmed at the clonal level

To confirm TIL responses to as-yet-undescribed HLA-peptide complexes, and to establish the significance of low TNF productions by some TIL (like that of TIL M199 to gp100/A*0101, see Fig. 1B), CD8 clones were obtained from TIL M28, M167, M171, and M199. As shown in Fig. 3, clones with most of the expected reactivities were obtained: Melan-A/B*3501, gp100/B*3501, and gp100/A*6801 from M28 TIL, gp100/A*0101, from M199 TIL, GAGE-2/GAGE-5/A*2402 from M167 TIL, and

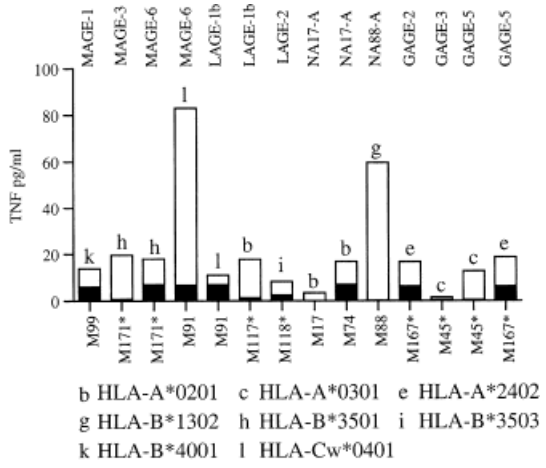

Fig. 2. Responses of TIL populations against tumor-specific antigens. COS cells were transfected with pcDNA3 vector coding for an HLA molecule and with pcDNA3 vector coding for an antigen. After 48-h incubation, COS7 cells are incubated 6 h at 37 °C with TIL populations. Then, TNF secreted in the supernatant was measured. (■) TNF secretion of TIL populations in response to COS7 cells transfected with the cDNA encoding the HLA molecule alone. (□) TNF secretion of TIL populations in response to COS7 cells transfected with cDNA encoding the HLA and the antigen. * Indicates TIL populations that recognized not described MHC/peptide complexes.

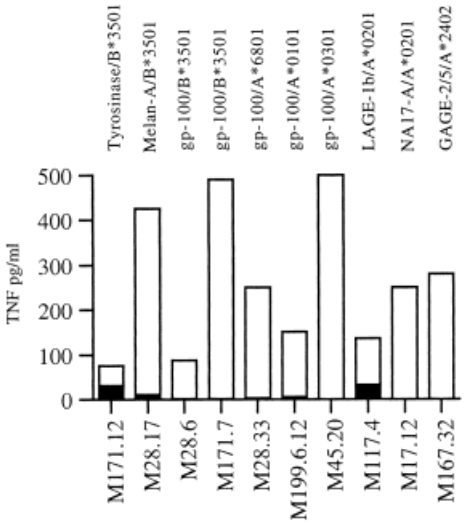

Fig. 3. Confirmation of polyclonal TIL responses at the clonal level. COS7 cells were transfected with a cDNA encoding the restriction element and a cDNA encoding the antigen. After 48-h incubation, COS7 cells were incubated 6 h at 37 °C with TIL populations and TNF secreted in the supernatant was measured. (■) TNF secretion by TIL clones in response to COS7 cells transfected with the cDNA encoding the HLA molecule alone. (□) TNF secretion by TIL populations in response to COS7 cells transfected with cDNA encoding the HLA and the antigen.

tyrosinase and gp100/B*3501 from TIL M171. We have previously derived clones from TIL M17, M45 and M117, that were specific for NA17–A/A*0201 [4], gp100/A*0301 (Fonteneau et al., in preparation) and LAGE-1b/A*0201 (unpublished data), respectively. The specificity of these clones thus confirmed the significance of TNF responses detected at the polyclonal level (see Fig. 1 and 2).

