Analyzing T Cell Responses

How to analyze cellular immune responses against tumor associated antigens

Edited by
Dirk Nagorsen and
F.M. Marincola

Springer

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Chapter 1

MONITORING ANTIGEN-SPECIFIC T CELL RESPONSES

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Abstract: Tumor immunology has made rapid progress since the discovery of tumor-associated antigens. Antigen-specific T cell responses can now be reliably induced by vaccination. Some preliminary studies suggest that induction of a specific T cell response correlates with the clinical response. Several methods have been successfully utilized for monitoring TAA-specific T cells in tumor patients. The ELISPOT assay, cytokine flow cytometry, and tetramers have emerged as standard first-line T cell assays. Further assays complement the analysis for specific scientific questions. T cell analysis is not yet a defined surrogate marker for clinical efficacy. However, it indicates the immunological potency of a vaccine. A detailed T cell analysis can shed light on the T cell phenotype and function and may thus promote the development of clinically more effective vaccines.

Key words: cancer, vaccine, monitoring, CD8

Tumor treatment basically relies on surgery, radiotherapy, and chemotherapy. Hormone therapy and passive antibody transfer are established additional options for a few malignant diseases. Despite great success in some areas, cancer treatment obviously remains a major challenge. Some clinical situations cannot be adequately addressed. These include, in particular, the prevention of recurrence after a complete response and the management of therapy-resistant carcinomas.

Fighting tumors with the host's own immune system is an idea that has intrigued researchers for many decades. It was, to our knowledge, as early as the mid-nineteenth century that the first scientific report was published on tumor shrinkage that was probably mediated by an immune response to

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therapeutic infection with erysipelas (1). Later Coley (2) developed his famous toxin from gram-positive *Streptococcus pyogenes* and gram-negative *Serratia marcescens* and was relatively successful in treating sarcoma patients (3). These and other attempts to stimulate the immune system against tumors were made by a handful of physicians and surgeons on the basis of observations in only a few patients. The scientific foundation for immunotherapy of cancer in humans was not laid until many years later. Particularly the discovery of tumor-associated antigens in the early nineteen-nineties (4, 5, also see Chapter 2) turned tumor immunology into a major field of research. Animal studies as well as observations in humans support the theory of immune surveillance against malignant cells (reviewed in 6, 7). Findings obtained by these investigations include an increased prevalence of certain tumors following immunosuppression and a correlation between intralesional T cells and an improved clinical outcome for various solid tumors (6), including malignant melanoma (8), colorectal cancer (9), esophageal carcinoma (10), and ovarian cancer (11). However, these studies do not indicate whether infiltrating T cells are directed against tumor-associated antigens.

Spontaneous T cell responses directed against specific TAA have been detected in peripheral blood or bone marrow of tumor patients (reviewed in 12) with various histotypes, including melanoma (13, 14), colorectal cancer (15), AML (16), breast cancer (17), and neuroblastoma (18). Findings in a few selected cases suggest a favorable clinical course in tumor patients with peripheral natural TAA-directed T cells (19, 20). However, in a recent study, again with a limited number of patients, we did not find a significant survival difference between colorectal cancer patients with and without TAA-directed T cell responses (21). There is not yet enough data to draw firm generalized conclusions about the clinical impact of spontaneous T cell responses against TAA.

Also, it is still unclear how spontaneous TAA-directed T cell responses influence the efficiency of vaccination therapies in cancer patients. Some investigators think that a detectable peripheral (precursor) T cell response might be necessary for an effective vaccination (22, 23), while others suggest that T cells with such responses might have lost their anti-tumor effectiveness (24). With improved vaccination schedules, TAA-directed T cell responses can be reliably induced using various vaccination approaches (reviewed in 25). Several reports have described a correlation between a vaccination-induced TAA-directed T cell response and a clinical response (26, 27, 28, 29). Preliminary data also suggest a possibly favorable clinical effect of vaccine-induced T cells in adjuvant vaccination therapy (30, 31, 32, 33). However, some studies have also described the coexistence of vaccine-induced TAA-specific T cells and TAA-bearing tumors (34). This paradox is

not fully understood. Tumor escape mechanisms and T cell dysfunctions are discussed as basic reasons (see chapter 3; 35).

Besides active vaccination, a further experimental treatment option using TAA-specific T cells is the autologous administration of isolated tumor-infiltrating TAA-specific cells after *in vitro* expansion and activation in patients with certain malignant diseases, e.g. melanoma (36). Some clinical success has been achieved in selected patients using specific T cell transfer, particularly after nonmyeloablative chemotherapy (37). Monitoring the presence and localization of adoptively transferred T cells is crucial for evaluating and improving these adoptive-cell-transfer therapies and of course also for gaining insights into the interaction between tumors and the immune system.

The role of T cells in host defense against tumors is not yet fully understood and thus requires a more comprehensive investigation. Several methods have been successfully utilized for monitoring TAA-specific T cells in tumor patients. The ELISPOT assay, cytokine flow cytometry, and tetramers have emerged as standard first-line T cell assays (38). Further methods such as qRT-PCR, proliferation assays, dimers, TCR analysis, cytotoxicity assays, or GFP-HLA complement the analysis for specific scientific questions (see Chapters 5, 6, and 13 through 16). First studies have also been performed on microarray analyses of enriched TAA-specific T cells (see Chapter 17, (39)). Each method has its own specific advantages and disadvantages. One major requirement for a first-line up-to-date monitoring technique is single-cell analysis. This can be done by ELISPOT, flow cytometric cytokine assays and tetramer staining. While functional cytokine assays (ELISPOT, intracellular cytokine flow cytometry) shed light on cytokine production in response to peptide in single cells, tetramers indicate specific binding between a single T cell and a target HLA/peptide complex irrespective of the functional state of the specific cell. Thus, even anergic or areactive specific T cells can be detected by tetramers.

Antibody-based TCR analyses can also be performed on a single-cell level. However, while antibody-based analysis can only detect 70% of the $\alpha\beta$ TCR repertoire, molecular analysis has the advantage of full coverage. Unfortunately, molecular methods cannot distinguish between T cell subsets. Their analysis requires previous separation by techniques such as tetramer staining or cytokine capture assay with subsequent cell sorting or separation by magnetic microbeads. This emphasizes the importance of combining different methods. A further example of successful combinations is the application of tetramer staining together with CD107 as a marker of degranulation (as described in Chapter 11). Most *ex vivo* functional assays are based on the measurement of epitope-induced cytokine production. Two further important functions of active T cells are their ability to proliferate

and their cytotoxic potential (Chapters 5 and 6). This book describes all relevant established techniques in separate chapters. In addition, we have included two techniques whose role has not yet been definitely determined: in situ MHC tetramer staining and peptide-HLA-GFP complexes. Though little data is available on these methods, they are very promising, and we expect them to provide important new information on tumor-infiltrating TAA-specific T cells and HLA-TCR interaction.

Scientists from other fields of immunology, especially virologists and rheumatologists, have a major impact on the development of better methods for T cell analysis. In particular, epitopes derived from CMV, EBV, influenza, and HIV are common targets for frequent "autologous" specific T cell responses. Moreover, our impression is that virus-specific T cells have a higher affinity to their targets. HPV vaccination, clinically successful in preventing cervical cancer (40, reviewed in 41), is an excellent example for overlapping questions and challenges in viral and tumor immunology.

There is a trend toward more and more detailed analysis of T cell responses. Especially for virus-specific T cell responses, several subsets of T cells have been described and related to specific functions and stages of development. In particular, CD45RA, CCR7, CD27, and CD28 have proven helpful for determining functional T cell subsets (42, 43, 44). Furthermore, analysis of cytokine expression within cell subsets, such as IL-4, IL-10, TGFβ, or IL-2, helps in assigning functional properties to T cells. The determination of further chemokine receptors on T cells is also of interest because, in combination with the local chemokine milieu, they are indicative of the homing ability of T cells (45).

All these latter analyses can be done by cytokine flow cytometric methods and tetramer staining. In contrast, ELISPOT assays, microarrays, molecular TCR analyses, and qRT-PCR do not allow further determination of T cell subsets unless preceded by a usually laborious cell separation. Before applying analysis methods, some investigators enhance *ex vivo* immunity by using protocols such as culturing T cells for 1-2 weeks with peptide and cytokines (IL-2, IL-7). This stimulation step is useful for analyzing extremely rare T cells and has its role in sensitive semiquantitative analyses. However, phenotypic, functional, and quantitative changes in T cells during *in vitro* stimulation obviously limit the information obtained after *in vitro* stimulation (46). Therefore, when reporting on an assay, it is important to differentiate between *ex vivo* T cell analysis and T cell analysis after *in vitro* stimulation.

There are basically three therapeutic situations in which TAA-specific T cells appear in cancer patients: spontaneously without prior immunotherapy, after active immunization, and after adoptive T cell transfer. Although some clinical success has been achieved, the actual role of antigen-specific T cells

in tumor patients remains to be determined, and the therapeutic niche of T-cell-targeted therapy has yet to be defined. However, first results are promising and strongly support the further development of immunotherapeutic approaches aimed at the induction or modification of T cell responses. Reliable and detailed immune monitoring of T cell responses is essential for further development of cancer vaccination and adoptive T cell transfer studies. Suitable utilization and further improvement of T cell analysis also serve other fields of immunology, including virology and rheumatology.

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Chapter 2

TUMOR ASSOCIATED ANTIGENS

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Abstract: The results of an extensive series of studies that were first carried out in the early 1990s have revealed that human tumor reactive T cells recognize a diverse array of antigens. These include antigens expressed in normal tissues, mutated gene products as well as novel epitopes encoded within alternative open reading frames, intronic sequences, as well as the products that result from protein splicing. This antigenic diversity provides opportunities as well as challenges for the development of more effective immunotherapies for the treatment of patients with cancer.

Key words: antigen, epitope, tumor association

1. INTRODUCTION

Studies initiated in the early 1980s demonstrated that CD8+ cytotoxic T cells (CTL) that recognized tumor cell in an MHC restricted manner could be generated by incubation of tumor infiltrating lymphocytes (TIL) with high doses of IL-2, as well as by the *in vitro* sensitization of peripheral blood mononuclear cells (PBMC) from cancer patients with autologous or HLA matched allogeneic tumor cells in mixed lymphocyte tumor cultures (MLTC). These studies lead to the identification of the first antigen recognized by human tumor reactive T cells, MAGE-1, in 1991 (1). Since that time, human tumor reactive T cells have been found to recognize peptides that are processed from the products of more than 50 distinct genes. These include antigens that are derived from normal gene products that are expressed in a tissue specific manner, products that are limited in their expression to tissues such as the testis that do not appear to represent

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immunologic targets, products that are over-expressed in tumors and mutated gene products. Several tumor reactive HLA class II restricted T cells have also been identified, many of which recognize epitopes from proteins that have also been shown to be recognized by HLA class I restricted T cells.

Several mechanisms influence the repertoire of T cells that is available to recognize antigens expressed on tumor cells. Tumor antigens recognized by TIL as well as by T cells generated by direct tumor stimulation have been shown in many cases to results from novel gene processing as well as antigen processing mechanisms that include the translation of RNA transcripts that contain unspliced introns (2), translation from alternative open reading frames (3, 4) and protein splicing (5, 6). The universe of potential antigens thus appears to be significantly larger than that which is based upon known or predicted translated gene products. At the same time, antigen processing mechanisms appear to result in the presentation of only a small proportion of the pool of peptides that are capable of binding to an individual HLA allele, and self-tolerance mechanisms may further limit the available repertoire of T cell specificities, hampering studies aimed at identifying immunogenic candidate T cell epitopes. Nevertheless, antigen identification studies carried out with tumor reactive T cells have provided a large array of targets that can be used for the development of cancer immuotherapies.

2. ANTIGEN IDENTIFICATION

A variety of methods have been employed to identify tumor antigen epitopes. Tumor antigens recognized by HLA class I restricted T cells have primarily been identified using expression cloning methods developed in the early 1980s that relied on the used of highly transfectable cell lines to introduce genes from tumor cell cDNA libraries. For this approach, the appropriate HLA class I restriction element involved with presentation of the T cell epitope must first be identified either by blocking T cell antigen recognition using antibodies that are specifically reactive with particular HLA alleles, or by assaying a series of tumor cells lines that either do or do not share particular HLA gene products with autologous tumor cells for their ability to stimulate the tumor reactive T cells. The appropriate HLA class I restriction element, once identified, is then used to transfected a target cell line such as the monkey kidney cell line COS or the human embryonic kidney cell line 293. Target cells are then transfected with pools of cDNAs generated from tumor cell mRNA, and transfectants assayed for their ability

to stimulate cytokine release from either tumor reactive T cell lines or T cell clones.

Additional studies have demonstrated that cell lines such as 293 could process and present antigens recognized by HLA class II restricted T cells. The HLA class II gene complex consists of an HLA-DR locus that contains from one to three functional genes that vary according to the haplotypes, along with the HLA-DP and DQ loci. Individual HLA-DR molecules consist of a highly polymorphic DRβ gene product that is associated with a nearly invariant DRα chain, while the HLA-DP and DQ products consist of polymorphic α and β chains. Transfected 293 cells that express HLA class II molecules as well as additional molecules associated with class II processing such as invariant chain and HLA-DM could efficiently process antigens recognized by HLA class II restricted T cells and, as discussed further below, has been used to identify unknown tumor antigens (7, 8). Alternatively, target cells generated by transfection of 293 cells with constructs encoding the appropriate HLA class II gene products as well as the HLA class II transactivator (CIITA) gene, which up-regulates the expression of multiple gene products associated with class II processing (9), have also been used to identify genes that encode antigens recognized by HLA class II restricted T cells (10, 11).

Biochemical approaches have also been utilized to identify antigens recognized by tumor reactive T cells (12, 13). Pools of peptides that were eluted from MHC class I molecules were fractionated using reversed phase HPLC columns and tested for their ability to sensitize target cells for T cells recognition. Additional fractionation steps were then carried out using a variety of columns or buffers, and peptides associated with T cell recognition were identified on the basis of their elution profile. The sequence of candidate peptides was then determined using tandem mass spectrometry, and synthetic peptides corresponding to these sequences were then generated and tested for their ability to be recognized by specific T cells. Antigen presenting cells such as dendritic cells (DC) and B cells can efficiently process whole proteins through an exogenous pathway that leads to presentation of HLA class II restricted T cell epitopes. An antigen recognized by HLA class II restricted T cells by fractionating proteins isolated from tumor cells using a variety of biochemical techniques (14).

The screening of tumor cell cDNA expression libraries with patient sera, designated SEREX (serological analysis of gene expression), has resulted in the identification of a large number of antigens, many of which appear to represent widely expressed auto-antigens that may not be recognized by tumor reactive T cells. Nevertheless, a small subset of the antigens identified using the SEREX technique have been found to be recognized by HLA class I and class II restricted T cells (15, 16).

Tumor antigens have also been identified through a process termed reverse immunology, whereby candidate epitopes are first identified from a protein that is over-expressed in a particular tumor type though the use of HLA binding motifs. The motifs represent consensus sequences that have been derived by direct measurement of MHC-peptide binding affinities (17), as well as by identifying common residues that appear at particular positions in known T cell epitopes and peptides that have been eluted from individual MHC molecules (18). The majority of HLA class I peptide epitopes consist of between 8 and 10 amino acids, although longer peptides have been shown to bind to certain HLA class I alleles. Amino acids that predominate at 2 or 3 positions, designated the anchor amino acids, directly interact with pockets in the MHC class I molecule and therefore strongly influence peptide binding affinity, although other secondary anchor positions that have a less important influence on binding have been identified. Peptides that bind to the HLA-A2 class I gene, which is expressed by approximately 50% of Caucasians and represents the most common allele expressed in this population, generally possess an aliphatic residue such as L or M at position 2 (P2) and a V or L residue at the carboxy terminus of the peptide (P9 or P10). The amino and carboxyl termini of HLA class II binding peptides are less well defined than class I binding peptides, as the class II peptide binding pocket has a more open structure than the class I binding pocket. Nevertheless, HLA class II binding peptides generally contain a core region of approximately 10 amino acids that contains two or three anchor positions. A general correlation has been noted between the peptide affinity and the ability to generate specific T cells (19); however, many of the peptides derived from normal, non-mutated gene products that are recognized by tumor reactive T cells appear to bind to HLA class I gene products with relatively low affinity. Presumably, the expression of certain antigens in normal adult tissues can lead to the deletion of T cells that recognize epitopes that bind to HLA molecules with high affinity.

3. ANTIGENS RECOGNIZED BY HLA CLASS I RESTRICTED, TUMOR REACTIVE T CELLS - DIFFERENTIATION ANTIGENS

The antigens recognized by human tumor reactive T cells can be categorized according to their expression profiles in normal tissues and tumors. The differentiation antigens are limited in their expression in the adult primarily to one tissue type and are dominated by the melanocyte differentiation antigens (MDA), which represent gene products that are expressed in melanomas as well as normal skin melanocytes and retinal

tissue. Epitopes have been identified from several MDA that play a role in the synthesis of melanin, including gp100, tyrosinase, TRP-1 and TRP-2, as well as proteins of unknown function such as MART-1 (Table 1).

Table 1. HLA Class I restricted shared tumor specific antigens.

GENE	HLA Allele/Epitope		Reference
BAGE-1:2-10	Cw16	ARAVFLAL	100
DAM-6:271-279	A2	FLWGPRAYA	101
GAGE-1/2/8:9-16	Cw6	YRPRPRRY	102
GAGE-3/4/5/6/7:10-18	A29	YYWPRPRRY	45
GnT-V(intron)	A2	VLPDVFIRCV	103
LAGE-1:157-165	A2	SLLMWITQC	42
LAGE-1:AORF*	A2	MLMAQEALAFL	104
LAGE-1:AORF	A31	LAAQERRVPR	43
MAGE-1:161-169	A1	EADPTGHSY	105
MAGE-1:96-104	A3	SLFRAVITK	106
MAGE-1:222-231	A68	EVYDGREHSA	106
MAGE-1:135-143	A24	NYKHCFPEI	107
MAGE-1:127-136	B37	REPVTKAEML	108
MAGE-1:258-266	B53	DPARYEFLW	106
MAGE-1:62-70	Cw2	SAFPTTINF	106
MAGE-1:230-238	Cw3	SAYGEPRKL	106
MAGE-1:230-238	Cw16	SAYGEPRKL	109
MAGE-2:112-120	A2	KMVELVHFL	110
MAGE-2:157-166	A2	YLQLVFGIEV	110
MAGE-2:156-164	A24	EYLQLVFGI	111
MAGE-2:127-136	B37	REPVTKAEML	108
MAGE-3:168-176	A1	EVDPIGHLY	39
MAGE-3:271-279	A2	FLWFPRALV	40
MAGE-3:112-120	A2	KVAELVHFL	49
MAGE-3:195-203	A24	IMPKAGLLI	112
MAGE-3:97-105	A24	TFPDLESEF	113
MAGE-3:167-176	B18	MEVDPIGHLY	114
MAGE-3:168-176	B35	EVDPIGHLY	115
MAGE-3:127-136	B37	REPVTKAEML	108
MAGE-3:114-122	B40	AELVHFLLL	94
MAGE-3:167-176	B44	MEVDPIGHLY	116
MAGE-3:143-151	B52	WQYFFPVIF	115
MAGE-4:169-177	A1	EVDPASNTY	117
MAGE-4:230-239	A2	GVYDGREHTV	118
MAGE-4:156-163	B37	SESLKMIF	119
MAGE-6:290-298	A34	MVKISFFPR	120
MAGE-6:127-136	B37	REPVTKAEML	108
MAGE-10:254-262	A2	GLYDGMEHL	121
MAGE-10:290-298	B53	DPARYEFLW	106
MAGE-12:271-279	A2	FLWGPRALV	40
MAGE-12:170-178	Cw7	VRIGHLYIL	122
NY-ESO-1:157-165	A2	SLLMWITQC	42
NY-ESO-1:AORF	A2	MLMAQEALAFL	104

GENE	HLA Allele/Epitope		Reference
NY-ESO-1:53-62	A31	ASGPGGGAPR	43
NY-ESO-1:AORF	A31	LAAQERRVPR	43
NY-ESO-1:94-102	B51	MPFATPMEA	123
NY-ESO-1:92-100	Cw3	LAMPFATPM	124
NY-ESO-1:80-88	Cw6	ARGPESRLL	124
SSX-2:41-49	A2	KSEKIFYV	16
TRP-2:intron	A68	EVISCKLIKR	46

The MART-1 antigen, also designated Melan-A, was independently cloned by 2 groups by the expression screening of melanoma cDNA libraries (20, 21). MART-1 represents an immunodominant antigen and is recognized by the majority of melanoma reactive TIL derived from HLA-A2 patients (22). Following the screening of 23 candidate MART-1 peptides that conformed to the HLA-A2 binding motif, a single nonapeptide, AAGIGILTV(MART-1:27-35), as well as two overlapping decapeptides, EAAGIGILTV (MART-1:26-35) and AAGIGILTVI (MART-1:28-36) were recognized by a MART-1 reactive T cell clone as well as three MART-1 reactive TIL. The MART-1:27-35 and MART-1:26-35 peptides both contain an alanine residue at the P2 primary anchor position which appears to be responsible for the relatively low affinity binding affinity of these peptides. Modifications of MART-1:27-35 peptide that resulted in enhanced binding to HLA-A2 generally generated peptides that are recognized less well than the unmodified peptide, whereas substitution of leucine for the alanine residue at the presumed P2 primary anchor position in the MART-1:26-35 peptide (ELAGAGILTV) resulted in the generation of a peptide with enhanced binding to HLA-A2, designated MART-1:26-35(2L) (23) Tumor reactive T cells were more readily generated when the modified MART-1:26-35(2L) peptide was used than when the un-modified peptide was used to carry out the *in vitro* sensitization of PBMC.

Soluble chimeric molecules that contain MHC class I or class II gene products bound to an appropriate peptide and that provide a direct measurement of the percentage of cells reactive with a particular epitope, termed MHC tetramers (24) have been used to characterize immune responses to MART-1 as well as additional tumor antigens. Low levels of cells reactive with the MART-1:26-35(2L) tetramer were detected in six out of ten normal donors and seven out of ten melanoma patients (25). Studies of the phenotype of tetramer positive cells indicated that those cells from normal donors were exclusively of the naïve phenotype, whereas in three out of the ten melanoma patients, cells expressed a more mature phenotype and expressed low levels of CD45RA and CD28, providing evidence that tumor cell stimulation may have lead to activation of the MART-1 reactive T cells.

Additional MART-1 epitopes recognized in the context of HLA-B35 (26) and B45 (27) encompass the MART-1 HLA-A2 epitope, suggesting that this region of the molecule may possess characteristics that lead to the efficient processing.

The gp100 glycoprotein, which was originally identified as an enzyme involved with melanin synthesis, was subsequently found to represent an antigen recognized by HLA-A2 restricted, melanoma reactive T cells (28). In one study, 13 out of 30 HLA-A2 restricted TIL were found to recognize gp100, and three peptides, KTWGQYWQV (gp100:154-162), ITDQVPFSV (gp100:209-217), and YLEPGPVTA (gp100:280-288) were recognized in the context of HLA-A2 by seven, six and six out of the thirteen gp100 reactive TIL, respectively (22). The gp100:280-288 peptide was also identified in an independent study in which peptides that have been eluted from the surface of melanoma cells were fractionated and used to sensitize target cells for T cell recognition (13). In this study, HLA-A2 expressing target cells pulsed with the gp100:280-288 peptide were recognized by five out of five melanoma reactive, HLA-A2 restricted T cell lines generated by in vitro stimulation with tumor cells.

The observation that the dominant gp100 epitopes contained non-optimal anchor residues lead to attempts to enhance the antigenicity as well as the immunogenicity of these peptides by substituting consensus anchor residues at these positions. The gp100:209-217 peptide contained a threonine at position two and possessed a relatively low HLA-A2 binding affinity of 172 nM; however, substitution of a leucine, methionine or isoleucine at the second position resulted in the generation of a peptide that had a significantly higher HLA-A2 binding affinity as well as an enhanced capacity to stimulate peptide and tumor reactive T cells *in vitro* (29). The results of in vitro sensitization studies carried out with PBMC from melanoma patients demonstrated that a variant containing a substitution of methionine for the threonine at position 2 of the native peptide, gp100:209-217(2M), was more effective at stimulating tumor reactive T cells than the parental peptide. Similarly, the gp100:280-288 peptide possessed a binding affinity of only 455 nM, and a peptide containing a substitution of valine for the alanine at position nine possessed a 10-fold higher HLA-A2 binding affinity than the parental peptide and was more effective at stimulating peptide and tumor reactive T cells than the parental peptide (29).

Several additional melanosomal proteins have been shown to represent targets of tumor reactive T cells. Results presented in an initial report indicated that melanoma reactive T cells recognized two HLA-A2 restricted epitopes of tyrosinase, the enzyme that catalyzes the first step in melanin synthesis from the amino acid tyrosine (30). One of the tyrosinase epitopes, MLLAVLYCL(tyr:1-9), was derived from the first 9 amino acids of the

tyrosinase signal peptide. The second epitope, YMNGTMSQV (tyr:369-377), was subsequently shown to contain a post-translational modification resulting from a substitution of aspartic acid for the asparagine residue at position 3 (31). The modified tyr:369-377 peptide was also recognized by CD4+ T cells (32), and antibody blocking studies demonstrated that the CD4 and CD8 co-receptors did not contribute to the recognition of this epitope, indicating that the CD4+ T cells may recognize this epitope with a relatively high avidity. A third HLA-A2 restricted epitope derived from the tyrosinase signal peptide, CLLWSFQTSA (tyr:8-17), was identified using a candidate epitope approach (33). Additional MDA that include TRP-1 (3) TRP-2 (34, 35) and AIM-1 have been identified as the targets of tumor reactive T cells, and tumor reactive T cells were generated by stimulating PBMC from melanoma patients with candidate epitopes from the OA1 (36) and P. polypeptide (37) proteins.

4. TUMOR SPECIFIC ANTIGENS

This category of tumor antigens, which represents non-mutated gene products that are not processed and presented on normal adult tissues, is predominantly comprised of members of the cancer/testis (C/T) family of genes (Table 2). The expression of C/T gene products is limited in adult tissues to the normal testis, which lacks expression of HLA class I and class II molecules. The prototype for this family, MAGE-1, represents the first antigen to be identified as a target of human tumor reactive T cells, and was cloned by transfecting an autologous tumor cell line that appeared to have lost antigen expression with a cosmid library containing genomic DNA from antigen positive tumor cells (1). The cancer/testis genes have been clustered into 10 families, 6 of which have been mapped to the X chromosome (38), and subsequent studies have lead to the identification of T cell epitopes expressed on multiple products derived from these gene families. The MAGE gene family includes 17 members, but additional families appear to contain only a small number of sequences, as the NY-ESO-1 families contains only 2 genes very closely related genes, NY-ESO-1 and LAGE-1. Members of these families are expressed at frequencies of up to 60% in melanoma, breast, lung, bladder and prostate cancers. Two epitopes recognized in the context of HLA-A1 have been identified from homologous regions of the MAGE-A1 and MAGE-A3 antigens (39). A reverse immunology approach was also used to identify an HLA-A2 restricted epitope of MAGE-A3 (40). This epitope may not generally be processed endogenously at levels sufficient to lead to T cell recognition, however, as

Table 2. HLA class I restricted differentiation antigens.

Gene	HLA Allele	/Epitope	Reference
AIM-1:41-50	A2	AMFGREFCYA	125
CEA:605-613	A2	YLSGANLNL	126
CEA:691-699	A2	IMIGVLVGV	49
CEA:61-69	A3	HLFGYSWYK	127
gp100:154-162	A2	KTWGYWQV	128
gp100:209-217	A2	ITDQVPFSV	128
gp100:280-288	A2	YLEPGPVTA	13
gp100:457-466	A2	LLDFTATLRL	128
gp100:476-485	A2	VLYRYGFSV	128
gp100:177,178-186	A2	(A)MLGTHTMEV	129
gp100:619-627	A2	RLMKQDFSV	130
gp100:639-647	A2	RLPRIFCSC	130
gp100:614-622	A3	LIYRRRLMK	130
gp100:17-25	A3	ALLAVGATK	131
gp100:intron	A24	VYFFLPDHL	81
gp100:182-191	A68	HTMEVTVYHR	132
gp100:71-78	Cw8	SNDGPTLI	35
mammaglobin-A:23-31	A3	PLLENVISK	133
MART-1:26,27-35,36	A2	(E)AAGIGILTV(A)	134
MART-1:32-40	A2	ILVILGVL	135
MART-1:24-33,34	B45	AEEAAGIGIL(T)	27
MC1R:244-252	A2	TILLGIFFL	136
MC1R:283-291	A2	FLALIICNA	136
OA1:194:202	A24	LYSACFWWL	36
PSA:165-174	A2	FLPKKLQCV	137
PSA:178-187	A2	VISNDVCAQV	137
P. polypeptide:427-435	A2	IMLCLIAAV	37
RAGE-1:11-20	B7	SPSSNRIRNT	138
SOX-10:331-340	A2	AWISKPPGV	139
TRP-2:180-188	A2	SYVYDFFVWL	140
TRP-2:360-368	A2	TLDSQVMSL	141
TRP-2:455-463	A2	YAIDLPVSV	125
TRP-2:197-205	A31,A33	LLGPGRPYR	142
TRP-1:AORF	A31	MSLQRQFLR	3
TRP-2:387-395	Cw8	ANDPIFVVL	35
TRP-2:403-411	A2	ATTNILEHV	139
TRP-2:288-296	A2	SLDDYNHLV	143
TRP-2:455-463	A2	YAIDLPVSV	125
tyrosinase:243-251	A1	KCDICTDEY	144
tyrosinase:146-156	A1	SSDYVIPIGTY	130
tyrosinase:1-9	A2	MLLAVLYCL	30
tyrosinase:369-377	A2	YMDGTMSQV	30
tyrosinase:8-17	A2	CLLWSFQTSA	33
tyrosinase:206-214	A24	AFLPWHRLF	145
tyrosinase:312-320	B35	LPSSADVEF	146
tyrosinase:192-200	B44	SEIWRDIDF	147

results presented in a second report indicated that T cells generated with this peptide failed to recognize untreated tumor cells (41).

The C/T antigen NY-ESO-1 was initially identified when serum from a patient with esophageal squamous cell carcinoma was used to carry out SEREX analysis (15). Subsequent studies have resulted in the identification of HLA class I restricted epitopes of NY-ESO-1 that are recognized in the context of HLA-A2 (42) and HLA-A31 (43). Antibodies directed against tumor antigens recognized by T cells have generally only been detected in a very low percentage of cancer patients; however, over 50% of patients bearing NY-ESO expressing tumors possess significant titers of anti-NY-ESO-1 antibodies (44). High titers of anti-NY-ESO-1 antibodies are correlated with the presence of bulky disease or multiple tumor metastases, and tumor regression in these patients was associated with a reduction of anti-NY-ESO-1 antibody titers, indicating that the presence of a substantial tumor mass is involved with maintaining anti-NY-ESO-1 antibody responses.

A relatively small number of antigens have been identified that are encoded by transcripts whose expression appears to be strictly limited to tumor cells. A T cell epitope was derived from a GnT-V gene transcript that is expressed only in tumor cells and that was initiated from the intron of a normal gene (45). A partially spliced transcript derived from the TRP-2 gene that appears to be expressed in melanoma cells but not in normal melanocytes also encodes an epitope recognized by tumor specific T cells (46).

5. ANTIGENS OVER-EXPRESSED IN TUMORS

Tumor reactive T cells have been shown in some cases to recognize proteins that are over-expressed in tumor cells relative to normal cells (Table 3). Several of the antigens in this category were initially identified by stimulating PBMC with candidate epitopes, although contradictory results have been obtained when investigators have evaluated the ability of T cells generated with certain peptides to recognize un-manipulated tumor cells. Initial studies indicated that candidate epitopes from the HER-2/neu protein, which is over-expressed in breast and ovarian carcinomas, resulted in the generation of tumor reactive T cells (47-49). In a subsequent clinical study, however, HLA-A2 positive patients with breast, ovarian, or colorectal cancer were immunized with the HER-2/neu:369-377 peptide in an attempt to raise the precursor frequency of T cells reactive with this antigen and hopefully also the frequency of tumor reactive T cells (50). Highly avid peptide reactive T cells could be readily generated from immunized patients;

however, the peptide reactive T cells failed to recognize either tumor cells expressing natural levels of HER-2/neu or HLA-A2 positive target cells that were transfected with a construct encoding HER-2/neu.

Table 3. HLA class I restricted antigens over-expressed in tumor cells.

GENE	HLA ALLELE/EPITOPE		REFERENCE
AIM-2:AORF	A1	RSDSGQQARY	90
AFP:542-550	A2	GVALQTMKQ	148
BING-4:AORF	A2	CQWGRLWQL	149
CPSF:250-258	A2	KVHPVIWSL	150
CPSF:1360-1369	A2	LMLQNALTTM	150
FGF-5:172-176/217-220 A3	A3	NTYASPRFK	54
G250:24-262	A2	HLSTAFARV	151
HER-2/neu:369-377	A2	KIFGSLAFL	47
HER-2/neu:5-13	A2	ALCRWGLLL	49
HER-2/neu:435-443	A2	ILHNGAYSL	49
HER-2/neu:952-961	A2	YMIMVKCMI	48
HER2/neu:665-673	A2	VVLGVVGFI	48
HER2/neu:689-697	A2	RLLQETELV	48
HER2/neu:654-662	A2	IISAVVGIL	152
HER-2/neu:754-762	A3	VLRENTSPK	127
iCE:AORF	B7	SPRWWPTCL	153
M-CSF:AORF	B35	LPAVVGLSPGEQEY	154
MUC-1:950-958	A2	STAPPAHGV	155
MUC-1:12-20	A2	LLLLTVLTV	155
p53:264-272	A2	LLGRNSFEV	156
p53:5-73	A2	RMPEAAPPV	157
p53:125-134	A24	TYSPALNKMF	158
p53:99-107	B46	SQKTYQGSY	159
PRAME:100-108	A2	VLDGLDVLL	92
PRAME:142-151	A2	SLYSFPEPEA	92
PRAME:300-309	A2	ALYVDSLFFL	92
PRAME:425-433	A2	SLLQHLIGL	92
PRAME:301-309	A24	LYVDSLFL	
PSMA:178-186	A24	NYARTEDFF	160
RU2AS:antisense	B7	LPRWPPPQL	161
survivin:95-104	A2	ELTLGEFLKL	162
hTERT:540-548	A2	ILAKFLHWL	91
hTERT:865-873	A2	RLVDDFLLV	163

Candidate epitopes identified from proteins that are over-expressed in many tumor cell types have also been utilized in attempts to generate widely applicable tumor antigen vaccines. Initial findings indicated that tumor reactive T cells could be generated following *in vitro* sensitization of PBMC with the telomerase:540-548 peptide (51). Results presented in an additional study, however, indicated that T cells generated using this peptide failed to

recognize target cells expressing the full length telomerase protein (52). Furthermore, results presented in this report indicated that this epitope could not be generated following incubation of purified proteasomes with longer precursors that encompassed this peptide, in contrast to the majority of naturally processed T cell epitopes (53).

