REVIEW

Human Melanoma Antigens Recognized by T Lymphocytes

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Abstract. Human melanoma antigens and their epitopes recognized by T cells have been identified using a variety of methods. These antigens are classified as 1) melanocyte specific melanosomal proteins (MART-1, gp100, tyrosinase and TRP-1), 2) proteins expressed in testis and a variety of cancers (MAGE-1, MAGE-3, BAGE and GAGE), 3) tumor specific mutated proteins (β -catenin, MUM-1 and CDK4), and 4) others (p15). Some of the HLA-A2 binding non-mutated melanoma epitopes contained non-dominant anchor amino acids and have relatively low HLA-A2 binding affinity, suggesting that these epitopes were likely to be subdominant or cryptic self determinants. The significant correlation observed between vitiligo development and IL2 based immunotherapy suggested that autoreactive T cells specific for these self peptides were involved in melanoma regression *in vivo*. In addition, since adoptive transfer into patients of CTL recognizing these epitopes resulted in tumor regression, these epitopes may be tumor rejection antigens. Melanoma reactive CTL were efficiently induced from PBL of patients by *in vitro* stimulation with PBMC pulsed with these melanoma epitopes and may be useful in adoptive transfer protocols for the treatment of patients with metastatic melanoma. An immunization trial using the MART-1 and gp100 peptides in conjunction with incomplete Freund's adjuvant is in progress. These identified antigens may be useful for the development of new immunotherapies for the treatment of melanoma patients as well as for understanding the mechanisms of anti-tumor immune responses and autoimmune disorders against melanocytes. (Keio J Med 45 (2): 100–108, June 1996)

Key words: tumor antigens, T cells, MHC class I, cryptic self determinants, immunotherapy

Introduction

T-cells play an important role in tumor regression *in vivo* in many animal tumor models. In contrast to B cells which can only recognize surface proteins on tumor cells, T cells can recognize peptides derived from intracellular proteins in tumor cells. In human melanoma, adoptive transfer of cultured cytotoxic T lymphocytes (CTL) derived from tumor infiltrating lymphocytes (TIL) resulted in tumor regression in 30–40% of patients.^{1,2} T-cell infiltrates in regressing tumors after IL2 based immunotherapies and the strong correlation between accumulation of injected T cells in tumor sites and clinical response to TIL therapy suggested an important role of T-cells in *in vivo* melanoma rejection.^{3,4} The molecular nature of human melanoma antigens recognized by these autologous T-cells has recently been identified using genetic or

biochemical techniques. Three strategies, 1) testing available candidate molecules, 2) isolation of peptides from HLA molecules on melanoma cells, 3) cloning genes encoding melanoma antigens, were used for the identification of melanoma antigens.^{5,6} The third method has been most successful in isolating tumor antigens. The isolated human melanoma antigens may be classified as 1) melanocyte specific melanosomal proteins (MART-1, gp100, tyrosinase and TRP-1), 2) testis specific proteins which are also expressed in a variety of cancers (MAGE-1, MAGE-3, BAGE and GAGE), 3) tumor specific mutated proteins (β -catenin, MUM-1 and CDK4), and 4) others (p15) (Table 1). In this review, we discuss these human melanoma antigens recognized by T-cells and their use for the treatment of patients with melanoma.

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Table 1 Human Melanoma Antigens Recognized by T Cells

Melanoma	Presenting MHC	Epitope
Melanocyte Lineage Proteins		
gp100	HLA-A2	KTWGQYWQV
	HLA-A2	ITDQVPFSV
	HLA-A2	YLEPGPVTA
	HLA-A2	LLDGTATLRL
	HLA-A2	VLYRYGSFSV
MART-1/Melan-A	HLA-A2	AAGIGILTV
	HLA-A2	ILTVILGVL
TRP1 (gp75)	HLA-A31	MSLQRQFLR
Tyrosinase	HLA-A2	MLLAVLYCL
	HLA-A2	YMNGTMSQV
	HLA-B44	SEIWRDIDF
	HLA-A24	AFLPWHRLF
	HLA-DR4	unknown
Proteins Expressed in Testis and Other Cancers		
MAGE-1	HLA-A1	EADPTGHSY
	HLA-Cw16	SAYGEPRKL
MAGE-3	HLA-A1	EVDPIGHLY
	HLA-A2	FLWGPRLV
BAGE	HLA-Cw16	AARAVFLAL
GAGE-1, 2	HLA-Cw6	YRPRPRRY
Tumor Specific Antigens		
β -Catenin	HLA-A24	SYLDSGIHF
MUM-1	HLA-B44	EEKLIVYLF
CDK4	HLA-A2	ACDPHSGHFV
Others		
p15	HLA-A24	AYGLDFYIL

underline: mutation.

