Unique Tumor Antigens: Evidence for Immune Control of Genome Integrity and Immunogenic Targets for T Cell – Mediated Patient-Specific Immunotherapy

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Abstract The molecular identification and characterization of antigenic epitopes recognized by T cells on human cancers has rapidly evolved since the cloning in 1991 of *MAGEA1,* the first gene reported to encode a CTL-defined human tumor antigen. In the expanding field of human tumor immunology, unique tumor antigens constitute a growing class of T cell – defined epitopes that exhibit strong immunogenicity. Some of these antigens, which often derive from mutation of genes that have relevant biological functions, are less susceptible to immunoselection and may be retained even in advanced tumors. Immunogenicity and constitutive expression of the unique tumor antigens provide a strong rationale for the design of novel, patient-tailored therapies that target such determinants. Here we discuss the immunologic relevance of unique tumor antigens in the light of the prospects for exploiting such epitopes as targets for patient-specific immune intervention strategies.

Tumor-specific transplantation antigens, leading to complete T cell – mediated elimination of a transplanted cancer by the syngeneic host, have been described on rodent chemical or UV-induced fibrosarcomas more than 40 years ago (1 – 4). The specificity of the immune response documented *in vivo* and *in vitro* against independently induced tumors led to the concept that the tumor-specific transplantation antigens were unique (i.e., expressed only by a syngeneic tumor but not by other tumors even when promoted by the same carcinogen and in the same mouse; refs. 1 – 4). After the identification of tumor-specific transplantation antigens, the search for the molecular basis of unique tumor antigens defined by T cells has been a major area of investigation for the understanding of the immune response to neoplastic cells. In 1995, the identification of a single amino acid substitution in cellular proteins (5, 6) provided evidence for the origin of this class of determinants from somatically mutated genes. These initial results confirmed earlier evidence on the mechanism that generated tumor-specific antigens after mutagen treatment of preexisting tumors (7). Somatic mutation was soon verified in human tumors as one of the mechanisms for generation of antigens recognized by T lymphocytes only on the autologous and not on HLA-matched tumors (8). This provided the evidence for the role of the immune system in surveillance of genome integrity. In the first study by Coulie et al. (8) describing the molecular basis of unique tumor antigens on a human tumor, $CD8^+$ T cells from a patient with metastatic melanoma were shown to recognize, in the context of the HLA class I allele HLA-A2, a nine-amino-acid-long peptide, which included a point mutation occurring in one intron of the *melanoma associated* (*mutated*) *antigen – 1* (*MUM1*) gene. This study, along with the previous work on the cloning of the first gene coding for a shared human tumor antigen (*MAGEA1*; ref. 9), also provided direct evidence for the power of the cDNA expression library as an effective strategy to identify genes encoding tumor antigens recognized by HLA-restricted T cells.

Over the past 15 years, a large variety of tumor associated-antigens has been identified. A number of these determinants were shown to be unique antigens encoded by somatically altered, and mainly point-mutated, genes (Table 1; refs. 8, 10 – 37). The investigation on the molecular nature of epitopes recognized by antitumor T cells (see ref. 38 for a listing of human tumor antigens recognized by T cells) has not only shed light on the genetic mechanisms that generate unique tumor antigens but has also allowed investigators to identify genes that, when altered, play a relevant role in tumor biology. In fact, unique antigens recognized by patient's $CD8^+$ or $CD4^+$ T cells have been shown to be encoded by genes that regulate several cellular processes such as cell cycle (11, 19, 20, 26), adhesion and motility (10, 21, 23, 30), apoptosis (28), stress response (16, 17, 36), RNA processing (20, 22), transcriptional activation and silencing (20, 32), and general metabolic pathways (refs. 13, 14, 19, 25, 29, 31; Table 1). Interestingly, in several instances, the genetic alterations that yield immunogenic T-cell epitopes are also responsible for inducing

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Table 1. HLA class I– and class II–restricted tumor-specific unique antigens recognized by T cells on single human cancers