The FGF-5 (5), iCE (54), and PRAME (55) antigens were cloned using PBMC that were sensitized *in vitro* with tumor cell lines, and thus contain epitopes that are naturally processed and presented on the cell surface. While expression of the FGF-5 and iCE gene products is not strictly limited to tumor cells, the level of expression observed in normal cells appears to be insufficient to allow T cell recognition of these antigens. The antigen PRAME antigen was expressed in a variety of tumor types but was also expressed in a variety of normal adult tissues that included testis, endometrium, ovary and adrenal tissues (55). The PRAME reactive T cells expressed high levels of the NK inhibitory receptor p58.2 that recognizes the HLA-Cw7 class I molecule, which was expressed on normal cells but not tumor cells from this patient. Thus, expression of p58.2 appeared to be at least partially responsible for inhibiting the response of PRAME reactive T cells to normal cells.

6. TUMOR ANTIGENS DERIVED FROM MUTATED GENE PRODUCTS

Tumor reactive T cells have been shown to recognize a variety of mutated gene products (Table 4). In general an individual mutation represents a relatively rare event that occurs in only one or a very small percentage of tumors, making identification of the HLA restriction element utilized for T cell recognition more difficult. Several mutations that are expressed in more than one tumor cell, however, have been identified. Expression screening carried out using a T cell clone that recognized a head and neck carcinoma resulted in the isolation of a caspase-8 (CASP-8) gene product containing a point mutation in the stop codon that resulted in the generation of an extension of the normal open reading frame (ORF) (56). The CASP-8 T cell epitope was encoded by sequences that spanned mutation, and amino acids 5 to 9 of the peptide FPSDSWCYF were encoded within the extended ORF. The screening of 150 additional tumor samples failed to result in the identification of additional tumor that expressed this mutation, indicating that this represented a relatively rare event. A mutated CDK4 gene product was cloned using tumor reactive CD8+ T cells, and 2 out of the 28 tumor cell lines that were analyzed expressed a product with the same mutation (57). Screening of an autologous tumor cDNA library

with an HLA-A24 restricted T cell clone resulted in the identification of a mutated β-catenin gene product (58). The analysis of β-catenin gene products expressed by multiple melanoma cell lines indicated that the identical mutation was observed in 4 of the 46 samples that were analyzed (59) and (Robbins, P., unpublished data). Use of an HLA-A11 restricted T cell to screen an autologous cDNA library resulted in the isolation of a transcript of the CKDN2A tumor suppressor gene locus containing a single nucleotide deletion, and the T cell epitope was derived from the frame-shifted region of this transcript (60). Two out of 18 additional melanomas that were screened expressed frame-shifted transcripts resulting from nucleotide deletions that encoded the T cell epitope.

Table 4. HLA class I restricted mutated antigens.

GENE	HLA ALLELE	EPITOPE	REFERENCE
α-actinin-4:118-127	A2	FIASNGVKLV	164
β-catenin:29-37	A24	SYLDSFIHF	58
BCR-ABL:926-934	A2	SSKALQRPV	66
BCR-ABL:922-930	B8	GFKQSSKAL	66
Caspase-8:476-484	B35	FPSDSWCYF	56
CDK-4:23-32	A2	ACDPHSGHFV	57
ELF2M:581-589	A68	ETVSEQSNV	12
ETV6-AML1:334-342	A2	RIAECILGM	165
HSP70-2M:286-295	A2	SLFEGIDIYT	166
	HLA-A*0201[b]		167
	HLA-A*1101[b]		60
KIAA0205:262-270	B44	AEPINIQTV	168
K-ras:7-15	B35	VVVGAVGVG	63
MUM-1:30-38	B44	EEKLIVVLF	2
MUM-2:123-133	B44	SELFRSGLDY	169
MUM-2:126-134	Cw6	FRSGLDSYV	169
MUM-3:322-330	A68	EAFIQPITR	170
N-ras:55-64	A1	ILDTAGREEY	64
Myosin-m:911-919	A3	KINKNPKYK	171
OS-9:438-466	B44	KELEGILLL	172
p14ARF:AORF[c]	A11	AVCPWTWLR	60
p16INK4a:AORF[c]	A11	AVCPWTWLR	60
MART-2:341-351	A1	FLEGNEVGKTY	173

a. The residue or residues that arise as a result of mutations are underlined.

b. Tumor specific T cells directly recognize the mutated class I HLA molecules.

c. Nucleotide deletions in the CDKN2a locus resulting in translation of the +1 and +2 open reading frames of the p14:ARF and p16INK4a gene products, respectively, result in generation of the T cell epitope.

Mutations that have been identified as a result of screening with tumor reactive T cells in many cases lead to functional alterations that may contribute to the tumorogenic phenotype. The mutated CDK4 gene product described demonstrated reduced binding to INK4a, a protein that is frequently mutated in melanomas and that plays a role in the inhibition of the activity of the normal CDK4 gene (61). The mutated caspase-8 gene product appeared to be attenuated in its ability to promote apoptosis, which may play a role in the loss of growth control in tumor cells that express this mutated transcript. Mutated β-catenin gene products can accumulate to abnormally high levels within the cell, which may lead to the activation of genes that are involved with the control of cell growth (62).

Candidate antigen approaches have also been used in attempts to generate tumor reactive T cells that recognize mutated gene products that are commonly expressed tumors of certain histologies. Common mutations that occur at either codons 12 or 61 result in the activation of members of the family of ras oncogenes, and tumor reactive T cells appeared to be generated following *in vitro* sensitization with peptides that encompass amino acid 12 in the K-ras gene product (63). In another study, the screening of melanoma reactive TIL that were stimulatedwith COS-7 cells that had been transfected with a mutated N-ras construct resulted in the isolation of T cell clones that recognized a single N-ras peptide that encompassed the mutated codon 61 residue (64). Chromosomal translocations that result in the generation of fused protein transcripts have been found to occur at relatively high frequencies in certain tumor types and may in addition lead to the generation of immunogenic T cell epitopes in the junctional region. A translocation resulting in fusion of exons derived from the BCR and ABL genes occurs at a high frequency in leukemias (65), and the *in vitro* stimulation of PBMC obtained from normal donors as well as patients with chronic myelogenous leukemia with a peptide encoded by sequences that spanned the break point resulted in the generation of HLA-A2 restricted, tumor reactive T cells (66).

7. HLA CLASS II RESTRICTED ANTIGENS

Multiple HLA class II restricted tumor antigen epitopes have been identified, many of which are derived from antigens initially identified as the targets of class I restricted T cells (Table 5). In one study, transgenic mice expressing the human HLA DRβ1*0401 molecule were initially immunized with recombinant human NY-ESO-1 protein (67). The T cells obtained from immunized mice were found to recognize 3 out of 8 candidate NY-ESO-1 peptides that were predicted to bind to this HLA class II allele and that were used to carry out in vitro sensitization. The *in vitro* sensitization of PBMC

from HLA-DRβ1*0401 positive melanoma patients with the peptide that appeared be most effective at stimulating T cells from immunized mice, NY-ESO-1:116-135, resulted in the generation of T cells that recognized HLA-DR4 positive tumor cells and target cells pulsed with the NY-ESO-1 protein.

Table 5. HLA Class II restricted antigens.

GENE	HLA ALLELE/EPITOPE		REFERENCE
Antigens arising from mutations[a]			
BCR-ABL:920-936	DR4	ATGFKQSSKALQRPVAS	80
CDC27:760-771[b]	DRβ1*0401	FSWAMDLDPKGA	7
dek-can:342-357	DR53	TMKQICKKEIRRLHQY	174
fibronectin:2050-2063	DRβ1*1501	MIFEKHGFRRTTPP	8
LDLR-FUT:315-324[c]	DRβ1*0101	WRRAPAPGAK	175
neo-PAP:724-734[b]	DRβ1*0701	RVIKNSIRLTL	95
PTPRK:667-682	DR10	PYYFAAELPPRNLPEP	79
TPI:23-37	DRβ1*0101	ELIGILNAAKVPAD	14
TGFβRII[d]	DPβ1*0301	SLVRLSSCVPVALMASAMTTSSSQ	176
Non-mutated antigens			
CEA:653-667	DR9	YACFVSNLATGRNNS	177
COA-1:372-385	DRβ1*0402	TLYQDDTLTLQAAG	178
COA-1:372-385	DRβ1*1301	TLYQDDTLTLQAAG	178
EphA3	DRβ11	DVTFNIACKKCG	10
gp100:44-59	DRβ1*0401	WNRQLYPEWTEAQRLD	74
gp100:174-190	DRβ1*0701	TGRAMLGTHTMEVTVYH	75
gp100:420-435	DRβ1*0701	TTEWVETTARELPIPE	76
Kallikrein 4:155-169	DR4	LLANGRMPTVLQCVN	179
Kallikrein 4:160-174	DR7	RMPTVLQCVNVSVVS	179
Kallikrein 4:125-139	DP4	SVSESDTIRSISIAS	179
LAGE-1:AORF	DR11,12	CLSRRPWKRSWSAGSCPGMPHL	51
MAGE-1,2,3,6:121-134	DR13	LLKYRAREPVTKAE	73
MAGE-1:281-292	DR15	EYVIKVSARVRF	180
MAGE-3,12:114-127	DR13	AELVHFLLLKYRAR	73
MAGE-3:267-282	DR1	ACYEFLWGPRALVETS	181
MAGE-3:149-160	DR4,7	VIFSKASSSLQL	182
MAGE-3:281-295	DR11	TSYVKVLHHMVKISG	183
MAGE-3:191-205	DR11	GDNQIMPKAGLLIIV	184
MAGE-3:247-258	DP4	TQHFVQENYLEY	185
MART-1:51-73	DRβ1*0401	RNGYRALNDKSLHVGTQCALTR	186
NY-ESO-1:119-130	DRβ1*0401	PGVLLKEFTVSG	67
NY-ESO-1:119-133	DRβ1*0401	PVGLLKEFTVSGNIL	68
NY-ESO-1:123-137	DRβ1*0401	LKEFTVSGNILTIRL	68
NY-ESO-1:80-109	DRβ1*0701	ARGPESRLLEFYLAMPFATPMEAEL	71
NY-ESO-1:134-148	DR1,3,4,7	TIRLTAADHRQLQLS	72
NY-ESO-1:115-132	DRβ4*01	PLPVPGVLLKEFTVSGNI	187
NY-ESO-1:121-138	DRβ4*01	VLLKEFTVSGNILTIRLT	187
NY-ESO-1:139-156	DRβ4*01	AADHRQLQLSISSCLQQL	187
NY-ESO-1:157-170	DPβ1:04	SLLMWITQCFLPVF	70
TRP-1:245-254	DRβ1*15	SLPYWNFATG	78

GENE	HLA ALLELE/EPITOPE		REFERENCE
TRP-2:241-250	DRβ1*15	ALPYWNFATG	78
tyrosinase:56-70	DRβ1*0401	QNILLSNAPLGPQFP	77
tyrosinase:448-462	DRβ1*0401	DYSYLQDSDPDSFQD	77
tyrosinase:386-406	DRb1*1501	FLLHHAFVDSIFEQWLQRHRP	188

a. The residue or residues that arise as a result of mutations are underlined.
b. Mutations outside of region encoding the T cell epitope appear to alter protein localization and lead to enhanced presentation of epitopes derived from these proteins.
c. A chromosomal translocation resulted from fusion of the LDLR gene with an inverted copy of the 2-alpha-L-fucosyltransferase gene, which encoded the T cell epitope.
d. A frame-shift mutation up-stream of the mutation resulted in generation of the epitope.

Human T cells that were stimulated with the NY-ESO-1:116-135 peptide also recognized the minimal T cell epitope NY-ESO-1:119-130, and peptides that overlapped with this epitope were independently shown to be recognized in the context of HLA-DR β1*0401 (68) as well as additional HLA-DR gene products (69). An NY-ESO-1 peptide was identified that is recognized in the context of two alleles of the HLA-DP locus that are expressed by approximately 75% of the population, HLA-DPβ1*0401 and *0402, and recognition of this epitope by T cells appeared to be associated with the presence of serum anti-NY-ESO-1 antibodies (70). The results of another study suggest that the presence of serum anti-NY-ESO-1 antibodies was associated with an enhanced precursor frequency in PBMC of T cells that recognize multiple HLA class II restricted NY-ESO-1 T cell epitopes (71). The majority of patients with significant serum anti-NY-ESO-1 antibody titers responded to the peptide NY-ESO-1:80-109, which appeared to be recognized in the context of multiple HLA class II alleles. An additional peptide, NY-ESO-1:134-148, was also shown to stimulate tumor reactive T cells that recognized this product in the context of multiple HLA-DR haplotypes (72); however, in this case no correlation was observed between the ability to generate peptide reactive T cells and serum anti-NY-ESO-1 antibody titers. Nevertheless, T cells that are reactive with certain class II restricted NY-ESO-1 epitopes may be involved with generating the anti-NY-ESO-1 antibodies that are observed in the serum of a large percentage of cancer patients bearing antigen positive tumors. Peptide as well as tumor reactive T cells have also been generated with HLA class II restricted T cell epitopes derived from one or more of the members of the MAGE gene family (73). Multiple HLA class II restricted gp100 epitopes have been identified (74-76), and CD4+ TIL were shown to recognize two epitopes of tyrosinase in the context of HLA-DRβ1*0401 (77). An epitope derived from the TRP-1 and TRP-2 proteins, which possess a high degree of sequence similarity, was also found to be recognized by HLA-DRβ1*1501 restricted, tumor reactive T cells derived from TIL (78).

Several mutated gene products have been shown to be recognized by HLA class II restricted, tumor reactive T cells. The screening of a cDNA library with HLA-DRβ1*1501 restricted T cells reactive with autologous tumor cells resulted in the isolation of a mutated fibronectin gene transcript (8). Formation of the fibronectin matrix was disrupted in cells expressing the mutated gene product, and results indicated that this alteration may have enhanced the metastasis of this tumor cell. A mutated product of the CDC27 gene [7], which represents a subunit of the anaphase promoting complex, as well as a mutated PTPRK protein tyrosine kinase gene product (79) were cloned by screening autologous cDNA libraries using tumor reactive T cells from melanoma patients. The expression of these mutated gene products may have played a role in the progression of tumors that was observed in these patients. In addition, a peptide that was encoded by junctional sequences present in BCR-ABL fusion transcripts appeared to be recognized by tumor reactive, DR4 restricted T cells (80).

8. MECHANISMS INVOLVED WITH THE GENERATION OF TUMOR ANTIGEN EPITOPES

Multiple transcriptional, translational, and post-translational mechanisms have been shown to be involved with generating the epitopes recognized by tumor reactive T cells. Intronic sequences that have been retained in a small percentage of the processed mRNA transcripts derived from certain genes appear to encode T cell epitopes. These include a mutated sequence derived from the intronic region of a gene that was termed MUM-1 (2), as well as non-mutated intronic sequences derived from the gp100 (81) and TRP-2 (46). Normal human melanocytes did not appear to express the intronic TRP-2 transcript at detectable levels *in vivo*, indicating that this actually represented a tumor specific antigen.

Several tumor antigens have been shown to represent the products of alternative open reading frames (ORFs), which in the majority of cases result from the initiation of translation at a methionine codon located downstream from the normal initiation codon. These include an HLA-A31 restricted epitope of TRP-2 (3), HLA-A2 restricted (4) and HLA-DR*11 and 12 restricted epitopes of LAGE-1 (51), and an HLA-A2 restricted epitope of the BING-4 molecule (82). In addition, nucleotide deletions resulting from mutations within the CDKN2A locus, which encodes 2 products that share a single exon 2 but that are translated in 2 open reading frames termed p14ARF and p16INK4a, have recently also been shown to result in the generation of a T cell epitope (60). A mutated p14ARF transcript containing a single nucleotide deletion in exon 2 gave rise to a product that was

translated in the third alternative open reading frame (AORF), and a T cell epitope that was recognized by an HLA-A11 restricted, tumor reactive TIL was present within this AORF. A melanoma that expressed a p16INK4a transcript containing a 2 base pair exon 2 deletion that resulted in the translation of the this AORF was also recognized by the HLA-A11 restricted TIL. Frame-shifted CDKN2A products are expressed at high frequencies in certain tumor types (83, 84), and thus this represents a highly shared mutated epitope that could be utilized as a target for immunotherapy in patients bearing certain cancers.

The proteasome plays an important role in protein processing steps that leads to the generation of MHC class I-binding peptides (85). The results of detailed analysis of processing suggest that many of the peptides that are produced are rapidly degraded by the proteasome (86), while at the same time, the proteasome plays an important role in generating the proper carboxy terminus of processed HLA class I restricted T cell epitopes. Thus, only a small percentage of candidate peptides from a given protein that are capable of binding exogenously to a particular HLA allele appear to be naturally processed endogenously (87, 88).

The importance of proteasomal processing mechanisms in generating the epitopes recognized by tumor reactive T cells has been demonstrated by several studies. The product of a short ORF of 11 amino acids encoded by a pseudogene that was similar to a portion of the 3' untranslated region of the homeoprotein HPX42B was recognized by HLA-B*1302 restricted, tumor reactive T cells (89). Recognition of the pseudogene was critically dependent on the presence of a stop codon in the pseudogene following the codon that encoded the carboxy terminal residue in the peptide epitope, which would generate a partially processed epitope. Similar mechanisms may also enhance the recognition of a subset of the tumor antigens encoded by short alternative ORFs (82, 90).

As mentioned above, some studies have failed to confirm initial reports indicating that T cells stimulated with certain candidate epitopes were capable of recognizing tumor cell targets. Initial reports indicated that tumor reactive T cells were generated following *in vitro* stimulation with an HLA-A2 restricted candidate epitope identified from MAGE-3, MAGE-3:271-279 (40). Subsequent studies indicated that T cells generated with this peptide failed to recognize tumor cell targets, and that incubation of long peptides that encompassed this epitope with purified proteasomes failed to generate the proper peptide carboxy terminus (41). Cultures of peptide reactive T cells recognized tumor cells that had been treated with the proteasomal inhibitor lactacystin, however, indicating that peptide bonds within this peptide sequence are normally cleaved by the proteasome, resulting in the inefficient recognition of un-manipulated cells. Initial results indicated that T

cells that were generated by stimulating PBMC *in vitro* with an HLA-A2 binding peptide from the human telomerase catalytic subunit hTERT, hTERT:540-548, recognized a variety of tumor cell targets (91). Subsequent studies failed to confirm the recognition of tumor targets and indicated that the peptide could not be generated by incubation of long peptide precursors with purified proteasomes (52).

Several recent studies have focused on identifying candidate T cell epitopes that can be efficiently processed by the proteasome. In one study, long peptides that encompassed candidate HLA-A2 restricted T cell epitopes derived from PRAME were incubated with purified proteasomes (92). The analysis of the peptide digests revealed that the appropriate carboxy terminal residue was generated from only 4 out of the 19 high affinity peptides that were subjected to degradation using this procedure. The T cells that were generated following *in vitro* sensitization with peptides that were identified using this approach appeared to efficiently recognize tumor cells that expressed PRAME and HLA-A2, indicating that this process might facilitate the identification of relevant peptide vaccine target epitopes. An epitope of the SSX-2 antigen that was initially identified by SEREX analysis was identified by incubation of long peptides with purified proteasomes (16).

Expression of the immunoproteasome, which has a distinct peptide cleavage specificity from the proteasome and which can be induced in a variety of cells by stimulation with interferon gamma (IFN-γ), may also affect the processing of certain T cell epitopes. The treatment of tumor cells with IFN-γ nearly eliminated the ability of those cells to stimulate T cells that recognize multiple HLA class I restricted T cell epitopes, including the gp100:209-217 and MART-1:26-35 peptides (93), while at the same time, the treated tumor cells did not demonstrate a diminished capacity to stimulate T cells that recognized additional epitopes. These findings are consistent with the altered cleavage specificity of the immunoproteasome. In contrast, IFN-γ treated, but not un-treated melanoma cells were capable of stimulating T cells that recognized a class I HLA-B40 restricted MAGE-3 epitope (94). Un-treated tumor cells that were transfected with the catalytic LMP7 subunit, which is only expressed in the immunoproteasome, were also recognized by the MAGE-3 reactive T cells, providing further evidence that an alteration in antigen processing was responsible for these findings.

Intracellular protein targeting mechanisms may influence the ability of T cells to recognize certain epitopes. A point mutation in the CDC27 transcript that was not directly involved with generating the HLA class II restricted T cell epitope resulted in re-localization of this nuclear protein to the cytoplasm, thereby presumably resulting in enhanced processing of a distal T cell epitope (7). A similar mechanism may be involved with the processing

of a mutated HLA class II restricted epitope derived from neo-poly(A) polymerase (95).

Post-translational processing has been shown to alter certain epitopes recognized by tumor reactive T cells. The HLA-A2 restricted tyrosinase epitope tyr:369-377 contains an asparagine residue at position 371 that represents an N-linked glycosylation site (31). HLA-A2 positive target cells that were pulsed with a peptide containing a substitution of an aspartic acid residue for asparagine residue, however, were recognized at significantly lower concentrations than target cells pulsed with the peptide that was encoded by the normal tyrosinase gene. A mammalian enzyme that removes oligosaccharide side chains from glycoproteins, PNGase (96), may be responsible for conversion of the amino acid sequence of the tyrosinase peptide. Subsequent studies indicated that tyrosinase molecules that are initially generated in the endoplasmic reticulum are subsequently transported into the cytoplasm where processing resulting in the generation of peptides that can then be transported back into the endoplasmic reticulum where they associate with HLA class I molecules (97).

Recent observations suggest that a process that has previously not been described in mammalian cells, protein splicing, is also responsible for generating T cell epitopes recognized by tumor reactive T cells. An epitope of the FGF-5 protein that was recognized by the HLA-A3 restricted T cells reactive with renal carcinomas resulted from the joining of sequences of 5 and 4 amino acids that were separated by 40 amino acids in the primary FGF-5 protein (54). Mechanisms such as alternative mRNA splicing or ribosomal skipping did not appear to be involved with generating this epitope, indicating that this peptide was derived by splicing of the FGF-5 protein. Two 3 and 6 amino acid peptides that were separated by a 4 amino acids in the gp100 protein appeared to be spliced to yield an epitope that was recognized in the context of HLA-A32 (6). Incubation of a 13 amino acid precursor with purified proteasomes resulted in the generation of the HLA-A32 restricted T cell epitope, indicating that peptide processing within this cellular compartment was capable of generating a spliced T cell epitope. Protein splicing also appeared to be involved with generation of a tyrosinase epitope that was recognized in the context of HLA-A24 (Robbins et al., manuscript in preparation). The tyrosinase epitope resulted from the linkage of a 5 amino acid peptide corresponding to residues 368 to 372 to a 4 amino acid peptide corresponding to residues 335 to 340. This T cell epitope appears to have result from splicing as well as re-ordering of the primary tyrosinase amino acid sequence. The HLA-A24 restricted tyrosinase epitope also contained 2 asparagines corresponding to residues 337 and 371 that represent N-linked glycosylation sites that appear to be converted to aspartic

acid residues during the processing of the T cell epitope, as described for the partially overlapping HLA-A2 restricted tyrosinase epitope (31).

9. INFLUENCE OF T CELL TOLERANCE ON TUMOR ANTIGEN RECOGNITION

Many of the T cell epitopes that have been identified from self antigens such as the MDAs bind to HLA class I gene products with relatively low affinities, and observations made in mouse as well as human studies indicating that the repertoire of T cells that is available for recognition of these antigens is at least partially influenced by tolerance mechanisms may be relevant to this finding. In one study, HLA-A2 transgenic mice that either expressed the normal mouse tyrosinase molecule or that failed to express this protein as a result of a radiation induced deletion of sequences that encoded this gene were immunized with the mouse analog of the tyr:369-377 peptide FMDGTMSQV (98). The results indicated that the precursor frequency of high affinity T cells reactive with the tyr:369-377 epitope was significantly reduced in normal mice relative the to knock-out mice. In a recent human study, an HLA-A24 restricted T cell epitope was identified from the OA1 melanocyte differentiation antigen by screening candidate epitopes from this protein, and the peripheral blood of a non-melanoma patient with ocular albinism who possessed a complete deletion of the OA1 gene, patient OAP-46, contained a higher precursor frequency of T cells reactive with the OA1 peptide epitope than peripheral blood obtained from normal donors (36). Tumor reactive T cells were readily induced from patient OAP-46 but not from either normal donors or melanoma patients, indicating that immune tolerance resulting from the recognition of OA1 expressed on normal melanocytes may have resulted in a partial deletion of high affinity T cells that recognize this epitope. These results raise the possibility that peptides that are expressed on normal cells and that bind to self MHC molecules with relatively high affinities may lead to an even more complete deletion of T cells reactive with these epitopes.

10. CLINICAL OBSERVATIONS

These findings indicate that expanded populations of T cells reactive with a wide variety of tumor antigens are present in cancer patients. The failure of these T cells to control tumor cell growth may result from a variety of factors that include the expression of immunosuppressive factors by tumor cells, as well as the lack of appropriate inflammatory signals within the

tumor environment. Approaches utilizing active tumor antigen immunization have to date met with only limited success, and new approaches may be needed to enhance or maintain sufficient levels of T cell activation required to eradicate what are in many cases bulky tumors. Dramatic regression of bulky metastatic lesions has been observed following the adoptive transfer of in vitro activated tumor reactive T cells with IL-2, but long term complete cures have still only been observed in a minority of patients treated using this approach. In a recent clinical trial in which patients were treated with non-myeloablative chemotherapy in order to enhance the engraftment of adoptively transferred tumor reactive T cells, nearly 50% of patients demonstrated complete or partial tumor regression (99). Recent findings suggest that only a minority of the *in vitro* cultured T cell clonotypes persist in these patients following adoptive transfer, and indicate that T cell persistence is significantly correlated with clinical response to therapy (Robbins et al., submitted for publication). A better understanding of factors that influence the *in vivo* activation of tumor reactive T cells, as well as the persistence of adoptively transferred T cells, will hopefully lead to the development of more effective cancer therapies.

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Chapter 3

IMMUNE ESCAPE

Tumor induced immune suppression and immune escape: Mechanisms and Possible Solutions

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Abstract: It has been a general experience that results of immunomonitoring of patients with malignant disease treated with active specific immunotherapy have poor, if any, predictive value. Furthermore, lack of clinical response and/or recurrence of disease in spite of induction and/or persistence of tumor antigen (TA)-specific immune responses appears to be the rule more than the exception in clinical trials. These disappointing results are likely to be caused, at least in part, by tumor cells' ability to evade immune recognition and destruction. In this paper after a description of the essential components required to generate an effective TA-specific T cell-based immune response, we have reviewed the potential mechanisms underlying the lack of correlation between immunological and clinical response in immunized patients. They include qualitative and/or quantitative defects in the generation and maintenance of TA-specific immune responses, changes in the antigenic profile of tumor cells because of their genetic instability and/or the potential negative impact of the tumor microenvironment on the interaction between host immune cells and tumor cells. Lastly, we have discussed potential strategies to overcome immune escape mechanisms utilized by tumor cells and improve the outcome of T cell-based immunotherapy in patients with malignant disease.

Key words: Tumor antigens, HLA expression, TCR-pMHC interactions, immune escape

Abbreviations: AICD, activation-induced cell death; APC, antigen-presenting cell; APM, antigen processing machinery; CTL, cytotoxic T lymphocytes; DC, dendritic cell; HLA, human leukocyte antigen; HNSCC, squamous cell carcinoma of the head and neck; HSP, heat shock proteins; IAP, inhibitor of apoptosis; IHC, immunohistochemistry; IL, interleukin; IFN, interferon; MHC, major

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histocompatibility complex; MSC, myeloid suppressor cell; NK, natural killer; PBL, peripheral blood lymphocytes; PMN, polymorphonuclear; RCC, renal cell carcinoma; ROS, reactive oxygen species; TA, Tumor antigens; TADC, tumor-associated dendritic cells; TAM, tumor associated macrophages; TCR, T cell receptor; TGF, transforming growth factor; TIDC, tumor-induced cell death; TIL, tumor infiltrating lymphocytes; Treg, regulatory T-cell; VEGF, vascular endothelial growth factor

1. INTRODUCTION

During the last three decades, there has been considerable enthusiasm for developing and applying immunotherapy to the treatment of malignant diseases, due to the limited efficacy of conventional therapeutic regimens (1,2) and the major progress made in the identification and molecular characterization of tumor antigens (TA) (3-5). Recent emphasis on active specific T cell-based immunotherapy (i.e. vaccination of against malignant cells) has emerged because of the disappointing results of the antibody-based trials conducted in the early 80's (6), the general belief that T cells play a major role in tumor growth control (7), the significant advances in our understanding of the molecular mechanisms leading to an immune response (5,7-10) and the availability of cytokines (11,12) and monoclonal antibodies (13) to modulate an immune response. Taking advantage of the many TA identified in malignant cells and of the multiple ways to modulate an immune response, an increasing number of immunization strategies have been developed and translated to clinical trials (5,14,15). Most, although not all, the immunization strategies tested have been found to be effective in eliciting a TA-specific cellular immune response (5,14,15). Contrary to the expectations, coordinate clinical and immunologic responses have only been observed in a limited number of the patients enrolled in immunotherapy-based clinical trials. In many cases, immune responses to vaccines have been weak or undetectable (5,14,15). In other cases, peptide- or TA-specific CD8+ cytotoxic T lymphocytes (CTL) are readily demonstrable in the patients' circulation prior to treatment, and their frequency increases following vaccine administration, but without any obvious or measurable impact on the clinical course of the disease (5,14,15). In fact, the experience has been that results of immunomonitoring assays in patients receiving TA-specific vaccines have poor, if any, predictive value (5,14,15) and that lack of clinical response and/or recurrence of disease occurs frequently, in spite of induction and/or persistence of TA-specific immune responses (5,14,15).

The lack of correlation between immune and clinical response in patients receiving active specific immunotherapy has been unexpected and cast doubt

regarding the ability of the host immune system to control tumor growth *in vivo*. These findings are in contrast to responses noted for anti-viral vaccines (16,18), and they do not fit with the evidence accumulated from a wide range of animal tumor models, where TA-specific T cells have been shown to play an active role in eliminating tumors and metastases as well as in inducing TA-specific T cell memory responses (7). Similarly, *in vitro* studies employing human peripheral blood lymphocytes (PBL) isolated from patients with malignant diseases have been reported to contain TA-specific CD8+ and CD4+ T cell precursors that are capable of killing tumor cell targets after appropriate *in vitro* activation (5,10,14,15). Therefore, one of the major challenges facing the field of modern tumor immunology is the understanding of why a TA-specific immune response, which can be detected in a variable percentage of patients, is not paralleled by a clinical response in the majority of immunized cancer patients. In view of the well-recognized immune escape mechanisms utilized by malignant cells (19-23) as well as their genetic instability (24) such disappointing clinical findings are likely to reflect the ability of malignant cells to escape immune recognition and destruction and/or their capacity to interfere with the differentiation, function and/or survival of immune effector cells (19-23). In this manuscript, we will first review the essential components required to generate an effective TA-specific T cell-based immune response. We will then discuss the possible mechanisms underlying the lack of correlation between immunological and clinical responses in immunized patients. Although a number of "immune escape" mechanisms have been identified and characterized we will focus on immune effector cell dysfunction in patients with malignant disease as well as on changes in the antigenic profile of tumor cells. Interested readers are referred to recent reviews (19-23) for additional information on immune escape mechanisms utilized by malignant cells. Lastly, we will discuss potential strategies to overcome "immune escape" mechanisms utilized by tumor cells and to improve the outcome of TA-specific immunotherapy in the future.

2. ESSENTIAL COMPONENTS OF AN EFFECTIVE TA-SPECIFIC T CELL-BASED IMMUNE RESPONSE

Although mechanisms of tumor-specific immunity and its role in the development and progression of cancer in man have been much debated, newer evidence suggest that the components necessary for mounting the anti-tumor immune response are present in cancer patients.

2.1 Tumor antigens

Tumor-antigens. Both the SEREX, T-cell-based and reverse immunology approaches have identified a variety of tumor antigens (TA), which can be broadly classified as tumor antigens that are capable of being recognized by the immunocompetent host: (a) cancer-germ line antigens such as MAGE 1 or 3, BAGE, GAGE and many others that are silent in normal tissues, with the exception of germ cells in the testes and ovaries but expressed in a variety of histologically distinct tumors (3-5); (b) differentiation-specific antigens exemplified by melanoma- and melanocyte-associated tyrosinase, MART-1/Melan-A or gp100 (3-5); and (c) unique antigens generated by point mutations in ubiquitously expressed genes, which regulate key cellular functions, such as MUM-1, CDK4, FLICE or b-catenin (3-5); (d) overexpressed antigens, such as p53, MDM2, CEA, which are components of normal cells but in tumor cells are greatly increased in expression (3-5,25). Despite the fact that the majority of the known TA, with the exception of mutation products, represent self epitopes and reactive T cells undergo normal thymus selection, the presence of both tumor-specific antibodies and T cells has been clearly documented in tumor-bearing humans and mice (3-5). Using tetramers, it has been recently possible to identify T cells specific for melanoma differentiation peptides, wild-type p53 peptides and others in the circulation of normal donors and patients with cancer (26,27).