Melanosomal Proteins

Many cultured CTL recognize autologous melanoma cells *in vitro* as well as allogeneic melanoma cells and cultured melanocytes which share HLA class I molecules which present antigens, but do not recognize other types of tissue cells.^{5,7-11} A correlation between vitiligo development and melanoma regression after IL2 based immunotherapy was observed in the NCI Surgery Branch, and others have reported that vitiligo development correlates with a favorable prognosis or response to chemotherapeutic in melanoma patients.¹²⁻¹⁴ These observations suggest that autoreactive T-cells specific for non-mutated peptides derived from melanocyte/melanoma specific proteins may be involved in tumor regression. Four melanocyte/melanoma specific proteins (MART-1, gp100, tyrosinase and TRP-1) have been identified as antigens for T cells. These proteins appear to be expressed in melanosomes and are involved in the synthesis of melanin pigment.

Tyrosinase

Tyrosinase was identified as a melanoma antigen by screening cDNA libraries using HLA-A2 restricted and HLA-A24 restricted, melanoma reactive CTL.^{15,16} Tyrosinase, a 529 amino acid melanosomal membrane protein, which has tyrosine hydroxylase, DOPA oxidase and DHI oxidase activity, and is involved in melanin synthesis.¹⁷ None of the HLA-A2 restricted TIL established in the NCI Surgery branch recognized tyrosinase, but HLA-A24 restricted TIL from 2 patients recognized tyrosinase.^{16,18} Adoptive transfer of one of the HLA-A24 restricted tyrosinase reactive TIL into an autologous patient resulted in complete regression of tumor, suggesting that tyrosinase may be a tumor rejection antigen. Tyrosinase has also been shown to be recognized by HLA-DR4 restricted CD4+ melanoma reactive T cells derived from TIL.¹⁹ Two HLA-A2 binding epitopes, MLLAVLYCL and YMNGTMSQV, and one HLA-A24 binding epitope AFLPWHRLF have been identified.^{18,20} An HLA-B44 binding epitope SEIWRDIDF has recently been identified.²¹ Peptide-specific CTL could be induced by stimulation of PBL in five out of six donors with one of the HLA-A2 binding peptides, YMNGTMSQV, which had relatively high HLA-A2 binding affinity; however, only three of the five CTL lines were found to recognize HLA-A2+ melanomas.²² The high concentrations of peptides used to stimulate the *in vitro* response might induce T cells bearing T cell receptors with low affinity. These T cells, while capable of recognizing cells pulsed with the peptide, can not recognize the relatively low levels of this peptide resulting from natural processing in melanoma cells.

MART-1/Melan-A

MART-1 (Melanoma Antigen Recognized by T-cells-1)/Melan A was isolated following the screening of a melanoma cDNA library with the HLA-A2 restricted melanoma-reactive CTL.^{9,23} MART-1 consists of 118 amino acids and contains a single putative transmembrane domain but does not appear to possess a signal sequence at the amino terminus. Northern blot analysis and immunohistochemical studies using anti-MART-1 antibody carried out on a variety of cell lines and tissues indicated that MART-1 was specifically expressed in most melanoma cell lines and melanocytes. Although the function of MART-1 is not yet known, melanocyte specific expression suggests that it may be involved in melanin synthesis.