Tumor	T-cell origin	Gene name by HUGO Gene Nomenclature Committee	Biological function of the normal protein
Melanoma	TIL	catenin (cadherin-associated protein), β1[†‡]	Cell-cell adhesion Wnt signaling
Melanoma	PBL	cyclin-dependent kinase 4[†§]	Cell cycle
Melanoma	TIL	hedgehog acyltransferase	Hedgehog signaling
Melanoma	PBL	melanoma associated (mutated) antigen-1[‖§]	Unknown
Melanoma	PBL	melanoma associated (mutated) antigen-2[‖§]	Protein transport
Melanoma	PBL	melanoma associated (mutated) antigen-3[‖§]	Nucleic acid metabolism
Melanoma	TIL	myosin class I	Cellular motility
Melanoma	PBL	peroxiredoxin 5	Oxidative stress
Melanoma	PBL	OS-9	Regulation of hypoxic responses
Melanoma	TIL	major histocompatibility complex, class I, A11[‡]	Histocompatibiliy antigen
Melanoma	TIL	growth arrest-specific 7[‖‡]	Cell cycle
Melanoma	TIL	glyceraldehyde 3-phosphate dehydrogenase[‖‡]	Energy metabolism
Melanoma	PBL	sirtuin-2[‖§]	Transcriptional silencing
Melanoma	PBL	glycoprotein (transmembrane) nmb[‖§]	Melanosomal protein
Melanoma	PBL	elongation factor Tu GTP binding domain containing 2[‖§]	RNA processing
Melanoma	PBL	zinc finger, UBR1 type 1[‖§]	Cell cycle
Melanoma	PBL	small nuclear ribonucleoprotein D1 polypeptide 16 kDa[‖§]	RNA processing
Melanoma	TIL	protein tyrosine phosphatase, receptor type, K[§]	Cell-cell adhesion
Melanoma	TIL	neo-poly(A) polymerase	RNA processing
Melanoma	TIL	fibronectin	Extracellular matrix component
Melanoma	TIL	low-density lipoprotein receptor/fucosyltransferase 4	—
Melanoma	TIL	triosephosphate isomerase 1	Energy metabolism
Melanoma	TIL	cell division cycle 27	Cell cycle
Melanoma	PBL	antigen recognized by Treg cells-1[**]	Unknown
Head and neck squamous cell carcinoma	PBL	caspase 8, apoptosis-related cysteine peptidase	Apoptosis
Lung non–small-cell carcinoma	PBL	eukaryotic translation elongation factor 2	Protein translation
Lung non–small-cell carcinoma	TIL	actinin, α4[§]	Adhesion
Lung non–small-cell carcinoma	PBL	malic enzyme[§]	Energy metabolism
Lung non–small-cell carcinoma	TDL	nuclear transcription factor Y, γ	Transcriptional activator
Bladder carcinoma	PBL	lysophosphatidylglycerol acyltransferase 1	Phospholipids biosynthesis
Renal cell carcinoma	PBL	major histocompatibility complex, class I, A2	Histocompatibility antigen
Renal cell carcinoma	PBL	integrator complex subunit 1	pre-mRNA processing
Renal cell carcinoma	TIL	heat shock 70-kDa protein 1B	Heat shock protein
Colorectal carcinoma	PBL	colorectal tumor-associated antigen-1	Unknown

Abbreviations: TIL, tumor-infiltrating lymphocytes; PBL, peripheral blood lymphocytes; TDL, tumor draining lymph nodes.
[*]Bold residues in the epitope indicate the mutation. The wild-type residue is shown in brackets. The mutations may create a novel HLA anchor residue or a TCR contact residue.
[†]Antigens encoded by genes of which the alteration, although rare, can be shared by more than one tumor.
[‡]Antigens recognized by TIL able to mediate tumor regression following adoptive transfer.
[§]Antigens recognized by T cells isolated from long-term survivors.
[‖]Antigens recognized by T cells of a single patient: melanoma-associated (mutated) antigen-1, -2, -3 identified by T cells from patient LB33; growth arrest-specific 7 and glyceraldehyde 3-phosphate dehydrogenase identified by T cells from patient 2098; sirtuin-2, glycoprotein (transmembrane) nmb, elongation factor Tu GTP binding domain containing 2, zinc finger UBR1 type 1, and small nuclear ribonucleoprotein D1 polypeptide 16 kDa identified by T cells from patient DT.
[¶]For these antigens, the mutation was not contained within the sequence of the antigenic peptide, but had an indirect effect involving antigen processing (22), relocalization (26), or generation of new proteins (27, 28).
[**]This antigen was recognized by HLA class II–restricted $CD4^+$ $CD25^+$ regulatory T cells.

Table 1. HLA class I– and class II–restricted tumor-specific unique antigens recognized by T cells on single human cancers (Cont'd)

HLA allele	Peptide*	Mechanism of generation	Wild-type allele	Reference
A24	SYLDSGIH**F** (S) HLA anchor	Mutation	Present	10
A2	A**C**DPHSGHFV (R) HLA anchor	Mutation	Present	11
A1	FL**E**GNEVGKTY (G) HLA anchor	Mutation	Present	12
B44	EEKL**I**VVLF (S) TCR contact	Mutation	Present	8
B44	SELFRS**G**LDSY (R) TCR contact	Mutation	Present	13
C6	FRS**G**LDSYV (R) TCR contact	Mutation		
A68	EA**F**IQPITR (S) HLA anchor	Mutation	Present	14
A3	**K**INKNPKYK (E) TCR contact	Mutation	Present	15
A2	LLLDD**L**LVSI (S) TCR contact	Mutation	Absent	16
B44	KELEGILL**L** (P)	Mutation	Absent	17
—	—	—	—	18
A2	SLADEAEV**Y**L (H) LADEAEV**Y**L (H)	Mutation	Present	19
A2	GIVEGL**I**TTV (M) GIVEGL**I**TT (M)	Mutation	Present	19
A3	KIFSEVT**L**K (P)	Mutation	Present	20
A3	TL**D**WLLQTPK (G)	Mutation	Present	20
A3	KLDAVVAQ**K** (E)	Mutation	Present	20
B7	**R**PHVPESAF (G)	Mutation	Present	20
B38	SHETV**I**IEL	Mutation	Present	20
DR10	PYYFAAELPP**R**NLPEP (G)	Mutation	Absent	21
DR7	Indirect effect**	Mutation	Present	22
DR2	MIFE**K**HGFRRTTPP	Mutation	Present	23
DR1	Fusion peptide	Chromosomal rearrangement	Present	24
DR1	GELIG**I**LNAAKVPAD (T) TCR contact	Mutation	Present	25
DR4	Indirect effect**	Mutation		26
DR1	Indirect effect**	Mutation	Absent	27
B35	Indirect effect**	Mutation	Present	28
A68	ETVSE**Q**SNV (G) TCR contact	Mutation	Present	29
A2	FIAS**N**GVKLV (K) TCR contact	Mutation		30
A2	FLDEFME**G**V (A)	Mutation	Absent	31
B52	AQQIT**K**TEV (Q)	Mutation	Absent	32
B44	AEPI**N**IQTW (D)	Mutation	Present	33
–	—	Mutation	—	34
A1	QT**A**CEVLDY (T)	Mutation	Present	35
A2	SLFEGID**I**YT (F) TCR contact	Mutation	Present	36
DR4, DR13	Indirect effect**	Mutation	Present	37