2.4 New MAA epitopes detected by TIL on transfected COS cells are naturally presented by melanoma cells

Because the COS cell line has been devised to permit a high expression of transfected genes [11], it was important to determine whether the CTL target epitopes seen on transfected COS cells could be presented naturally by tumor cells at a sufficient density to induce a T cell response. To answer this question, we investigated whether TIL clones readily recognized tumor cell lines expressing the appropriate HLA and MAA. As shown in Fig. 4, recognition of several melanoma cell lines established that the B*3501-restricted epitope of Melan-A/MART-1, the A*6801-restricted epitopes of gp100 and the A*0201-restricted epitope of LAGE-1b could be presented naturally by melanoma cells for which expression of the corresponding antigen had been detected by RT-PCR or by immunolabeling (data not shown).

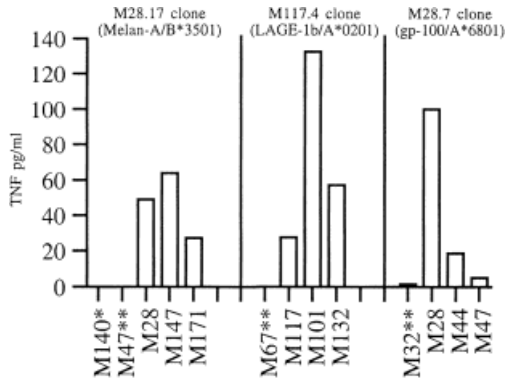

Fig. 4. Confirmation that epitopes recognized by TIL on transfected COS cells are also expressed by melanoma cell lines expressing the target antigen and the restriction element. TNF secretion by antigen-specific clones upon stimulation with melanoma cells. Negative controls were performed using melanoma cell lines that did not express the antigen and/or the restriction element. (■) TNF amount spontaneously secreted by tumor cells. (□) TNF amount secreted by TIL-derived clones in response to melanoma cells. * HLA-B*3501-negative cell line. ** Antigen-negative cell lines.

2.5 Summary of the data on MAA recognition by TIL and on restrictions of this recognition

Table 1 summarizes the HLA contexts in which MAA recognition by TIL have been tested and the results obtained. TIL were tested for 81 potential HLA-A restricted responses to MAA, in 7 different HLA-A contexts. Nineteen TIL responses were observed in HLA-A contexts, most of these in the context of HLA-A*0201. Five distinct MAA were recognized in this context. Only 1 or 2 TIL were restricted by HLA-A-*0101, *0301, *1101, *2402, and A68. No response was observed in a HLA-A29 context but the precise allele used for transfection could have been inappropriate for the 4 TIL tested. gp100 was recognized in the context of 5 different HLA-A alleles.

TIL were tested for 68 potential HLA-B-restricted responses to MAA, in 12 different HLA-B contexts. Only 5 of the HLA-B alleles tested (underlined) were found to restrict MAA recognition by 8 of these TIL. HLA-B*3501 restricted the recognition of 5 MAA by 3 TIL populations. HLA-B*4001 restricted the recognition of 3 MAA by 2 TIL populations. Finally, the other 3 HLA-B alleles restricted MAA recognition of a single TIL population. No response was observed in the context of many HLA B alleles, including some quite frequent alleles such as B*0701, B*0801, B*4402 and B*2705. Tyrosinase was recognized in the context of 3 distinct HLA-B alleles.

TIL were tested for 91 HLA-Cw/MAA combinations. Only 4 out of 12 HLA-Cw alleles (underlined in Table 1) were found to restrict MAA recognition, each one by one TIL population. No response was observed in the context of HLA-Cw*0701, which was the most frequent allele among the TIL under study. gp100 was the antigen most frequently recognized in an HLA-C context (in 3 alleles). The other MAA recognized by a TIL population in an HLA-C context were MAGE-6, LAGE-1b and gp75.