MART-1 was found to be an immunodominant melanoma antigen and was shown to be recognized by the majority of HLA-A2 restricted melanoma reactive TIL established in the NCI Surgery Branch²⁴ as well as by a large percentage of melanoma reactive T cell clones

derived from the PBL of HLA-A2+ melanoma patients.²⁵ By screening 28 MART-1 peptides (8-10-mers) synthesized on the basis of the HLA-A2.1 binding motifs, a 9-mer peptide, AAGIGILTV (M9-27) was identified as an epitope. This peptide was found to be an immunodominant epitope and was recognized by all HLA-A2 restricted MART-1 specific CTL.²⁴ Another MART-1 epitope, ILTVILGVL, (M9-32) was isolated from HLA-A2 molecules on melanoma cells.²⁶ By stimulating PBL from melanoma patients with the M9-27 epitope potent melanoma reactive CTL could be generated.²⁷ The cytolytic activity of the CTL generated *in vitro* with the M9-27 peptide was 50 to 100 fold greater than conventional TIL expanded with IL2 alone, indicating that these CTL may be useful for adoptive immunotherapy. Tumor regression was not observed in several patients who received the TIL reactive to MART-1. However, some evidence of tumor regression was observed in 3 of 23 patients who were immunized with the M9-27 peptide in conjunction with incomplete Freund's adjuvant in a recent clinical trial carried out in the NCI Surgery Branch, suggesting that the M9-27 peptide may represent a tumor rejection antigen.

Gp100

Gp100 was identified as a melanoma antigen for T cells by the three different methods described above.^{8,28,29} gp100 was found to represent the antigen recognized by melanoma reactive murine monoclonal antibody HMB45 which is commonly used for the diagnosis of melanoma in pathology laboratories.³⁰ gp100 is a membrane glycoprotein consisting of 661 amino acids with a leader sequence and a single transmembrane domain. gp100 was found predominantly in the membrane and filamentous matrix of melanosomes and is suspected to be involved in the late steps of melanin synthesis.^{31,32}

Gp100, like MART-1, also appears to represent an immunodominant antigen, since it was recognized by many HLA-A2 restricted melanoma reactive CTL,²⁴ and five gp100 epitopes have now been identified.³³ Three of the gp100 epitopes, G9-154 (KTWGQYWQV), G9-209 (ITDQVPFSV), G9-280 (YLEPGPVTA) were recognized by CTL from different patients, suggesting that these represented immunodominant common epitopes. Melanoma reactive CTL could be induced by *in vitro* stimulation with these gp100 peptides, but not as efficiently as with the MART-1 peptide.³⁴ Longer culture and more restimulations were necessary for the induction of melanoma reactive CTL with gp100 epitopes, indicating that the precursor frequency of CTL specific for the gp100 epitopes may be lower than the frequency of the MART-1-specific T cells. Somewhat paradoxically, the MART-1 M9-27 epitope has a lower HLA-A2 binding affinity than the gp100 epitopes. Conceivably, however,

the gp100 peptides may be expressed at higher levels on the cell surface *in vivo*, than the MART-1 peptide, which could actually lead to partial tolerance induction. However, gp100 epitopes on melanoma cells were more efficiently recognized by activated gp100 specific T-cells. There was a correlation between the clinical response to TIL therapy and the recognition of gp100, but not MART-1, by TIL.³³ In a clinical immunization protocol using G9-154, G9-209 or G9-280 peptide in conjunction with incomplete Freund's Adjuvant is ongoing in the Surgery Branch, NCI. The existence of multiple HLA-A2 binding epitopes in gp100, probably related to its large size suggests that gp100 may contain more T cell epitopes for various alleles of HLA. Thus, gp100 represents an attractive candidate antigen for the development of effective anti-tumor vaccines.

TRP1 (tyrosinase related protein-1, gp75)

TRP1 was isolated by cDNA expression cloning techniques using an HLA-A31 restricted melanoma reactive CTL (35) which was administered to the autologous patient and induced tumor regression.³⁶ TRP-1 was previously identified as an antigen recognized by IgG antibodies found in the serum of a patient with melanoma.³⁷ Passive immunization with a mouse monoclonal antibody against murine TRP1 has recently been reported to result in tumor rejection of TRP1+ B16 murine melanoma.³⁸ These results suggest that TRP1 may represent a good target for immunotherapy. TRP1 consists of 527 amino acids and is a melanosomal glycomembrane protein which has DHI-2-carboxylic acid oxidase activity involved in melanin synthesis.³⁹ Surprisingly, the epitope MSLQRQFLR was not found in the TRP1 protein sequence, but instead, was found in an alternative open reading frame.⁴⁰ T cell clones recognizing this epitope also recognized HLA-A31+ melanocytes as well as melanoma cells. Although the mechanism and generality of this phenomenon is not entirely clear, it is important to consider the possibility that T cells may recognize epitopes from alternative open reading frames.