significant change of function in the corresponding protein. For example, the mutated proteins may behave as a dominant oncogene *in vivo* (11), or disrupt tumor suppressor pathways (10), or affect tumor migration and metastasis (refs. 23, 30; Table 1). For these reasons, the investigation on the molecular basis of unique tumor antigens can be placed at the crossroads of cancer genetics, tumor biology, and tumor immunology.

Definition of unique tumor antigens. Table 1 lists the somatically altered and mainly point-mutated epitopes recognized by T lymphocytes from peripheral blood or tumor site of cancer patients that are "strictly" unique (i.e., only expressed in the neoplastic cells from a single tumor). By this strict definition, unique tumor antigens include also the idiotypic determinants of the rearranged immunoglobulin of B-cell malignancies (39). It is to be pointed out that such classification of unique tumor antigens is not straightforward as it seems. In fact, in few instances, the "uniqueness" of the tumor antigen is dictated by the HLA constraint on T-cell recognition, rather than by the presence of the altered gene only in one tumor. For example, identical mutations in *CDK4* and

β-catenin genes (Table 1) have been described in more than a single melanoma. However, such mutations will not lead to the generation of the antigenic determinant in patients that do not express the required HLA restricting element for presentation of the relevant epitope.

Furthermore, gene alterations like mutations occurring in oncogenes or tumor suppressor genes, or chromosomal translocations encoding novel fusion proteins, can yield additional classes of tumor-specific epitopes (40–49). These determinants cannot be classified as "unique tumor antigens" because the mutations are frequently shared by several tumors (representative examples are listed in Table 2). These tumor-specific, mutant epitopes include the shared antigens resulting from mutations in *KRAS*, *NRAS*, and *BRAF* oncogenes or generated by mutations in *p53* tumor suppressor gene (40–42, 50). The available evidence indicates immunogenicity, *in vivo*, of at least some of these determinants. This has been documented not only by the presence of antigen-specific T cells in patients but even by the results obtained in clinical trials of immunotherapy targeting such antigens. In fact, recently, significantly longer survival has been described in cancer patients with positive versus negative CTL responses after immunization against mutant p53 and mutant KRAS peptides (50). Epitopes generated by chromosomal translocations include those derived by fusion proteins, such as BCR-ABL, DEK-CAN, and ETV6-AML1 in different leukemias (45, 47, 48), SYT-SSX in synovial sarcomas (44), and PAX-FKHR in alveolar rhabdomyosarcomas (49). In some instances, immunogenicity of these determinants has been shown *in vivo* based on a high frequency of antigen-specific T cells found in a fraction of patients (45, 48). In addition, in a recent clinical trial in synovial sarcoma patients vaccinated with SYT-SSX–derived peptides, peptide-specific CTLs were induced in four of six patients and suppression of tumor progression was documented in one of six patients (51).

Immunogenicity and immunodominance: frequent features of unique tumor antigens. One of the key questions, with respect to the antigenic repertoire of neoplastic cells, is which of the various classes of antigens thus far identified is the most relevant for tumor rejection and for the development of antigen-specific immunotherapy approaches. The coexpression of unique and shared antigenic determinants on neoplastic cells, which has been reported three decades ago, has offered the possibility to compare these two classes of determinants. In fact, early evidence obtained in the mid 1970s indicated that murine plasmacytomas and chemical carcinogen–induced fibrosarcomas expressed both unique and shared tumor-associated antigens (52–54). The finding that a single experimental tumor could express multiple tumor-associated antigens (TAA) of different classes (unique and shared) prompted investigations on the relative immunodominance of these determinants. The murine models investigated by Schreiber and colleagues in the early 1980s indicated that a single tumor could express multiple tumor-specific antigens and that there was a hierarchy of immunodominance among the epitopes. In fact, some of the TAA could be recognized only when the immunodominant epitopes had been lost through T cell–mediated immunoselection (55, 56). By exploiting such

Table 2. Examples of shared tumor antigens generated by gene mutation or chromosomal translocation