2.2 Antigen presenting cells (APC)

Most of the immunization strategies, which have or are being tested in clinical trials today, share the common goal of inducing TA-specific CTL capable of lysing malignant cells. However, it should be stressed that TA-specific (CD8+) CTL and helper (CD4+) T cells as well as antibody-secreting B cells are essential for anti-tumor effector functions (5,7-10). Moreover, as in most chronic diseases, both non-specific and specific components of the host immune response play a role in the control of tumor growth and metastasis, with some components, e.g., natural killer (NK) cells, polymorphonuclear cells (PMN) and macrophages, thought to participate in the early phase of the response, prior to the appearance of T or B cells. It is noteworthy that NK cells are likely to play a role in the elimination of tumor cells which fail to express major histocompatibility complex (MHC) molecules and thus are not recognized by TA-specific CTL (28).

The structural basis of tumor cell recognition by CTL occurs through the interaction of T cell receptor (TCR) with class I Human Leukocyte Antigens (HLA) complexed to TA-derived peptides (i.e., HLA class I-TA peptide complexes) generated by the antigen processing machinery (APM) (Figure

1) (29). These complexes can be presented to T cells directly by tumor cells through a process defined as direct priming, although the tumor cell is no longer considered as the central antigen-presenting component of an ongoing TA-specific immune response. Alternatively, TA can be captured by professional antigen presenting cells (APC) and processed for indirect priming of CTL via T helper cells. Tumor cells can also transfer TA to APCs via apoptotic or necrotic tumor cells as well as tumor-derived exosomes. TA-derived peptides are then presented to T cells through a process defined as cross priming (30). Heat shock proteins (HSP) can also transfer TA to APC by chaperoning TA-derived peptides which are eventually loaded onto HLA class I antigens and presented to CTL (31).

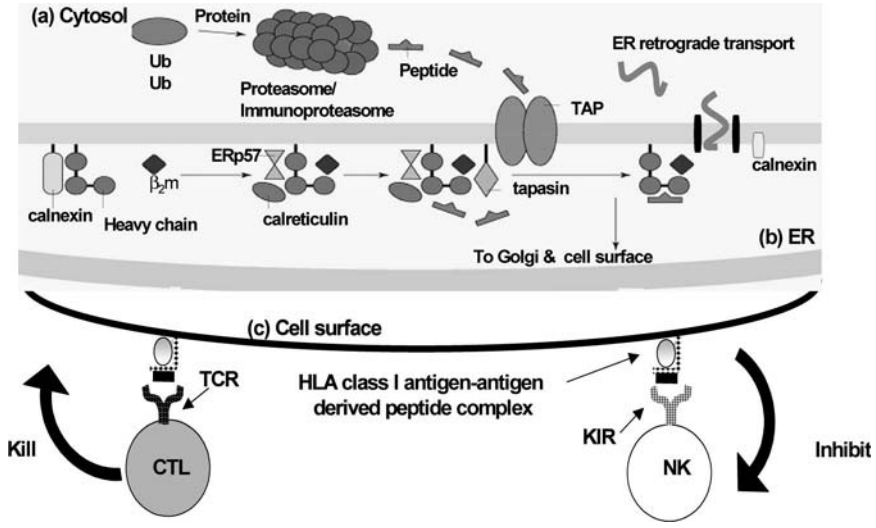

Figure 1. Generation and interaction of HLA class I antigen-antigen derived peptide complexes with T cells and NK cells. (A) Intracellular protein antigens, which are mostly endogenous, are marked for ubiquitination within the cytosol and subsequently degraded into peptides by the proteasome. Peptides are then transported into the ER through TAP. (B) Nascent, HLA class I antigen heavy chains are synthesized in the ER and associate with the chaperone immunoglobulin heavy chain binding protein (BiP), a universal ER chaperone involved in the translation and insertion of proteins into the ER. Following insertion into the ER, the HLA class I heavy chain associates with the chaperone calnexin and the thiol-dependent reductase ERp57. Calnexin dissociation is followed by HLA class I heavy chain association with β2m, tapasin and the chaperone calreticulin. Calnexin, calreticulin and ERp57 play a role in folding of the HLA class I heavy chain. Tapasin brings the HLA class I heavy chain-β2m-chaperone complex into association with TAP and plays a role in both quantitative and qualitative peptide selection. (C) The trimeric HLA class I heavy chain β2m peptide complex is then transported to the plasma membrane where it plays a major role in the interactions between target cells and activation of peptide-specific CTL through TCR or with NK cells through killer inhibitory receptors (KIR).

Dendritic cells (DC) are the most potent APC. DC have a high surface density of HLA antigens and costimulatory molecules and can produce immunostimulatory cytokines and chemokines (32). Furthermore, DC are efficient in processing exogenous TA via the HLA class I antigen pathway, cross-presenting TA to CD8+ T cells and also interact with cells of the innate immune system, i.e., NK and NKT cells (33-36). After internalizing TA at the tumor site, chemokine receptor 7-positive (CCR7+) DC traffic to the tumor-draining lymph nodes, where they interact and influence the maturation of T cells within the paracortical cords and of Ab-producing plasma cells in the medullary cortex (32,38). DC may also be present at the tumor site and are referred to as tumor-associated DC (TADC). TADC cross-present TA to recruited CD8+ T cells, potentially inducing their activation, proliferation and maturation into TA-specific effector cells. In addition, TADC, when appropriately activated, mediate the sensitization of naïve T cells that may have been recruited into the tumor site. Thus, interactions between the tumor-infiltrating T lymphocytes (TIL) and TADC are essential for driving and maintaining the local TA-specific immune response. Optimal biologic functions and survival of T cells and DC may be enhanced by reciprocal signaling between these two cell types via HLA class I-peptide complexes and co-stimulatory receptor-ligand pairs (39).

Interaction of TCRs with HLA class I antigen-peptide complexes, together with help from activated $CD4^+$ T cells, leads to activation and clonal expansion of TA-specific $CD8^+$ CTL. Based on our current understanding, both CD8+ and CD4+ T lymphocytes can be categorized into at least three functional subsets, depending on the cytokines they produce (40-42). These subsets include i) Tc1/Th1 (Type-1) cells which produce IFN-γ and IL-2; ii) Tc2/Th2 (Type-2) cells which produce IL-4 and IL-5, and iii) Th3/T regulatory (T-reg) cells which produce IL-10 and/or TGF-β. It is worth noting that Type-1-biased immune responses are strongly supported by IL-12p70 (43) and are associated with the host's ability to control and eliminate intra-cellular pathogens and tumors. On the other hand, Type-2-biased immune responses inhibit Type-1 responses and do not favor the development of cellular tumor immunity. Once TA-specific T cells are activated, they leave the lymph node environment and make their way via the lymphatics to tumor site(s), arriving as primed, but not necessarily fully differentiated effector cells. CTL are expected to induce programmed cell death of malignant cells which express targeted HLA class I antigen-peptide complexes, through the perforin-granzyme mechanism and/or the Fas/Fas ligand pathway (44). The latter requires the expression of Fas receptor on target cells and Fas ligand on effector CTL.

2.3 Cytokine milieu

Through secreted chemokines and cytokines, tumors can induce and amplify non-HLA-restricted, inflammatory responses in the host, leading to the accumulation of immune cells at the tumor site. Moreover, cytokines and chemokines play a key role in shaping functional attributes of both T cells and DC in a tissue microenvironment. Like T cells, DC are functionally heterogeneous and the polarization of DC into distinct subsets, i.e. DC1, DC2 and DC3, appears to correspond to the functional T cell subsets they interact with, i.e. Type-1, Type-2 and T-reg (38,40). For example, DC matured in the presence of Th1 type cytokines such as IFN-γ are polarized to secrete IL-12p70, a cytokine which promotes Th1-type responses (DC1); DC matured in the presence of the Th2 type cytokines such as IL-4 and IL-5 are polarized to promote Th2-type responses (DC2), and DC matured in the presence of regulatory T cell (T-reg) cytokines such as IL-10 and TGF-β, might assume a down-regulatory activity (DC3). The context of DC polarization and their ability to "switch" their functional potential in response to a new cytokine cocktail are being extensively studied at present, and both *in vitro* and *in vivo* experiments indicate a remarkable plasticity of this cellular population, which is clearly driven by cytokines and chemokines (45,46). Overall, it is clear that cytokines dictate the nature of the locoregional immune response, depending on the activation signals received by the T cells and DC infiltrating the tumor microenvironment (47).

Clearly a number of components are necessary for the generation and maintenance of an effective TA-specific T cell-based immune response including TA, APC, immune effectors and cytokines. The role each of the components plays in the development of TA-specific T cell-based immune responses as well as the cellular interactions envisioned to take place within the tumor microenvironment are summarized in Figure 2.

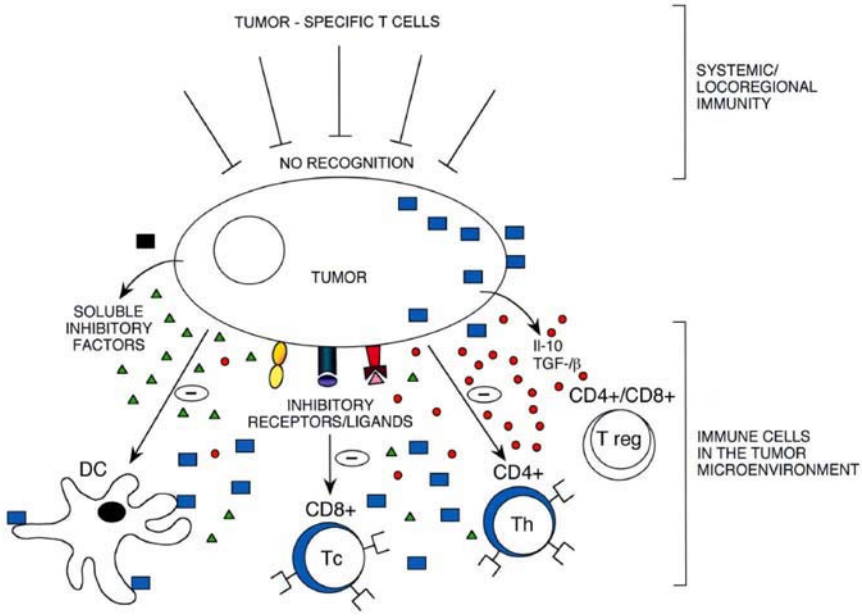

Figure 2. Schematic representation of the cellular interactions that may occur within the tumor microenvironment. Immune escape mechanisms utilized by tumor cells include secretion of immunoinhibitory factors (blue rectangles) and cytokines (e.g., IL-10, TGF-β) produced by the tumor (Red circles); and/or loss of TA (Green triangles). Together, these effects promote a tumor microenvironment suppressive for immune cells (DC, Tc, Th) and enriched in regulatory T cells (T reg).

3. MECHANISMS UNDERLYING THE LACK OF CORRELATION BETWEEN CLINICAL AND IMMUNE RESPONSES

To date, a large number of TA-specific immunotherapy trials have been conducted in patients with malignant disease. It is clear from these studies that the various types of tumor vaccines i) have limited or no toxicity and ii) are able to induce TA-specific immune responses and/or augment already established TA-specific immune responses in immunized patients. Nevertheless, the results of these studies have highlighted two challenges facing tumor immunologists and clinical oncologists. The first is the selection of the most effective immunotherapeutic strategy, since the various available strategies have not been systematically compared. Unfortunately,

this information is not likely to become available in the near future given the prohibitive costs of clinical trials. The second is the understanding of why a TA-specific T cell-based immune response, which can be detected in a variable percentage of patients, is not paralleled by a clinical response in the majority of immunized patients, despite the presence of all the necessary components for the successful development of a TA-specific immune response, i.e., TA, APC, immune effector cells and cytokines, in immunized patients. A number of mechanisms may account for the lack of correlation between immunological and clinical responses in immunized patients. They include i) qualitative and/or quantitative defects in the generation and maintenance of TA-specific immune responses and ii) changes in the antigenic profile of tumor cells because of their genetic instability.

3.1 Evidence for locoregional and systemic immune dysfunction in cancer

The presence of leukocytes in human tumors has led to speculations that these cells play a positive role in the control of tumor growth. Numerous reports in the literature have attempted to correlate the degree or type of these cellular infiltrates with prognosis and/or patient survival, without reaching a consensus (48,49). Nevertheless, it seems reasonable to predict that it is not the presence *per se*, but the functional potential of the infiltrating cells confronting the tumor that determines their utility in host defense.

Most TIL are activated T cells containing variable proportions of CD8+ and CD4+ T cell subsets, which are almost exclusively CD45RO+ memory T cells (21,23,48,49). In comparison to autologous PBL or those isolated from tissues distant from the tumor, TIL have been consistently found to be poorly responsive or unresponsive to traditional T-cell activating stimuli (21,23,50,51) (Table 1). It has been suggested that the paucity of Th1 cytokines (i.e., IL-2, IFN-γ and IL-12) at the tumor site or tumor-draining lymph node as well as the prevalence of T-reg cytokines (i.e., IL-10 or TGF-β), appear to condition evolving TA-specific T cells toward the less efficacious Th2 or T-reg functional phenotypes. In fact in patients with malignant disease, TIL have been shown to display a predominant Type-2 or/T-reg functional phenotype associated with the local production of IL-4 or IL-10 rather than the mixed Type-1/Type-2 responsiveness observed in normal donors (52). It is noteworthy that although a proportion of TIL may be anergic *in vivo*, they can be reactivated and polarized to Th1/Tc1-type functionality after being removed from the tumor microenvironment and appropriately stimulated *in vitro* in the presence of high concentrations of cytokines, especially IL-2 or IL-15 (5,7,11,53). These findings suggest that

even in the face of tumor-induced immune deviation, these lymphocytes can be salvaged and may be of clinical benefit, if an appropriate treatment is administered.

Table 1. Immune deviation in T cells present in the tumor microenvironment.

1. Activation of proteolytic enzymes in tumor-infiltrating leukocytes: rapid degradation of cellular proteins (21,23)
2. Signaling defects in TIL and PBL-T
   (a) NFκB abnormalities (56,58)
   (b) ζ chain defects: either low expression or absence (63,67,73,75,78)
   (c) Ca++ flux alterations (60)
3. Cytokine expression: absent/decreased Th1-type cytokines (103-105)
4. Inhibition of lymphocyte proliferation, cytotoxic activity or cytokine production (21,23,60)
5. Inhibition of leukocyte migration (174)
6. Induction of T-cell apoptosis (21,23,95)
7. Expansion of immunosuppressive macrophages (111,176)

Alterations in systemic TA-specific T cell immunity also occur in patients with malignant disease. In the early 1990's Mizoguchi and colleagues (54), studying dysfunctional T cells from long-term tumor bearing mice, demonstrated a marked decrease in the expression of CD3ζ chain, and of $p56^{lck}$ as well as $p59^{fyn}$ tyrosine kinases, all of which play a critical role in the signal transduction events that lead to T cell activation (50). These changes were accompanied by a decreased tyrosine kinase phosphorylation and diminished calcium influx. These findings provided for the first time a molecular basis for T cell dysfunction in cancer patients. Additional studies demonstrated that T cells derived from patients with renal cell carcinoma (RCC) and from long-term tumor bearing mice were unable to translocate NFκBp65 nuclear transcription factor, resulting in a predominance of NFκBp50/50 homodimer known to act as a repressor of the IFN-γ gene (55-59). In fact, cytokine production during the progressive growth of tumors in mice demonstrated a Th1 response (IL-2 and IFN-γ) early after tumor implantation, followed by an increased production of Th2 cytokines (IL-4 and IL-10) after three weeks (50,60). More recent studies in patients with malignant disease confirmed these initial observations in murine models. In this regard, T cells and NK cells from approximately half of the patients with carcinoma of the head and neck (50,60,61), breast (50,60,62), stomach (50,60) colon (50,60,63,64), kidney (50,60,65,66), ovary (50,60,67), cervix (68,69) and prostate (70), as well as non-Hodgkins lymphoma (71), Hodgkins lymphoma (72), and melanoma (73,74) demonstrate a decreased CD3ζ chain expression and a decreased *in vitro* response to antigens or mitogens. We have also demonstrated that circulating T cells are biased in their cytokine profile or otherwise functionally

compromised in patients with malignant disease (60,61,75,76). Interestingly, alterations in circulating T cell function, as determined by CD3ζ chain expression, proliferative index or NFκB activity, are associated with the extent of alterations in TIL function and with tumor stage (50,60,75,76). These observations suggest that CD3ζ chain expression may be a marker of immune competence in patients with malignant disease and individuals who have normal CD3ζ chain expression are most likely to respond favorably to biotherapy (61). It is noteworthy that changes in signal transduction molecules are not limited to CD3ζ chain. Kolenko and colleagues demonstrated that Jak-3, a tyrosine kinase associated with the γ chain, a common element to IL2, IL4, IL7 and IL15 cytokine receptors, was also decreased in T cells from RCC patients (77). Moreover, T cells from RCC patients also had a diminished ability to translocate NFκBp65 (58,59). Regardless of the specific defect, the presence of such systemic alterations may explain, in part, why vaccines and other immunotherapies induce objective clinical responses in only a small minority of patients with malignant disease.

Functional defects in systemic immunity as well as TIL appear to be clinically significant. Alterations in T cell signal transduction are assocaited with advandced tumor stage in colon carcinoma (64) and RCC (57). Moreover, we have demonstrated that the level of CD3ζ chain expression in TIL from patients with oral carcinoma is an independant predictive parameter of 5-year survival rate (figure 3) in patients with advanced stage disease (78). This study is the first report of a direct association between reduced CD3ζ chain expression in TIL-T cells, disease progression and patient survival. It is noteworthy that abnormalities in T cell signal transduction might also occur early in disease, as diminished CD3ζ chain expression has been found in patients with in situ cervical carcinoma (68,69). The role CD3ζ chain defects play in the clinical course of malignant disease is highlighted by the association between CD3ζ chain expression levels and response to treatment. Thus, levels of CD3ζ chain expression increase in T cells of non-Hodgkins and Hodgkins lymphoma patients who respond to chemotherapy, while levels of CD3ζ chain expression are reduced in patients who have recurrence of the disease (72). In addition, limited data from clinical trials in colon carcinoma, RCC, ovarian carcinoma and melanoma indicate that CD3ζ chain expression levels are increased in patients receiving IL-2 based therapies (57,74,79).

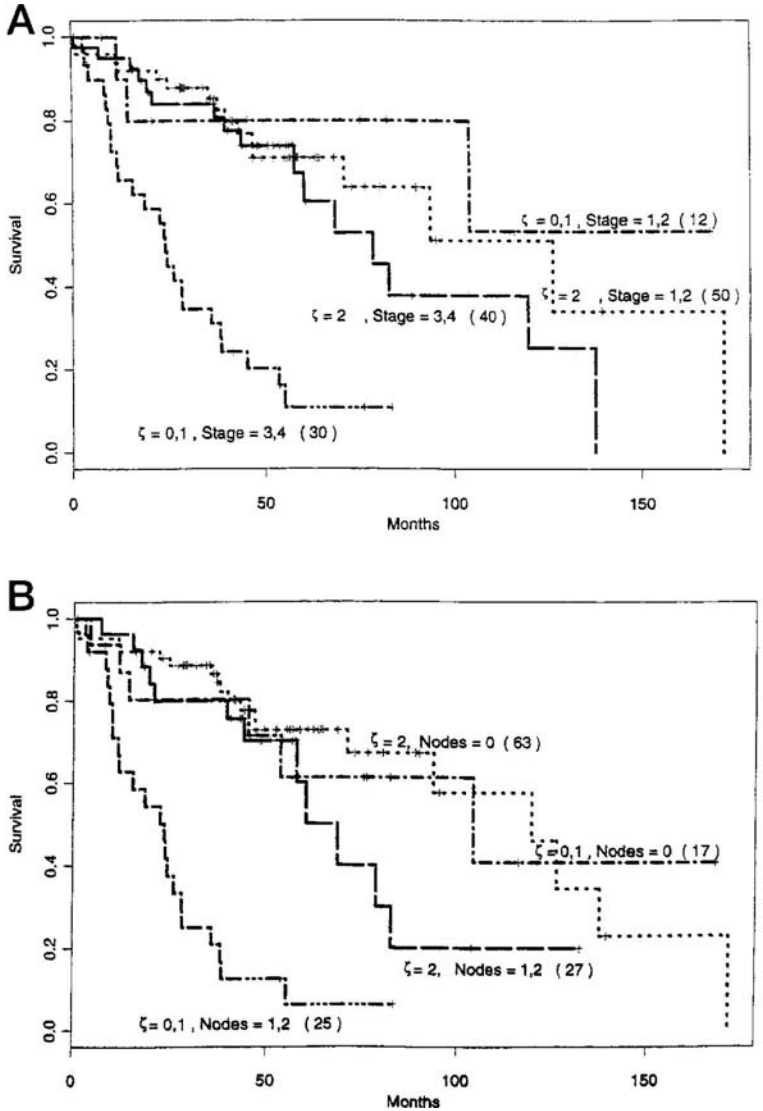

Figure 3. Association of ζ chain expression in TIL, tumor stage and lymph node involvement with survival in oral squamous cell carcinoma patients. Reduced survival is seen in patients with oral squamous cell carcinoma whose lesions demonstrate reduced ζ chain expression in TIL and (A) high tumor stage or (B) high degree of lymph node involvement. Numbers in parentheses indicate numbers of patients in each category (low ζ, stage I-II; normal ζ, stage I-II, low ζ, stage III-IV; and normal ζ, stage III-IV). Reproduced from ref. 71.

However, this did not always coincide with a full recovery of T cell function, since tyrosine kinase activity is not completely restored.

Functional impairments have also been noted for alternate effector cells that accumulate within tumor sites. Several recent reports indicate that TADCs are functionally defective, especially in their antigen-presenting capacity (80, 81). Moreover, tumor-associated macrophages (TAM) also exhibit functional defects relative to their counterparts obtained from tumor-uninvolved inflammatory sites in the same patient (81,82). However, the functional consequences of these impairments have not yet been elucidated.

3.2 Mechanisms of immune dysfunction in patients with cancer

3.2.1 Tumor-derived suppressive factors

Production of inflammatory molecules by tumor cells may play a role in modulating the host immune response within the tumor microenvironment. In fact, early experiments, dating back more than 30 years, provided evidence that tumor-derived factors can alter the normal functions of immune cells *in vitro* (83). More recently, serum derived from patients with malignant disease has been shown to interfere with DC differentiation and T-cell activation and to induce apoptosis in activated T cells (80,84-86). Over the years, a number of tumor-derived factors with immunosuppressive activity have been identified (Table 2). They include the caspase-8 inhibitor cellular FLICE inhibitory protein cFLIP (87), gangliosides (88), IL-8 and IL-10 (89,90), soluble MIC (91-93), prostagladin E2 (90), TGF-β (90), and VEGF (80,94). These molecules have been demonstrated to downregulate T cell activity. Recent evidence suggests that the non-classical HLA class I molecule HLA-G (21) may also play a significant role in downmodulating T cells' activity against tumor cells. It is noteworthy that tumors vary in their ability to express and produce inhibitory molecules, and not all of the immunosuppressive factors listed in Table 2 are made by all tumors of different histotype.

Table 2. Tumor-associated suppressive factors[a]

1. The TNF family ligands: induce leukocyte apoptosis via the TNF family receptors (reviewed in 21)	
FasL	Fas
TRAIL	TRAIL-Rs
TNF	TNFR1
2. Small molecules	
Prostagladin E2 (PGE2)	Inhibits leukocyte functions through increased cAMP (175)
Histamine	Inhibits leukocyte functions through increased cAMP (175)
Epinephrine	Inhibits leukocyte functions through increased cAMP (175)
INOS	Promotes or inhibits Fas-mediated apoptosis by regulation of NO levels (175)
H_2O_2	Has pro-oxidant activity, increases cAMP levels, causes apoptosis in NK cells, inhibits tumor-specific CTL (175)
3. Enzymes	
Indoleamine 2, 3 dioxygenase	Suppresses T-cell responses (177)
Arginase I	Impairs T-cell functions, decreases ζ chain expression (178)
4. Cytokines	
TGF-β	Inhibits perforin and granzyme mRNA expression; inhibits
IL-10	Inhibits production of IL-1β, IFN-γ, IL-12 and TNFα (179,180)
GM-CSF	Promotes expansion of immunosuppressive tumor-associated macrophages (176)
5. Tumor-associated gangliosides	Inhibits IL-2 dependent lymphocyte proliferation or induce apoptotic signals (88)

[a] A partial list of immunosuppressive factors selected to demonstrate their diversity and a wide spectrum of effects on immune cells.

3.2.2 Apoptosis of T cells in patients with cancer

Many studies have demonstrated that a high frequency of T cell apoptosis occurs in patients with cancer (21,23,60). TIL are often TUNEL positive, i.e., demonstrate evidence of DNA fragmentation, whereas few apoptotic lymphocytes are observed in control normal tissues or tumor-uninvolved tissues (*figure 4*). It should be noted that apoptosis of T cells is not limited to the tumor site, since an increased percentage of apoptotic T cells is found in the peripheral blood of patients with HNSCC, breast carcinoma and melanoma (21,23,95,96).

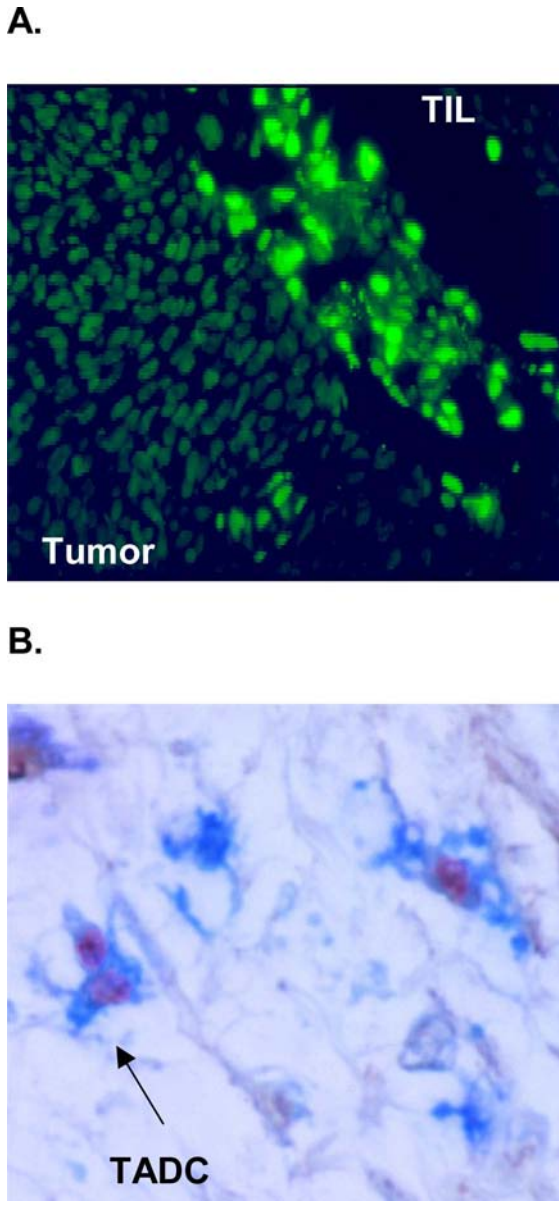

Figure 4. Apoptosis of lymphocytes and dendritic cells in the tumor microenvironment. Surgically removed sections of (A) human oral carcinoma and (B) prostate carcinoma in situ were analyzed for the presence of (A) lymphocyte apoptosis by TUNEL staining and (B) dendritic cell apoptosis by determination of caspase activity. TIL=tumor infiltrating lymphocytes; TADC=tumor associated dendritic cells. Black arrow denotes caspase+ (red) DC (blue = CD83+). Figure 4B was provided by Dr. Michael Shurin. See also Color Plates page 305.

In general, human malignancies seem to be characterized by rapid T cell death and equally rapid inactivation of naïve T cells. The TREC (T-cell receptor excision circles) analysis (97) or telomere-length studies (98) confirm the rapid lymphocyte turnover in patients with cancer. Furthermore, TUNEL staining of TIL and Annexin V binding to circulating T cells suggest that CD8+ rather than CD4+ T cells selectively undergo apoptosis at the tumor site and in the peripheral circulation. Thus, the fate of these two T-cell subsets may differ due to their divergent sensitivity to apoptosis. Also, the effector subpopulations of $CD8^+$ T cells (e.g., CD8+CD45RO+CD27- and CD8+CD28-) are targeted for apoptosis in patients with cancer (21,23). Taken together, these findings suggest that a loss of effector T cell function through targeted apoptosis might severely compromise anti-tumor functions of the host immune system and contribute to tumor progression. On the other hand, it is noteworthy that T lymphocytes or monocytes/macrophages isolated from tumors, LNs and PBMC of patients with cancer are not all irreversibly damaged, as recovery of proliferative and cytolytic functions can occur in response to cytokines *ex vivo*. Limiting-dilution studies with human TIL indicate that only a subset of TIL-T cells retain or recover their ability to proliferate in response to IL-2 in culture (21,23). Therefore, some T cells or their precursors resist tumor-induced dysfunction and can be rescued and expanded following removal from the tumor site or upon exposure to exogenous cytokines *ex vivo*. Indeed, adoptive immune therapy with *ex vivo* expanded TIL in patients with melanoma or ovarian carcinoma resulted in objective clinical responses in a minority of the patients (21,23).

Different mechanisms may account for the high frequency of T-cell apoptosis observed in patients with cancer. Binding of Fas ligand (FasL) to the Fas receptor has been known for some time to induce apoptosis of T cells responding to autologous antigens and maintain tolerance to normal tissue antigens. Furthermore chronically stimulated T cells are likely to undergo activation-induced cell death (AICD) mediated by the Fas/FasL pathway, or they may die because appropriate cytokines are not secreted (99). AICD is induced by repeated or chronic antigenic stimulation, and neither co-stimulatory molecules nor Bcl-2 family members can rescue T cells from AICD. In this regard, TIL, LNL or peripheral T cells in patients with cancer experience chronic or repeated antigenic stimulation with TA and often express CD95 on the cell surface (60,95). Therefore, the chronic or acute systemic dissemination of TA may result in an excess of Ag and “high dose” tolerance of specific T and B cells, making them particularly susceptible to AICD. It is noteworthy that sensitivity to Fas-mediated death is a regulated phenomenon, in which both IL-2 and antigenic stimulation also play a crucial regulatory role (100-103). In AICD, which is an essential part of any normal immune response, IL-2 is a potentiating cytokine. At appropriate

concentrations and in the presence of a relevant antigen, IL-2 enhances the Fas/FasL pathway in activated T cells leading to expression of CD95 (99-103). However, IL-2 expression has been shown to be low or absent in tumor cells both at the message and at the protein level (103,104). Furthermore, TIL *in situ* do not appear to produce IL-2 or express IL-2R (104,105), and translation of IL-2 mRNA is defective in TIL isolated from breast carcinoma lesions (105). Therefore, if IL-2 is required for the assembly or function of the Fas death complex in T lymphocytes (100-103), then AICD may not be solely responsible for the demise of these cells in the tumor microenvironment.

A somewhat different but related mechanism may be envisioned, in which the tumor not only induces lymphocyte dysfunction, including the reduced ability to produce IL-2 and IFN-γ (21,23), but also capitalizes on the expression of TNF family receptors on its own surface. A variety of freshly harvested or cultured human tumor cells have been found to express mRNA for FasL as well as surface and/or cytosolic FasL protein (100-103). In addition, microvesicles, which are presumably derived from tumor cells and, contain biologically active FasL are present in sera of patients with carcinoma of the head and neck, of those with ovary carcinoma and in those with melanoma (84-86). These structures can mediate the apoptosis of Fas+ lymphocytes at sites distant from tumor lesions. Therefore, tumors that express Fas-ligand or shed Fas-ligand into serum could induce apoptosis in T cells infiltrating the site of tumor as well as in circulating T cells, and thus effectively escape from the effector arm of the immune response (90). On the other hand, Fas-expressing malignant cells are themselves resistant to apoptosis. Some of the mechanisms identified include the over-expression of key anti-apoptotic proteins such as two members of the inhibitors of apoptosis family (IAP) family (survivin and ML-IAP), FLICE inhibitory proteins (FLIP), Bcl-2 and the ability to produce inducible nitric oxide synthetases (iNOS) which may play a role in inhibiting apoptosis (90).

3.2.3 Apoptosis of DC in patients with cancer

An additional mechanism that may also explain the dysfunction, and ultimately, death of T cells *in situ* might result from the impairment in TADC functions (80,81,106). TADC not only process and present TA, but are important sources of IFN-α, IL-1, IL-12, IL-15, IL-18, IL-23 and IL-27, among other cytokines. They are also rich in co-stimulatory molecules (CD80, CD86, OX40, 4-1BBL) necessary as second signals or growth factors for T-cell differentiation, proliferation and memory development (107). Therefore, if TADC are not able to perform normally, as suggested by data in the literature (80,81), or if they also undergo apoptosis *in situ*, as

shown in *figure 3B*, then TADC-TIL interactions are not likely to be optimal for productive TA-specific immunity. In this regard, the role of apoptosis in the regulation of DC differentiation and elimination of tumor cells as well as the molecular mechanisms regulating this process have yet to be systematically addressed. We and others have recently demonstrated that elimination of DC or DC precursors in the tumor microenvironment is an important element of tumor-induced immune suppression and may, in part, contribute to an ineffective TA-specific T cell immune response in patients with malignant disease (80,81,108-111). However, the mechanisms responsible for induction of apoptosis and protection of different DC subpopulations and DC precursors from death signals are poorly understood. The molecular pathways that may be involved include: i) downregulation of the anti-apoptotic Bcl-2 family proteins in DC (111,112); ii) accumulation of ceramides which may interfere with PI3K-mediated survival signals (111), or iii) production of nitric oxide species (NO) by tumor cells which suppresses expression of cellular inhibitors of apoptosis proteins (cIAPs) (111,113) or cFLIP. Analysis of gene and protein expression in DC and DC precursors in the tumor microenvironment has demonstrated that expression of several intracellular signaling molecules is reproducibly altered in DC co-incubated with tumor cells, including IL-2Rγ, IRF2, Mcl-1, and small Rho GTPases among others (80,81,111). It appears that both intrinsic and extrinsic apoptotic pathways are involved in tumor-induced apoptosis of DC, as determined by an increased resistance to apoptosis of DC genetically-modified to overexpress XIAP, Caspase 8, Bcl-xL or FLIP. Finally, we have shown that DC genetically engineered to overexpress Bcl-xL induce potent TA-specific T cell immune responses and inhibit tumor growth in murine tumor models *in vivo* (114). Taken together, these data suggest that efficient protection of endogenous DC in patients with malignant disease or prior modification of DC for vaccination of these patients should significantly increase host TA-specific immune responses and inhibit tumor growth.