Proteins Expressed in Normal Testis and a Variety of Cancer

This group of antigens contain the four proteins MAGE-1, MAGE-3, BAGE and GAGE, which have been isolated as melanoma antigens recognized by CTL clones established from a single melanoma patient who was repeatedly immunized with mutagenized autologous tumor cells.⁴¹

MAGE family

MAGE-1 and its epitope EADPTGHSY was identified

using an HLA-A1 restricted melanoma reactive CTL clone.^{42,43} An additional MAGE-1 epitope SAYGEPRKL which was presented by HLA-Cw16 was also identified.⁴⁴ In addition to normal testis, MAGE-1 was also expressed in approximately 40% of melanomas, as well as a smaller percentage of breast, lung, laryngeal, gastric cancers and sarcomas. Another member of the MAGE gene family, MAGE-3, was subsequently shown to be recognized by an HLA-A1 restricted CTL clone and EVDPIGHLY was identified as an epitope.^{45,46} Since MAGE-3 is expressed in almost 70% of melanomas, this antigen may represent a better target for immunotherapy of melanoma than MAGE-1.

Although melanoma reactive CTL could be induced from the PBL of melanoma patients or normal donors by stimulation with the HLA-A1 binding epitope of MAGE-1 or MAGE-3, it was not as efficient as CTL induction with the MART-1 peptide.^{45,47} In one study, melanoma reactive CTL was generated from PBL of only one of six melanoma patients.⁴⁷ An HLA-A2 binding MAGE-3 peptide FLWGPRALV was identified by inducing CTL *in vitro* with this peptide.⁴⁸ However, this CTL recognized melanoma cells only after they were treated with interferon- γ which increased HLA expression. The role of MAGE in *in vivo* tumor rejection is unclear, however, active immunization trials using the MAGE-1 and MAGE-3 HLA-A1 binding epitopes are in progress.

BAGE and GAGE

BAGE and GAGE antigens were found to be expressed in a pattern similar to that found with members of the MAGE family and were isolated as melanoma antigens using T cells.^{49,50} An epitope AARAVFLAL recognized by an HLA-Cw16-restricted T cell clone was identified. The GAGE was found to be a member of a multi-gene family, and two of the members of the family GAGE-1 and GAGE-2 were found to encode the peptide YRPRPRRY, which was recognized by the HLA-Cw6 restricted CTL. Expression of the GAGE gene was observed in 25% of sarcomas, 24% of melanomas, as well as a variety of additional cancers.

Tumor-specific Antigens

Since cancer cells accumulate many genetic changes, mutated peptides may be recognized by T cells as tumor-specific antigens. In melanoma, about 70% of patients responding to TIL therapy did not develop vitiligo and some T cell clones appeared to recognize only autologous melanoma cells *in vitro*. These observations indicated the existence of tumor specific mutated peptides as melanoma antigens. Although the majority of melanoma epitopes so far identified are non-mutated normal self

peptides, three mutated peptides derived from MUM-1, CDK4 and β -catenin have been identified as melanoma specific antigens.

MUM-1

The gene MUM-1 (melanoma ubiquitous mutated) was isolated using an HLA-B44 restricted melanoma reactive CTL⁵¹ and appeared to be unrelated to any known genes. This gene was expressed in a variety of normal tissues such as liver, colon, muscle and heart. A single point mutation found in this sequence gave rise to the mutated peptide epitope containing a substitution from serine to isoleucine at a position in the sequence EEKLIVVLF. This epitope also appeared to spur an intron/exon boundary in an alternatively spliced gene product.