Fig. 5 illustrates the frequency of recognition of the different MAA under study in the 31 HLA contexts used. Melanocytic differentiation antigens were the most frequently recognized, as shown in Fig. 5A (Melan-A/MART-1>gp100>tyrosinase). The cancer-testis antigen LAGE-1b may be recognized as frequently as tyrosinase, but only 17 TIL populations were tested for recognition of this antigen. We showed that Melan-A/MART-1 recognition was mainly restricted by the HLA-A*0201 molecule, whereas the recognition of gp100 or tyrosinase occurred in various HLA contexts. Fig. 5 B shows that the HLA contexts in which antigen recognition was frequently found were: A*0201, B*3501, A*0301, B*4001, A*2402, Cw*0401 and 0501.

Table 1. HLA restrictions and frequency of MAA recognition

HLA-A	Fraction of specific TIL[a)]	MAA		HLA-B	Fraction of specific TIL[a)]	MAA		HLA-C	Fraction of specific TIL[a)]	MAA	
A*0101	1/10	gp-100	(1)[b)]	B*0702	0/10			Cw*0102	0/4		
A*0201	13/36	Melan-A	(11)	B*0801	0/9			Cw*0202	0/8		
		gp-100	(2)	B*1302	1/2	NA88-A	(1)	Cw*0303	0/5		
		Tyrosinase	(1)	B18	0/9			Cw*0401	1/14	MAGE-6	(1)
		NA17-A	(2)	B*2705	0/5					LAGE-1b	(1)
		LAGE-1	(1)	B*3501	3/11	Melan-A	(1)	Cw*0501	1/11	gp-100	(1)
A*0301	2/12	gp-100	(2)			gp-100	(3)			gp-75	(1)
		GAGE-5	(1)			Tyrosinase	(1)	Cw*0602	1/5	gp-100	(1)
		GAGE-3	(1)			MAGE-3	(1)	Cw7	0/11		
A*1101	1/7	gp-100	(1)			MAGE-6	(1)	Cw*0701	0/17		
				B*3503	1/2	LAGE-2	(1)	Cw*0802	1/5	gp-100	(1)
A*2402	1/9	GAGE-2	(1)	B*3508	0/1			Cw*1402	0/5		
		GAGE-5	(1)	B*4001	2/4	Tyrosinase	(1)	Cw15	0/2		
A29	0/4					gp-75	(1)	Cw*1601	0/4		
A68	1/3	gp-100	(1)			MAGE-1	(1)				
				B*4002	0/1						
				B*4402	0/9						
				B*4403	1/5	Tyrosinase	(1)				

a) Number of antigen-specific TIL populations/number of TIL populations sharing the same HLA molecule.
b) Total number of antigen-specific responses.

Table 2 shows that 9 TIL populations recognized several MAA in the same HLA context. Eight TIL populations recognized two melanoma antigens in HLA-A*0201, A*0301, B*3501, B*4001, Cw*0501 or CW*0401 contexts, and 1 population (M171) recognized 4 antigens in the HLA-B*3501 context.

3 Discussion

We performed a systematic screening of the MAA recognized by 59 melanoma TIL populations using transfected COS cells to present the epitopes from 28 MAA, capable of binding to 31 different HLA contexts. This method had been used to screen viral epitopes, as targets of joint infiltrating lymphocytes from chronic rheumatoid arthritis patients [12] and more recently, to look for melanosomal protein recognition by melanoma TIL, in 5 HLA-A contexts [9].

About half melanoma TIL populations (23 out of 59) contained T cells that recognized 1–3 of the MAA tested, in 1–3 different HLA contexts. Nonetheless, this remains certainly an underestimation, since (i) some MAA-specific TIL could have been lost during the culture, reaching levels too low to be detected, as described elsewhere (Pandolfino et al., submitted), (ii) only 31 HLA contexts were tested, out of the 80 expressed by these 59 TIL populations, and (iii) additional MAA likely exist. In support of this, 8 TIL populations failed to recognize COS cells, but recognized the autologous melanoma cells (Pandolfino et al., submitted).