3.2.4 Myeloid suppressor cells

Recent studies have demonstrated that a group of CD11b (+), Gr-1 (+) cells known as myeloid suppressor cells (MSC), play an important role in tumor unresponsiveness by suppressing TA-specific T cell immune responses (111,115). These cells may be an important factor contributing to the failure of tumor vaccines in patients with malignant disease. MSC represent a heterogeneous population that includes mature granulocytes, monocytes and immature cells of the myelomonocytic lineage. There is ample evidence that tumor growth in all types of patients with malignant disease and all types of tumor-bearing mice is associated with an accumulation of MSC (111,115). *In vitro* studies indicate that MSC purified

from tumor-bearing mice can suppress CD8(+) T cells. This suppression is nitric oxide independent, antigen specific, requires direct cell-cell contact and is dependent on MHC class I antigens. In contrast, MSC derived from naïve mice are able to suppress CD8(+) T cell activity. The molecular mechanisms underlying this phenomenon are unclear, although several studies indicate that reactive oxygen species (ROS) may play a significant role. In this regard, many human tumors and cell lines are capable of secreting cytokines not limited to but including GM-CSF, IL-3, IL-6, M-CSF and VEGF. When these cytokines are released in large amounts systemically by growing tumors or immune distress they are capable of expanding the myeloid cell pool. Furthermore, these factors may also lead to an increase in ROS production and arginase activity in MSC (111,115). The latter enzyme is involved in the hydrolysis of L-arginine and indirectly increases the ROS level by decreasing L-arginine concentrations. Constant production of these factors could lead to the different levels of ROS observed in MSC from tumor-bearing and tumor-free mice. In fact, the ROS level has been found to be 3 fold greater in MSC derived from tumor bearing mice than in non-tumor bearing mice (111). The main target for ROS in T cells has been shown to be CD3ζ (116). Specifically, oxidative stress as well as granulocyte derived ROS are involved in the suppression of CD3ζ and IFN-γ expression in advanced cancer patients. ROS are short-lived substances, exerting their effects on cells in close proximity, which may explain the need for direct MSC-T cell contact and also explain the antigen-specific nature of the inhibition (116). Antigen-specific interactions between T cells and APC are much more stable and last longer than interactions in the absence of antigen. MSC express MHC class I molecules, but have low or undetectable levels of MHC class II antigens(111,116). This phenotype may explain the lack of CD4(+) T cell suppression by MSC.

3.2.5 CD25(+) regulatory T cells

Accumulating evidence indicates that a population of CD25(+) T regulatory (Tregs) cells may at least be partially responsible for T cell dysfunction. CD25(+) T cells prevent the induction of a variety of autoimmune diseases in murine models (117). Therefore, it has been hypothesized that these cells play a role in the regulation of immune responses to tumors, since tumor immunity can be thought of as an autoimmune process. In this regard, *in vivo* studies performed in murine models have shown that CD25(+) T cells, which comprise 5-10% of CD4(+) T cells in naïve mice, can inhibit the generation of TA-specific T cell immune responses (118). Depletion of CD25(+) T cells has been shown to promote rejection of tumors in mice (118). This rejection is dependent on CD8(+) or CD4(+) T cells alone or both, depending on the mouse strain and

tumor model. In human tumors, TIL have been reported to be enriched in Tregs (118). *In vitro* studies suggest that CD25(+) T cells may suppress CD8(+) and CD4(+) T cells in an antigen non-specific fashion through the production of TGF-β(118,119). Clearly, future studies should be directed at understanding the role CD25(+) T regulatory cells play in regulating TA-specific T cell immune responses in patients with malignant disease.

3.3 Antigenic changes on malignant cells

Tumor cells can evade the host's immune response by being poor stimulators of T cells or being poor targets for effector CTL. Because of their genetic instability (24), malignant cells may change in the expression of molecules such as TA, HLA class I antigens as well as APM components, each of which plays a crucial role in the generation of the HLA class I antigen-TA peptide complex (20,21). The latter mediates the recognition of tumor cells by the host's CTL.

3.3.1 TA abnormalities

Abnormalities in TA expression as well as a variable degree of inter- and intra-lesional heterogeneity characterize many tumors. As a result, peptides may not be generated from TA or may be formed in very low amount and the corresponding HLA class I antigen-TA peptide complexes are not formed in spite of the expression of the relevant HLA class I allospecificity. The phenomenon of TA loss has been mainly described in melanoma. Melanocytic differentiation proteins (MDPs, e.g. gp100, MART-1, TRP-1, and tyrosinase) have been found to be lost in metastatic lesions in patients with melanoma independently of the treatment with immunotherapy (120-122). More recently, loss of the newly identified melanoma associated antigen, melanoma inhibitor of apoptosis protein (ML-IAP) was reported in a recurred intestinal metastasis in conjunction with a lack of lymphocyte infiltration, following immunotherapy utilizing GM-CSF-secreting autologous tumor cells as immunogens (123). In SCID mice, expansion of MART-1-loss variant of human melanoma cells was causally linked to the presence of adoptively transferred MART-1-specific CD8+ T cells (124). In other types of tumors, e.g., breast cancer, MUC-1 was found to be downregulated in progressive mammary tumors with c-Neu expression in a mouse model (125). Whether the latter finding has a human counterpart remains to be seen. TA mutations which result in a loss of CTL-recognized TA epitopes may also occur in tumors. A mutated TA may still be expressed but the location of the mutation can abolish the generation of epitopes to be recognized by its cognate CTL. The latter is supported by the identification

of such a mutation in the p53 protein which inhibits proteasome mediated generation of the p53-derived peptide ($p53_{264-272}$). This peptide is immunogenic and is known to be recognized by HLA-A*0201-restricted CTL in squamous cell carcinoma of the head and neck (SCCHN) (126). Lastly, tumor cells may present TA-derived peptide analogs with antagonist activity resulting in suboptimal T cell activation (90). These defects due to genetic instability of tumor cells have been found to render malignant cells ineffective targets for TA-specific T cells.

3.3.2 HLA class I antigen abnormalities

A large body of evidence indicates that malignant transformation of cells is associated with changes in classical HLA class I antigen expression (20). As shown in *figure 5* these changes range from total loss or downregulation of all HLA class I allospecificities expressed by one cell to selective loss or downregulation of a single HLA class I allospecificity, from loss or downregulation of the gene products of HLA-A, B or C loci to loss of one haplotype, i.e. loss of the HLA class I allospecificities encoded by the genes located in one of the two copies of chromosome #6. The latter carries the major histocompatibility complex in human (127).

The most common types of solid tumors for which more than 100 surgically removed primary lesions have been analyzed include head and neck squamous cell carcinoma (HNSCC), carcinomas of the breast, lung, colon, liver, kidney, cervix and prostate, melanoma, leukemia and lymphoma (20,128-130). For other tumors including stomach (131), pancreatic (132,133), bladder (134,135), ovarian (136,137), germ cell (138) and basal cell (139-141) carcinomas much less information is available regarding HLA class I antigen expression since the number of lesions analyzed is too low for one to draw definitive conclusions. With the exception of liver carcinoma (142-144), leukemia (129) and lymphoma (128,130), the frequency of HLA class I antigen loss and/or downregulation has been found to range between 16% to 80% of the various types of tumors analyzed with mAb recognizing monomorphic determinants. The frequency of this phenotype varies significantly among different malignancies with breast carcinoma and prostate carcinoma demonstrating the highest frequency and RCC and melanoma demonstrating the lowest frequency (20).

While it is clear that abnormalities in classical HLA class I antigen expression do occur in malignant lesions, it should be stressed that the frequency of HLA class I antigen abnormalities reported in the

literature for each particular type of malignancy has been found to vary considerably (20).

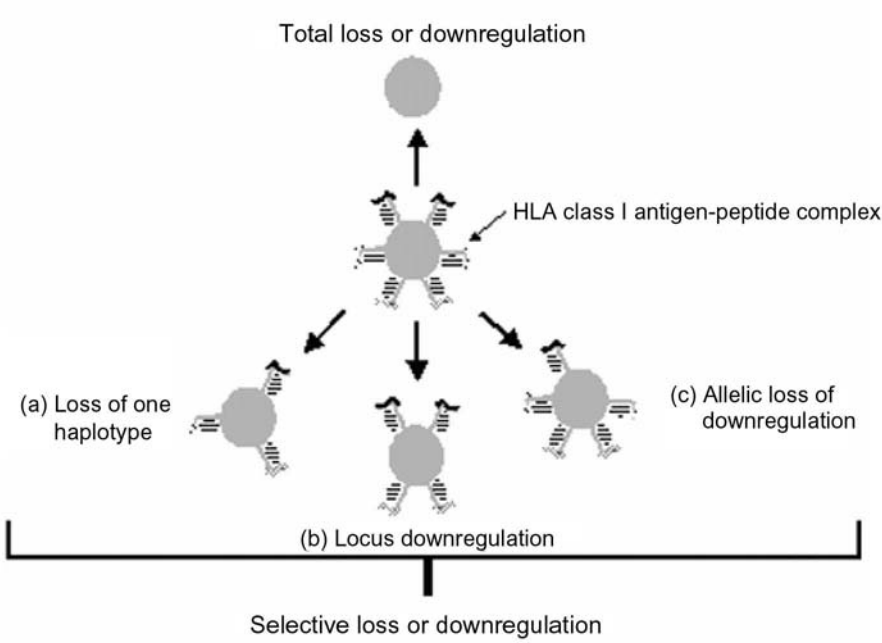

Figure 5. Defective HLA class I phenotypes identified in malignant cells. The phenotypes identified in tumor cells include total loss and/or downregulation of the gene products of the HLA-A, B and C loci; (a) total loss of all HLA class I antigens encoded in one haplotype; (b) selective downregulation of the gene products of one HLA class I locus; (c) selective loss or downregulation of one HLA class I allospecificity; or complex phenotypes resulting from a combination of two or more of the described phenotypes.

Therefore the frequency of HLA class I antigen abnormalities reported in the literature should be interpreted with caution. The reason(s) for differences in the frequency of classical HLA class I antigen defects in various types of tumors is (are) not known. It is likely that these differences partly reflect the sensitivity of the immunohistochemical reaction to detect HLA class I antigens and/or the subjective evaluation of IHC staining. Moreover, there is no information regarding the reproducibility of IHC staining results among different laboratories, although there is some data which demonstrates that variations in the percentage of stained cells enumerated by two experienced observers within the same laboratory is less than 10% (145). Additional sources of variability are represented by differences in the characteristics of the patient population and/or histologic classification of the particular type of tumor analyzed, e.g. ductal *vs* lobular

breast carcinomas with tubular, mucinous, papillary, medullary or adenoid cystic histology, since the role of these variables have not been investigated. In fact, upon review of the literature it is evident that nearly all the studies reported have failed to stratify malignant lesions of a particular tumor according to histologic type. The few studies that have stratified malignant lesions according to histologic type have noted differences in the frequency of HLA class I antigen expression among serous and mucinous-adeno ovarian carcinomas (146) as well as squamous cell, small cell, adeno and large cell lung carcinoma (147). Lastly as we have discussed elsewhere (148), it is our opinion that variable frequency of HLA class I antigen abnormalities observed among tumors of different histotype also reflects the type of immune selective pressure imposed on tumor cell populations, their genetic instability and time length between onset of tumor and diagnosis.

Studies performed mostly with long term culture cell lines derived from surgically removed malignant lesions have identified a number of distinct molecular mechanisms underlying the abnormal HLA class I antigen phenotypes of malignant cells (20). These abnormalities do not represent artifacts of *in vitro* cell culture, since several of them have also been identified in surgically removed tumors. These mechanisms which are differentially present in different types of tumors include defects in β_2-microglobulin (β_2m) synthesis, defects in HLA class I heavy chain synthesis, epigenetic alterations involving the HLA class I heavy chain loci, defects in the regulatory mechanisms that control HLA class I antigen expression, abnormalities in one or more of the APM components. This topic has been reviewed by us. We refer the interested reader to our recent papers (20,21).

4. MECHANISMS FOR PROTECTION OF IMMUNE CELLS AND PREVENTION OF TUMOR ESCAPE

Immune escape mechanisms utilized by malignant cells represent major obstacles in the outcome of immunotherapy in patients with malignant disease because of their multifaceted nature. Therefore, there is a need to develop strategies to counteract the immune escape mechanisms utilized by malignant cells in order to enhance the efficacy of T-cell based immunotherapies. At present, various vaccination strategies are being evaluated in phase I clinical trials. In this regard, polyvalent vaccines may be preferable to monovalent vaccines, in order to counteract the selective loss of the target antigens. Moreover, polyvalent vaccines may lead to the activation of CD4+ T lymphocytes, which appear to be an important immune component in the eradication of tumor cells (148). The choice of multivalent vaccines is also supported by the reports of beneficial effects on the clinical course of the disease in the trials conducted in patients immunized with

polyvalent vaccines (149-153). However, whether the currently available TA have the potential to serve as tumor rejection antigens and whether the currently used strategies are optimal to identify TA to be used for immunotherapy remains to be determined (154). It is debatable whether metastases and CTL obtained from patients who have failed to reject their cancer represent the most appropriate source of TA and/or most appropriate probes to identify TA effective in mediating inhibition of tumor growth by CTL. It might be more informative to examine the TA profile of tumors from patients with a highly favorable course of the disease as well as TA with preferential expression in primary lesions. It is also noteworthy that TA-specific CTL responses detected in immunized patients have been mostly monitored in peripheral blood. This evidence proves the vaccines' ability to induce a TA-specific immune response, but provides no indication about the ability of these cells to traffic, infiltrate, function and persist in the tumor microenvironment. These questions have been challenging to address due to the difficulty of relating studies of the immune response *ex vivo* to events that occur within human solid tumors. However, answers to these questions are critical to assess the potential effectiveness of these vaccines. Therefore, there is a need to develop appropriate methodology to monitor the immune response in malignant lesions in situ.

Strategies also need to be developed to reverse the immune dysfunction of T cells observed in cancer patients as well as to protect and augment functions of DC in the tumor environment. In this regard, the rationale behind cytokine therapies such as IL-2, -12 or IFN-γ has been the predicted up-regulation of TA-specific CTL functions, especially those associated with T cells specific for TA (11,12,155-157). In retrospect, it seems that attempts to up-regulate the functionality of dying cells are not likely to succeed. Moreover, by simply increasing the number of activated TA-specific CTL, one may merely prompt their rapid removal, with help from circulating tumor-derived factors. Therefore, new strategies for cytokine delivery and perhaps new cytokines themselves (e.g., IL-15) need to be applied to rescue dying cells and to protect viable cells from death-inducing signals (158). Such therapeutic strategies based on creative and novel use of cytokines are both rational and practical. While the precedent exists for clinical applications of these cytokines in humans, the novelty of the suggested approach is that it targets biotherapy to the specific pathways or even affected molecules and is designed to carefully evaluate the impact of these corrective measures on tumor-specific functions of immune cells in treated patients.

Another caveat to consider is the possibility that immature and mature DC might have differential sensitivity to TICD, and that tumors might inhibit DC generation or differentiation and also contribute to DC demise. It has been previously reported that tumor-shed gangliosides (GD2, GD3 and GM2) alone or in combination with other factors inhibit DC differentiation

in vitro, and that specific neutralization of these factors diminishes their adverse effects on DC maturation. Tumor-derived gangliosides have also been shown to induce defective NFκB activation and the heightened sensitivity of T cells to apoptosis (159). Thus, gangliosides appear to be implicated in the dysregulation of both T-cell and DC functions in individuals with malignancies. Based on these data, it is expected that protection of both immature and mature DC from TICD will be necessary to improve the efficacy of DC-based immunotherapies. While it is not clear at present which subset of DC has the best therapeutic value, evidence indicates that immature DC are better at the uptake of soluble proteins and whole cells and at antigen processing via membrane-associated peptidases (32). In contrast, mature DC not only migrate better *in vivo* but are also more effective in presenting antigenic epitopes and secreting cytokines and chemokines, which are necessary for the generation and direction of memory T cell responses (160). Thus, *in vivo*, it will likely be necessary to protect both of these subsets in order to obtain the most effective therapeutic results, and it now becomes essential to characterize tumor-derived factors that preferentially target DC subpopulations.

The hypothesis that DC can protect T cells from apoptosis forms a basis for another therapeutic strategy which is based on an extensive series of experiments with human cells *in vitro* (161-163). Although the mechanisms responsible for the protection are not known, it is now recognized that DC, which take up apoptotic cells undergo maturation and secrete cytokines involved in T-cell polarization. The therapeutic implications of this finding are profound. In effect, this concept shifts the emphasis from the well-recognized APC role of DC to their role as T-cell protectors from signals mediated by the TNF family of receptors/ligands or other apoptosis-inducing signals. If confirmed, this observation offers a novel approach to the therapy of cancer and other diseases (e.g., AIDS), in which apoptosis of T cells in tissues or in the peripheral circulation is a common feature (164). Thus, the number and function of DC or that subset of DC, which is responsible for the protection of T cells from apoptosis, now become the primary objective of biologic therapies in cancer patients, where premature death of tumor-specific CD8+ (and CD4+) T cell effectors occurs.

Attention must also be focused on immunized patients' tumor cells susceptibility to immune recognition and destruction. As discussed above, defects in HLA class I antigen expression have been convincingly documented in melanoma large number of malignant lesions of different histotype (20). Although not conclusive, the available evidence suggests the clinical relevance of these defects, since they are associated with a poor course of the disease in several malignancies, are present with increased frequency in malignant lesions of patients treated with T cell-based immunotherapy (20). These findings have been interpreted to indicate the outgrowth of malignant cells which have acquired the ability to escape T cell

recognition because of HLA class I antigen defects. If this interpretation is correct the data we have summarized imply that, given the high rate of mutations in tumor cell populations already in primary lesions, T cell-based immunotherapy, even when successful, is not likely to be able to eradicate a patient's malignancy. The eventual outcome is likely to be the outgrowth of tumor cells that have developed multiple escape mechanisms. The latter can be counteracted by immunotherapeutic strategies that utilize multiple TA and multiple immune effector mechanisms.

Although not conclusive, the information we have reviewed has been useful to focus investigators' attention on the potential role of HLA class I antigen defects in tumor cell escape and in the lack f clinical responses in spite of a detectable T cell response Furthermore the observations we have reviewed have contribute to stimulate oncologists and tumor immunologists' interest the analysis of HLA class I antigen expression in surgically removed malignant lesions, especially in patients treated with T cell-based immunotherapy. Although providing useful information, the latter studies do not address the analysis of HLA class I antigen-peptide complexes, which play a crucial role in the interaction between HLA class I antigen restricted, TA-specific CTL and tumor cells. Therefore, it will become important to monitor the expression of actual peptide complexes, that is complexes formed between HLA class I antigens and TA-derived peptides on tumor cell membranes, especially since increasing evidence suggests lack of correlation between expression of a HLA class I allospecificity and that of the HLA class I-TA derived peptide complex used as a target by T cells.

5. TUMOR ESCAPE AND FUTURE APPROACHES TO CANCER IMMUNOTHERAPY

Recent progress in tumor immunology has led to novel insights regarding the functions and interactions of immune cells (T, B, NK, M and DC) and the molecules expressed on these cells, which are linked to the development and efficacy of TA-specific immune responses. In addition, a better understanding of the molecular signals and mechanisms involved in the generation of productive immune responses in general has focused attention on those molecular events that occur or do not occur in the tumor microenvironment. The realization that immune cells undergo apoptosis in tumors has led to a search for the mechanism(s) responsible for this death and was instrumental in identifying the TNF family of receptors and ligands as instrumental in mediating tumor-induced apoptosis (165-167). This realization was prefaced by the recognition of the Fas/FasL pathway and its role in maintaining the immune privilege at sites such as the anterior chamber of the eye, the brain, the testis or the thyroid gland (168). The

notion that tumors might also be able to protect themselves from immune effector cells by inducing their death was both appealing and supported by the extensive evidence that these effector cells are dysfunctional in the tumor microenvironment (21,23, *Table 1*). It also provided a reasonable explanation for the noted limited success of adoptive immunotherapies employing activated effector cells in patients with cancer (53). However, it has now become necessary to convincingly demonstrate that the newly identified mechanisms leading to apoptosis of immune cells apply to tumor-effector cell interactions. If immune effector cells die in the tumor, and if the rate of their demise exceeds that of survival, then it might be surmised that tumor-induced apoptosis of immune cells might be an important prognostic parameter. This hypothesis has to be tested. The potential ability of immunotherapies to protect effector cells from apoptosis might be related to the clinical response of patients, and this hypothesis can also be formally examined. The question of how to best protect immune cells from premature tumor-induced apoptosis becomes an essential, but so far inadequately explored, goal of cancer immunotherapy.

Extensive immunization trials are on-going worldwide in patients with breast, colon, renal cell, ovarian and prostate carcinomas as well as melanoma and other malignancies. These clinical trials, largely initiated in patients with advanced metastatic disease, have been able to induce clinical responses in not more than 20% of patients. They are unlikely to yield the desired results, if only a proportion of the immunization-induced, TA-specific effector cells survive *in vivo*. Moreover, if a proportion of specific T cells or DC are preferentially killed, and if the level of apoptosis exceeds that of effector cell influx into the tumor or their generation within the secondary lymphoid sites, the immunization strategies (as currently applied) may prove ineffective. Likewise, it may be counterproductive to generate TA-specific effector cells in a situation where the tumor is resistant to that type of immune cell, i.e., poised to escape specific immune interventions. To avoid additional disappointments in the clinic, it will be necessary to combine immunization strategies with therapies providing: a) protection of T-cells from tumor-induced apoptosis and b) improved recognition of tumor cells (by interfering with their mechanisms of immune escape). Preliminary experiments suggest that effectively (Type-1) polarized DC as well as survival cytokines can protect TA-specific immune effector cells from apoptosis in the tumor microenvironment and, at the same time, alter expression of key components of the antigen processing machinery in the tumor, thus making tumor cells susceptible to immune attack. Therefore, the rationale exists for the implementation of this approach with the objective of defining the molecular and cellular signals responsible for the observed dysfunction of immune cells and/or for tumor escape. The resulting

information is expected to be useful to optimize studies to investigate in pre-clinical *in vitro* models as well as in clinical trials the possible beneficial effects of DC and/or cytokine administration on protection of immune cells from apoptosis.

A better understanding of the fate of anti-tumor effector cells generated *in vivo* or those that are adoptively transferred into patients with advanced malignancies is of critical importance to the optimization of effective cancer immunotherapy in the future. Although several mechanisms that could be responsible for the development of immune cell dysfunction in tumor-bearing hosts have been identified, none can systematically account for the range and magnitude of dysfunction and immune cell death observed in patients with cancer (58,67,156,169). Many unanswered questions remain, and research is critically needed in order to distinguish between those mechanisms that might favor tumor escape through modification leading to greater resistance of tumor cells vs. those attributable to effects the tumor exerts (directly or indirectly) on immune effector cells. With new insights into proliferation and death of immune effector cells in the tumor microenvironment (170), cytokine and growth factor biology (171,172), the role of DC in antigen cross-presentation (32), regulation of target-cell killing (44,173) and mechanisms underlying the apoptosis of immune cells (21,23), it has been possible to formulate a series of testable hypotheses regarding the nature of the mechanisms that mediate tumor escape or tumor-induced cell death. Further, novel therapeutic strategies that could prevent tumor evasion and protect immune effector cells from apoptosis in the tumor microenvironment need to be tested for efficacy in changing the fate of TA-specific immune effector cells and thus improving outcome.

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Chapter 4

VIRUS SPECIFIC T-CELL RESPONSES

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Abstract: CD8+ and CD4+ T-cells play a key role in the maintenance of our immunity against viruses. Recent technological developments, such as the use of MHC-peptide tetrameric complexes, have permitted significant improvements in the study of these cells. It is now possible to assess precisely frequencies as well as phenotypic and functional features of virus specific T-cells from the onset of many viral infections onwards. Different virus specific T-cell populations exhibit distinct functional characteristics and can be positioned at different stages of a process of post-thymic development, which we are drawing near to understanding the significance. Still, further work is needed before consensus is reached as regards what defines and how to induce the optimal virus specific T-cell response which will confer long lasting immunological protection in humans.

Key words: Viral infection, CD8+ T-cell, CD4+ T-cell, tetramer, function, cytotoxic, differentiation, protective immunity.

1. INTRODUCTION

1.1 The importance of CD8+ T-cells in viral infections

The principal constituents of the adaptive cellular immunity arm are helper CD4+ T-cells, which participate in antigen recognition and in regulation functions; and cytotoxic CD8+ T-cells (CTL), which can kill virus infected cells and thus prevent viral spread. The importance of CD8+ T-cells in controlling viral infections was first illustrated in experiments of adoptive transfer with virus specific CTL, which conferred protection to mice exposed

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to lethal doses of various viruses (e.g. influenza virus (1), respiratory syncytial virus (RSV) (2), herpes simplex virus (3), Sendai virus (4) and Moloney murine sarcoma virus (5). In simian immunodeficiency virus (SIV) primate models, depletion of CD8+ T-cells during both acute and chronic SIV infection in monkeys results the complete loss of viral control (6, 7). A conclusive role for CTL in the control of human viral infections has been more elusive. Nevertheless, influenza specific CD8+ T-cell levels were reported to correlate with protection after challenge with life virus (8), and adoptive transfer of Epstein-Barr virus (EBV) or cytomegalovirus (CMV) specific CTL were shown to represent a benefit in the treatment of EBV and CMV associated complications respectively (9, 10). Moreover the majority of persistent viruses have developed strategies to escape CD8+ T-cell recognition, for instance through specific mutations in the epitopes recognized by cytotoxic T lymphocytes or by blocking the process of antigen presentation, which also argues in favor of a strong role for CTL (11). Overall, it is clear that T-cell mediated immunity serves as the one of the principle defense mechanisms against virus infections and is essential for human survival. Its study is central to medical research.

Since the discoveries by Rolf Zinkernagel and Peter Doherty that CD8+ T-cells were able to recognize lymphocytic choriomeningitis virus-(LCMV) infected cells in an MHC-dependent manner (12, 13), and by Alan Townsend and Andrew McMichael that influenza specific CTL recognize target cells presenting virus derived peptides (14), our understanding of the mechanisms involved in cellular immunity has expanded considerably. Considerable knowledge about the dynamics of CTL responses in viral infections has been obtained from studies in mouse models, however I will concentrate in this chapter on the characterization of virus specific T-cells in humans, which is principally related to the focus of this book.

1.2 Methodological development in the study of CD8+ T-cells in humans

1.2.1 Measuring CD8+ T-cell activity

For obvious questions of practicality, detail analyses have been more difficult to perform in humans than in mice. Progress in the study of human CTL responses and of their involvement in the control of virus infections has been largely dependent on the development of several methods. The early established methods are based on the ability of CTL to lyse appropriate target cells *in vitro*. This can be performed under limiting dilution conditions (limiting dilution assay, LDA) to provide a quantitative

measurement of the antigen-specific CTL precursor cells which are able to grow and divide *in vitro* (15). The use of other recently-developed techniques including the measurement of cytokine production by CD8+ T-cells (by intracellular cytokine staining- ICS) (16) and the Interferon-gamma (IFN-γ) ELISpot assay (17), has shown that the LDA may significantly underestimate the true frequency of circulating CTL with specificity for viral antigens. However, all these techniques represent methodological limitations. They address different aspects of antigen-specific CD8+ T-cell activity. Limiting dilution analysis measures the lytic ability of specific CTL, but is also dependent on their ability to grow and divide in tissue culture conditions. ELISpot and ICS assays examine the ability of antigen-specific CD8+ cells to secrete a single cytokine, usually IFN-γ, on contact with their cognate antigen, but do not address their lytic function; it is also clear that not all antigen-specific CD8+ cells are able to produce IFN-γ upon specific stimulation or secrete other soluble factors. Moreover, since *in vitro* manipulation may significantly alter the composition and functional properties of the populations of interest, a detailed analysis of virus specific CD8+ T-cells is difficult to perform.

1.2.2 The tetramer era

In 1996, after more than 20 years of abortive efforts to measure the CD8+ T-cell response with precision, immunologists at last had at their disposal a tool which enabled a direct visualization of antigen specific CD8+ T-cells: the introduction of peptide-MHC class I tetrameric complex (tetramers) technology initiated a profound revolution in the field of cellular immunology (18). The use of tetramers to stain antigen-specific CD8+ T-cells is based on the ability of their T-cell receptor to interact specifically with a complex of the appropriate HLA molecule assembled with a relevant peptide with sufficient avidity to allow read-out by flow cytometry. The analysis of tetramer positive CD8+ T-cells has provided a method that reliably quantitates the number of specific CD8+ T-cells present in peripheral blood and secondary lymphoid organs (19, 20). The benefit of such a technology is not only limited to the precise quantification of these cells. The next step in the use of tetramers is the detailed analysis of the characteristics of the antigen-specific T-cell population. Together with the technological progress of multicolor flow cytometry (21), the reliable identification of virus-specific CD8+ T-cells by tetramers offer the possibility of obtaining additional information about their phenotype through co-staining for cell surface markers, including markers of activation (22), T-cell receptor Vβ usage (23), and other differentiation and homing markers (e.g. CD family and chemokine receptors) (24). Moreover, the combination

of intracellular labeling techniques with the use of tetramers enables us to characterize in detail the function of virus-specific CD8+ T-cells by assessing practically all aspects of the life of the cell (25). Caveats compelled with tetramer studies in humans still remain, and include generally the analysis of a limited number of epitopes per virus, and its restriction to the peripheral blood compartment. Nevertheless, the tetramer technology and its combination with cellular functional analysis provides for the first time the mean to uncover specific T-cell attributes associated with anti-viral protection.

2. STUDYING VIRUS SPECIFIC CD8+ T-CELLS

2.1 Frequency

The number of tetramers available for the study of CD8+ T-cell populations specific for various viruses is increasing [e.g. influenza (26), HIV (19), EBV (27), CMV (28), hepatitis B virus (HBV) (29), hepatitis C virus HCV (30), human papillomavirus (HPV) (31), human T-cell lymphotrophic virus type 1 (HTLV-1) (32), RSV (33) and dengue virus (34)], covering a large range of HLA types. Together with these technological advances, a better identification of individuals in early stages of viral infections have facilitated more detailed studies of virus specific CD8+ T-cells found in human peripheral blood from primary infection to the establishment of viral latency. During primary infection, most viruses induce a substantial antigen-driven activation and expansion of CD8+ T-cells, referred to as the "effector phase" (35). The use of tetramers has revealed that T-cell frequencies in diverse viral infection are much higher than estimated previously. The expansion during the primary infection is such that a population specific for one single viral epitope (from an EBV lytic protein) was shown to reach up to 44% of the total CD8+ T-cells at that moment [27]. Responses specific for more than one viral epitope are usually elicited. Subsequently as the viral burden is lowered or cleared, the majority of the CTLs undergo apoptosis, giving place during the "memory phase" to a long lasting pool of resting T-cells, which provide a faster and more effective response to reinfection or virus rebound, upon reactivation and expansion of these cells. The frequency of virus specific CD8+ T-cells detected following primary infection vary according to the virus considered. For instance HIV, EBV and notably CMV seem to induce particularly strong responses; during chronic infection, it is not unusual to detect 1% to 10% CD8+ T-cells specific for these viruses. This phenomenon is most certainly

related to continuous antigenic stimulation in the light of persistence of these viruses. In HIV infection, suppression of viral replication using drugs results in a decline of HIV specific T-cell numbers (36). Moreover, T-cell frequencies for viruses that cause limited infection of the host (e.g. influenza) are usually low (<0.1%) after resolution of primary infection (26). There is no consensus as regards a correlation between frequencies of virus specific CD8+ T-cells and viral control. It is certain though that the presence of virus specific CD8+ T-cells *per se* does not prevent viral spread and the development of virus associated complications in some patients. This has led to hypothesizes of dysfunctional CTL enable to suppress viral replication, for instance due to poor effector functions, anergy or exhaustion, and to direct studies on the functional and phenotypic characteristics of the CD8+ T-cells to verify this hypothesis.

2.2 Activation, proliferation and apoptosis

In vivo, infection by pathogens leads to a marked activation of T-cells whose eventual consequences can be as diverse as proliferation or apoptosis of the cells (35). CD8+ T-cells can also apoptose following lysis of their targets (37). Assessing these features is essential to understand the dynamics of the T-cell response. The activation status of antigen-specific CD8+ T-cells *ex vivo* can easily be monitored by staining for cell surface activation markers (e.g. CD38, HLA class II molecules) (22, 38). The nuclear antigen Ki67 is a commonly used marker to assess cell proliferation *ex vivo*; its expression coincides with activation markers. Following permeabilisation, tetramer positive T lymphocytes can easily be stained for Ki67, thereby providing a measurement of the proliferation of virus-specific CD8+ T-cells, for example during acute viral infection or viral reactivation (39, 40). Apoptosis is conducted through two main pathways: the first involves the engagement of death receptors such as Fas (or CD95) through interactions with their ligands (e.g. Fas ligand) (41) and the second pathway is governed by the Bcl-2 family which includes both pro-apoptotic (e.g. Bax, Bik) and anti-apoptotic (e.g. Bcl-2, Bcl-xL) members, promoting or preventing death signals from diverse cytotoxic stimuli (such as cytokine deprivation, DNA or mitochondrial damage) (41, 42). The measurement of the expression of cell surface death receptors such as CD95 *ex vivo*, as well as various intracellular mediators of the apoptotic pathway such as members of the Bcl-2 family provides information as regards the susceptibility to apoptose of the cells. Their expression is also closely linked to the level of T-cell activation. Overall, the expression of these molecules serve as *ex vivo* indicators of the reactivity of virus specific CD8+ T-cells to a viral challenge. As a consequence of active viral replication during primary infection and

secondary infection or viral rebound, virus specific CD8+ T-cell populations exhibit a similar phenotype of activated antigen experienced cells, expressing CD38, HLA-DR, Ki67 and low Bcl-2 levels (38, 43) (Figure 1).