Genetic alteration	Present in	Source of T cells	*In vitro* stimulation with	HLA-restriction element	Recognition of HLA-matched targets	Reference
Mutations						
$NRAS^{Q61R}$	Melanoma	PBL from patients	Autologous tumor	A1	$NRAS^{Q61R}$ melanomas	(40)
$KRAS^{G12V}$	Pancreatic adenocarcinoma	PBL from patients	Mutated peptides	B35	$KRAS^{G12V}$ pancreatic adenocarcinomas	(41)
$BRAF^{V600E}$	Melanoma	PBL from patients	Mutated peptides	DR4	$BRAF^{V600E}$ melanomas	(42)
CDKN2A	Melanoma		Autologous tumor	A11	$p14^{ARF-ORF3}$- and $p16^{INK4A-ORF3}$ melanomas	(18)
Microsatellite instability (MSI)- induced frameshift mutations in different genes	Colorectal, gastric, and endometrial carcinomas	PBL from healthy donors	Frameshift peptides	A2	MSI^{+} cancer cells	(43)
Chromosomal traslocations						
SYT-SSX	Synovial sarcoma	PBL from patients	Fusion peptide	A24	Synovial sarcoma cell lines	(44)
BCR-ABL	Chronic myeloid leukemia	PBL from healthy donors	Fusion peptides	A2, A3, A11, B8	Leukemic cells	(45)
PML/RARα	Promyelocytic leukemia	PBL from healthy donors	Fusion peptide	DR11	Autologous LCLs transduced with the fusion gene	(46)
DEK/CAN	Acute myelogenous leukemia	PBL from healthy donors	Fusion peptide	DR53	Dendritic cells loaded with killed leukemic cells	(47)
ETV6-AML1	Acute lymphoblastic leukemia	PBL from healthy donors	Fusion peptides	A2	Leukemic cells	(48)
PAX-FKHR	Alveolar rhabdomyosarcoma	PBL from healthy donor	Fusion peptide	B7	Rhabdomyosarcoma cells	(49)

approach of CTL-mediated immunoselection, on a chemically induced tumor, Dudley and Roopenian (57) showed that the same tumor expressed two unique and one shared epitope. However, the shared epitope was the weakest, and the response against such determinant could be revealed only in immunoselected variants lacking the two strong unique antigens. These initial studies provided also the earliest evidence for the shaping effect of the immune system on tumor immunogenicity, a concept that Dunn et al. (58) have later placed at the basis of the cancer immunoediting hypothesis.

In agreement with results initially obtained in experimental tumors, it soon became clear that even human tumors can coexpress both unique and shared determinants and that the immune repertoire of patients was not homogeneously distributed against the different classes of TAA. For example, the clonal analysis of the CTL-mediated response to human melanoma cells provided the evidence for coexpression of at least four classes of TAA on the same tumor (59, 60). These classes included operationally-defined "unique" antigens. Such epitopes were defined by the reactivity of T cells that recognize only autologous tumor and not large arrays of allogeneic HLA-matched normal or neoplastic cells from different tissues. By a limiting dilution analysis designed to discriminate the recognition of melanoma cells and of normal HLA-matched melanocytes, we found that the HLA-A*0201–restricted $CD8^+$ immune repertoire to melanoma, in terms of precursor frequency, was mostly directed to antigens expressed on neoplastic cells and not on normal melanocytes (60). These results indicated not only coexpression of different classes of antigens on human melanoma cells but also immunodominance of the epitopes expressed only on the neoplastic cells over those shared with normal melanocytes (60). Similarly, the analysis of HLA-A3–restricted CTLs from a single patient indicated the simultaneous expression of both unique and shared TAA not found on HLA-matched melanocytes (61). In agreement with these results, Lennerz et al. (20) have recently identified several new differentiation antigens and new gene mutations yielding at least five new unique antigens expressed by the neoplastic cells of a single melanoma patient. Interestingly, the T cell–mediated response from blood lymphocytes isolated over a 4-year interval from this patient showed that the response was dominated by T cells directed to the unique mutation-derived antigens (20), suggesting immunodominance of such epitopes over the shared antigens expressed by the same tumor. It is, however, to be pointed out that an immune response to immunodominant epitopes is not necessarily associated with protective immunity. This is true not only in the antitumor response but it has been shown even in viral infections (62).

A peculiar opportunity to compare unique and shared human tumor epitopes for immunogenicity and immunodominance has been offered by analysis of response to the idiotypic surface immunoglobulin expressed by B-cell malignancies. In this instance, the immunoglobulin heavy chain encodes both T cell–defined unique epitopes that derive from the hypervariable segments (63) and shared epitopes that derive from nonmutated, germ-line framework sequences (64). In a recent study, Baskar et al. (65) looked at the fine specificity of the $CD4^+$ and $CD8^+$ T-cell response elicited in follicular lymphoma patients by vaccination with tumor-derived immunoglobulin conjugated to keyhole limpet hemocyanin and mixed with granulocyte macrophage colony-stimulating factor. The authors found that most of the vaccinated patients exhibited a vaccine-specific T-cell response. However, interestingly, such T-cell response was mainly directed to multiple epitopes within the hypervariable regions (CDR2 and CDR3) of the tumor immunoglobulin, and not to peptides derived from the framework regions of the same idiotypic protein (65). In agreement with previous results obtained in experimental models (57), this evidence suggests that unique, idiotypic epitopes of the tumor immunoglobulin are immunodominant over the shared determinants contained within the sequence of the same immunoglobulin. Moreover, these results suggest that the T-cell response to such unique antigens may contribute, together with anti-idiotypic humoral immunity (66), to the clinical efficacy of idiotypic vaccination documented in B-cell tumor patients (39, 67).