HLA contexts of TIL responses were diverse (15 out of 31 of the HLA alleles tested). Recognition was more frequent in HLA-A, than B or C contexts. This prevalence of HLA-A restriction was due in part to the recognition of the Melan-A/MART-1 peptides 26–35 or 27–35 bound to HLA-A*0201. Furthermore, all but one HLA-A alleles

Table 2. A single HLA molecule frequently restricts the recognition of different MAA by TIL of a given tumor

HLA	TIL populations	Melanoma-associated antigen[a)]
	M101	Melan-A; gp-100
A*0201	M102	Melan-A; gp-100
	M117	Melan-A; LAGE-1b
A*0301	M45	gp-100; GAGE 3/5
	M28	Melan-A; gp-100
B*3501		
	M171	gp-100; Tyrosinase; MAGE-3; MAGE-6
B*4001	M99	gp-75; MAGE-1
Cw*0501	M45	gp-100; gp-75
Cw*0401	M91	MAGE-6; LAGE-1b

a) MAA recognized by TIL populations.

tested restricted TIL responses, while this was observed for less than half of the HLA-B and C alleles tested. Therefore, MAA presentation seems to be more efficient on HLA-A than on HLA-B or C alleles and HLA-A*0201 is the HLA-A allele the most efficient for MAA presentation (38% of HLA-A*0201 TIL recognized MAA in this context). HLA-B*3501 and HLA-B*4001 also appeared more efficient than other HLA-B alleles tested for presenting MAA peptides to TIL, since 3 of 11 and 2 of 4 TIL expressing these alleles recognized MAA in these contexts, respectively. HLA contexts most frequently involved in MAA recognition by TIL were those associated with the presentation of several MAA. Five MAA were recognized in the HLA-A*0201 context (Melan-A/MART-1, gp100, Tyrosinase, NA17-A and LAGE-1b) and in the B*3501 context (Melan-A/MART-1, gp100, tyrosinase, MAGE-3 and MAGE-6). It may be that the HLA alleles most frequently involved in MAA recognition bind to a wider diversity of peptidic sequences than other HLA.

Melanosomal proteins were the MAA most frequently recognized. Twenty TIL populations recognized at least one of these proteins and the majority of these TIL recognized either Melan-A/MART-1 or gp100, or both. A high frequency of recognition of these two antigens has also been observed using TAP-deficient cells pulsed with A-0201-restricted peptides [8–10].

Melan-A/MART-1 was clearly the MAA most frequently recognized by HLA-A*0201 TIL. In the present study, this recognition was observed by 30% of these TIL lines and