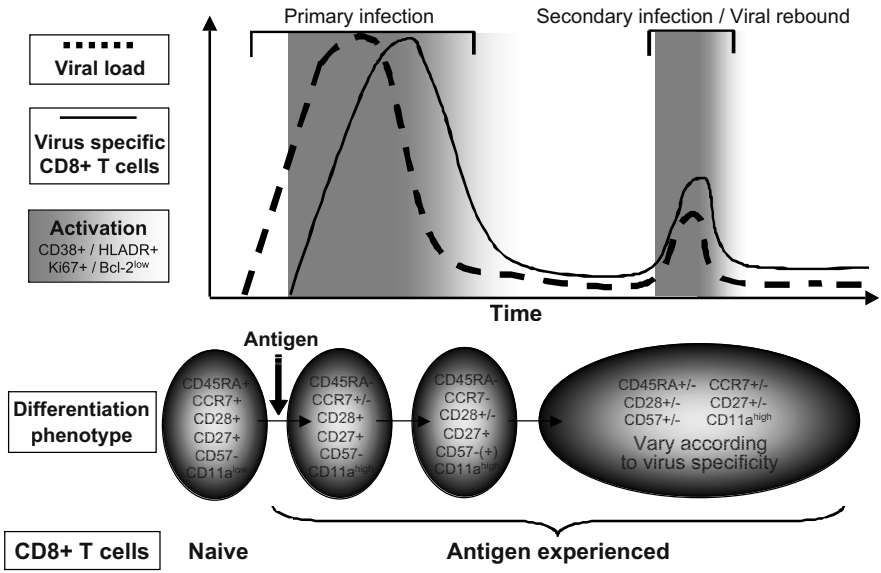

Figure 1. Common pattern of CD8+ T-cell response in human viral infections.

An important technical advance has been the possibility of monitoring cell division, for up to 8 discrete cycles, by following the serial halving of the dye CFSE (carboxyfluorescein diacetate succinimidyl ester) (44). CFSE can easily be loaded into lymphocytes, which retain their normal cell function throughout the assay. Following diffusion into the intracellular environment, the molecule loses its permeant ability and becomes highly fluorescent. CFSE is then partitioned with remarkable fidelity between daughter cells: proliferation results in progressive dilution and ultimately extinction of the dye, observed by flow cytometry. Using CFSE labeling in combination with tetramer staining *in vitro*, it was shown in the context of HIV-1 infection that non progression towards the disease is associated with the presence of HIV specific CD8+ T-cells capable of proliferating rapidly upon antigenic stimulation (45). This is in keeping with mouse studies suggesting that protective immunity is principally conferred by CD8+ T-cells displaying a strong proliferative capacity (46). The limitation of this technique is that this dye cannot be used *in vivo* in humans, restricting its use to *in vitro* assays. Nevertheless, it may be only a matter of time before innovative techniques, such as deuterated glucose and water labeling (47),

which enable the study of proliferation and turn over *in vivo* in humans, are combined with the tetramer technology, and make possible the study of virus specific T-cell populations.

2.3 Effector function

A major issue in the understanding of the relationship between T-cell response and anti viral control resides in the assessment of the CD8+ T-cell effector characteristics. Virus-specific CD8+ T-cells can mobilize two main effector mechanisms: cytolysis of infected cells and production of cytokines, chemokines and microbicidal molecules. The exploration of the capacity to execute these functions is particularly important in pathologies in which failure of immune surveillance is postulated to be significant (e.g. HIV infection).

2.3.1 Production of anti-viral soluble factors

CD8+ T-cells produce various soluble factors, including cytokines and chemokines, which play an active role in the immune response. For instance, IFN-γ plays an important role in the induction of cellular antiviral proteins (48) and through its ability to activate macrophages (49); IL-2 is a strong inducer of T-cell proliferation; and TNF-α is able to inhibit viral gene expression and replication (48). The CC-chemokines RANTES, MIP-1α and MIP-1β coordinate chemoattraction of lymphocytes and macrophages to the infection site, and have direct inhibitory effects against HIV infection (50). CD8+ T-cells produce cytokines rapidly upon stimulation (51), and reliable methods have been developed to measure intracellular cytokines by flow cytometry (52) following *in vitro* antigenic stimulation. Intracellular cytokine staining (ICS) for IFN-γ, TNF-α, IL-2, and MIP-1β can be successfully combined with tetramer staining. Staining with tetramers prior to activation can help significantly in preserving a reasonable level of tetramer staining, which is usually reduced due to activation triggered downregulation of the T-cell receptor (53). While few cells seem able to secrete IL-2 *ex vivo*, a majority (>50%) of CMV, HIV and EBV specific CD8+ T-cells can producing IFN-γ, TNF-α and MIP-1β during chronic infection, suggesting that these cells are functional, at least on the basis of cytokine production (54-56). This capacity to produce IFN-γ seems to be altered with the time in HIV infection; its reduction has been reported with late stages of the infection (57). HCV specific CD8+ T-cells have been shown to produce little IFN-γ, thus designated as stunned cells (30). A difficulty with the study of cytokine production relates to the interpretation of the results: although we know for certain that these cytokines have a role

in anti viral immunity, the importance of their production by CD8+ T-cells is not completely solved. For instance, what is the suitability of measuring IFN-γ secretion as a marker of effector function? What are the most relevant cytokines secreted by CD8+ T-cells to suppress viral replication? Relevance may even vary from one viral infection to another.

2.3.2 Cytotoxic potential

CD8+ T-cells mediate cytotoxicity through two main pathways: the perforin-dependent cytotoxic pathway and the Fas/FasL pathway (58). FasL (Fas ligand) present on the CD8+ T-cell surface binds to target cell-surface death receptor Fas (or CD95), which induces apoptosis of the target cell. Unfortunately the study of FasL expression on virus specific CD8+ T-cells remains difficult, due principally to a lack of sensitivity of the method or low levels of expression in the cells. Perforin is contained in lytic granules, and is thought to form pores in target cell membranes to enable the entry of granzymes which activate an apoptotic cascade resulting in cell death (59, 60). These molecules can be stained intracellularly. It is also possible to monitor the presence of intracellular lytic granules using monoclonal antibodies specific for TIA-1 (or GMP-17), a protein found in the granule membrane (61, 62), or the ability to degranulate by surface staining for CD107 (or LAMP) (63), also present in the membrane of cytotoxic granules, but which is transiently expressed onto the cell surface upon antigenic stimulation and degranulation. These tools provide an indication as regards the cytotoxic potential of T-cells *ex vivo*. Increasing evidence suggest that the majority of virus specific CD8+ T-cells exhibit such cytotoxic potential (with lytic granules containing perforin and granzymes) (62-65), even though this one may vary in intensity, as various levels of perforin and granzymes are found in different virus specific populations. The significance of these differences in cytotoxic potential for viral control remains to be understood. Moreover, staining for these molecules is only indicative of a potential, but is not a direct proof of cytotoxic efficiency *in vivo*. *Ex vivo* lysis assays (using chromium loaded target cells) with fresh PBMCs may be thought to provide a better view of cytotoxicity; however a recent study in the mouse actually suggests that these assays are not a true reflection of *in vivo* cytotoxic capacities (66), and so these measurements may be misleading as to what constitutes a protective “effector cell”.

2.4 CD8+ T-cell differentiation in viral infections

2.4.1 Post thymic development

With the large number of monoclonal antibodies against a variety of molecules (e.g. co-stimulatory receptors, chemokine receptors, adhesion molecules) available for flow cytometry, analysis of CD8+ T-cell subsets has been very extensive in human studies, resulting in the characterization of many phenotypically distinct subpopulations. The phenotype of a naïve cell is agreed by all and is characterized by expression of: CD45RA, the lymph node homing receptors CCR7 and CD62L, and the co-stimulatory receptors CD28 and CD27, low expression of the integrin CD11a, and lack of expression of markers such as CD57, granzymes and perforin (67). However, beyond the phenotype of naïve cells, complexity begins, with the description of a multitude of antigen experienced subsets characterized by distinct pattern of expression for the above cited markers (62, 68, 69). Nonetheless, there seems to be consensus that the expression of many of these molecules follows a common pattern, thus marking a linear pathway of CD8+ T-cell differentiation, as depicted simply in Figure 2. Such model is supported by longitudinal *ex vivo* analysis of virus specific CD8+ T-cells in human viral infections (Figure 1) (38, 43), as well as telomere length (see later) measurement in the different subsets and *in vitro* data following naïve T-cell priming (70). The *in vivo* significance and role of these distinct subpopulations has become a matter of intense debate, for instance with the use by different investigators of terms as varied as effector, senescent or suppressor cells to designate a same subset. In order to avoid confusion with the use of any particular terminology, the pathway of differentiation described above may be seen simply as the post-thymic development of T-cells, along which T-cell subsets are more or less differentiated; the attribution of particular functions or protective properties to these subsets remains unclear.

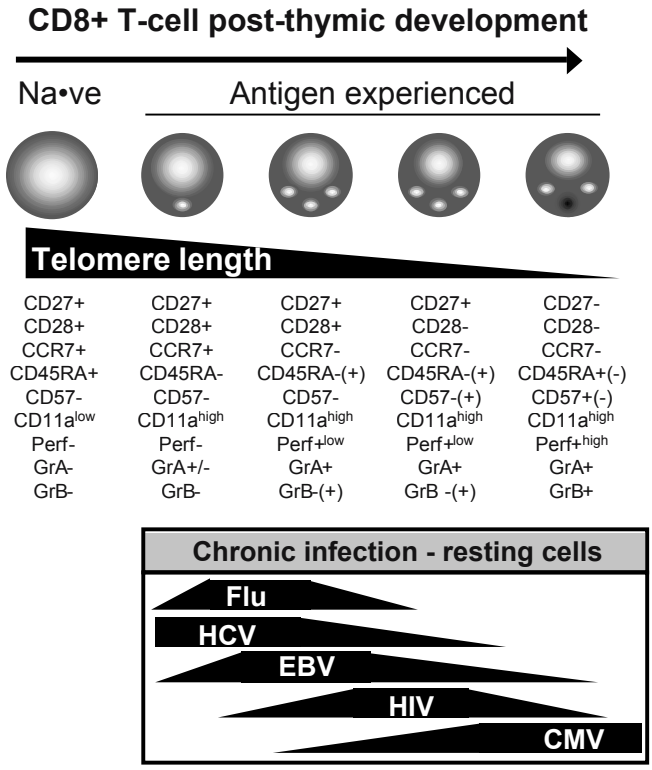

Figure 2. Distribution of virus specific CD8+ T-cells along T-cell differentiation during chronic virus infections. Partially reproduced from Appay V, Rowland-Jones SL. Lessons from the study of T-cell differentiation in persistent human virus infection. Seminars in Immunology. 2004 Jun;16(3):205-12 -Eelsevier Publishers.

2.4.2 Virus specific CD8+ T-cell differentiation

During the primary stage of most viral infections, virus specific CD8+ T-cells are present at early stages of differentiation with expression of CD27, as well as CD28, and no expression of CCR7 and CD45RA (43, 62, 71-73). During chronic infection, however, the picture is less simple: considerable heterogeneity is observed in the extent of differentiation of the CD8+ T-cell populations, and interestingly, this appears to be related to the viral specificity of the cells (62, 74, 75). For instance, the pattern of expression of differentiation markers varies between HCV-, EBV-, HIV- and CMV-specific CD8+ T-cells suggesting that these populations are at distinct stages of post thymic development (Figure 2). Resting Influenza A specific CD8+ T-cells usually show an early differentiated phenotype. Additional

differences reflecting particular antigen specificity have also become apparent. CD8+ T-cells specific for EBV latent proteins present a less differentiated phenotype that those specific for EBV lytic proteins (72). Cells recognizing the HLA-B8 restricted HIV nef epitope (FLKEKGGL) have been reported to be further differentiated compared to other HIV specific CD8+ T-cells (57). Nonetheless, this diversity is not as striking as those associated with different viral specificities. The basis for these differences and their significance in viral control remain to be determined. One hypothesis would be that the different differentiation states observed in peripheral blood for particular viral infections may relate to compartmentalization of some virus specific CD8+ T-cells in secondary lymphoid organs or at sites of viral persistence. This may be particularly relevant in the context of chronic HCV and HBV infection, where a significant number of virus-specific cells may be located in the liver.

Alternatively, the differentiation state of anti-viral T-cells with different viral specificities may reflect distinct conditions associated with particular viral infections, such as variations in antigen load, persistence, anatomical location and antigen presentation. The level of stimulation may be an important factor in this process (70). Elevated CD8+ T-cell activation seems to drive more extensive differentiation, as seen in both *in vitro* experiments using priming of naïve CD8+ T-cells and *ex vivo* analysis of virus specific CD8+ T-cells. In this context, antigen specific CD8+ T-cells may be subjected, from priming onwards, to variable intensity of activation or co-stimulation which differs in different infections, thereby dictating their development (i.e. expansion and differentiation). In this scenario, CMV would constitute a particularly potent stimulus for CD8+ T-cells, promoting extensive differentiation of CMV-specific cells. The regulation of T-cell differentiation is likely to be multi-factorial, and may also depend on the cytokine environment, the presence of T regulatory cells and the involvement of viral immune evasion strategies.

3. PERSPECTIVES

3.1 The importance of virus specific CD4+ T-cells

Giving focus only on virus specific CD8+ T-cells is only half a story told. CD4+ T-cells play also a major role in the establishment of effective anti viral immune responses, performing helper or co-ordination functions. The presence of virus specific CD4+ T-cells has been associated with long term non progression in HIV infection (76, 77), with control of CMV

replication after transplantation (43), and with the resolution of HCV viremia (78). The characterization of CD4+ T-cell populations specific for viral antigens represents a major challenge in human immunology. There is a substantial gap in our knowledge between CD8+ T-cells and CD4+ T-cells. This is in part due to the apparent complexity relative to the nature of these cells. On the basis of the pattern of cytokines produced, CD4+ T-cells have been divided into distinct functional subpopulations: T helper-0 (Th-0) cells , Th-1 cells and Th-2 cells; in addition, there are subsets of CD4+ T-cells that have regulatory – suppressing functions, or also cytotoxic potential. A further difficulty relates to the lack of tools adapted to their detailed study. Current approaches to study virus specific CD4+ T-cells rely on particular functions of the cells, such as proliferation and cytokine (often IFN-γ) production. This way, it is possible to detect CD4+ T-cell activity specific for most human viruses; although antigen specific CD4+ T-cells are usually present at significantly lower frequencies compared to their CD8 counterparts, which may be related to a slower division rate of the CD4+ T-cells, and represents an additional obstacle for their study. Moreover, these techniques do not permit *ex vivo* characterization of T-cell populations with a defined peptide specificity; the development of MHC class II /peptide tetrameric complex technology, adapted to CD4+ T-cell population analysis, has been more difficult than for CD8+ T-cells (79). At the cost of intensive efforts, the generation and use of reliable class II tetramers have recently begun (80); it should not be long before in depth characterization of virus specific CD4+ T-cells is performed. Furthermore, in order to overcome the problem of low frequency and to distinguish populations of interest from background, investigators have combined tetramer staining with magnetic bead capture techniques, thus enriching the tetramer positive cells to obtain satisfactory data (78).

In the recent years, the study of CD4+ T-cell subsets has also become more intensive and reveals a differentiation process, resembling CD8+ T-cell differentiation. Similarities involve phenotypic as well as functional changes that occur along the post-thymic development pathway: for instance, loss of expression of molecules such as CD27, CD28 and CCR7, gain in expression of CD57 as well as perforin, re-expression of CD45RA, and reduced capacity to proliferate (81, 82). Eventually, highly differentiated CD4+ T-cells, which have become cytotoxic, show striking resemblances to late differentiated CD8+ T-cells. Strong CD4+ T-cell differentiation seems particularly likely to occur under similar conditions of strong or chronic activation, such as in HIV-1 infection (83). Interestingly, virus specific CD4+ T-cells also exhibit distinct differentiation phenotypes in different infections: HCV, HIV and EBV specific CD4+ T-cells are less differentiated than CMV specific CD4+ T-cells (78, 84, 85), in parallel with the

observations made in CD8+ T-cells. Although these observations remain to be understood, this suggests that similar mechanisms are involved in the differentiation of these two lineages. Much work is needed to detail the exact implication of virus specific CD4+ T-cells in conferring protective immunity, as well as the mechanisms involved.

3.2 Understanding senescence and the maintenance of proliferative capacity

Since recent studies both in humans and in mice suggest an association between the capacity of virus specific T-cells to proliferate and the establishment of protective immunity (45, 46), it is most important to further our understanding of the loss and maintenance of such capacity by T-cells. Continuous activation can drive clonal exhaustion (86) as well as aging of lymphocytes, which eventually reach a state of replicative senescence (87); this process seems to be associated with T-cell differentiation, with highly differentiated drawing near senescence (70). Various mechanisms, under investigations, are involved in the onset of replicative senescence (88). One possible way to address the aging of T-cells resides in the measure of telomere length (89). Cellular expansion, involving extensive numbers of divisions, results in shortening of hexameric DNA sequences found at the end of chromosomes referred to as telomeres. With each division, 30 to 60 base pairs of telomeric DNA are lost, due the inability of DNA polymerase to replicate fully the extreme ends of chromosomes. Telomeres are responsible for maintaining chromosomal stability and integrity, and their shortening may eventually lead to cell cycle arrest and replicative senescence (90). The measurement of telomere length by flow cytometry is possible by flow-FISH (fluorescence in situ hybridization by flow cytometry) (91, 92), a technique recently associated with staining of virus specific CD8+ T-cells by tetramers (93). During primary infection and despite intensive T-cell proliferation, telomeres are not shortened, due to the expression of the telomerase (the enzymatic complex necessary for the maintenance of telomere length) (94). However, the induction of human telomerase expression appears to decrease in T-cells thereafter, when cells are stimulated again (95), resulting in telomere shortening. Interestingly, constitutive expression of telomerase by transduction in an HIV-1-specific CTL clone resulted in enhanced proliferative capacity and cytolytic capabilities in *in vitro* experiments (96).

3.3 Concluding remark

Understanding protective immunity is the gateway to vaccine development. Long term immunological protection is likely to depend on both the quantity and the quality of the antigen specific CD4+ and CD8+ T-cells that are generated. The level of antigen provided and the manner of presentation do certainly impact on both these parameters. However what defines exactly these parameters and how to govern them are not known yet. Although we are becoming better at inducing antigen specific T-cell responses *in vivo* using various vaccine strategies (e.g. antigen with adjuvants, antigen presenting dendritic cells, recombinants viral vectors, DNA), interrogation and controversy still remain as regards which are the most relevant T-cell phenotypic subpopulations or functional characteristics (i.e. effector functions as well as T-cell receptor usage and affinity - avidity) to generate. The path is still long before we reach a complete understanding of all T-cell mediated immunity mysteries; the study of virus specific T-cells is necessary to shed light on these points.

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Chapter 5

CYTOTOXICITY ASSAYS

Killer Lymphocytes in Cancer

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Abstract: The central theme of this chapter is to assess the type, mode of action and the monitoring of killer lymphocytes in the cancer patient. Both adaptive (α/β and γ/δ T cell) and innate (NK and NKT cell) cell-mediated immune responses are evoked by syngeneic tumors. The primary mechanisms used by both adaptive and innate effectors in vitro involve the perforin/granzyme and Fas pathways of lysis induction. Data from in vitro and animal studies suggest that while cytotoxicity may be only one of a number of immune defenses to cancer, it is likely to be an important one, and close monitoring of tumor-specific cytotoxicity can provide important information about the progress of both the tumor and the host response in vivo. Detecting killer lymphocyte (mostly CTL) activity is an indication of an ongoing tumor-specific immune response against the patients' own tumor. CTL activity can also be a valuable sign for a favorable response to a tumor vaccine given to the patient, often along with biological response modifiers such as adjuvants or cytokines. Due to the relatively low frequency of effector cells in many cancer patients, a combination of methods may be required for a full assessment of a patient's responding T cells, including CTLs. Although the predictive value of detecting killer cells in the cancer patient can be easily overestimated, it at least provides a clue to an ongoing process of anti-tumor activity, which may indicate response to therapy and consequently a favorable prognosis.

Key words: CTL, NK cells, gamma/delta T cells, chromium release,

1. HOST CELLULAR IMMUNE RESPONSES TO TUMORS

There are several situations in which cells in the body become targets for direct cytolytic attack by cells of the immune system, for example in

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autoimmunity, in infection by intracellular parasites, and in cancer. Unlike autoimmunity and immune responses to intracellular parasites, where humoral mechanisms can play a major role in host defense, the effective immune response to cancer is almost entirely cellular. The cellular immune response consists of both innate (NK/NKT cells and macrophage) and adaptive (both CD4 and CD8 T cell, and possibly γ/δ T cell) components. These cells can exert direct, contact-induced cytotoxic damage against tumors, and release inflammatory cytokines that further compromise tumor survival.

Early studies suggesting the response to syngeneic tumors (e.g. chemically induced fibrosarcomas) is cell-mediated were carried out by George Klein and his associates. They found that mixing tumor cells with lymphocytes from syngeneic tumor-immune animals prior to implantation in a new syngeneic host greatly reduced or abolished the ability of the tumor cells to form a new tumor, whereas pretreatment with immune serum had no such effect (1, 2). Cell-mediated immunity against virally induced tumors was established shortly thereafter (3, 4).

1.1 CTL in cancer

That in mice the cells responsible for syngeneic tumor rejection, whether chemically or virally induced, are at least in part T cells was suggested by studies showing such rejection was seriously compromised by neonatal thymectomy (5, 6). The first test of an in vitro effect of lymphocytes sensitized to syngeneic tumor antigens in vivo involved 'colony inhibition' of target cells (7), but was followed soon thereafter with demonstrations of antigen-specific cytotoxicity as defined in allograft systems (8, 9). Cytolytic T lymphocytes (CTL) specific for a number of different syngeneic human tumors have been isolated from patients and cloned (10-13). These CTL can be shown to kill cognate tumor cells in vitro with varying degrees of efficiency.

In vivo, tumor antigen-specific CD8 cells can be identified with great precision at the tumor site in concentrations significantly greater than in normal tissues (14, 15). One convincing proof that CTL can be effective in syngeneic tumor control would be to show that tumor-specific CTL, when adoptively transferred to an animal bearing an established tumor, cause regression of that tumor. Preclinical studies showed that in a few cases in mice, especially when the tumor is of viral origin, and often when combined with chemotherapy (16), adoptive transfer of tumor-specific CTL could be effective in causing tumor regression, and even cure (17). Clinical trials in humans showed occasional successes (18, 19), particularly in EBV-induced lymphomas (20). But in the case of non-virally induced tumors, adoptive

transfer of CTL generated in vitro in mixed lymphocyte-tumor cultures, particularly when expanded with repeated stimulations in the presence of IL-2, had little or no effect on growth of the tumor , even though their in vitro cytolytic effect was unusually strong (21, 22).

Even the use of highly lytic CTL clones specific for tumor antigens gave at best transient tumor regressions in patients. However, recent advances in our understanding of what happens in adoptive transfer of tumor-specific CTL offers hope that this approach may yet yield important benefits (23). For example, Shimizu et al (24) described a CD4 cell bearing the CD25 marker whose removal greatly increased tumor immune responses in mice. This regulatory CD4/CD25 cell probably acts in a suppressive fashion on CD4 helper cells needed to promote anti-tumor responses, and its removal enhances both CTL and NK cell function. Patient treatments aimed at reducing this cell population, even transiently, improved development of cellular responses to their tumors, and in combination with adoptive transfer of tumor-specific CTL showed the strongest tumor regressions yet seen (25, 26).

1.2 NK and NKT cells in cancer

The innate arm of the immune response can discriminate between tumor and normal cells. The ability to detect and cause the elimination of tumor cells in vivo without prior sensitization was the founding definition of natural killer (NK) cells. The evidence that they do so is overwhelming, but until recently only indirect (27). Tumors sensitive to NK killing in vitro grew more slowly, or were rejected, in vivo (28, 29). Because many of the features of NK cells are shared with other lymphocyte subsets, it has been difficult to make definitive correlations between NK cell activity *in vitro* and their putative in vivo functions. Nevertheless, there is persuasive indirect evidence for the role of NK and NKT cells in controlling tumors. For example, IL-12 has been shown to be a potent stimulator of defenses against many tumors in mice. This effect is equally strong in wild-type and RAG2-deficient mice (30); the latter have no α/β or γ/δ T cells, but do have NK and NKT cells. The protective effect of IL-12 in both types of mice is lost when NK cells are depleted.

The closest we have to an NK "knockout" mouse is a mouse strain made transgenic for Ly49A under a Granzyme A promoter (31). Granzyme A is present in all NK cells. Ly49A delivers a negative signal to NK cells when it encounters murine class I molecules such as H-2D^d (32). Spleen cells from these mice are unable to kill standard murine NK targets such as YAC leukemia cells in vitro. However, they are also unable to kill MHC class I-negative targets such as RMA-S. These mice in fact have very few

"classical" NK cells in any lymphoid tissues. On the other hand, NKT cells were present and appeared functionally normal. Ly 49A was expressed on about half of the T cells in these mice, but these T cells showed normal activity against all cells except those displaying H-2D^d, the ligand recognized by Ly49A. Thus, the presence of Ly49A in lymphoid cells does not cause a generalized immune paralysis. In vivo, these mice were unable to clear YAC and RMA-S, leukemias that are quickly rejected by wild-type mice. Most importantly, these tumors were rejected in the transgenic mice if the mice were reconstituted with spleen cells from syngeneic SCID mice, which have NK cells but no T or B cells. Using other tumor systems, it was shown that these NK cells are also involved in suppression of metastases. These data are the most direct evidence we have to date that NK cells do in fact play a major role in tumor control in vivo.

1.3 γ/δ T cells in cancer

γ/δ T cells may be involved in tumor control. In some situations, γ/δ cells may be at least as important as α/β T cells (33). The Vγ9/Vδ2 subset is associated with hematological malignancies (34), while the subset using Vδ1 seems to be involved with solid tissue tumors (35). The Vδ2 subsets are particularly effective against class I-deficient tumor targets (36). Like α/β T cells, γ/δ cells are attracted to reaction sites by chemokines (37). γ/δ Ţcells interact with a variety of peptide and non-peptide structures on cell surfaces, not always in the context of classical or even non-classical MHC structures, and in some cases it is not even clear that recognition is via the T cell receptor (TCR). Perhaps most important for tumor control, γ/δ T cells recognize stress-related proteins, such as MICA and MICB (MHC class I chain-related proteins A and B) and/or heat shock peptides, which signal that something is wrong inside a cell. Tumor cells are under a great deal of metabolic stress, driven to divide constantly often in the face of insufficient nutrients and oxygen, and they display many of the same stress signals as parasitically infected cells.

γ/δ T cells can lyse autologous tumor targets in vitro (37, 38). There appears to be a good correlation between the ability to mobilize γ/δ T cells and tumor progression or regression (39), but there is no direct data suggesting that it is the cytotoxic function of these cells, rather than their production of cytokines, that is critical. Advantage has been taken of the ability of γ/δ T cells to respond to certain microbial products (40), and to natural products such as bis-phosphonates (34), to sensitize otherwise unrecognized tumor cells for lysis. As more information is gathered on the biology of γ/δ T cells, it is entirely possible they will turn out to be a major

defense against tumor growth in vivo, and one that can be manipulated for therapeutic benefit.

2. THE ^{51}CR-RELEASE ASSAY AND OTHER METHODS FOR ASSESSING THE LYTIC ACTIVITY OF KILLER LYMPHOCYTES

By far the most common assay for measuring lymphocyte-mediated killing of tumor cells in vitro is the ^{51}Cr-release assay. Early studies on the lytic action of cytocidal lymphocytes were based on direct microscopic observations of the destruction of tumor target cells cultured in Petri dishes. This subjective method of estimating target cell death continued to be used for several years, but had obvious quantitative drawbacks. Uptake of vital stains such as trypan blue by killed (but not live) target cells was used in an attempt to improve quantification, and worked reasonably well in many situations. However, this assay, which requires counting large numbers of stained vs. unstained cells under a light microscope, is tedious. Progress toward a complete definition of CTL-mediated lysis required a rapid, reproducible, and highly precise assay for recording target cell death. This was provided by the ^{51}Cr-release assay.

The release of trapped intracellular radioactive chromium (introduced into cells in the form of the anion $^{51}CrO_4^{-2}$ (commercially available as sodium chromate), a weak gamma emitter with a half-life of 29 days, had been used to assess the viability of red blood cells in hemolytic anemias as early as 1950, and ascites tumor cell viability in 1958. Arnold Sanderson (41) and Hans Wigzell (42) first used ^{51}Cr release to quantitate the lysis of lymphocytes induced by antibody and complement. Over the next several years, several authors adapted this assay for use in measuring lymphocyte-induced target cell death (43-46). It proved to be rapid and highly accurate, for both adherent and non-adherent target cells. Moreover, it correlated well with other assays of target cell destruction such as trypan blue uptake, loss of cell cloning potential, and the release of endogenous macromolecules.

An important advantage of the ^{51}Cr assay is that chromium released from lysed target cells is not reincorporated into CTL, or into target cells that have not yet been killed, which would greatly complicate quantitation of the assay results. This failure to reincorporate released chromium is thought to be due to a reduction in the oxidation state of ^{51}Cr inside living cells. Alternatively, the released chromium may be bound to other released cellular components, which are not readily taken up by other cells. Martz (47) found that greater than 90 percent of the ^{51}Cr released during target lysis by CTL had a molecular mass of less than 4,000 daltons, but larger than ^{51}Cr itself. Some

of the chromium initially taken up by target cells also binds to cellular components that are not released upon the death of the cell. Hence, the proportion of non-releasable chromium (between 20-40 percent for various cells depending on the lytic agent or means - e.g. detergent, freeze-thaw– used to induce cell death) must be determined in each assay for precise quantification. Nevertheless, the ^{51}Cr-release assay has proved superior to all other methods of assessing target cell death, and is used by nearly all laboratories studying lymphocytotoxicity. It should be pointed out that ^{51}Cr release measures only one aspect of target cell damage by killer lymphocytes: membrane damage. Killing by cytotoxic effector cells induces apoptotic cell damage and death in target cells, including DNA fragmentation. This damage in fact precedes the plasma membrane disruption resulting in ^{51}Cr release.

To quantitate the lysis of adherent cells such as fibroblasts or epithelial cells growing as monolayers, cultures are incubated (usually overnight) with medium containing 1-5 μCi/ml $Na_2{}^{51}CrO_4$ per ml. The labeled cell monolayers are then carefully washed several times, and a suspension of putative effector cells is plated over the labeled targets. After incubation, the overlying medium is centrifuged to remove any detached but non-lysed target cells, and radioactivity in the supernates is assessed in a gamma counter. Lytic assays of adherent target cells can take up to 24 hours, and are referred to as "long-term" assays. Whether the longer time required for lysis is related to the fact that the cells are in a monolayer, or to the distinct nature of the cells themselves, is unclear.

Non-adherent target cells such as leukemia cells or ConcanavalinA-stimulated (for 3 days) normal lymphoblasts are labeled with 100-200 μCi ^{51}Cr in a small volume of 0.1-0.2 ml for 1-2h at 37°C and then washed 2-3 times. CTL populations are mixed with labeled target cells in varying proportions, using 5,000- 10,000 target cells per assay well, depending on the degree of labeling. 1000-2000 cpm per well are required and sufficient for statistically valid data. However, up to 5000 cpm may be required to detect statistically significant differences in the range of five percent among experimental groups, and between experimental groups and controls.

Typically, assays are carried out in 96-well V- or U-bottom microtiter plates or in small tubes in a volume of 100-200 μl. The plates are centrifuged at room temperature to initiate the binding of CTL and target cells (conjugate formation, ref. 48), and incubated for up to several hours at 37^0C. At the end of the assay, identical quantities of supernates (usually 100 μl) are collected after centrifugation, and the radioactivity counted. Separate sets of control samples (no effector cells, or non-immune lymphocytes from a source similar to the effector cells) are included for determination of the total amount of ^{51}Cr in the assay system, and the amount of radioactivity that is

released spontaneously from target cells during the assay period. The data are usually presented as the percentage of target cell lysis at varying E: T ratios.

The most commonly used equation to calculate the percentage of lysis in either assay is:

$$\% \text{ specific Cr release} = \frac{[\text{experimental release} - \text{spontaneous release}]}{[\text{maximum release} - \text{spontaneous release}]} \times 100$$

where experimental release = cpm released from a sample during a fixed assay period; spontaneous release = cpm released from a control sample not incubated with killer cells during the same period; maximum release = cpm released by e.g. detergent. "Spontaneous release" reflects ^{51}Cr that spontaneously leaks out of living cells, and/or it may reflect target cells that die naturally during the assay period. Either way, it is not radioactivity whose release is driven by CTL. Lytic assays showing substantial target cell destruction in 2-4 hours are referred to as "acute" lytic assays.

Caution must be exercised in interpreting ^{51}Cr-release data, especially when comparing degrees of target-cell lysis by different effector cell populations, and particularly where extended incubation is required and spontaneous release is high. Inspection of the above equation suggests that high spontaneous release values (e.g. greater than 25%) could compromise the statistical value of the derived percent lysis. Moreover, the value for the maximum cpm in the system is subject to variability, depending on whether or not one corrects for radioactivity that is not truly available for release. Failure to include values of spontaneous release, in addition to the derived percent lysis values, prevents interpretation and comparison of lysis among experimental groups (49).

Determining the lytic activity of poorly cytocidal populations, or where target cells are refractory to lysis as is the case with most human non-leukemia cancers, when maximal lysis caused is low (e.g. under 30 %), is always problematic and prone to serious statistical error. One of the better ways to compare the lytic activities of lymphoid populations that vary widely in lytic potency against a given target is to derive a quantity called lytic units (LU) (50). One LU is defined as the number of effector cells required to lyse a defined fraction (usually 30-50%) of the target cells, and is deduced from lytic data obtained with serial dilutions of effectors tested against a fixed number of target cells in a fixed assay period. The number of LU per 10^6 cells is then calculated for each population, and is directly comparable between populations.