T-cell response to unique tumor antigens: evidence from regressing lesions and from long-term survivors. *In vivo* immune response to unique human tumor antigens has been documented in melanoma and lung cancer patients by looking at the presence, frequency, and function of antigen-specific T cells in regressing lesions or in long-term survivors. Clearly, this evidence does not necessarily mean that such immune response had a protective effect against tumor growth. However, the T-cell response to an immunogenic tumor antigen is expected to generate a high frequency of antigen-specific T cells and/or to yield differentiated T cells endowed with fast, secondary expansion *in vitro* on rechallenge with antigen. In agreement with these predictions, in a patient showing spontaneous regression of a primary melanoma, Zorn and Hercend (15) found a highly expanded $TCRBV16^+$ T-cell population directed to a unique tumor antigen generated by a point mutation in a *myosin* gene. This finding suggests a role of the response to such unique determinant in the observed tumor regression. In another study, $CD4^+$ T cells directed to a unique melanoma antigen generated by a mutant receptor-like tyrosine phosphatase κ were found not only in a metastatic lesion but even in peripheral blood lymphocytes obtained almost 6 years later, when the patient was still disease-free (21). Interestingly, T cells from peripheral blood lymphocytes and from tumor site showed antigen-specific IFN-γ production in ELISPOT, without prior *in vitro* activation, consistent with previous priming *in vivo* and differentiation to effector stage (21). Presence in peripheral blood of a high frequency of T cells directed to unique tumor antigens and/or rapid T-cell expansion *in vitro* on restimulation with antigen has been documented even in long-term survivors after surgery for lung cancer. Thus, in a disease-free lung cancer patient, by HLA tetramer analysis, antigen-specific T cells directed to a mutated malic enzyme have been found at frequencies of 0.1% to 0.4% of the $CD8^+$ cells up to 10 years after initial surgery for squamous cell carcinoma (31). In another patient, remaining disease-free after removal of a large-cell carcinoma of the lung, low-frequency T cells specific for a mutated *α-actinin-4* gene, isolated 4 years later from peripheral blood, could readily be expanded *in vitro* on restimulation with antigen (30). Interestingly, when *α-actinin-4*–specific T cells from tumor site and peripheral blood were compared, the authors found that only the antigen-specific T cells with high functional avidity (i.e., T cells showing effective target lysis and/or cytokine secretion in response to low antigen doses) were expanded at tumor site whereas a

low-avidity T-cell clone was expanded only in periphery (68). Taken together, these studies provide strong evidence consistent with immunogenicity of unique tumor antigens expressed in human tumors and for selective expansion at tumor site of functional T cells directed to such epitopes. They also suggest that immune response to unique tumor antigens may even contribute to the favorable clinical evolution observed in the patients in whom such epitopes have been identified.

Immune selection: shared versus unique tumor antigens. Tumor escape mechanisms are a major hurdle for successful immunotherapy, and several studies support the evidence for the generation of antigen-loss variants in tumors subjected to the selective pressure of the immune response. Evidence consistent with such process has been frequently obtained in melanoma patients treated with immunotherapy targeting shared differentiation antigens. In an early study, in melanoma patients treated with immunogenic peptides from melanoma differentiation antigens gp100 or Melan-A/Mart-1 (+/− interleukin 2), Riker et al. (69) found an increase in the frequency of gp100- or Melan-A/Mart-1 negative lesions after therapy. In addition, pretherapy lesions with high expression of gp100 were more frequent in responding patients than in nonresponding ones (69). These initial results suggested that vaccination against a class of nonmutated self-antigens can result in immunoselection *in vivo*, and that antigen expression in pretherapy neoplastic cells may be relevant in predicting the response to therapy. In a subsequent study in patients vaccinated against gp100, the same authors found that immunoselection occurred in responding lesions, without interfering with tumor regression, and did not occur in nonresponding lesions (70). Appearance of antigen-loss variants has been described even after adoptive immunotherapy with Melan-A/Mart-1 and gp100-specific T-cell clones (71). On the other hand, in a recent study, immunohistochemical analysis of antigen expression in recurrent tumors of melanoma patients vaccinated with a gp100 "anchor-modified" peptide did not provide conclusive evidence on the generation of antigen-loss variants, in spite of the generation of high levels of tumor antigen–specific T cells in all these patients (72).