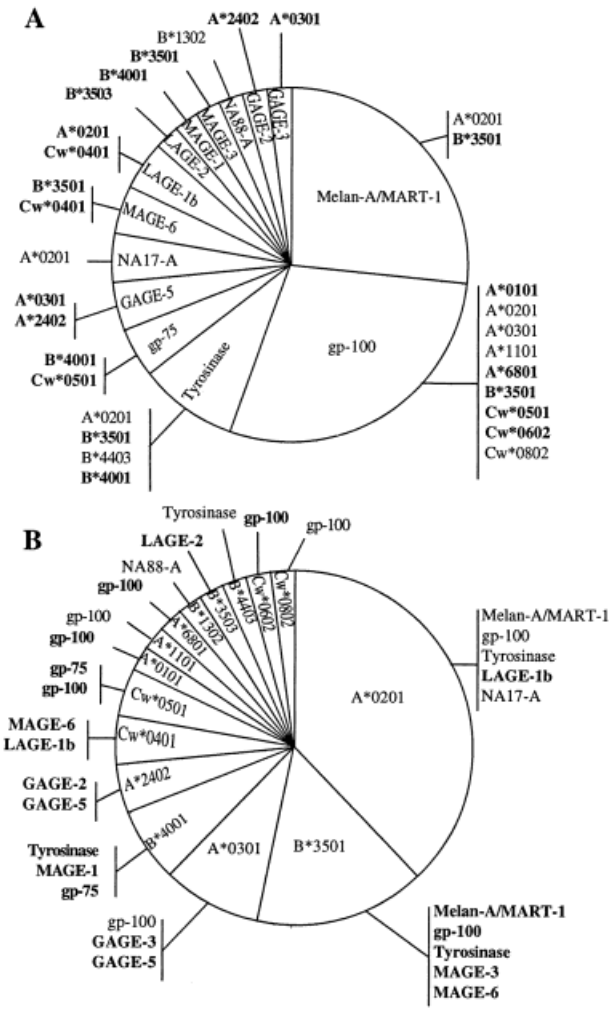

Fig. 5. Relative frequencies of recognition of different MAA by TIL and corresponding HLA restriction. (A) Relative frequencies of HLA TIL restrictions and (B) corresponding antigens. TIL populations indicated in bold characters recognized not described MHC/peptide complexes.

was directed against the same epitope(s), Melan-A/MART-1 26–35 and/or 27–35. Somewhat higher frequencies of recognition of these epitopes by TIL (between 40 and 56%) have been reported using peptide pulsed T2 cells [9, 10]. This suggests that lower peptide densities are induced on transfected COS cells by endogenous protein processing than on TAP-deficient T2 cells by peptide addition. Low-avidity T cell populations would, therefore, remain undetected by the COS assay. In support of this, we found one TIL population that reacted against Melan-A peptide pulsed-T2 cells but failed to react to transfected COS cells (data not shown).

The high immunodominancy of HLA-A0201-restricted Melan-A/MART-1 epitopes remains as yet unexplained. It

may be that the specific repertoire is especially large, which could be due to cross-reactivity with an environmental antigen. In support of this, remarkable peptide mimicry has been described [13]. Melan-A/MART-1 was also recognized by one TIL population in the B*3501 context, which suggests the existence of a new Melan-A epitope.

Unlike Melan-A/MART-1, gp100 was recognized by TIL in many different HLA contexts, and the previously described HLA-A*0201 binding gp100 epitopes [14] were not frequent targets of these. Only 2 out of 36 HLA-A*0201 TIL recognized these peptides, whereas 5 of these TIL recognized gp100 peptides in other HLA contexts. This suggests that HLA-A*0201-restricted gp100 peptides are not efficiently presented, or recognized. This is at variance with the high frequency of recognition of these peptides by TIL, reported by Kawakami et al. [8]. As stated above, for Melan-A/MART-1, this discrepancy may be due to lower HLA-peptide complex densities reached by the endogenous pathway of peptide presentation. Therefore, in many HLA-A*0201- and gp100-expressing melanomas gp100 is recognized more efficiently in non-HLA-A*0201 contexts, especially in the B*3501 context (3 out of 11 TIL expressing this allele). Among gp100 epitopes recognized in the present study, those restricted by A*0201, A*0301, A*1101 and Cw*0802 could correspond to epitopes already described [15–19]. The data show the existence of gp100 epitopes presented in new HLA contexts (A*6801, B*3501, Cw*0501, and CW*0602).

The third melanosomal protein, tyrosinase, although expressed by melanomas at a frequency similar to Melan-A/MART-1 and gp100 [10], was recognized much less frequently than these two proteins. Three of these responses were in already described HLA contexts: A*0201 [20], B*4403 [21] and B*3501 [22]. The other one was restricted by HLA-B*4001, a context not described so far. Since the recently described B*3501-restricted tyrosinase epitope [22] was not recognized by our TIL clone, these data show the existence of two new tyrosinase epitopes. They also show that known HLA-A*0201-restricted tyrosinase epitopes did not efficiently stimulate specific TIL in HLA-A*0201- and tyrosinase-expressing tumors, as observed for gp100.

gp75 recognition has been described before in the context of HLA-A31. Here we show that this melanosomal protein can also be recognized in two other HLA contexts (B*4001 and Cw*0501).

Collectively, the above data show that a high number of as-yet-undefined melanosomal protein epitopes can induce melanoma TIL responses. Since these proteins are frequently expressed in melanoma, identification of these peptides will provide useful tools for the development of new vaccination assays.