A useful procedure to measure lymphocytotoxicity that applies when extended incubation (6 –18h) is required is the end-labeling method, often

done with ^{35}S-methionine to quantify the number of residual, adherent viable cells incorporating the label into proteins. With the end-labeling procedure, problems emerging from high spontaneous ^{51}Cr -release due to the extended incubation are circumvented. However, remaining lymphoid cells, which incorporate the label, constitute a serious problem. Release of label from ^{35}S-methionine pre-labeled target cells can also be used to assess cytotoxicity.

Fluorogenic methods to measure cell viability are used as well, as is the FACS-based technique to measure killed target cells in CTL-target conjugates (51). A recent method for quantifying CTL action employs cell-permeable fluorogenic caspase substrates activated upon killer lymphocyte-induced apoptosis in the target cell (52). Last but not least is the JAM test of cell death which measures DNA fragmentation of cells whose DNA was pre-labeled by ^{3}H-thymidine (53); it is noteworthy that DNA fragmentation, a hallmark of apoptosis, precedes ^{51}Cr-release in CTL mediated lysis. Hence, the JAM test, in fact, is a measure CTL-induced target cell apoptosis and not necessarily of lysis.

3. KILLING MECHANISMS USED IN HOST RESPONSES TO SYNGENEIC TUMORS

It is now clear that killer lymphocytes can use several mechanisms to destroy aberrant self-cells including tumor cells. The most vigorous of these are based on the perforin and Fas pathways, with perhaps a lesser contribution of TNF and related pathways (54). One of the first comprehensive looks at the role of perforin in rejection of syngeneic tumors was carried out by van den Broek et al (55, 56). They looked at rejection of a wide range of lymphoid and non-lymphoid tumors in perforin knockout mice, compared to normal controls. The results varied widely, with no obvious relation to tumor type. A melanoma was equally lethal in mice with or without perforin. A fibrosarcoma grew about ten times more readily in perforin-deficient mice, although both the knockout mice and normal controls developed stable tumors. Both mice rapidly rejected a modified adenovirus-induced tumor. In general, among lymphoid tumors, perforin deficient mice were 100-1000 times more susceptible to some, and completely unable to control others. However, at least one tumor, RMA leukemia, was controlled equally well in both types of mice. Among some of the tumors controlled by perforin-deficient mice, the presence of Fas was shown to be critical. On the other hand, RMA-S, a class I-deficient variant of RMA whose rejection is presumably under control of NK cells, had previously been shown to grow without restriction in perforin knockout mice, whereas it is adequately controlled in normal mice. Finally, MCA-

induced tumors were found to develop sooner and grow faster in perforin knockout mice (55, 56).

The impact of perforin on the onset of spontaneous tumor growth was examined in another study. Researchers maintaining perforin knockout mouse colonies had not observed an unusual incidence of spontaneous tumors, but the numbers under observation were generally small, and few mice were kept more than a year or so. When observed in large numbers and over a longer time span, perforinless mice developed a high incidence of tumors of lymphoid cells (lymphomas), particularly of T, NKT and B cells. These tumors were also highly metastatic. Because mutations of p53 are commonly found in tumors of both mice and humans, it was decided to look at the incidence of tumors in p53-mutant mice that were also perforin-deficient. Both the incidence and time of onset of lymphomas were greatly accelerated in the doubly mutant mice. Surprisingly, a much lower proportion of the lymphomas were metastatic. Other types of tumors, principally sarcomas, began showing up in the doubly deficient mice as well, but this was likely due more to the lack of p53 rather than of perforin. Control of primary tumor growth appeared to be under control of CD8 cells, and the impact of perforin suggests it is the cytotoxic function of these cells that is critical (57 58). In a separate study, these same authors had shown that the key cell type involved in perforin-associated control of metastasis is the NK cell (59).

The role of perforin in syngeneic tumor control must be evaluated carefully. In the BALB/c kidney tumor system RENCA, where tumor control is dependent on CD8 cells, disruption of the perforin gene resulted in a major decrease in killing of tumor cells in vitro, but had little impact on tumor control in vivo (60). Although Fas appeared to be used for in vitro killing in this system, neither Fas nor TRAIL appeared to have any effect on tumor growth vivo.

CTL can use perforin alone to kill target cells in vitro (as evidenced by ^{51}Cr release), but the target-cell DNA fragmentation normally accompanying CTL killing requires the participation of granzymes (61). The granzymes studied most intensely in this regard are granzymes A and B. In GrzA knockout mice, DNA fragmentation is unaffected, while in GrzB-deficient mice, DNA fragmentation is slowed considerably. In doubly granzyme-deficient mice, there is no DNA fragmentation. In situations where perforin appears to be critical for tumor control in vivo, the absence of granzymes in one report appeared to make no difference, whether the putative effector cells were CTL or NK cells (62). However, a subsequent report using some of the same tumor cells lines and essentially the same knockout mice, reached the opposite conclusion (63). At present, this discrepancy is unresolved.

Perforin also plays a role in innate cellular responses to syngeneic tumors, most likely mediated by NKT cells (64). In RAG2-deficient mice, where tumor control was exerted largely by NK and/or NKT cells (30), the efficacy of these cells was shown to be dependent on perforin, but not on Fas. However, numerous other studies have shown that NK and NKT cells also utilize the FasL-related molecule TRAIL (TNF-related apoptosis inducing ligand) in controlling tumors (65). TRAIL in soluble form appears to be highly selective for tumor cells in vitro cytotoxicity tests and in vivo (66). IFNγ-stimulated NK cells, especially those found in liver, are strong expressers of TRAIL, and blocking of TRAIL in some cases can profoundly inhibit the ability of NK cells to block tumor development (67). TRAIL knockout mice were found to be more susceptible to both transplanted and spontaneous tumors (68, 69). TRAIL is now thought to be a major component of tumor resistance in both mice and humans, and trials to test the efficacy of the TRAIL ligand alone as an anti-tumor agent in humans are underway (70, 71).

It is also possible that granulysin could play a role in tumor control. Granulysin has been shown to kill tumor cells in vitro (72, 73). Its expression in tumor-infiltrating lymphocytes correlates with progression or regression more closely than that of perforin (74). However, at present, absent a granulysin knockout mouse, there is no definitive evidence for a role for granulysin in tumor suppression in vivo, and little insight into how granulysin might selectively target tumor cells in NK cells.

Taken together, the data in perforin and granzyme knockout mice confirm that cytotoxicity plays an important role in tumor surveillance and control, but as in other immune defense systems, it may be only one mechanism at the disposal of host animals to guard against aberrant and potentially life-threatening tumor cells.

4. CANCER VACCINES THAT ELICIT CTL

The consensus that cytotoxic T lymphocytes play a major role in tumor control led to efforts to design vaccines that specifically induce or amplify tumor-specific CTL. Key to production of such vaccines is the identification of tumor antigens recognized by CTL. In humans in particular, the vast majority of tumors arise sporadically, and the associated antigens are highly variable and difficult to predict. Early attempts to define tumor antigens based on antibody reactivity to tumor surface structures seemed of limited use, particularly once it was realized that CTL do not recognize native antigen at cell surfaces.

One of the more efficient ways to identify tumor-associated antigens and peptides is by CTL screening (75, 76). CTL (bulk or cloned; from peripheral blood or isolated from the tumor) known to lyse a particular tumor are used as effector cells, and ^{51}Cr-labeled, MHC-compatible normal cells, transfected with pooled cDNA from the cognate tumor, are used as targets. A target cell that is lysed is assumed to express one or more peptides recognized by the CTL, and candidate peptides are eluted from target MHC molecules and analyzed until the triggering peptide (and parent protein) is found. In many instances, the frequency of tumor-peptide-specific CTL is considerably higher in patients bearing the tumor, reinforcing the notion that such peptides were recognized in vivo and served to expand the corresponding T-cell population (77-79).

Using these and other approaches, a large number of tumor-associated peptides have been identified (80-82). Some are derived from proteins normally expressed at low levels in a limited number of cells, but over-expressed in tumor cells. Some represent proteins (differentiation antigens) normally present only at restricted stages of development, but expressed – again, often at very high levels – in tumor cells. So-called "tumor-unique antigens" may be the most promising targets for vaccine development. These are proteins unique to the oncogenic state, such as oncogenes or other mutated cell-cycle proteins, or in fact any mutated protein associated with, even if not causative of, oncogenesis (83-85).

Tumor-unique antigens occur randomly and unpredictably, either in genes or in their associated introns (86) or even in control-region sequences. Thus the number of unique peptides that could be associated with any given cancer is potentially huge. Moreover, the heterogeneity in class I structures within a population makes it difficult, even with non-mutated tumor proteins, to predict which peptides will interact well with individual MHC structures, or to prepare peptide "cocktails" that will cross-react with sufficiently large numbers of alleles to make this approach economically feasible. On top of all that, selected peptides must find CTL with cognate receptors to be effective as vaccines. In spite of these difficulties, some striking results have been obtained with this approach, and are discussed briefly herewith.

In humans, extensive work has been done to define peptides associated with malignant melanoma such as MART-1 and gp100 (reviewed in 82; 87), and clinical trials have been underway for the past several years. Clinical trials using a modified gp100 peptide administered together with IL-2 yielded promising results. Objective tumor regressions were obtained in 42 percent of HLA-A2-positive patients with advanced melanoma (88). IL-2 appeared to promote sequestration of responding CTL at the tumor site (89). In an unrelated trial a selected peptide from MART-1 was shown to induce

significant CTL responses in melanoma patients, which correlated with a prolonged time to relapse (90).

Vaccine trials with peptides representing other cancers including breast (91), cervical (92), and pancreatic (93), are underway, and have given broadly similar results where reported. As with the melanoma trials, these are all Phase I/II studies, restricted to patients with advanced cancer who have failed conventional treatments. However it must be admitted that at present, although CTL and NK cell responses can often be elicited by these vaccination procedures, success has been considerably less than with melanoma. As more peptides are identified, and modified where appropriate to broaden class I affinities, or to enhance responses within individuals, and as treatment is extended to patients with less advanced disease, it is reasonable to expect the present encouraging results will improve still further.

Dendritic cells are key to induction of CD8 T-cell responses to tumors. DC are attracted to tumor sites by cytokines released either by the tumor itself, or nearby DC, NK cells or other elements of the innate immune system. After ingestion of potentially antigenic material, the DC migrate to nearby lymph nodes where they in turn activate CD4 and CD8 T cells to peptides displayed on their MHC molecules (94, 95). Peptide-presenting DC can be prepared in the laboratory either by peptide pulsing or by transfection with cDNA encoding key peptides. It is also possible to present larger antigenic protein structures to DC, and allow them to process these naturally into peptides for MHC loading. A promising recent approach involves introducing tumor antigen genes into hematopoietic stem cells, and then infusing these back into mice where they travel to bone marrow and produce antigen-laden DC. The advantage is better homing of such cells to lymph tissues where they can interact with T cells (96). Key to many current DC-based vaccines is inclusion of substances to activate the DC, such as microbial products. The route of administration of peptide-bearing DC may be critical to the effectiveness of this approach (97). And as with viral vaccines, inclusion of activating cytokines such as GM-CSF as part of the vaccine protocol may further enhance the response (98, 99). Indeed, provision of GMCSF alone to tumors greatly increases CTL activation and tumor reduction (100).

These approaches to tumor vaccination have produced encouraging results in animal models (e.g., 101). Clinical trials are now underway with pulsed DC as vaccines to immunize patients against their own tumors. Again, most such trials involve melanoma patients (25, 102-105). Other trials using DC are aimed at prostate and bladder cancer (106-108). The prostate trials have been disappointing, but a trial using DC pulsed with B-cell lymphoma antigen achieved a response rate of 75 percent (109).

Vaccines based on cell-cycle proteins are typified by Her-2, an oncogene expressed in a wide range of human and animal tumors (110). A recent Phase I trial using DC pulsed with Her-2 peptides resulted in production of CTL able to lyse Her-2-transfected cells in vitro, and positive DTH responses in about a third of patients (111). A major drawback with the use of DC for vaccination procedures is the difficulty in harvesting large numbers of these cells from patients to use in their own vaccines. This limitation may be overcome by using non-dendritic cells geneticaly modified to function as DC (112, 113).

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Chapter 6

MONITORING T CELL PROLIFERATION

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Abstract: Many methods have been developed to monitor lymphocyte proliferation to detect prior exposure to antigen, or to monitor an ongoing response. These methods include detection of DNA synthesis, or recognition of replicative phases of the cell cycle. More recently fluorescent dye dilution methods are used to reveal the division history of individual cells. When combined with other flow cytometric methods, dye dilution enables a comprehensive picture of how cell proliferation and differentiation proceed simultaneously. Furthermore, relatively simple techniques allow estimates of cell proliferation rates, responding cell precursor frequency and cell survival changes with cell division.

Key words: Profliferation, cell division, CFSE, Thymidine, BRDU

1. INTRODUCTION

Cell proliferation is essential for the adaptive immune response (1). Detection of dividing cells is therefore an important indicator of lymphocyte activation, both *in vivo* and *in vitro*. In contrast to the relatively simple original formulation of Burnet, however, the control of T cell proliferation is a complex, highly regulated process that requires cell cooperation and receipt of cell contact-delivered, or cell secreted, regulatory signals.

For these reasons the detection of T cell proliferation to monitor Ag stimulation and exposure is an important skill for the immunologist. Many methods have been developed that can be adapted to different situations. The following discussion reviews some important issues when monitoring T cell proliferation *in vitro*, presents an overview of methods for detection, focussing particularly on the use of dye based cytometric methods, and

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concludes with a discussion of graphical methods for monitoring the kinetics and quantitative features of T cell proliferation.

2. REGULATING T CELL PROLIFERATION

Many regulators of T cell proliferation have been identified and should be considered when designing experiments.

2.1 IL-2

The T cell growth factor IL-2 was discovered almost 30 years ago and shown to be produced by T cells themselves (2-5). Antigen stimulation of resting T cells results in both secretion of IL-2 and expression of the IL-2 receptor on the surface of the activated cells. IL-2 sustains T cell survival and growth and its source may be from the same T cell (autocrine) or neighboring T cells (paracrine). For T cell proliferation *in vitro* the presence of IL-2 is usually essential as antibody directed against IL-2, or its receptor, abrogates T cell proliferation. IL-2 production from CD4 T cells is usually much greater than that of CD8 T cells even though both are dependent on the factor for growth. Therefore, a simultaneously occurring CD4 T cell response results in an elevated CD8 response through shared IL-2 (T-T collaboration). *In vivo* T-T collaboration may also occur through additional mechanisms (6).

While the importance of IL-2 in inducing T cell activation *in vitro* has been clearly demonstrated, it is not essential *in vivo*. T cells from IL-2 knockout mice show decreased proliferation *in vitro*, but are able to mount effective, although reduced, cytotoxic T lymphocyte (CTL) responses *in vivo* when the mice are challenged with virus (7-9). It is thought that other cytokines *in vivo* engage receptors that replace IL-2 in this setting.

2.2 Costimulators and antigen presenting cells

In addition to IL-2, many other receptors on the T cell surface have been identified that can promote T cell proliferation when engaged. Typically these receptors are referred to as ‘costimulatory’. Among them, CD28 is probably the most potent and the best studied. CD28 ligation is not essential for the initiation of T cell responses but it markedly increases the amount of IL-2 secretion in cultures, thereby promoting sustained survival and proliferation (10). CD28 and many other costimulatory signals can also mediate direct effects on T cell responses such as promoting survival or reducing division times. Typically the combinations of costimulatory

signals, and those delivered by soluble mediators such as IL-2 can add together in a powerfully synergistic manner to greatly enhance T cell proliferation (11, 12).

T cells detect antigen as peptide fragments that are presented by molecules of the major histocompatibility complex (MHC) on the surface of antigen-presenting cells (APC). APC play more than a passive role and are able to engage a number of co-stimulatory receptors on the T cell surface, as well as secrete some of the growth factors and cytokines that alter properties of the responding T cell. *In vivo* mature dendritic cells (DC) are crucial players in the activation of naive T lymphocytes but under certain circumstances macrophages and B cells can also function as professional APC. *In vitro* many systems have been developed to circumvent the need for professional APC and/or the presentation of antigen. Some of these will be discussed further in context below.

3. PRINCIPLES OF T CELL PROLIFERATION

T cell activation and proliferation follows a defined sequence (illustrated in Figure 1). Resting T cells are usually identified by surface expression of high CD62L and low CD44. Upon activation CD62L is downregulated and the cells express new molecules on the surface, such as the activation marker CD69 and the IL-2 α-chain (CD25). If the stimulus is strong enough the cells enlarge and begin proliferating. These dividing T cells are often referred to as T cell blasts and are considered 'primed'. Without further exposure to antigen T cells will divide a number of times before stopping and beginning to die by apoptosis. The number of divisions cells undertake in this manner varies for CD4 and CD8 (13).

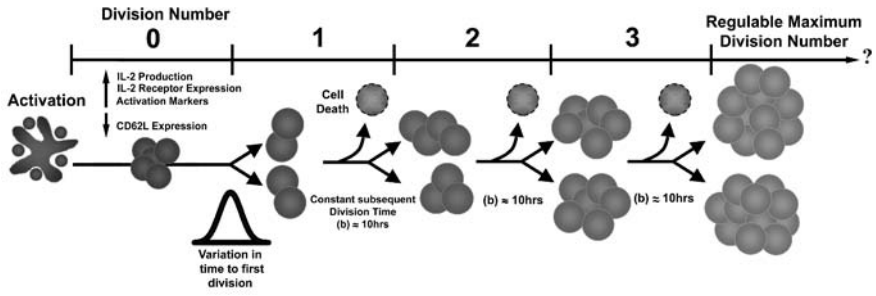

Figure 1. Key events in T cell Proliferation Dynamics – Resting T cells receive activation signals and undergo numerous changes including production of IL-2 and expression of IL-2 receptor. Activated cells grow in size and eventually divide. Time to the first division can show a broad distribution. Subsequent divisions are much shorter. Under some conditions a

proportion of cells will die as shown. Quantitative methods using division tracking can help estimate the values of kinetic parameters.

Primed cells can be triggered by antigen to undertake effector activities. However, when primed, T cells are more sensitive to activation by the TCR alone and less dependent on costimulation than naïve cells.

3.1 Design Principles for *In Vitro* Proliferation Assays

T cell proliferation *in vitro* is widely used to either detect prior exposure of T cells to antigen or to study the biology and control of naïve T cell proliferation. It should be noted that proliferation assays only indicate cell expansion, and give no indication of effector functions the lymphocytes mediate. Hence proliferation assays are often combined with other readouts such as cytokine production, cytotoxicity assays and quantitation of apoptosis to accurately gauge the T cell response.

T cell stimulatory protocols can be polyclonal or antigen specific. Polyclonal methods, allow non-antigen dependent stimulation of most T cells in culture. A widely used method is stimulation through anti-TCR component antibodies, such as anti-CD3. Other polyclonal methods include certain lectins and the pharmacological agents phorbol myristic acetate (PMA) and ionomycin.

Stimulating resting T cells with antigen presents problems as antigen specific cells are typically extremely rare. More commonly transgenic T cells with defined specificity are used for examining primary responses. Reactivation of *in vivo* primed cells, or antigen specific memory cells, can be carried out *in vitro* with good results.

If antigen specific T cells are to be stimulated, a method for antigen presentation or source of APC must be included in culture. The simplest arrangements are to prepare total lymphoid cells from lymphoid tissues or blood and add them to culture together with the antigen or stimulus. The population of endogenous APCs process and present antigen to stimulate the T cell response. However, making quantitative comparisons between different experimental groups using this method can be difficult as many uncontrolled variables affect the final level of proliferation. These variables include APC type, number and maturation state, the frequency of responding T cells, the presence of other cell types that may inhibit proliferation through elaboration of mediators, and the rate of accumulation of growth factors such as IL-2. Improved quantitation and consistency is provided by controlling the number and type of APC added to cultures of purified T cells and providing a saturating concentration of IL-2 (although this should only be done if the T cells are purified resting cells otherwise the ‘background’ proliferation can be unacceptably high).

Highly purified $CD4^+$ or $CD8^+$ T cells suitable for *in vitro* culture can be prepared from mononuclear cell suspensions using any of a number of well established techniques including positive and negative selection with paramagnetic beads (Miltenyi® or Dynal®), complement depletion, panning or fluorescence activated cell sorting (FACS).

4. MEASURING PROLIFERATION

Methods to measure proliferation can be broadly categorised into four groups:

1) Measurements of cell number (direct and indirect).
2) Quantitation of DNA synthesis,
3) Detection of cell-cycle associated antigens, and
4) Monitoring cell division by fluorescence.

4.1 Measuring cell number

The most direct method to establish proliferation is to sample cells from culture and counting live cells under a microscope using a haemocytometer. Increases in cell number over time establish that cell division has occurred and can give an indication of the rate and approximate time between divisions. It should be remembered, however, that the cell number increase can be offset by the death of cells and that often both processes will occur in a T cell culture. It is possible and common for example, to detect evidence of proliferation by one of the methods described below, even though the cell number is decreasing.

A popular colorimetric method for assessing live cell numbers without harvesting the cells from the culture vessel uses the tetrazolium salt 3-(4,5-dimethyethiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) (14, 15). MTT is converted to a colored insoluble formazan product by metabolically active cells. MTT is usually used for monitoring growth and viability of cells cultured in 96-well format. The salt is added to each well and the resulting insoluble product dissolved by direct addition of isopropanol to each well. Tetrazolium methods are toxic and are now less popular due to the development of other less invasive and safer methods such as alamarBlue™ detection. AlamarBlue™, is sensitive to the reducing environment of proliferating cells but is non-toxic, soluble and stable in culture medium therefore allowing continuous monitoring of the cells. AlamarBlue™ measurements can be performed both spectroscopically or fluorometrically.

Measuring expansion of antigen specific cells *in vivo* is possible if T cell receptor clonotypic probes or tetrameric MHC/peptide complexes suitable

for detecting the specific cells are available. Experimental detection of increases in, for example, specific T cell number/organ over time may indicate growth but is clearly complicated by the possibility of cell migration/sequestration and death that is difficult to simultaneously assess *in vivo*.

4.2 DNA synthesis

4.2.1 Tritiated thymidine incorporation

A widely used method to monitor polyclonal or antigen-specific proliferation *in vitro* is the incorporation of ^{3}H-thymidine into nascently synthesized DNA of actively dividing cells. The method is extremely robust and sensitive, and can be modified to suit many different applications.

Typically cultures are prepared in 96 well tissue culture plates and left to incubate for 3-5 days. After this time a small amount of ^{3}H-thymidine is added, usually 1 μCi per well. The labeling period varies for different labs, but is usually between 4 and 18 hours. Quantitation is performed by 'harvesting' the contents of each well onto glass fiber filters by aspiration and measuring the ^{3}H content per well by automated scintillation counting methods. Note that once the pulse with ^{3}H-thymidine is completed the labeled DNA is stable. Therefore, culture plates can be stored at –20°C and harvested whenever convenient.

In general the counts per minute (cpm) measured for each culture well are linearly related to the number of dividing cells. This method gives a very broad dynamic range and extreme sensitivity at the low end. Typically counts can vary from 50 to 500,000 giving a 10,000-fold range of detection.

4.2.2 BrDU incorporation

An alternative to the use of radioactive ^{3}H-thymidine is the incorporation of 5'-bromo-2'-deoxyuridine (BrdU), a thymidine analogue (16). BrdU incorporation can be detected using BrdU-specific antibodies coupled to fluorochromes and analyzed by immunohistochemistry or flow cytometry. In contrast to thymidine incorporation, this method has the advantage that it can be combined with other flow cytometry tools. Used in conjunction with the staining of cell surface markers and analysis of cytokine production (intracellular staining or ELISPOT) BrDU labeling can help provide a comprehensive description of a T cells' activation and proliferative status.

4.3 Detection of Cycling Cells

The presence of cells in S, G2 or M phases of the cell cycle is diagnostic of an actively dividing population. These phases of the cell cycle are marked by increased DNA levels and can be detected by flow cytometry with any of a large range of fluorescent DNA intercalating dyes including prodidium iodide (PI), 7-actinomycin-D (7-AAD), and Hoechst 33258.

Proliferation is also associated with the synthesis of new proteins. One of these, Ki-67, is expressed during S, G_2 and M but not during G_0 and G_1 phases of the cell cycle (17). In contrast proliferating cell nuclear antigen (PCNA), rises in the late G_1 phase – immediately before DNA synthesis – reaching a maximum during S phase. Both Ki-67, and PCNA can be detected with monoclonal antibodies within fixed and permeabilized cells. These methods are particularly useful for detecting proliferating cells in tissue sections where other methods are unavailable.

4.4 Monitoring division history with fluorescent dyes

A number of relatively new techniques allow cell division history to be monitored over time. Cells are stably labeled with a fluorochrome that is distributed equally between daughter cells allowing detection of up to 7-10 divisions (18). One such dye, 5(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), works extremely well for monitoring T cell proliferation and has found many applications both *in vitro* and *in vivo* (Figure 2).

If CFSE labeled cells are transferred *in vivo* they have been followed for months if they do not divide (19). CFSE molecules are permeable to cell membranes and, when incubated with live cells, they are enzymatically processed by endogenous esterases and attached to the cytoplasmic and even nuclear proteins. CFSE treated cells display identical spectral characteristics to FITC-labeled proteins, and as such can be detected and measured with all single and multiple laser flow cytometers. Another division tracking dye is the red PKH26, which, in contrast to CFSE, becomes integrated into the cellular lipid membrane. While it also appears to be kept relatively stable within the cell and is partitioned equally between daughter cells upon cell division, division peaks are usually more poorly resolved than with CFSE.

Division tracking is proving to be a very powerful method for assessing T cell responses when used in conjunction with other techniques. Further technical details on the use of this dye is given below.

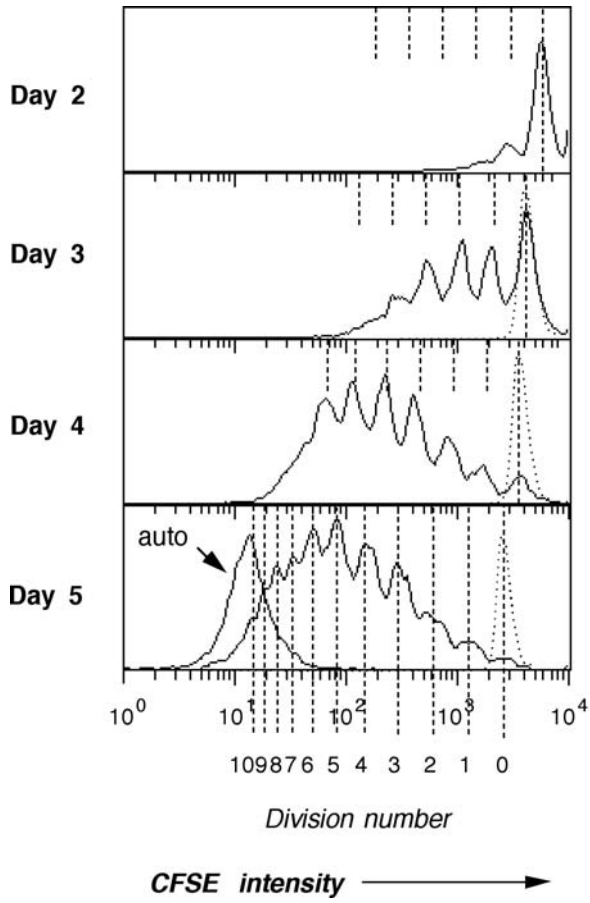

Figure 2. Analysis of CFSE Data – For accurate analysis of CFSE data it is necessary to have the correct controls. The figure represents typical data for a CFSE time course experiment. Proliferating lymphocytes (bold line) show the progression of cells through division number indicated by 2-fold serial dilutions of fluorescence intensity. The dotted line represents CFSE labelled lymphocytes with no proliferation activating stimulus. These cells serve as a control to locate the undivided cell population. Note that over time the intensity of this population is dimishing. The autofluorescence control, which consists of stimulated lymphocytes that are not labelled with CFSE, is indicated by the arrow. The CFSE profile on day 5 emphasises the importance of these controls. In this case the cells have undergone a substantial level of proliferation. The consequence of this is that there is no reference point for the undivided cell population and the peaks representing the later divisions progressively converge upon the autofluorescence level (Adapted from (Hodgkin, Lee et al. 1996) with kind permission of the publisher).

5. DIVISION TRACKING WITH CFSE

5.1 CFSE labelling protocol

Achieving consistent division tracking results requires attention to detail and careful maintenance of reagents. The following protocol has been successfully used in our laboratory for many years.

A 5mM stock solution of CFSE (Cat # 1157, Molecular Probes, Eugene, OR) is prepared in DMSO, aliquoted into convenient volumes (e.g. 10 µl) and stored at -20°C. Stock solutions can be kept frozen for over a year. Aliquots should be thawed only once and then discarded.

Cells to be labeled are suspended at 10^7/ml in PBS (pH7) containing 0.1% Bovine Serum Albumin (BSA). Cells should be well suspended with no aggregates. The small amount of BSA in the staining buffer greatly improves post-staining cell viability. It is important that the labeling medium not contain FCS, since it markedly reduces CFSE labeling.

To each ml of cell suspension, 20 µl of 1:10 diluted CFSE stock solution is added to yield a final concentration of 10 µM CFSE. The resolution of cell division peaks requires uniform staining so it is helpful to mix the cell suspension well (use of a vortex is recommended) at the moment of addition of CFSE and also periodically during incubation. Cells are incubated for 10 min at 37°C in a water bath, and the labeling stopped by adding five volumes of ice cold RPMI/10%FCS. After two further washes with RPMI/10%FCS the labeled cells are resuspended in the desired culture medium and placed in culture as soon as possible.

Choosing the correct concentration of CFSE is crucial, as high concentrations can be toxic and reduce cell viability whereas lower concentrations will limit the number of cell divisions that can be followed. Therefore, a preliminary titration of a number of CFSE concentrations for optimal labeling is recommended. This should be done for each new stock solution as some batch to batch variation has been noted.

5.2 Tracking cell divisions

Immediately after labeling, the intensity of CFSE within cells is extremely bright. The high fluorescence of freshly labeled cells diminishes rapidly over the first 24 hours, presumably due to the excretion of activated but unconjugated CFSE, as well as the catabolism of many labeled proteins. The initial strong intensity of staining often complicates flow cytometer measurement. Early time points in a time series may be off scale, and cannot be used in conjunction with other fluorochromes such as PE due to the

impossibility of appropriate compensation. Therefore, for the analysis of time points earlier than 24 hours, it is advisable to lower the CFSE concentrations for labeling or run the samples at different detector gain settings than used for later time points. The rate of CFSE loss through catabolism is constant in dividing and non-dividing cells, thereby maintaining the 2-fold difference between peaks (Figure 2).

The parameters that limit the number of divisions that can be followed are the labeling intensity, which is governed by concentration of CFSE and, the autofluorescence emitted by live cells. Since the autofluorescence emission is constant, it is added to the CFSE intensity, thus bringing peaks closer and closer together as CFSE is diluted. This is illustrated in the Figure 2. To resolve this problem, we recommend the use of peak-fitting software available in a number of commercial packages such as Flowjo (Tree Star Inc.) or Weasel (http://www.wehi.edu.au/cytometry/WEASEL.html).

To help determine the division numbers of CFSE it is recommended to have the following *in vitro* culture controls:

1. CFSE labeled cells either unstimulated in culture or stimulated in such a way to maintain viability without inducing division. For example, IL-4/IL-7 will keep resting T cells alive for several days of *in vitro* culture, without inducing proliferation. This control is especially important if stimulation conditions are such that all cultured cells might progress through cell divisions, making it difficult to identify the fluorescence level of undivided cells which are required as a reference point for assigning correct division numbers to each peak.
2. An autofluorescence control: Cells which have not been CFSE labeled, but are stimulated to proliferate in the same way as the experimental samples. Autofluorescence intensity can change depending on how the cells are stimulated and should be measured for each culture condition.

5.3 Combining CFSE with other methods

Tracking cell division with CFSE is well suited for use in conjunction with other flow cytometric methods.

5.3.1 Surface marker analysis

CFSE labeling can be combined with measurement of the expression of activation and differentiation markers on the surface of stimulated T cells to monitor their changes with division. This requires the use of multiple fluorochromes in combination with CFSE. As mentioned above, the intensity

of CFSE can be significantly higher than the average FITC level, especially in undivided cells making compensation with PE or other fluorochromes difficult. It is important, therefore, to have a full set of single color control samples to adjust compensation before analysis. CFSE is well suited for use in combination with dyes excited by a second laser such as allophycocyanin.

5.3.2 Intracellular staining for cytokines

By intracellular staining of CFSE labeled T cells, division history and ability to secrete cytokines can be studied simultaneously (Figure 3). This technique was used to show the importance of division in promoting the differentiation changes associated with division itself (12, 20-22). Due to the labile nature of the CFSE fluorochrome, however, not all fixation and permeabilisation methods are suitable for intracellular staining. In our hands fixation with 2% paraformaldehyde for 30 minutes at 4°C and permeabilisation with 0.1% Tween-20 or 0.1% saponin overnight at $+4^0$C generally gives a satisfactory result (22, 23) (Figure 3).

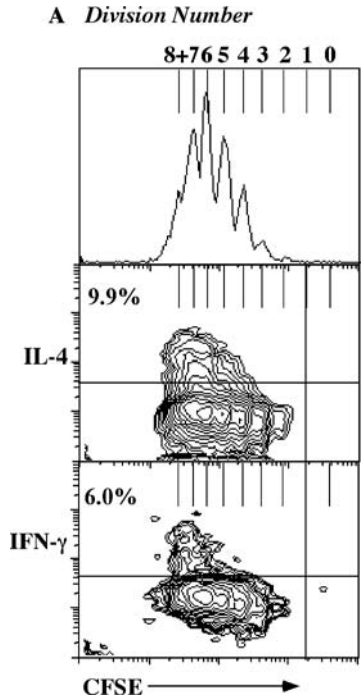

Figure 3. Intracellular staining for cytokines – CFSE labelled T cells were stimulated with anti-CD3, IL-2 and IL-4 for 4 days and analysed for proliferation (A) and differentiation of the cytokines indicated (B & C). Cells were restimulated with PMA and ionomycin for 6

hours in the presence of the secretion inhibitor brefeldin A. They were then stained with the appropriate PE labelled anti-cytokine antibodies (Adapted from (Gett and Hodgkin 1998) with kind permission of the publisher).