In contrast to the results obtained by looking at immune selection in the response to shared tumor antigens, different studies suggest that at least some unique tumor antigens may be resistant to immune selection in spite of their immunogenicity. Recently, in a melanoma patient in whom we identified a unique tumor antigen, recognized by HLA-A*0201–restricted T cells, we had the opportunity to evaluate immunogenicity and retention in neoplastic cells of unique and shared TAA expressed by the same tumor (16). The unique antigen was encoded by a mutated *peroxiredoxin 5* (*Prdx5*) gene expressed in a tumor lacking the wild-type allele (16). Because the patient expressed HLA-A2, we had the opportunity to compare the response against the unique antigen with that against a shared antigen (Melan-A/Mart-1_{26-35}) recognized on the same tumor in the context of the same HLA restricting element. By HLA tetramer analysis, we found that mutant Prdx5-specific T cells were present in a tumor-invaded lymph node at a frequency similar to Melan-A/Mart-1–specific T cells, but much higher than gp100-specific T cells (16). Moreover, >50% of the T cells directed against Prdx5, or against Melan-A/Mart-1, showed a differentiated "$T_{Central\ memory}$" or "$T_{Effector\ Memory}$" phenotype consistent with previous priming and differentiation *in vivo* (16). We also found that the mutant peroxiredoxin enzyme retained its antioxidant activity, protecting cells from oxidative stress (16). Moreover, the mutated protein was expressed in all cells from a lymph node metastasis whereas expression of other shared antigens (Melan-A/Mart-1, tyrosinase, and gp100) showed strong intratumor heterogeneity (16). These results suggest that unique tumor antigens that behave as mutant Prdx5 may, in principle, be considered as attractive targets for immunotherapy for at least two reasons: immunogenicity and "resistance" to loss of expression by immunoselection even in advanced cancers. Evidence consistent with resistance of at least some unique antigens to immunoselection has recently been described in patients vaccinated against the B-cell lymphoma idiotype (73). In fact, in six mantle cell lymphoma patients treated with a B-cell idiotype–specific vaccine (73), administered after chemotherapy plus rituximab, the analysis of relapsing tumors indicated no mutations or change of expression of the surface immunoglobulin in spite of significant tumor-specific T-cell responses elicited in 87% of the 26 patients (73). Although previous studies (74) had shown the emergence of idiotype-negative B-cell tumors, after immunotherapy with anti-idiotypic monoclonal antibodies, the more recent results (73) suggest that it is possible to boost *in vivo* the T-cell response to a unique tumor antigen without inducing antigen-loss variants of the tumor.

Evidence for immune recognition of unique tumor antigens in patients responding to immunotherapy. Immunogenicity, immunodominance, and evidence for resistance to immunoselection are attractive features of tumor antigens to be used as targets of immunotherapy approaches. The previously discussed evidence suggests that at least some unique tumor antigens may have such characteristics. On the other hand, the need for identifying the genes coding for such determinants in each patient has often hampered the development of clinical studies of immunotherapy aimed at boosting immunity to such class of epitopes. For this reason, and with the significant exception of idiotype vaccines in B-cell malignancies (39, 65–67), most immunotherapy studies thus far have targeted the class of shared tumor-associated antigens (75, 76) or have been designed to induce response to the whole array of determinants expressed by a tumor, without prior knowledge on the expression of unique determinants (77–79). In spite of these limitations, results from different clinical studies have indicated that patients subjected to active or adoptive immunotherapy (10, 18, 19, 80–84) may develop responses to unique tumor antigens (Table 3). Early clinical trials of adoptive T-cell transfer in melanoma patients indicated that significant clinical responses were associated with the infusion of T cells recognizing different antigens, including melanocyte lineage differentiation antigens, cancer testis antigens, and unique antigens (Table 3; refs. 10, 81–84). More recently, remarkable clinical responses have been observed in patients who received adoptive transfer of tumor-reactive tumor-infiltrating lymphocytes and nonmyeloablative chemotherapy (85). In these patients, a systematic approach to identify the tumor antigens recognized by the adoptively transferred T cells has indicated that unique tumor antigens are a relevant class of epitopes targeted by T cells that persist *in vivo* in responding patients (Table 3; refs. 18, 19, 80, 86, 87). Even in tumors different from melanoma, response to unique antigens has been found to be associated with clinical response. In a metastatic renal cell

carcinoma patient vaccinated with granulocyte macrophage colony-stimulating factor–transduced autologous tumor cells, and showing strong clinical response to the vaccine, Zhou et al. (35) found induction of a heterogeneous $CD8^+$ T-cell repertoire directed not only to shared antigens but also to a new unique antigen encoded by a *mutated integrator complex subunit 1* (*KIAA1440*) gene. Finally, current evidence indicates that even strategies involving active immunization with known melanocyte lineage differentiation antigens or cancer testis antigens can boost/prime response to previously unrecognized unique tumor antigens. Lurquin et al. (88) investigated the frequency of T cells in peripheral blood and tumor site of a melanoma patient vaccinated against a shared cancer testis antigen (MAGEA3). The results indicated that postvaccine lesions had a dramatic increase in frequency of T cells directed not to the MAGEA3 epitope but to other TAA. Interestingly, one of the T-cell clonotypes that were found at high frequency in post-vaccine lesions, compared with prevaccine metastases or peripheral blood, was directed to a potential new unique antigen generated by mutation of a caseinolytic protease (88). These latter results suggest that vaccination against shared tumor antigens may, at tumor site, provide conditions that favor activation of T cells directed to several different TAA, including unique TAA.