As suggested in previous studies [9], tumor-restricted MAA were recognized by TIL much less frequently than melanosomal proteins, (10 instead of 20 specific TIL populations), and none of these antigens were recognized by more than 2 TIL populations. Furthermore, only 10 out of the 28 tumor-restricted antigens analyzed were found to be TIL targets (LAGE-1b, 2, GAGE-2, 3, 5, MAGE-1, 3, 6, NA88-A and NA17-A).

MAGE- and GAGE-specific responses were developed by only few TIL populations (3 and 2 out of 59 tested, respectively). These data, together with the limited number of studies reporting MAGE-specific TIL [23, 24], indicate that lymphocytes specific for these antigens are uncommon in melanoma tumors.

Remarkably, TIL responses to MAGE, LAGE, and GAGE proteins were in new HLA contexts and were, therefore, directed against undefined epitopes. This underscores the usefulness of the “COS” method for studying the recognition of tumor-associated antigens, since a classical screening with candidate peptides would have failed to detect these responses.

The antigen NA17-A was recognized by two TIL populations in the HLA-A0201 context. RT-PCR, performed for a large series of melanoma patients for inclusion in an NA17-A peptide-based vaccination program, showed that this antigen is expressed by about 60% of melanomas (N.L., unpublished). The frequency of NA17-A recognition by TIL of patients expressing both this antigen and the HLA-restricting element is around 10%.

Two main hypothesis could explain the less frequent recognition of tumor-specific than of melanosomal proteins: (i) tumor-specific proteins are expressed at lower levels than melanosomal proteins, which gives rise to an expression by tumor cells of peptide densities unable to induce specific TIL expansion, or (ii) the T cell repertoire specific for non-self proteins is of higher avidity, and therefore more susceptible to tolerance induction by mechanisms such as clonal deletion or anergy than the repertoire specific for melanosomal proteins. It is critical that these questions are now addressed.

This study also led to the unexpected finding that recognition by TIL of different MAA is frequently restricted by a single HLA in individual tumors (see Table 2). This suggests that, in these tumors, peptide presentation is more efficient on a given HLA molecule. This could result from an over expression of this HLA molecule inside the tumor. In support of this, the dominant HLA restriction in one patient was the HLA-A*0301 allele, which was homozygous.

Finally, we observed a large diversity of antigen-derived epitopes recognized by TIL from different tumors, except for Melan-A/MART-1 antigen. This was especially clear for tyrosinase and gp100, two antigens expressed by a majority of melanoma tumors. Indeed, despite the existence of many HLA-A*0201-restricted peptides derived from these antigens, TIL responses were preferentially directed against new peptides presented in other HLA contexts.

In conclusion, this study presents important new data on the capacity of TIL to recognize most recently detected MAA in different HLA contexts. The heterogeneity of recognized epitopes between tumors and the frequent dominance of an HLA-restriction context in individual tumors would imply that immunization strategies developed so far against one or few epitopes may have failed to target the most immunogenic epitope in a majority of patients. This could be one of the reasons of the relative inefficiency of these therapies. Our data suggest that individually designed immunotherapies targeting HLA/peptide complexes that are dominant in the patient's tumor could be required in the future to treat melanoma patients.

The present approach is also applicable to many other tumor models to obtain a systematic inventory of T cell antigens, which is a necessary step for the development of immunotherapeutic treatments.