5.3.3 Cell cycle and proliferation analysis

BrdU staining in combination with CFSE labeling allows the identification of recently, or currently dividing cells in the context of cell division history (Figure 4.A, B). This method was used to show that T cells in all divisions divide at a similar rate once they have entered their first division round (21). Double staining with DNA intercalating dye 7-actinomycin D 7-AAD provides additional information on the current cell cycle phase of each cell (24, 25) (Figure 4C, D).

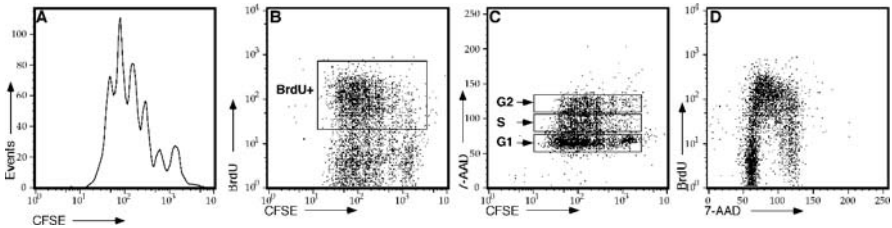

Figure 4. Simultaneous use of BrdU and 7-AAD staining in CFSE labelled cells - (A) CFSE labelled lymphocytes were stimulated for 3 days in culture. Cells were then fixed and permeabilized and analysed for intracellular components. (B) Labelling of bromodeoxyuridine (BrdU) after a 1 hour pulse allows actively dividing cells to be identified. (C) 7-AAD staining identifies the progression of cells through each step of the cell cycle in relation to division number. Cells from different division can be gated and examined BrdU and 7-AAD labelling. (D) Cells in S-phase clearly are BrdU positive. BrDu negative cells in G2 phase of cell cycle had completed S-phase before BrdU was added and have not divided before the 1 hr pulse ended.

Since intracellular staining increases background fluorescence, the following controls are important: 1) Cells not pulsed with BrdU but treated and stained as if they were; 2) fixed and permeabilized cells stained with secondary conjugates only if the BrdU specific antibodies are not directly conjugated to fluorochromes.

The DNA intercalating molecule 7-AAD is excited at 488nm and fluoresces at 647nm. Therefore, this agent is compatible with both CFSE and PE in a single laser flow cytometer. This channel should be collected in linear acquisition mode to differentiate nuclear phases. Another DNA dye DAPI can also be used in combination with CFSE and BrdU method, by using a flow cytometer with UV laser.

5.3.4 Cell sorting for functional studies

A major advantage of the CFSE labeling technique is the ability to recover viable cells that have undergone a defined number of cell divisions by FACS. Sorted cells can be re-cultured and monitored using functional assays to measure cytokine secretion or surface marker expression in response to different stimuli (21, 23). These cells can also be re-stained with CFSE to assess further proliferative potential. Sorted cells can be used as a source of RNA or DNA to assess levels of specific message for cytokines and other products, or to determine genomic recombination events and changes in DNA methylation patterns associated with differentiation.

5.4 QUANTITATIVE ANALYSIS OF CFSE DATA

Fluorescent division tracking data can be analyzed in a semi-quantitative or fully quantitative manner. Visual inspection of histograms such as that shown in Figure 2 can reveal whether division has occurred, and whether cells in one culture have divided further than another. Simply inspecting division peaks can be misleading when comparing cultures as CFSE profiles provide no indication of cell numbers. For example, two different culture conditions might exhibit similar CFSE profiles; however, if there is an increase in death rate in one of the cultures, the total cell numbers can be very different (26). To undertake more quantitative methods it is necessary to learn some of the kinetic and stochastic principles of T cell proliferation. A surprisingly, small number of rules can be used to acquire a great deal of information about a T cell culture. The following is a guide to quantitative analysis methods used in our laboratory.

5.4.1 Measuring average time between divisions

One of the most striking features of experiments using CFSE is the spread of cells in different divisions at each time point as noted in Figure 2, 3 and 4. Put more simply, lymphocytes are not all behaving identically, even when the stimulus is polyclonal and growth factors such as IL-2 are provided in saturating concentrations. What is the source of the variability? Experiments using BrDU pulsing show that the division rates of cells after their first entry to division are relatively constant. Similarly, when cells were sorted from different divisions (1-7) and placed back in culture, they underwent a uniform progression through division (Gett and Hodgkin unpublished). Thus, once T cells are stimulated into division they divide at a relatively even rate. These experiments indicate that the major source of

variation in division progression arises from cells exhibiting *different times of entry into their first division.*

We investigated the shape of the distribution governing the variation in time for cells to enter their first division and found it to be similar to a Gaussian probability distribution (21). This has proved to be true under a variety of different stimulation conditions. These data help reveal a relatively simple kinetic model of T cell proliferation (illustrated in Figure 1). The model divides T cell proliferation into two phases. There is the time of passage to the first division that is typically quite slow and takes an average of 30-60 hours with a relatively broad standard deviation of 10-15 hours. Once cells enter their first division they divide much more quickly (usually in the range of 8-12 hours between divisions). Importantly there is no evidence that cells that are fast or slow to enter the first division 'inherit' the tendency into the next division rounds. Experiments reveal that division times (both to first division and subsequently) are strongly influenced by the conditions of culture and the presence of cytokines and co-stimulators. To help assess quantitative differences between T cells responding to different culture conditions or treatments we developed a relatively simple graphical method for estimating average division times (Illustrated in Figure 5) (21).

5.4.2 The Cohort Method: Time to first division and division times

The cohort method is a graphical method for determining the quantitative values of the division times assuming normally distributed times to the first division. The cohort method relies on the principle of removing cell expansion from the cell number data and following the dividing cells as a normally distributed population.

Method

Typically, a time course of CFSE cell division data must be collected. As an example, T cells would be CFSE labeled and placed in identical cultures harvested at 5, 18, 24, 36, 48, 60, and 72 hours. To increase the accuracy cultures are harvested in triplicate. Before harvesting cells, a small amount of PI is added to the culture (final concentration 10µg/ml) and a known number of CaliBRITE™ beads (BD Biosciences, San Jose, CA) in 10µl for a 200µl culture. Care should also be made to keep the total number of beads >10% of the total number of events collected in a FACS file. The samples are run immediately and 10,000-20,000 event files collected.

Data Analysis

PI enables a live cell gate to be set while the addition of beads allows the total live cell number to be determined using the following calculation:

$$\frac{\text{Number (\#) of beads counted}}{\text{Number of cells counted}} \times \text{total cell \# added to culture} = \text{total cell \# in culture}$$

The next step is to analyze CFSE peaks and determine the proportion of the total live cells found in each division number. Regions can either be drawn manually or fitted using software packages such as Flowjo (Tree Star Inc.) or Weasel (http://www.wehi.edu.au/cytometry/WEASEL.html). From these proportions and the total live cell number the number of cells in each division can be determined. Plots of these numbers themselves are useful and can show the progression of cells over time.

The first step is to convert the total cell numbers in each division from our time series data to "cohort" data. Cohorts in our terminology are the number of starting cells now found together in a particular division number. Thus, if 1000 cells are measured in the 3rd division then the number of cohorts in division three is given by $1000/2^3$ to give 125 division three 'cohorts', or cells from the original cell inoculum. If the number of cohorts in all divisions greater than 0 are added together an estimate of the total number of starting cells that have divided so far can be made. This number divided by the starting number is an approximation to the precursor frequency (problems with this simple calculation are discussed further below).

An example of cohort data plotted against division numbers is shown in Figure 5B. In this plot the cohort values are normalized to a percentage of the total number in the time period. As can be seen the plot of cohort numbers against division number appears normally distributed. We need to fit a normal curve to the data and find the mean. A normal distribution curve can be fitted to data in different ways. Software packages such as Prism (Graphpad, CA) have a suitable fitting function for this purpose. We have constructed an Excel spreadsheet capable of calculating these values and plotting the necessary graphs to complete the analysis (available on request). Note in Figure 5B that with time the normal distribution moves to the right and that the standard deviation of the distribution does not markedly change, consistent with a constant subsequent division time.

Fitting of the normal curve must "ignore" the undivided population and concentrate on the shape of the dividing cells as can be seen in Figure 5B. Often the early time points yield limited information about the shape of the normal curve and a true fit cannot be applied. Therefore, the bulk of the information generated from this analysis will come from later time points in the time series.

When this analysis has been completed the mean division number of the fitted cohort plots can be graphed against their corresponding harvest time.

The result is usually a linear relationship (Figure 5C). From this line it is possible to calculate both the division time (*b*) and the mean time to 1st division (MTTFD). The gradient of the line is equal to the reciprocal of the division time (*b*) i.e. slower dividing populations will have a shallower slope while faster dividing populations will have a steeper slope. In this situation, division times are assumed to be constant across divisions; however, in practice they slow due to the utilization of media or because of a 'programmed' limitation in maximum division number. Extrapolating this fitted line back to the intersection with division 1 provides the value of MTTFD. Finally the standard deviation of the times to first division can be calculated from the information so far. The standard deviation of the fitted cohort plots is expressed in division numbers. This number multiplied by the calculated value of b yields the required value for standard deviation in time.

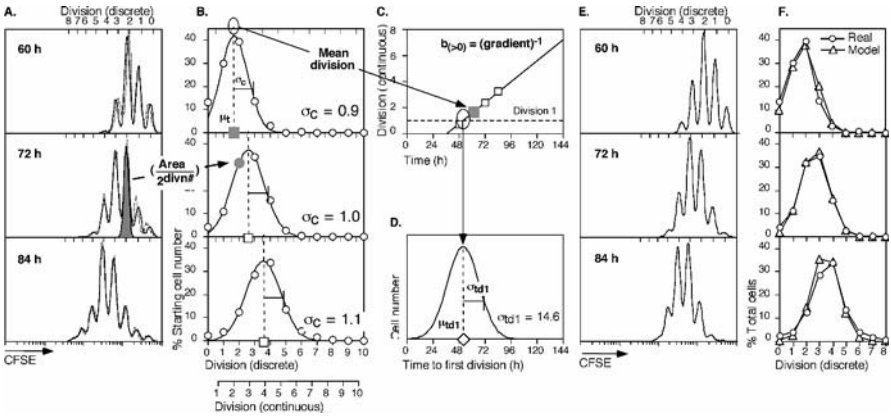

Figure 5. Cohort Method for Quantitating T cell Proliferation – (A) CFSE labelled T cells were stimulated and proliferation was tracked for 60, 72 and 80 hours. (B) The number of cells in each division number were divided by $2^{\text{division number}}$ and converted to a percentage of the total (circles). Normal distributions were fitted to the cohort data in order to obtain both a mean (μ_t) and standard deviation (σ_c) for each harvest time. Note how with time the Gaussian curve moves evenly to the right. Also note that the mean is calculated in reference to a 'continuous' division scale as cells in division peak, for example 3, are on average in division 3.5. (C) Plot of μ_t versus harvest time. Plots generated a linear relation for which the inverse of the slope gives the average division cycle time (b). The intercept of the line with division 1 is the average time the population of T cells took to reach the first division (μ_{td1}). (D) The distribution representing the variation in entry to the first division for the T cell population was then plotted using μ_{td1} (generated in c) and the standard deviation (σ_{td1} x $b_{(>0)}$). (Reproduced from (Gett and Hodgkin 2000) with the kind permission of the publisher).

This method has some shortcomings. The estimation of division times assumes that there is little death of dividing cells. In reality the proportion of cells that survive each successive division can itself be continuously regulated in similar manner to division times (12). If only a small percent of cells are being lost (<15%) there is little impact on the results of the above version of the cohort analysis. A larger proportional loss of cells through each round of division will alter the result of the cohort analysis such apparent time taken for subsequent divisions will be underestimated. Another problem is that the distribution of entry into division is actually lognormal (12), meaning that it is normally distributed on a log time axis. Fortunately, however, under many situations the standard deviation of T cells entering division is sufficiently small that a normal distribution can be as a successful approximation.

More complete analyses that take into account rates of cell death and changes in other parameters such as precursor frequency require more complex specialized tools for data analysis (12).

5.4.3 Precursor frequency

It is often useful to know how many of the starting cells have been stimulated into division. This 'precursor frequency' is a familiar parameter that can help compare groups. As mentioned previously an estimate is obtained by calculating the sum of the cohort values for cells in divisions greater than one. While useful there are also some problems. The final number does not take account of cells that may have died while in passaging division, and therefore could underestimate the true pF. Similarly the broad range of entry times to first division make it unlikely that all the cells that will enter division will have done so by day 3 or 4, when many estimates are taken. A further problem, and one that is extensively discussed previously (12) is that many potential 'precursors' will die before the first division and so cannot be counted by the cohort calculation. Despite these problems the simple device of counting cohort numbers is a useful and easily performed comparative statistic.

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Chapter 7

ELISPOT ASSAY

Assessment of Cellular Immune Responses to Anti-Cancer Vaccines

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Abstract: The ELISPOT assay is widely used for monitoring of immune responses to anti-tumor vaccines. It is a versatile, robust assay, which can be formatted to measure the frequency of antigen-specific T cells in peripheral blood or in culture and to assess the generation of effector cells upon ex vivo priming. The assay performance, results interpretation and its limitations are described.

Key words: ELISPOT, immune monitoring, T-cell response, T-cell frequency, cytokine production

1. INTRODUCTION

Monitoring the immune status of patients who receive anti-tumor vaccines or other biotherapies is an important component of therapy. As indicated in this volume, a wide variety of immune assays designed to measure anti-tumor responses is available. Consequently, the selection of the most appropriate assay or assays for monitoring of patients receiving anti-tumor vaccines is not easy. Clearly, assays are needed which can accurately measure vaccine-induced changes in the frequency and function of anti-tumor effector cells. Further, these assays need to be adapted to serial testing and to a large-scale monitoring with a minimal loss of accuracy. Among single-cell assays available today for immune monitoring, the enzyme-linked immunospot (ELISPOT) assay is perhaps most widely used.

The ELISPOT assay was developed and initially applied to the detection of immunoglobulin-secreting cells (1). More recently, it has been adapted to

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enumerate T lymphocytes secreting a cytokine, generally interferon-γ (IFN-γ), in response to an antigenic or mitogenic stimulus (2, 3). The ELISPOT assay belongs to a category of single-cell assays, because it measures production and release of the cytokine by individual T cells in a plated population of cells. Although ELISPOT has all but replaced limiting dilution analysis (LDA) for frequency determinations of antigen-responding cells, it is important to remember that in ELISPOT, T cells are counted based on their functional response to a cognate antigen. The ELISPOT assay exists in several different formats, which can be adapted to individual experimental situations (4, 5). However, only the ELISPOT assay performed with T cells directly isolated from blood or tissues, and not expanded *ex vivo*, can be used for estimations of the frequency of antigen-specific T cells, as they exist *in vivo*. Since the frequency of such T cells is generally low in the peripheral blood, the assay used for their detection has to be sensitive. The ability of ELISPOT to detect 1/100,000 of antigen-responsive cells (4) in the population, and its specificity defined by antibodies (Abs) used for capture and detection of the secreted cytokine, qualify it for an acceptable monitoring assay. In addition, its inter- and intra-assay reproducibility are satisfactory (6), and this reproducible performance, together with the assay robustness, seem to account for its popularity. By far the most important attribute of the assay in terms of serial monitoring is its application to cryopreserved specimens. Early comparisons of fresh with cryopreserved human peripheral mononuclear cells indicated that ELISPOT can be reliably performed using cryopreserved and thawed cells (4). This allows that pre- and post-vaccine specimens to be tested in a single assay, providing a reliable measure of vaccine-induced changes.

In this chapter, the principle, performance and interpretation of the ELISPOT assay will be discussed. The overall objective is to acquaint the reader with the options ELISPOT offers to its users and with possible pitfalls in results interpretation. Consequently, the emphasis will be on the direct and indirect formats of the assay and limitations inherent to each.

2. ASSAY PRINCIPLE

The general principle of the ELISPOT assay involves plating responder cell populations into wells of a 96-well nitrocellulose plate that have been pre-coated with an anti-cytokine Ab. Although the ELISPOT assay can be formatted to detect secretion, a variety of different cytokines, currently the interferon gamma (IFN-γ)-based assay has the widest application (7, 8). Antigens, stimulator cells or mitogens are added to replicate wells and incubated for defined time periods (usually 18 to 24 hours). Any cytokine

produced in response to the stimulation will be captured in the local environment of the cytokine-secreting cell by the membrane-bound capture Ab. All cells are subsequently removed by washing, and the captured cytokine is detected using a biotinylated, cytokine-specific detection Ab followed by the addition of an avidin-conjugated enzyme. Visualization of the resulting complex is achieved through the addition of an appropriate enzyme substrate, producing a colored spot, representing an individual cell that secreted the specific cytokine (Figure 1). The number of cytokine-secreting cells present within the population of plated responders can then be calculated. The ELISPOT assay has been reported to the capable of detecting one IFN-γ secreting cell per 100,000 cells plated (4).

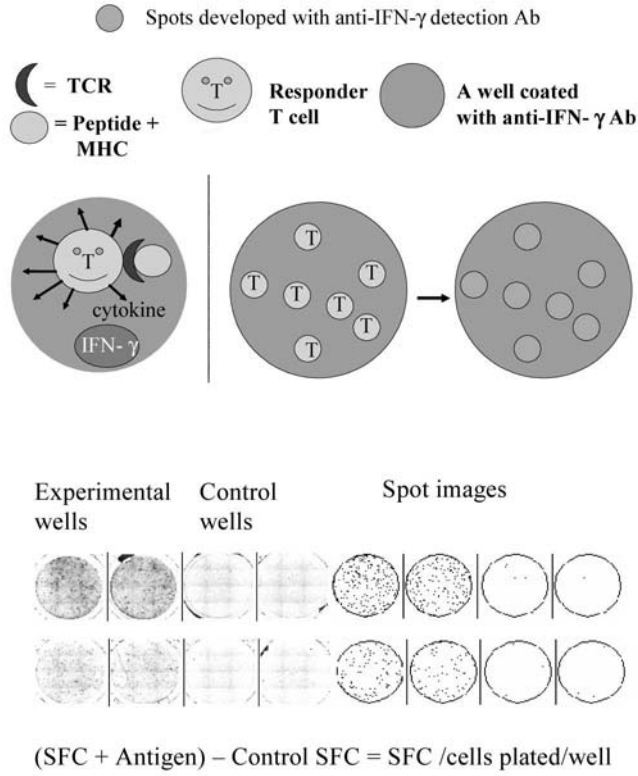

Figure 1. The principle and schematic of an ELISPOT assay for assessing the frequency of T cells producing IFN-γ in response to stimulation with a cognate antigen. In A,schematic representation of a single responder T cell placed on the nitrocellulose filter coated with the capture Ab specific for IFN-γ. The cell is shown interacting via T cell receptor (TCR) with the antigenic epitope presented by a relevant MHC molecule (left). This activated T cell

produces IFN-γ, which is captured by the Ab, forming a spot containing AgAb complexes. In the well containing a known number of T cells, only those cells recognizing the antigenic epitope will be activated and will produce spots (right). The spots are counted, providing the number of epitope-responding, spot-forming T cells among those plated. In B, the actual experimental wells with spot-forming cells (SFC) and control wells are shown (left). Also shown are images of the experimental and control wells obtained using an analyzer (right). The formula used for computation of the number of SFC/plated cells /well is provided.

3. PROCEDURE

3.1 Prior to the Assay

A number of critical steps are required to assure the assay success. The assay template has to be prepared to assure a maximum number of test samples per plate and to facilitate plating of samples. The template serves as the assay worksheet. The number of assay plates needed has to be determined. The plates are coated with the capture Ab. The final concentration of this Ab for coating is usually 10µL/mL, but may vary and needs to be pre-determined by individual laboratories. Coating of the plates is an important step: the use of a repeating pipettor is obligatory, making sure that each well receives the same volume of the capture Ab and that the entire surface of the well is covered. Label and date each plate. Store it in a sealed plastic bag at 4°C for no longer than 3 days.

3.2 Day of the Assay

The assay plates are washed several times (3 – 4 x) with phosphate buffered saline, using the flicking motion to remove the wash. Block the plate by adding 200µL/well of RPMI medium plus 10% (v/v) fetal calf serum (FCS) to all wells. Incubate the plate at 37°C for 1 hour. Use the plate immediately, do not store it.

3.2.1 Preparation of Cells

The use of a normal control cell preparation with each assay is recommended. This normally consists of a viable cyropreserved aliquot of PBMC from a normal donor with previously documented responsiveness to polyconal activation by PMA/ionomycin or, alternatively, OKT3 Ab. Every assay plate should include wells for the stimulated control, the spontaneous cytokine release control, and the assay medium control, each plated into triplicate wells. Preparation of responder and antigen-presenting cells (APC)

will vary greatly depending upon specific protocols. A range of 10,000 to 200,000 responder cells per well is normally plated, depending on whether freshly-isolated or cultured responder cells are used. Specific antigen stimulation is accomplished through the addition of either purified peptides or peptide/antigen-pulsed stimulator cells. For peptide stimulation, individual or pooled synthetic 9-mer or longer peptides of >80% purity are used at a final concentration of 1 to 2 μg/mL. Appropriate controls for the assay include responder cells alone, responder cells plus unpulsed stimulator cells, stimulator cells alone, and responder cells plus stimulator cells pulsed with an irrelevant antigen. Regardless of the stimulation strategy, all experimental conditions and appropriate controls are run in triplicate wells. When cryopreserved cells such as PBMC are used as responder cells in the ELISPOT assay, it is recommended that thawed cells be cultured for 2-4 h or overnight at 37°C (5% CO_2) in a polypropylene, 50-mL centrifuge tube containing 2 x 10^6 cells/mL in assay medium. The purpose of this step is to permit those cells stressed during the cryopreservation and thawing process to undergo apoptosis. The viability assessment after this brief culture period will be more accurate than one performed immediately post-thaw, and will improve the overall sensitivity and precision of the ELISPOT assay.

3.2.2 Plating of Cells

Use the prepared assay template as a guide in plating the assay. Prior to the addition of cells to the assay, remove the blocking solution by flicking the contents into the sink. Wash the plate once by adding 200 μL of assay medium and immediately flicking the contents into the sink. Using a repeating pipettor, add the cells slowly to the wells, ensuring even coverage in the wells. Always plate responder cells first, followed by either peptides or stimulator cells. Finally, label the plate by recording the date, protocol number, and plate number on both the lid and plate. Incubate the plates for 18 to 24 hours in a 37°C, 5% CO_2 incubator. During incubation, do not repeatedly open and close the incubator once the incubation has begun, as this can interfere with overall spot clarity.

3.3 Spot Development

Remove the plates from the incubator and wash six times with PBS-Tween-20. For manual washing, soak 15 to 20 minutes for the first wash and three minutes for each of the subsequent washes. Prepare the biotinylated detection Ab during the washing steps at a working concentration of 1 μg/mL in PBS/0.5% BSA. Add 50 μL of detection Ab to each well and incubate at 37°C for 2 hours.

During the last 10 minutes of the incubation, prepare the ABC reagent according to the instructions (see below). At the end of the 2 hour incubation, flick the contents of the plate and wash 6 times with PBS/Tween-20, with 3 minute soaks between each manual wash. After the final wash, flick out the contents of the plate and add 100 µL of ABC reagent per well. Incubate for 1 hour at room temperature. At the end of the 1 hour incubation, flick out the contents of the plate and wash three times with PBS/Tween-20 as above. Continue washing 3 more times with nonsterile PBS wash. Add 100 µL of AEC substrate to each well, and allow the color to develop for 5-10 minutes at room temperature in the dark. Spots in the positive control wells should be clearly visible. Rinse the plates 6 times with sterile water to stop color development. Allow the last rinse to soak for 2 minutes. Immediately remove the undertray from the plate and blot the bottom of the wells with a paper towel. Air-dry the plate completely at room temperature overnight in the dark. The plates must be completely dry before counting spots.

3.4 Spot Imaging and Data Analysis

The spots can either be counted manually using a standard dissecting microscope or imaged electronically using an automated imaging system. Several automated imagers are commercially available. Automated imaging systems are highly recommended for those laboratories dealing with a high-assay volume. Spots are counted according to specific manufacturer's recommendations. Each system has its individual means of dealing with differences in spot size and resolution of individual spots. The images for each experiment can be stored electronically and archived for future reference. Data are transferred into a spreadsheet format, and calculations are performed according to specific assay protocols. Among the calculated data fields are: Mean spot numbers, standard deviation of triplicate wells, % CV, background corrected mean spots (i.e., mean experimental value minus mean background value), SD, and corrected mean spots per 10^5 or 10^6 responder cells. Various statistical tests can be performed to determine the significance of the test value compared to the background.

4. COMMENTS REGARDING ELISPOT REAGENTS

Attention is called to selection / formulation of several key reagents. First, the selection of capture and detection Abs is the most crucial aspect of the ELISPOT assay. Not all anti-cytokine Abs perform equally well for

cytokine capture when coated onto a solid surface, and the biotinylated detection Ab must be able to recognize the cytokine complexed to the capture Ab. Once selected, both Abs need to be titrated to determine dilutions for optimal reactivity. The selected Abs should be purchased in bulk to avoid repeated optimization required for each new lot of Abs for ELISPOT. A wide variety of Ab pairs suitable for ELISPOT assays are now commercially available, but each pair needs to be evaluated and its performance validated in individual laboratories. Preparation of enzymes, chromogens and buffers is also important, and should be accomplished as follows:

- Avidin-biotin complex (ABC) diluent (0.1% Tween-20 in PBS): Add 0.1 mL Tween-20 to 100mL sterile PBS, sterilize through a 0.2μm filter, and store at 4°C for up to three months.
- ABC reagent (avidin biotinylated horseradish peroxidase complex): For each plate, add one drop of Reagent A and one drop of Reagent B to 10 mL of ABC diluent. Mix immediately and allow reagent to stand for 30 minutes at room temperature before use. Prepare fresh for each assay and discard any unused portion in a sanitary drain.
- AEC (3-amino-9-ethylcarbazole) substrate for peroxidase: For each plate, add four drops of buffer solution to 10 mL of sterile water and mix. Next, add six drops of AEC stock solution and mix. Finally, add four drops of H_2O_2 and mix. Use immediately after preparation and discard any unused portion in a sanitary drain.

Options exist, of course, for selection of different enzyme and chromogen combinations, but in all instances the assay will require optimization.

5. ELISPOT ASSAY FORMATS

The ELISPOT assay can be used for analysis of various types of specimens (6, 7, 8). The responder cells may be whole PBMC, isolated CD4+ and/or CD8+ T cells, cultured PBMC activated by *in vitro* sensitization (IVS) with a stimulating antigen or cultured T-cell lines. Antigen-presenting cells (APC) may consist of autologous PBMC, dendritic cells (DC) or a specific T-cell line such as T2 cells.

Broadly, the two widely-used formats of ELISPOT can be distinguished as direct or indirect assays. The former refers to a 24 h assay performed with responder cells (either PBMC or separated CD4+ or CD8+ T cells) obtained from the donor and tested without any IVS (Figure 2). The latter involves IVS and culture of responder cells in the presence of an antigen presented by APC (Figure 3). Aside from methodologic differences between

the two types of the ELISPOT assay, information provided by them is distinct.

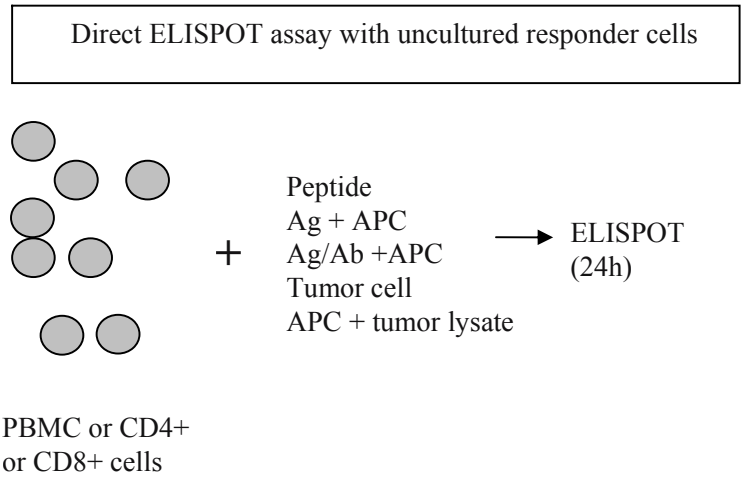

Figure 2. A schematic representing the direct or one-step ELISPOT assay. The possibilities for using a variety of stimulating cells or antigens and different types of uncultured responder cells in this assay format are indicated.

The direct assay measures the frequency of antigen-primed T cells in the plated population of lymphocytes obtained from the tested subject. Short-term (24 hour) stimulation with a cognate antigen induces cytokine production from such *in vivo* primed T cells. In contrast, the indirect ELISPOT assay depends on stimulation of naïve precursor T-cells with an appropriately presented antigen and their expansion during a 7-day culture. These expanded T-cells are re-stimulated with the antigen in a 24 h ELISPOT assay to yield the number of antigen-responsive, cytokine-secreting lymphocytes present in the culture (Figure 3). Thus, the one-step (direct) and two-step (indirect) ELISPOT assays detect entirely different immune responses to an antigen: cytokine production by *in vivo* primed T cells re-stimulated in ELISPOT vs. cytokine production by effector cells generated upon *in vitro* stimulation of naïve precursor T cells with an antigen and their expansion in culture, which are followed by re-stimulation in the ELISPOT assay.

ELISPOT after IVS with Ag or Ag/Ab on DC

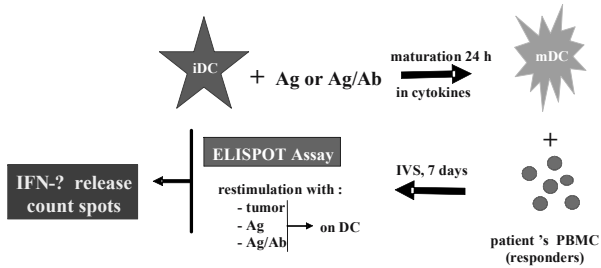

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Figure 3. An indirect or two-step ELISPOT assay consists of an in vitro sensitization (IVS) step, in which PBMC or other responder cells are cultured in the presence of an antigenic epitope presented on APC, such as mature dendritic cells (mDC). Following a 7-day culture, which allows for expansion of epitope-specific responder T cells, these cells are re-stimulated with the same antigenic epitope in ELISPOT assay (step 2) to determine their frequency in the culture.

6. RESULT INTERPRETATION

The distinction between the one-step and two-step ELISPOT assays is extremely important, and it appears to be often misunderstood, leading to incorrect result interpretation. The direct ELISPOT assay is a measure of the frequency of antigen-primed T cells among those plated and, because such T cells are rare in un-immunized donors, it is often negative. The indirect ELISPOT assay addresses not the frequency but the ability and efficiency to generate antigen-specific effector T cells from the host's naïve T cells upon IVS. Because IVS amplifies the frequency of such effector cells, indirect ELISPOT is often positive when direct ELISPOT is not. Hence, there is a natural tendency of investigators to resort to IVS and by-pass the direct ELISPOT. While this is reasonable, the results of such indirect assays cannot be directly correlated to *in vivo* immune responses and must be interpreted accordingly (5).

Whether the one-step or two-step ELISPOT assay is used, a positive response is defined in terms of the relationship between the numbers of spot-forming cells in experimental wells versus those appearing in corresponding background wells. Although, at present, there is no uniform definition of positive ELISPOT responses, a number of conventions are in widespread use. The most common of these uses the criteria of two- or three- fold increases in spot numbers above the background as a threshold for response positivity. Each testing laboratory must establish its own criteria, based on validation data derived within that laboratory. If, for example, the laboratory is expected to use an ELISPOT assay specifically formatted to measure the cellular response to a candidate peptide-based anti-tumor vaccine administered to patients with cancer, a part of the assay validation procedure must include testing of responses in PBMC obtained from 20 to 30 healthy, non immunized volunteers in order to establish the level of background 'noise' in the specific assay. According to the results obtained, the laboratory can then modify the criteria for a definition of the positive response

7. PROBLEMS AND SOLUTIONS

The ELISPOT assay provides a sensitive means of detection and enumeration of antigen-specific T cells in freshly harvested or cryopreserved PBMC or other lymphocytes and in IVS cultures. However, the following problems may arise limiting the assay utility:

- Specimen quality. The specimens have to be collected, transported, processed and stored under conditions established to assure that cellular functions are not compromised.
- Sample processing. A loss of precursor T cells during processing, cell separation or washing steps will negatively affect the assay results (i.e., give "false negative" results).
- Sample cryopreservation. When using cryopreserved and thawed PBMC, the viability must be 80% and the recovery should not be less than 75%.
- Quality of the capture and detection Abs. This is an Ab-based assay, and selection of antibodies determines its
- Number of plated cells/well. "Crowding" of cells in wells will compromise spot detection and enumeration. Plating of increasing numbers of responder cells/well should result in a linear increase of spots/well.

- Cell delivery. The numbers of total responder cells/well should be identical in triplicate or quadruple wells and should not exceed 2 x 10^5/well.
- Type of APC. The selection of APC is especially important when proteins as opposed to peptides are used for stimulation.
- Antigen/peptide concentration. Insufficient concentration will underestimate the response. An excess of antigen could induce unresponsiveness.
- Ratio of stimulator to responder cells. This is a critical aspect of the assay, that has to be pre-determined for every APC-responder cell system.
- Duration of the assay. While an 18- to 24-hour assay is optimal for IFN-γ, the IL-5 ELISPOT requires 48 hours. Optimal performance has to be pre-determined for every cytokine/cell product measured.
- Color development. It is necessary to optimize color development. Overdeveloped or underdeveloped color will interfere with the spot counts.
- Background. High number of background spots makes the assay uninterpretable. Background levels may vary considerably, but should not exceed 50 spot-forming cells per 10^6 responders. If high backgrounds persist, reagent quality or contaminations of reagents need to be carefully checked. Also, responder lymphocytes obtained from some patients with cancer might spontaneously release cytokines and give high background levels.
- Low frequency of precursor cells in the sample. When the frequency of antigen-responsive T cells is lower than 1/50,000, results of one-step ELISPOT may not be reliable. Amplification of the response may be necessary, using *in vitro* sensitization (IVS).
- Phenotypic identity of responder cells. In ELISPOT assays performed with PBMC, the identity of responder cells is not certain. However, it is possible to isolate CD4+ or CD8+ T cells or remove natural killer cells using immunobeads prior to ELISPOT assays. An ELISPOT assay combining cytokine and the responder phenotype detection has also been introduced (9).