Strategies for the identification and targeting of unique tumor antigens in solid tumors. The general strategy for the identification of genes coding for tumor-associated antigens recognized by T cells depends on the availability of tumor-specific T-cell clones (see Fig. 1A for an outline of the strategy to identify unique tumor antigens defined by $CD8^+$ T cells). In the instance of T cells that are thought to be directed to unique tumor antigens, T-cell clones are initially selected on the basis of reactivity only against the autologous tumor and not against panels of HLA-matched allogeneic tumors of the same histologic origin. The approach shown in Fig. 1A has been extensively used in the past to characterize most of the known unique tumor antigens described thus far (see Table 1) and is based on the expression, in appropriate recipient cells (such as COS cells), of cDNAs isolated from autologous tumor together with the HLA restricting element. This strategy has a significant limitation: only one antigen at a time can be identified. More recently, this approach has been modified and improved by Lennerz et al. (ref. 20; Fig. 1B). The first change was the usage of polyclonal T-cell lines obtained by isolation of $CD8^+$ T cells from short-term autologous mixed lymphocyte-tumor cell cultures (MLTC). The second modification of the cloning strategy was the introduction of an additional screening for recognition of COS cells transfected with known tumor antigens (to rule out recognition of previously identified antigens), as well as with each of the different HLA class I alleles of the patient (Fig. 1B). In addition, the read-out system (based on cytokine production by T cells) was modified by introducing a quantitative assay (ELISPOT). On the basis of frequency of cytokine-producing T cells, such quantitative assay allows to discriminate distinct effectors, present even in the same polyclonal T-cell line and directed against different tumor-associated antigens (20). By applying such technique, Lennerz et al. showed that it was possible to use polyclonal MLTC T-cell lines for the screening of COS transfectants, without prior knowledge of the relevant HLA restricting elements used by these effectors, among those expressed by the patient (20). This strategy allowed the identification not only of three novel epitopes derived from melanoma differentiation antigens (tyrosinase and gp100) but also of five new unique antigens generated by somatic point mutations in different genes in the same melanoma patient (20).

In spite of these technical improvements, in most patients with solid tumors, the identification of unique tumor antigens for immunotherapy purposes remains a difficult and

Table 3. Identification of antigens recognized by adoptively transferred T cells persisting in peripheral blood of clinically responding melanoma patients

Adoptively transferred TIL*	Category of antigens	Specificity	HLA restriction	Reference
2035†	CTA	MAGEA6	HLA-A23	(80)
	CTA	NY-ESO-1	HLA-A	(80)
	Unknown antigens		HLA-B or HLA-C	(80)
2098†	Unique	Mutated growth arrest-specific 7	HLA-A2	(19)
	Unique	Mutated glyceraldehyde-3-phosphate dehydrogenase	HLA-A2	(19)
1913†	Unique	Mutated HLA-A11	HLA-A11	(18)
	Unique	Frameshift $p14^{ARF}/p16^{INK4A}$	HLA-A24	(18)
1290‡	Unique	Mutated β-catenin	HLA-DRβ1*1502	(10)
	MDA	TRP-1	HLA-DRβ1*1502	(81)
	MDA	TRP-2	HLA-DRβ1*1502	(81)
	MDA	Gp100	HLA-A24	(81)
	MDA	Gp100	HLA-A24	(82)
	MDA intron-retaining transcript	Gp100-in 4	HLA-A2	(82)
	MDA	Tyrosinase	HLA-A24	(83)
	Normal gene product	P15	HLA-A24	(84)

Abbreviations: CTA, cancer testis antigen; MDA, melanocyte lineage differentiation antigen.
*Name of the TIL cultures used for adoptive cell transfer.
†These TIL corresponded to the T-cell clonotypes that persisted *in vivo* in clinically responsive patients.
‡These TIL were transferred into the patient, but persistence *in vivo* was not verified, although the patient was responsive to treatment.