CDK4

A mutated gene product ACDPHSGHFV derived from the cyclin-dependent kinase 4 gene (CDK4) was identified as a melanoma epitope recognized by HLA-A2 restricted melanoma reactive T cell clones.⁵² This mutation is a C to T transition which is typically caused by UV irradiation, a major inducer of melanoma.⁵³ The mutation was found to result in a change from an arginine to a cysteine at the primary anchor position of the HLA-A2.1 binding peptides. When the mutated epitope ACDPHSGHFV and the normal self peptide ARDPHSGHFV were tested for their ability to sensitize targets, the mutated peptide was found to sensitize HLA-A2+ indicator cells at a 100-fold to 1000-fold lower concentration than that required for sensitization with the normal peptide. The mutated peptide, but not natural peptide, appeared to have high binding affinity for HLA-A2. CDK4 forms a complex with cyclin D1 which promotes cell cycle progression from G1 to S phase. Binding of the mutated CDK4 to the CDK4 inhibitors p16INK4a and p16INK4b as well as its kinase activity was reduced. Thus, this mutation in CDK4 may be involved in the malignant transformation of the melanoma cells.

β -catenin

β -catenin has recently been isolated using an HLA-A24 restricted melanoma reactive TIL which induced complete tumor regression when administered into the autologous melanoma patient.⁵⁴ The isolated cDNA was found to contain a single mutation C to T transition, which was also observed in the CDK4 gene, and this may represent another example of a UV-induced mutation.⁵³ This mutation resulted in a change of amino acid residue from serine to phenylalanine in P9 residue of the T cell epitope, which created a typical HLA-A24 binding motif

which contained tyrosine at the second position and phenylalanine at the last position.⁵⁵ The mutated peptide SYLDSGIHF appeared to sensitize HLA-A24 expressing targets at 10⁶ fold lower concentration than the natural peptide. This appears to have resulted from the enhanced binding of the mutated epitope to HLA-A24. This newly created peptide with high HLA-A24 binding ability induced HLA-A24 restricted melanoma reactive CTL.

β -catenin has been shown to interact with the cellular adhesion molecule E-cadherin and the tumor suppressor gene APC product. Mutations of these proteins may be involved in generation of the tumor phenotype,⁵⁶ indicating that mutation of the β -catenin may be involved in oncogenesis in this melanoma. The identification of mutated epitopes as T cell antigens in CDK4 and β -catenin which may be involved in the tumor phenotype, suggest that these mutations may be important for the growth of these tumor cells.

p15

p15 was isolated using an HLA-A24-restricted TIL.⁵⁷ This gene was expressed in most normal tissues and the identified T cell epitope, AYGLDFYIL, was encoded by a non-mutated gene. Since TIL used to isolate p15 does not recognize most cells which expressed p15, post transcriptional events such as a difference between antigen processing in tumor cells and normal cells may allow expression of this epitope only on the tumor cell surface.

The Nature of Human Melanoma Antigens Recognized by T Cells

Both normal self peptides and tumor specific mutated peptides were identified as human melanoma antigens recognized by T cells. However, all of the melanoma epitopes identified in melanosomal proteins as well as testis specific proteins represented non-mutated self peptides (Table 1). MAGE, BAGE, and GAGE were isolated using CTL derived from a single patient who was repeatedly immunized with mutagenized melanoma cells and thus may not represent antigens which are normally recognized by T cells from most patients. Self peptides derived from melanosomal proteins, however, appear to represent highly immunogenic antigens, since they are recognized by T-cells derived from many patients.²⁴ One question raised by these results is why T cells which recognize mutated peptides do not predominate. It may be related to the high immunogenic nature of melanosomal proteins. It has been reported that p53 mutations were not found in the regions encoding HLA-A2 binding peptides in HLA-A2+ lung cancer cells, suggesting that tumor cells expressing HLA-A2 binding mutated p53 peptides may have been eliminated before by T-cells.⁵⁸ Melanoma cells which express highly immunogenic

mutated peptides may be generally eliminated by immunosurveillance.