4 Materials and methods

4.1 Cell line and TIL culture

Melanoma cells lines were established from fragments of metastasic tumors or tumor invaded lymph nodes, cultured in RPMI 1640 (Biowhittaker, Walkersville, USA) medium containing 10% FCS (Gibco-BRL, Cergy-Pontoise, France), penicillin (10 μg/ml)/streptomycin (10 U/ml) (Sigma, St. Louis, USA) and L-glutamine (2 nM) (Sigma). Mouse fibrosarcoma WEHI 164 clone 13, used for TNF production assay, and COS-7 cells were obtained from T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). COS-7 cells were cultured in DMEM medium (Biowhittaker) containing 10% FCS, antibiotics and L-glutamine. The EBV-B-transformed cell line LAZ 338 was a gift from T. Hercend (Vertex Pharmaceutical, Abingdon, England). Polyclonal TIL were obtained from melanoma-invaded lymph nodes of stage IIb patients, by a 12-day culture in RPMI 1640 (Sigma) medium containing 8% human serum, antibiotics and L-glutamine and 150 U/ml recombinant IL-2 (Chiron, Amsterdam, Netherland). Polyclonal TIL amplification was performed using phytohemagglutinin (PHA-P) (Difco, Detroit, USA). Briefly, TIL were seeded in 96 multiplates at about 1,000 TIL per well, in the presence of irradiated feeder cells (2×10^4 LAZ cells and 10^5 PBL/well), PHA-P (15 μg/ml) and recombinant IL-2 (150 U/ml). Production of TIL clones was done in the same way but using limiting dilution culture of TIL.

4.2 Transfection of COS-7 cells

The COS cells were transfected with cDNA coding for the HLA alleles expressed by each patient and with the cDNA coding for the 28 following melanoma-associated antigen (MAA): Melan-A/MART-1, Tyrosinase, gp100, gp75**,** MAGE-1, 2, 3, 4, 5, 6, 12, BAGE, PRAME, GAGE-1, 2, 3, 4, 5, 6, RAGE-1, 3, 4, LAGE-1a, LAGE-1b, LAGE-2/NY-ESO-1, NA17-A, NA88-A and Her-2 neu. The cDNA coding for HLA molecules were obtained from T. Boon (LICR, Brussels, Belgium) and from E. Houssaint (Unité 463 INSERM, Nantes, France). Most cDNA coding for MAA were also obtained from Thierry Boon, NA88-A was cloned in our laboratory [5] and Her-2 neu was a gift from Kostas Kosmatopoulos (Unité 484 INSERM, Villejuif, France). Transfection procedures were performed by the DEAE-dextran-chloroquine method [20, 25]. In brief, 16.5×10^3 COS-7 cells were co-transfected with 100 ng of plasmid coding for an HLA and 100 ng of plasmid coding for an MAA. Transfected COS cells were then used to stimulate patients TIL or TIL-derived clones.

4.3 T cell stimulation assay

TIL (5×10^3–5×10^4), or T cell clones (2×10^3–10^4), were added to transfected COS-7 cells 24–48 h after transfection. For T cell stimulation by melanoma cells, T cell clones (10^4 cells) were stimulated in triplicate cultures by melanoma cells (3×10^4). Culture supernatants were harvested 6 h later and tested for TNF content. TNF determination was done by a biological assay measuring the cytotoxicity of culture supernatant on the highly sensitive WEHI 164 clone 13 in a MTT colorimetric assay, as previously described [11].

4.4 RT-PCR assays for the detection of melanoma antigen RNA expression

Total RNA was extracted from tumor-invaded lymph node fragments or from tumor cell lines by the guanidinium-cesium chloride procedure. Reverse transcription was performed on 2 μg total RNA in a reaction volume of 20 μl with 4 μl of 5× reverse transcription buffer (Life Technologies), 2 μl of 20 μM oligo (dT-15) primer solution (Life Technologies), 4 μl of 10 mM dNTP mix (Boehringer Mannheim, Germany), 20 U RNAsin (Promega, Madison, WI), 2 μl 100 μM DTT and 200 U M-MLV reverse transcriptase (Life Technologies). The reaction was incubated for 60 min at 42°C, and the volume was then adjusted to 100 μl with distilled water. PCR amplifications of human β-actin, gp100 tyrosinase Melan-A/MART-1 and LAGE-1 cDNA were performed as previously described [3, 26]. Ten microliters of PCR products were then size-fractionated on 1% agarose gel.

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