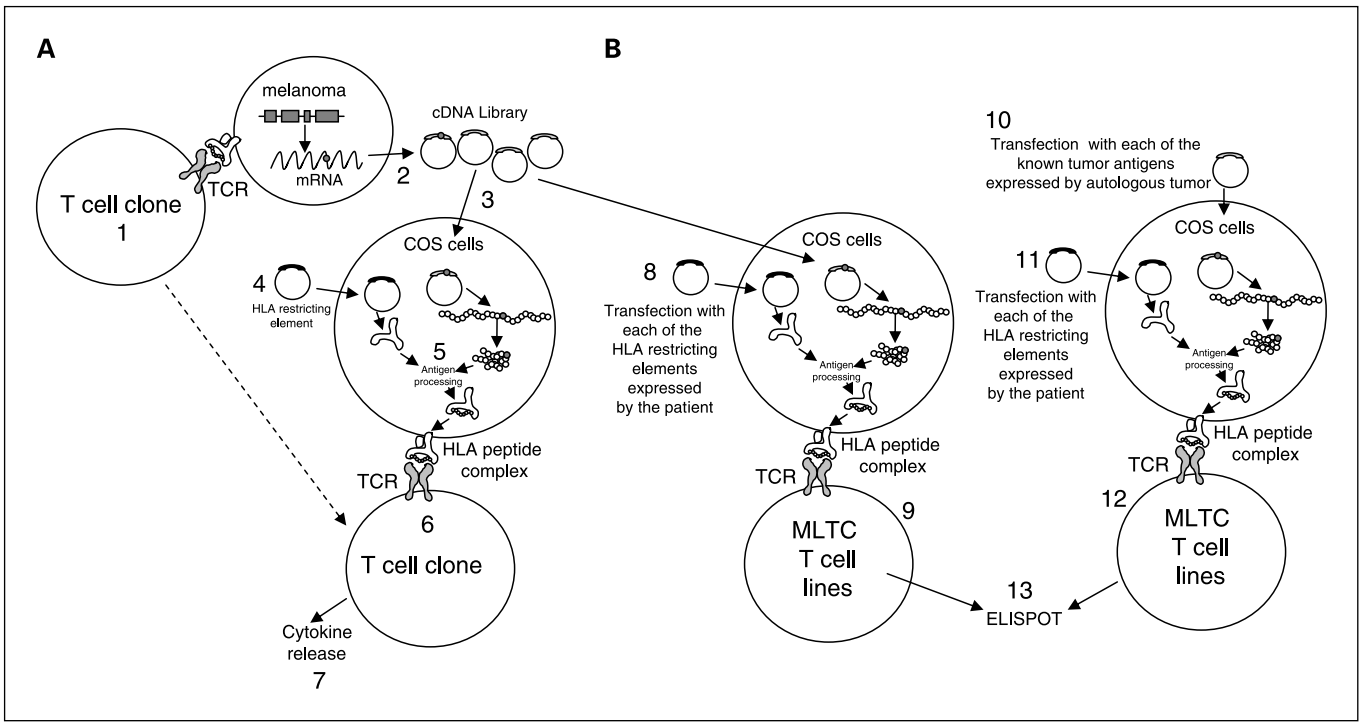

Fig. 1. Strategies for the identification of genes coding for unique tumor antigens recognized by $CD8^+$ T cells. *A,* the first step (*1*) requires the identification of a T-cell clone that recognizes in an HLA-restricted fashion only the autologous tumor and not panels of HLA-matched allogeneic tumors of the same histologic origin. mRNA isolated from the autologous tumor (*2*) is then used to generate a cDNA library (*3*) in appropriate expression vectors. Pools of plasmids from the library are then used to transfect COS cells together with an expression vector coding for the relevant HLA restricting element required by the T-cell clone (*4*). Such HLA restricting element can associate with peptides generated by the intracellular degradation of proteins resulting from the expression of the transfected cDNA segments (*5*). The COS transfectants are then cocultured with the specific T-cell clone (*6*) and T-cell recognition of an antigen-positive transfectant is verified by cytokine release assays (*7*). *B,* the previous strategy has been recently modified by Lennerz et al. (20) to allow the screening of COS cells transfected with each of the HLA class I alleles of the patient (8) and with pools of plasmids from the cDNA library generated from the autologous tumor. Such transfectants can be screened by a quantitative assay, such as the ELISPOT, for recognition by polyclonal short-term cell lines isolated by MLTC (9). A distinct set of COS transfectants are generated that can express each of the known tumor-associated antigens expressed by the autologous tumor together with each of the HLA class I alleles of the patient.

time-consuming task. Therefore, the available approaches for targeting unique tumor antigens in clinical studies of patient- and tumor-specific immunotherapy are those based on adoptive cell transfer of *ex vivo* activated tumor-specific T cells (85, 89, 90) or on vaccines containing the whole repertoire of tumor antigens from the autologous tumor (77–79). Such vaccines in turn may express known shared tumor antigens as well as unknown unique epitopes. Several vaccines of this kind have been developed and novel approaches are being pursued including tumor-derived heat shock proteins, RNA to load antigen-presenting cells, or exosomes (91, 92) as a novel multiepitope antigen delivery system.

Conclusions

As reviewed recently by Rosenberg et al. (76), the overall objective response rate seen in clinical studies of vaccination involving 1,306 patients with metastatic cancers was 3.3%. A large fraction of these studies have not been designed to target specifically unique tumor antigens but have attempted to promote/boost immune response to the shared determinants identified in several human tumors over the past 15 years. Several reasons can contribute to explain these disappointing results. These processes include tumor escape mechanisms, inefficient priming of $CD4^+$ and $CD8^+$ T cells, suppressive activity of regulatory T cells, inadequate T-cell differentiation to effector stage, and impaired homing of antigen-specific T cells to tumor site (93). Such processes will likely hamper the efficacy of any vaccine targeting shared or unique tumor antigens. However, the previously discussed specific features often expressed by unique tumor antigens (such as immunogenicity, immunodominance, and resistance to immunoselection) might represent key factors for the efficacy of immune intervention strategies aimed at targeting this class of epitopes.

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Unique Tumor Antigens: Evidence for Immune Control of Genome Integrity and Immunogenic Targets for T Cell–Mediated Patient-Specific Immunotherapy

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