In order to evaluate the immunological nature of non-mutated melanoma epitopes, the HLA-A2 binding T cell epitopes identified in the melanoma antigens were evaluated for their MHC binding affinity and the ability to induce CTL responses *in vitro*. In contrast to peptides isolated from HLA-A2 on cells or previously identified viral epitopes, most of which contained leucine or methionine at the second position (P2) and valine at the C-terminus (P9) (dominant anchor amino acids in HLA-A2.1 binding motif) and had actually high binding affinity to HLA-A2.1,⁵⁹⁻⁶² melanoma epitopes generally contained non-dominant amino acids at the primary anchor positions (alanine or threonine at P2 of AAGIGILTV, KTWGQYWQV and ITDQVPFSV, and alanine at P9 of YLEPGPVTA) and possessed relatively low (intermediate) binding affinity for HLA-A2 (Table 2).³³ These results indicate that it may be important to screen peptides which contain non-dominant anchor amino acids and have intermediate MHC binding affinity when attempting to identify tumor antigenic epitopes.³³ Binding affinity is one of the major factors influencing the cell surface density of epitopes along with other factors including the amount of protein and efficiency of enzymatic cleavage and peptide transport to the endoplasmic reticulum. These results suggest that melanoma epitopes may be subdominant or cryptic self determinants which are expressed at very low density on cell surfaces and for which tolerance is not completely induced.⁶³⁻⁶⁵ When PBL from melanoma patients were stimulated to induce CTL *in vitro* with a number of MART-1 peptides which had various HLA-A2 binding affinities, CTL could be induced only with peptides which had intermediate affinities, but not with high affinity peptides.²⁷ T cells specific for high affinity peptides may be tolerated by normal cells such as melanocytes which express these peptides at high density on the cell surface. Murine experiments

Table 2 HLA-A2 Binding Melanoma Epitopes

Antigen	Epitope	HLA-A2 Binding Affinity
gp100	KTWGQYWQV	high
	ITDQVPFSV	intermediate
	YLEPGPVT <u>A</u>	intermediate
	LLDGTATLRL	intermediate
	VLYRYGSFSV	high
MART-1	AAGIGILTV	intermediate
	ILTIVLGVL	intermediate
Tyrosinase	MLLAVLYCL	intermediate
	YMNGTMSQV	high

Underline: Non-dominant amino acids at primary anchor positions.

have suggested that T cell tolerance was induced only to peptides which appeared to be expressed at high density on the cell surface (dominant self-determinants)^{63,66}

These CTL specific for cryptic determinants which were usually silent were tumor reactive. Overexpression of antigen or MHC molecules on tumor cells might increase expression of these epitopes on cell surfaces and tissue destruction and inflammation around tumors might induce cytokines which activate anti-tumor T cells directly or augment the expression of MHC/antigen and adhesion molecules necessary for T-cell activation. These *in vivo* antigen primed CTL might be further activated and expanded *in vitro* when cultured with IL2.

Immunotherapy of Patients with Melanoma Using the Identified Melanoma Antigens

Mutated peptides which are expressed in one or a small number of patients' tumors may be difficult to use for immunotherapy unless they are common mutations or new techniques are developed for the rapid identification of tumor antigens. Non-mutated epitopes, particularly HLA-A2 binding epitopes (HLA-A2 is expressed in about a half of patients) appear to be most useful for immunotherapy. When these normal self peptides are used for immunotherapy, autoimmune problems may occur similar to Vogt-Koyanagi-Harada syndrome which represents an autoimmune disease against melanocytes. Patients may develop symptoms such as vitiligo, poliosis, uveitis, dysacusis or meningismus as a result of melanocyte destruction.⁶⁷ However, dramatic tumor regression was observed in some patients who received TIL therapy only with occasional vitiligo.⁸ Therefore, autoreactive T cell responses can be used for the treatment of melanoma patients without severe adverse effects on normal melanocytes.^{64,65,68,69}

In order to induce T-cells specific for cryptic self epitopes, the use of methods which allow high expression of these epitopes on the antigen presenting cell (APC) surface is important (Table 3).^{64,65} This may be accomplished through the use of systems which result in high levels of expression such as recombinant viral vectors containing tumor antigen genes, or by exogenous peptide loading on APC. Recombinant vaccinia virus, fowlpox virus and adenovirus containing MART-1 or gp100 have been constructed and will be used in clinical trials in the NCI Surgery branch.⁷⁰ Viral vectors have potential risks and it may be difficult to boost immune response because anti-viral T-cell responses may decrease the efficacy of subsequent immunizations. Immunization with "naked DNA" is safe and repeatedly usable, however, the efficacy of immunization is weaker than with recombinant viruses.⁷¹ Peptides may be used for immunization in conjunction with adjuvants or lipids.⁷² Clinical

Table 3 Reagents for Active Immunization of Cancer Patients

1. Antigenic peptides or whole proteins
(with adjuvants, combined with lipids or liposomes, with gp96, Hsp70 or Hsp90)
2. Recombinant viruses containing tumor antigen genes
(adenovirus, fowlpox virus, vaccinia virus)
3. Naked DNA encoding tumor antigen genes
(intramuscular or by "gene gun")
4. Recombinant bacteria containing tumor antigen genes
(BCG, Salmonella, Listeria)
5. Cells expressing tumor antigens
(dendritic cells pulsed with epitopes, cells transfected with tumor antigens, HLA and B7 genes, cells transfected with epitope/HLA fusion genes)
6. Cytokines augmenting immunization
(IL2, IL12)

trials using MART-1 or gp100 peptides along with incomplete Freund's adjuvant are being conducted in melanoma patients in the NCI Surgery Branch. Some evidence of tumor regression has been observed in 3 of 23 melanoma patients who were immunized with the MART-1 M9-27 peptide. In addition, professional APC such as dendritic cells pulsed with the peptides may be used as immunogens. Heatshock proteins such as gp96, hsp90 or hsp70 may be useful as peptide delivery vehicles for APC.⁷³ Potent melanoma reactive T-cells more (~50 fold) than the conventional CTL established from TIL by culturing with IL2 could be induced from the PBL of patients by repeated stimulation with PBMC pulsed with the melanoma epitopes.²⁷ These CTL may be more effective if used for adoptive transfer protocols.

Since many melanoma epitopes have relatively low MHC binding affinity and MHC binding affinity usually correlates with the immunogenicity of epitopes in the case of foreign antigens,⁷⁴ the natural melanoma epitopes may not be efficient immunogens. By changing amino acids of melanoma epitopes, particularly at primary anchor positions, we could make peptides which had significantly better HLA-A2 binding ability and could induce melanoma reactive CTL *in vitro* more efficiently than the native epitopes.⁷⁵ For example, the G9-280-9V peptide in which alanine at P9 was substituted to valine could induce potent melanoma reactive CTL from PBL of all 7 patients tested, whereas the natural G9-280 peptide could induce weak melanoma reactive CTL only in 2 of 7 patients. The G9-209-2M peptide in which threonine was replaced with methionine at P2 could also induce melanoma reactive CTL more efficiently than the native G9-209 peptide. Therefore, these modified peptides are more immunogenic and may be useful for the induction of melanoma reactive T-cells *in vitro* and *in vivo*. Clinical trials using these modified peptides is in progress in the

NCI Surgery branch. These amino acid changes may also be incorporated in the gp100 cDNA which could be used for the construction of recombinant viruses or naked DNA vaccines. Since the concept of the modified peptides which induce T-cells specific for cryptic self determinants is similar to the concept of molecular mimicry of foreign peptides to initiate autoimmune diseases, these modified peptides may also induce more autoimmune problems. Another system which results in expression of melanoma epitopes on APC surface at high density is also being exploited. Melanoma epitopes have been covalently linked to HLA-A2.1 molecules with a spacer to prevent dissociation of peptides from the groove of HLA-A2 molecules. Melanoma reactive CTL could be induced *in vitro* from PBL by stimulation of B-cells transfected with this MART-1 M9-27 epitope-HLA-A2.1 fusion DNA (Kang XQ *et al*, unpublished data).

Administration of cytokines such as IL2, IL12 or interferon may increase the efficacy of immunotherapies by improving immunization as well as improve tumor recognition at the effector phase by increasing epitope/MHC complexes on the tumor cell surface.^{71,76}

Since melanocytes and melanoma cells may tolerize T-cells *in vivo*,⁷⁷ repeated immunization may be necessary to induce complete regression of tumors. In order to maintain anti-tumor T cell response it may be necessary to sequentially immunize patients with multiple antigens using different immunization methods. In addition, as tumor cells may escape from T cell recognition by a variety of mechanisms including loss of antigens, MHC, β 2-microglobulin or altered antigen processing ability,⁷⁸ it may be important to combine, with immunotherapy, therapies with different anti-tumor mechanisms such as chemotherapy.

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