8. CONTROLS FOR ELISPOT ASSAYS

Two types of controls are necessary for adequate result interpretation: (a) assay controls and (b) controls for functional integrity of responder cells. To ensure that ELISPOT is in control, a response of normal donor cells to a polyclonal activator such as PMA/ionomycin should be measured. An

alternative is to use a donor with documented T-cell reactivity against a defined antigen such as an MHC class I-restricted CMV peptide. In either case, by cryopreservation of multiple PBMC aliquots obtained from such a donor, respnsiveness of control cells can be assessed in each assay.

Negative and positive controls are obligatory for evaluation of functional integrity of responder cells. The use of PMA/ionomycin or OKT3Ab as stimulators of responder cells obtained from each tested subject defines their capability to secrete a cytokine under the assay conditions. This is an important control, because in patients with cancer, responder T cells may be functionally compromised and, therefore, unresponsive or only weakly responsive to polyclonal activators. Negative controls consist of responder cells simulated with a scrambled (nonsense) peptide or an irrelevant antigen presented in the same way as a relevant epitope.

9. CONCLUSION

The ELISPOT assay has evolved into a useful and broadly applicable monitoring tool for serial assessments of immune responses in patients receiving antitumor vaccines. When optimally performed, the ELISPOT assay is robust and reproducible. It lends itself well to a large-scale testing of samples obtained in the course of clinical research protocols. Its various formats offer an opportunity for assessment of CD8+ and/or CD4+ T-cell responses to peptides, antigens or even whole tumor cells or their lysates. The assay can be formatted to accommodate different APC (e.g., autologous PBMC, DC or MHC class I- or class II-restricted cell lines). Flexibility in the assay formating allows for exploration of various aspects of the immune response to vaccinating or tumor-associated antigens (i.e., priming *in vitro* using IVS, culture and ELISPOT vs. measuring the *in vivo* frequency of antigen-responsive T cells). At the same time, the assay is technically demanding, and its performance under carefully controlled conditions is necessary for meaningful result interpretation.

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Chapter 8

MODIFIED ELISPOT

Modifications of the Elispot Assay for T Cell Monitoring in Cancer Vaccine Trials

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Abstract: The use of the IFN-γ ELISPOT assay to evaluate cellular immune responses has gained increasing popularity, especially as a surrogate measure for CTL responses. We developed and validated some modifications of the IFN-γ ELISPOT assay to optimize immunological monitoring of various cancer vaccine trials. Taking into consideration that the main mechanism of cell-mediated cytotoxicity is the release of cytolytic granules that contain, among others, cytolytic protein Granzyme B (GrB), we developed the GrB ELISPOT assay. Extensive studies demonstrated that the GrB ELISPOT assay is specific, accurately measures the rapid release of GrB, is more sensitive than the 51Cr-release assay, and that it may be successfully applied to measuring CTL precursory frequency in PBMC from cancer patients. Assuming that immunological assays that demonstrate recognition of native tumor cells (tumor-specific) may be more clinically relevant than assays that demonstrate recognition of tumor protein or peptide (antigen-specific), we developed and validated the Autologous Tumor IFN-γ ELISPOT assay using PBMC from idiotype vaccinated lymphoma patients as effectors and autologous B cell lymphoma tumor cells as targets. The precursor frequency of tumor-reactive T cells was significantly higher in the postvaccine PBMC, compared with prevaccine samples in all patients tested. Furthermore, the specificity of these T cells was established by the lack of reactivity against autologous normal B cells. These results demonstrate the feasibility of evaluating tumor-specific T cell responses when autologous, primary tumor cells are available as targets. Modifications of ELISPOT assay described in this chapter allow more comprehensive assessment of low frequency tumor-specific CTL and their specific effector functions and can provide valuable insight with regards to immune responses in cancer vaccine trials.

Key words: granzyme B, ELISPOT

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1. INTRODUCTION

Active specific immunotherapy is a promising but investigational modality in the management of cancer patients. Currently, several different cancer vaccine formulations such as peptides, proteins, antigen-pulsed dendritic cells, whole tumor cells, etc. in combination with various adjuvants and carriers are being evaluated in clinical trials (1-3). To determine the optimal cancer vaccine strategy, a surrogate immunological end-point that correlates with clinical outcome needs to be defined, since it would facilitate the rapid comparison of these various formulations. Traditional immunological assays such as ELISA, proliferation and cytotoxicity assays can detect immune responses in vaccinated patients but are not quantitative. In contrast, novel assays such as enzyme-linked immunospot (ELISPOT) assay, intracellular cytokine assay and tetramer assay can quantitate the frequency of antigen-specific T cells. Of these, the ELISPOT assay has the lowest detection limit with $1/10^5$ peripheral blood mononuclear cells (PBMC) and has been determined to be one of the most useful assays to evaluate immune response to cancer vaccines (4). However, the IFN-γ ELISPOT assay is not an exclusive measure of cytotoxic T-lymphocyte (CTL) activity as non-cytotoxic cells can also secrete IFN-γ. Additionally, CTL with lytic activity do not always secrete IFN-γ (5). A more relevant approach to assess functional activity of cytotoxic lymphocytes would be to measure the secretion of molecules that are associated with lytic activity.

One of the major mechanisms of cell-mediated cytotoxicity involves exocytosis of cytoplasmic granules from the effector toward the target cell. The granules contain a number of proteins, including the pore-forming protein perforin and a family of serine proteases called granzymes, including Granzyme B (GrB). Granzyme B is present mainly in the granules of $CD8^+$ CTL and natural killer (NK) cells (6,7) and may be involved in the lethal hit that kills virus-infected and tumorigeneic cells (8-11). Therefore, the release of Granzyme B in response to the appropriate target may be used to evaluate cell-mediated cytotoxicity by specific antitumor CTL generated by vaccination.

The GrB ELISPOT assay was previously shown to measure GrB release by GrB transfected CHO cells, T-cell lines and PBMC from patients with AIDS (12). In our study we demonstrated that the GrB ELISPOT assay could accurately detect GrB secretion by CTL in different model systems and that this assay correlated with the ^{51}Cr-release assay (13). We also compared the GrB ELISPOT assay with the IFN-γ ELISPOT assay that is routinely used as a surrogate indicator of CTL precursor frequency in immunological monitoring.

The desired outcome of cancer vaccination is to induce a potent T cell response which can specifically recognize and eliminate autologous tumor cells in *vivo*. Therefore an immunological assay that demonstrates recognition of native tumor (tumor-specific) may be a more clinically relevant assay to assess T cell responses following cancer vaccination, compared with assays that demonstrate recognition of tumor protein or peptide presented on appropriate antigen-presenting cells (antigen-specific).

Standard IFN-γ ELISPOT assay has been primarily used for the detection of T cell responses against vaccine components by using peptide or protein pulsed antigen-presenting cells as surrogate T-cell targets (14-16). Here, a modified IFN-γ ELISPOT assay for the direct quantitation of T cell responses against autologous primary tumor cells is described (17). To develop the assay, follicular lymphoma patients vaccinated with tumor-derived idiotype (Id) protein were used as a model, since we have previously shown that Id vaccination induces tumor-specific T cell responses in these patients (18).

2. GRANZYME B ELISPOT ASSAY

2.1 Material and Methods

2.1.1 Target cell lines

K562 (Human myelogenous leukemia cell line, ATCC, Manassas, VA) was cultured in complete medium (CM) consisting of RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 2mM glutamine, 1mM pyruvate, 100 U/ml Penicillin and 100 µg/ml Streptomycin (Gibco BRL Life Technologies, Grand Island, NY). The C1R.A2 human plasma leukemia cell line which expresses a transfected genomic clone of HLA-A2.1 was cultured in CM supplemented with 500 µg/ml of G418 (Invitrogen, Carlsbad, CA). The T2 human lymphoblastoma Tap-deficient cell line (ATCC, Manassa, VA) was cultured in IMDM medium (BioWhittaker, Walkersville, MD) supplemented with 20% FBS (Hyclone), 100 U/ml Penicillin and 100 µg/ml Streptomycin. All target cell lines were grown at 37°C, 5% CO_2. Prior to use in assays, C1R.A2 or T2 target cells were pulsed with HLA-A2 binding peptides for 2 h at 37°C, 5% CO_2. Flu-Matrix peptide (FMP, NH_2-G-I-L-G-F-V-F-T-L-COOH, American Peptide Company, Inc., Sunnyvale, CA) and melanoma common tumor antigen MART-1 (NH_2-A-A-G-I-G-I-L-T-V-COOH), kindly provided by

Dr. Jay Berzofsky, NIH, Bethesda, MD) were utilized as relevant and irrelevant HLA-A2 binding peptides, respectively.

2.1.2 Preparation of human PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from venous blood of normal human volunteers by buoyant density centrifugation over Ficoll-Paque (Pharmacia, Piscataway, NJ). Aliquots of effector cells were cryopreserved in the vapor phase of liquid nitrogen for future use in functional testing and flow cytometric analysis. PBMC were cryopreserved in RPMI 1640 (BioWhittaker) supplemented with 10% FBS (Hyclone), 2 mM L-glutamine, 25 mM HEPES (Gibco) and 7.5% DMSO (Fisher Chemical, Fair Lawn, NJ). Samples of cryopreserved PBMC from cancer patients were kindly provided by Dr. Steven Rosenberg (NIH, Bethesda, MD).

2.1.3 Generation of human anti-flu-matrix peptide (αFMP) CTL and $CD8^+$ cells depletion

PBMC from normal donors were screened for HLA-A2 expression by flow cytometry using direct staining with anti-HLA-A2 FITC-conjugated monoclonal antibody (One Lambda Inc., Canoga Park, CA). PBMC ($3x10^6$) were resuspended in 2 ml of CM supplemented with 1000 U/ml of IL-7 (Peprotech, Rocky Hill, NJ) and seeded in 24 well plates (Costar, Corning, NY). FMP was added to the PBMC at a final concentration of 5 μg/ml. The CTL were grown at 37°C, 12% CO_2. At day 2 and day 5, 20 IU/ml of IL-2 (Hoffmann-LaRoche, Basel, Switzerland) were added. Cells were assayed at day 7 of culture.

Depletion of $CD8^+$ cells from CTL cultures was performed as previously described (19). Briefly, $2x10^7$ PBMC were incubated for 40 min on ice with 100 μl anti-CD8 (Becton Dickinson Immunocytometry Systems, San Jose, CA), washed and mixed with magnetic beads coated with sheep anti-murine IgG (Dynabeads, Dynal A.S., Norway), at a ratio of 1 lymphocyte to 30 beads. Following 30 min incubation at 4°C with occasional gentle rotation, positive cells were removed by magnetic selection. The depleted population contained <3% $CD8^+$ cells as measured by flow cytometry using anti-CD8 mAb (Becton Dickinson).

2.1.4 ^{51}Cr-release cytotoxicity assay

Cytotoxicity of effector cells was assessed using the standard ^{51}Cr-release assay. Briefly, one million T2 target cells were labeled at 37 °C for 1 h with

100 µCi $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Ma). Target cells were washed and resuspended in CM at $5x10^4$ cells/ml. Five thousand target cells per well (100 µl) were added to a 96 well plate (Costar, Cambridge, MA) following the appropriate number of effector cells (100 µl/well). The defined effector to target (E:T) ratios were plated in triplicate. Cytotoxicity assays were performed at 37 °C for 4 h. After incubation, cell-free supernatants were collected using a Skatron harvester and analyzed in a gamma counter (LKB-Wallac CliniGamma 1272, Wallac, Finland). Percent specific lysis was calculated using the following equation:

(ER – SR)/(MR – SR) x 100,

where ER = experimental release, SR = spontaneous release and MR = maximum release.

2.1.5 Granzyme B ELISPOT assay

MultiScreen-IP plates (PVDF membrane, Millipore, Bedford MA) were coated overnight at 4°C with 100 µl/well of anti-human GrB capture antibody (clone GB-10, PeliCluster, Cell Sciences, Norwood MA) diluted to 7.5 µg/ml in PBS. After incubation, coated plates were washed 4 times with PBS and blocked with 200 µl/well of assay medium consisting of RPMI-1640 (BioWhittaler), 10% FBS (Hyclone), 2 mM L-glutamine, 20 mM HEPES, 0.1 mM NEAA, 1 mM sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco) for 2 h at 37°C, 5% CO_2. Targets (C1R.A2) were pulsed with 5 µg/ml FMP or 3 µM MART-1 peptide for 2 h at 37°C prior to use in assay. Unpulsed C1R.A2 and C1R.A2 pulsed with MART-1 peptide as well as K562 cells served as controls. Effector cells (100 µl/well) were added to triplicate wells at specified concentrations followed by $5x10^4$ target cells per well (100 µl) for 4 h at 37^0C in 5%CO_2. Negative controls consisted of effector cells in the absence of target cells, target cells in the absence of effector cells and medium only.

After the incubation, the plates were washed 6 times with PBS/0.05% Tween 20 and 100 µl/well of biotinylated anti-human GrB detecting antibody (clone GB-11, PeliCluster, Cell Sciences) diluted to 0.25 µg/ml in PBS/1% BSA/0.05% Tween 20 was added. Plates were incubated for 3 h at room temperature, washed 4 times with PBS and 50 µl of Streptavidin-Alkaline Phosphatase (Gibco BRL Life Technologies) diluted 1:1500 in PBS/1% BSA was added. After 1 h incubation at room temperature, the plates were washed 4 times with PBS and the spots visualized with 100 µl/well of filtered BCIP-NBT phosphatase substrate (KPL, Gaithersburg, MD). Plates were developed for 30 min at room temperature in the dark and the reaction stopped by rinsing plates with distilled water. The membranes were air-dried and spots were subjected to automated evaluation using the

ImmunoSpot Imaging Analyzer system (Cellular Technology Ltd, Cleveland, OH).

2.1.6 IFN-γ ELISPOT assay

MultiScreen-IP plates (PVDF membranes, Millipore) were coated overnight at room temperature with 50 µ/well of anti-human IFN-γ capture antibody (Biosource, International, Camarillo CA) diluted at 20 µg/ml in PBS. After the incubation, coated plates were washed and blocked as stated above. Effector cells (100 µl/well) were added to triplicate wells at specified concentrations followed by $5x10^4$ target cells per well (100 µl). After effector and target cells were incubated for 4 h at 37°C, the plates were washed with PBS/0.05% Tween 20 and 50 µl/well of biotinylated anti-human IFN-γ detecting antibody (PharMingen, San Jose, CA) diluted to 1.3 µg/ml in PBS/1% BSA/0.05% Tween 20 was added. Plates were incubated with detecting antibody for 2 h at room temperature, washed 4 times with PBS and 50 µl of Streptavidin-Alkaline Phosphatase (Gibco BRL Life Technologies) diluted 1:1500 in PBS1% BSA was added. After 1h incubation at room temperature, the plates were washed and the spots visualized and enumerated as stated above.

2.1.7 Statistical analysis

Statistical analysis was performed using Pearson correlation coefficient (R^2).

2.2 Results and Discussion

Granzyme B is a potential candidate molecule for measuring tumor specific T-cell responses via the ELISPOT method. This molecule is present in CTL and NK cells and is constitutively expressed in memory but not naïve CTL (20-22). Upon effector-target interactions, GrB is rapidly released by cytolytic lymphocytes in a calcium dependent manner and therefore may be used to assess cell-mediated cytotoxicity.

Unlike the IFN-γ ELISPOT which is widely utilized, the application of the GrB ELISPOT has been limited (12). Our laboratory has optimized the GrB ELISPOT assay for various cytolytic cells including αEN-EBV and αJY CTL cell lines and have shown that the GrB ELISPOT assay is capable of measuring MHC restricted cytolytic activity quantifying frequency of specific effector cells (13). Optimal concentration of effector cells ranged between 10^4 and 10^3 cells per well based on several experiments (13).

Since the GrB ELISPOT assay can accurately measure the specific secretion of GrB by CTL cell lines, we generated αFMP-CTL as a more clinically relevant model system to assess whether the GrB ELISPOT assay can reliably detect effector cell responses to specific peptides. We tested CTL reactivity against FMP-pulsed C1R.A2 (specific targets) as well as non-pulsed and MART-1 pulsed C1R.A2 cells (non-specific targets) in the GrB ELISPOT assay (Figure 1). K562 were utilized as a control for NK activity (data not shown). Granzyme B secretion was antigen specific as only wells with CTL and FMP-pulsed CIR.A2 contained a substantial number of spots.

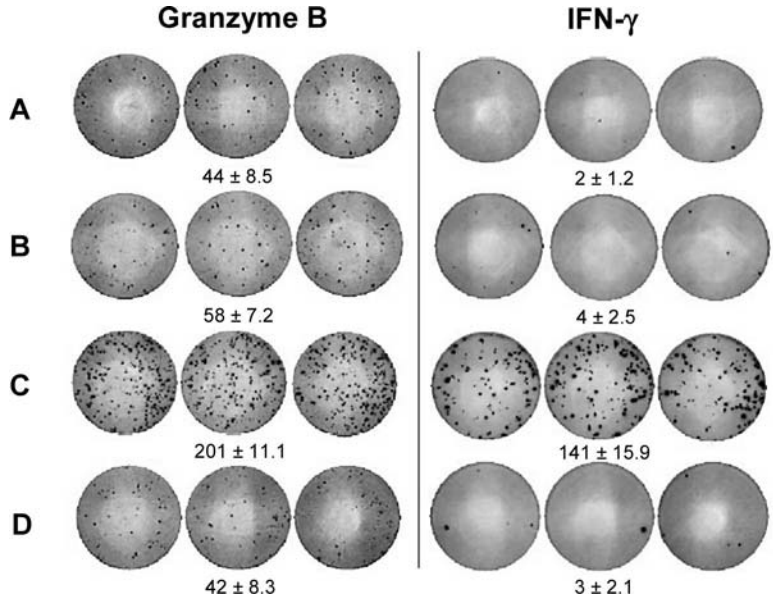

Figure 1. Specificity of GrB and IFN-γ secretion by αFMP-CTL in the ELISPOT assays. Human αFMP-CTL (7 day culture, 5x103 cells per well) were run alone (A) or against various target cells (5x104 cells per well): C1R.A2 (B), C1R.A2 pulsed with 5 μg/ml FMP (C) or C1R.A2 pulsed with 3 μM MART-1 (D). Effector and target cells were incubated for 4 h at 37°C. Image from the plate scan generated by the CTL Analyzer is shown. Data is presented as spots per well ± SD and is representative of 3 experiments (13).

When the GrB ELISPOT assays were performed with a constant number of target cells but varying numbers of effectors, a strong correlation between the number of effector cells and spots per well was observed (R^2=0.9642, Figure 2).

Cell-mediated cytotoxicity has conventionally been measured using the standard ^{51}Cr-release assay (22), which assesses CTL and NK cell functions

via lysis of radioisotope-labeled target cells. This assay is considered to be the gold standard to evaluate CTL lytic activity. Recently, the IFN-γ ELISPOT assay has become a surrogate measure of CTL responses. We compared the GrB ELISPOT assay to the ^{51}Cr-release and IFN-γ ELISPOT assays to evaluate its ability to quantify CTL lytic responses (Figure 2). Both ELISPOT assays were significantly more sensitive than the ^{51}Cr-release assay. At effector to target ratios of 50:1-12:1, specific lysis was measurable via the ^{51}Cr-release assay, but the spots per well in the ELISPOT assays were too numerous to count accurately (data not shown). Significant IFN-γ and GrB secretion was evident even at E:T ratios as low as 0.2:1, below the level of sensitivity of the ^{51}Cr-release assay. However, when the optimal number of CTL are used in each individual assay, the amount of GrB and IFN-γ secreting cells in the ELISPOT assays and cytotoxicity in the ^{51}Cr-release assay have shown excellent cross-correlation with R^2 above 0.95 for all three assays.

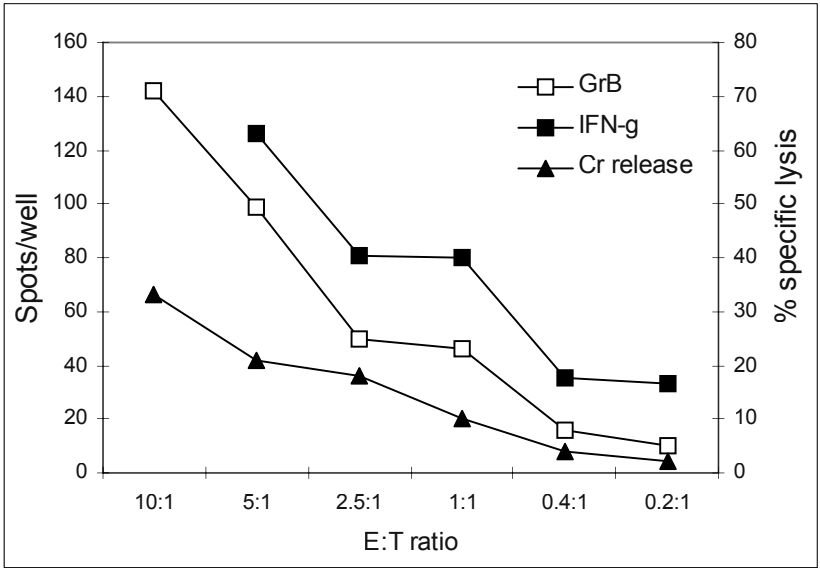

Figure 2. Correlation of GrB and IFN-γ secretion in the ELISPOT assays with cytotoxicity in 51Cr-release assay. Human α-FMP-CTL (7 day culture) were used as effector cells. Target cells were C1R.A2 (ELISPOT) or T2 (51Cr-release) pulsed with FMP, 5x103 cells per well. Incubation time was 4 h at 37°C for all three assays. At the 10:1 effector to target ratio the spots in the IFN-γ ELISPOT assay were too numerous to count. Background (CTL alone) was subtracted from the results. Data is representative of 3 experiments. Modified from (13)

To further confirm that we were measuring CTL activity, we removed $CD8^+$ cells from the cultures using anti-CD8 mAb and magnetic beads. After

depletion, the percentage of $CD8^+$ cells in the cultures was decreased from 24.8±2.9% to 2.7±0.4% and an abrogation of GrB secretion was observed (Figure 3). These results show that at least in this system GrB is secreted by $CD8^+$ cells.

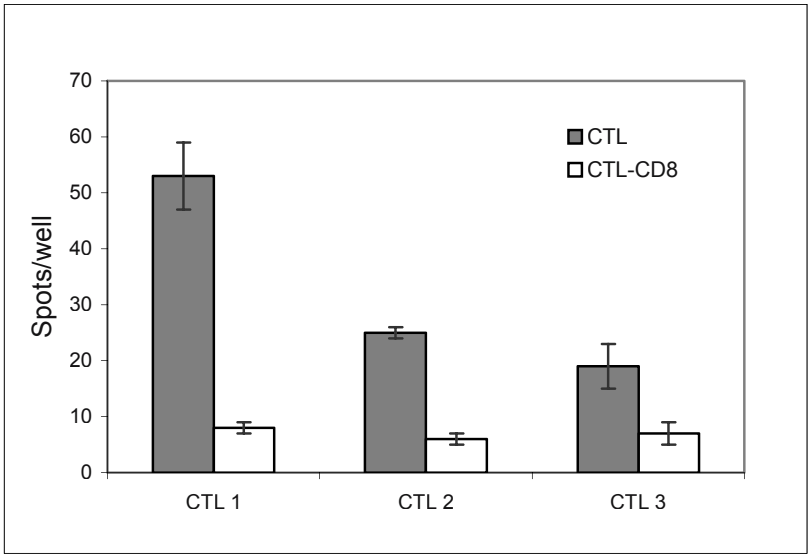

Figure 3. Effector cells secreting GrB in the ELISPOT assay. Human αFMP-CTL (7 day culture) before and afterdepletion of CD8+ cells were used as effector cells (5x103 cells per well). Target cells were C1R.A2 pulsed with FMP (5x104 cells per well). CD8+ cells were removed from effector cell cultures using anti-CD8 mAb and magnetic beads. Effector and target cells were incubated for 4 h at 37°C. Data is representative of 3 experiments. Modified from (13).

The dynamics of GrB and IFN-γ secretion differ. Granzyme B secretion was detectible as early as 10 min after initial contact of effectors and targets in most experiments. Significant amounts of GrB spots were observed within 30 min of incubation with maximal secretion at 4 h. In contrast, measurable amounts of IFN-γ spots were found only after 1 h of incubation with significant IFN-γ secretion measured at 4 h (13).

The difference in the observed pattern of GrB and IFN-γ secretion parallels the well-defined dynamics of CTL effector functions. When CTL interact with target cells, IFN-γ. is secreted within hours whereas GrB is released very rapidly (within minutes) from preformed granules. Using effector cells with NK activity, we have shown that treatment with Brefeldin A, a protein secretion blocker, significantly decreases IFN-γ but not GrB secretion. In contrast, BAPTA-AM, which sequesters intra-cellular calcium

and therefore inhibits degranulation, abrogated GrB secretion (manuscript in preparation). The fact that the number of effectors spontaneously secreting GrB is higher than the number secreting IFN-γ (Figure 1), suggests that the GrB measured in the ELISPOT could be present in preformed granules. However, the spontaneous release of GrB could also be due to the activation state of cytotoxic cells. Granzyme B is consistently expressed in activated cytolytic cells, especially $CD8^+$ CTL (20,21).

Although all three assays correlated, there are numerous advantages to utilizing the ELISPOT assays over the standard ^{51}Cr-release assay. The ELISPOT assays use a lower number of effector cells to accurately assess activity. The high sensitivity and specificity of the ELISPOT assays are beneficial for monitoring clinical trials where frequently there are limited numbers of patients' cells available. The ELISPOT assays also enumerate antigen specific lymphocyte frequency by measuring secretion of a specific immune protein. As such, the ELISPOT assays are both qualitative and quantitative. Additionally, the problems associated with the labeling efficiency of targets and the isotope use are not a concern with the ELISPOT assays.

Therefore, both the GrB and IFN-γ ELISPOT assays are superior alternatives to the ^{51}Cr-release assay to test CTL response. However, when compared with the IFN-γ ELISPOT, the GrB ELISPOT assay is more rapid and may be a more direct measure of antigen specific CTL lytic activity.

While IFN-γ ELISPOT is widely used for cancer vaccine trials monitoring, we did not find any information on GrB ELISPOT clinical use except for testing AIDS patients (12). Our preliminary research using PBMC samples from cancer patients suggests that the GrB ELISPOT assay may be successfully applied to evaluate CTL precursor frequency. We have shown that the results obtained from the GrB ELISPOT and the standard ^{51}Cr-release assays tended to correlate (R^2=0.9927, Figure 4).

The role of perforin in immune surveillance and rejection of tumors has been well established (22), but the role of GrB is still controversial. A recent study demonstrated that GrB is not critical for CTL perforin-mediated rejection of spontaneous or experimental tumors in mice (23). These findings are in contrast to previous studies that have suggested that GrB plays a critical role in controlling tumors *in vivo* (24-26). Regardless of the role of GrB in cell-mediated killing, GrB expression is restricted to CTL and NK cells and therefore the release of GrB is a more specific measure of cytotoxic lymphocytes than IFN-γ (20-22). As such, simultaneous use of the IFN-γ and GrB ELISPOT assays may provide important immunological insight into patient responses to cancer vaccines that may then be directly assessed against clinical outcome.

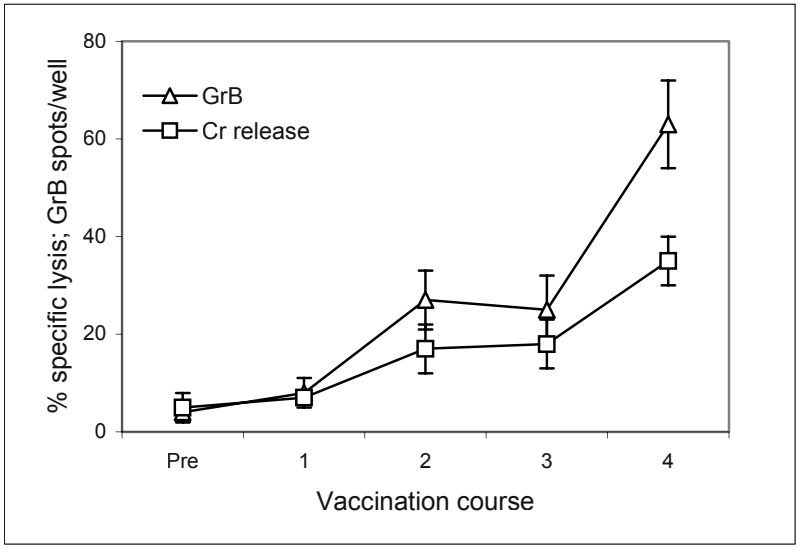

*Figure 4.*Correlation of Granzyme B release by PBMC from vaccinated patients in the ELISPOT assay and cytotoxicity in the 51Cr-release assay. PBMC from melanoma patients immunized with gp100:209-2M peptide were tested pre- and at different time points post vaccination against gp100:209 peptide. No reactivity against control peptide gp100:280 was found. Average from 7 patients that showed response in 51Cr-release assay. All samples tested blinded. Samples were kindly provided by Dr. S. Rosenberg, NIH, Bethesda.

3. AUTOLOGOUS IFN-γ ELISPOT ASSAY

3.1 Material and Methods

3.1.1 Preparation of PBMC

Blood samples were obtained from patients with Stage III or IV follicular center cell lymphoma grade 1 or grade 2 at various time points before and after vaccination with the patient-specific tumor-derived Id protein incorporated into liposomes along with recombinant human IL-2 as described previously (27). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation with Ficoll Isopaque (ICN Biomedicals Inc., Aurora, OH) and cryopreserved for immunological assays. To perform the testing, pre- and postvaccine PBMC were thawed, washed

and resuspended to a concentration of 1-3x10^6 cells/ml in RPMI 1640 medium (Invitrogen Corp, Carlsbad, CA), supplemented with 5% FBS (Hyclone, Logan, Utah), 1mM sodium pyruvate (BioWhittaker, Walkersville, MD), 20 mM HEPES buffer (Invitrogen), 50 μM β-mercaptoethanol (Sigma, St. Louis, MO), 2 mM l-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Five ml/well of the cell suspensions were plated into 6 well plates (Corning, Inc. Corning, NY) and rested overnight at 37ºC, 5% CO_2. The next day, PBMC were harvested, washed and counted prior to use in the ELISPOT assay.

3.1.2 Activation of tumor cells and normal B cells

Cryopreserved cells from the lymph node biopsy specimen were enriched for tumor cells by depletion of T cells with CD3 microbeads over a magnetic column (Miltenyi Biotec, Auburn, CA) using the manufacturer's protocol. Autologous normal B cells were isolated from PBMC by magnetic cell separation method using the B Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. The purity of the isolated tumor and normal B cells was > 95%. Tumor cells and normal B cells were activated for 3 days with 800 ng/ml of recombinant human soluble CD40 ligand trimer (sCD40Lt, Amgen, Thousand Oaks, CA) and recombinant human IL-4 (2 ng/ml, Peprotech, Rocky Hill, NJ). Activated tumor cells and normal B cells were harvested and washed prior to co-culture with PBMC in the ELISPOT assay.

3.1.3 Autologous tumor IFN-γ ELISPOT assay

Several variables were evaluated to optimize the signal to noise ratio for the detection and quantitation of tumor-specific T cells. These variables included: type of microtiter plate, activation of tumor cells with sCD40Lt, freshly thawed versus overnight cultured PBMC, effector to target cell ratio, length of incubation time of the assay, culture medium, and finally, substrate development system (17). Optimizes protocol is presented below.

MultiScreen-IP opaque 96-well plates (High Protein Binding Immobilon-P membrane, Millipore, Bedford, MA) were coated overnight at room temperature with 50 μl/well of 20 μg/ml mouse anti-human IFN-γ mAb (BioSource, Camarillo, CA) in DPBS (Invitrogen). After overnight incubation, the plates were washed three times with 200 μl DPBS per well and blocked with 200 μl/well of RPMI 1640 supplemented with 10% human AB serum (Mediatech, Herndon, VA), 25 mM Hepes, 2 mM L-glutamin, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) for 2 h at 37ºC, 5% CO_2. Harvested effectors were added to the plates in triplicates at 10^5

cells/well with either culture medium alone or with $2x10^5$ cells/well sCD40Lt activated autologous tumor cells, and cultured for 48 h at 37ºC, 5% CO_2. The plates were washed manually 6 times with 200 µl/well of DPBS/0.05% Tween. 50 µl/well of 2 µg/ml mouse anti-human IFN-γ-biotinylated mAb (BD Pharmingen) in DPBS/1%BSA/0.05% Tween was added and the plates were incubated for 2 h at room temperature. Plates were manually washed 4 times with DPBS and 50 µl/well of streptavidin HRP (BD Pharmingen) diluted 1:2000 in DPBS/1% BSA was added for 1 h at room temperature. The plates were washed 4 times with DPBS. Spots were visualized by adding 100 µl/well of True Blue Peroxidase Substrate (KPL, Gaithersburg, MD) for 2 min. Plates were scanned and counted using the ImmunoSpot analyzer (Cellular Technology, Ltd., Cleveland, OH) to determine the number of spots/well. The precursor frequency of tumor-specific T cells was determined by subtracting the background spots in tumor alone and PBMC alone from the number of spots seen in response to tumor cells.

3.1.4 Statistical analysis

Statistical analysis was performed using Student's t-test for paired mean values and Pearson correlation coefficient (R^2).

3.2 Results and Discussion

Activation of B cell tumor cells with CD40L upregulated various costimulatory molecules and MHC class I and class II molecules on the surface of tumor cells. It is associated with enhanced antigen presenting capability (28). Our pilot experiments indicated that activation of tumor cells with sCD40Lt markedly enhanced the sensitivity of the ELISPOT assay by increasing the IFN-γ production by responding T cells as compared to non-activated tumor cells (17). We have therefore used sCD40Lt activated tumor cells as stimulators to evaluate T cell responses in this assay.

Triplicate wells demonstrating the IFN-γ spots produced by pre- and post-vaccine PBMC analyzed in parallel are shown in Figure 5. Significantly higher numbers of IFN-γ spots were detected in the postvaccine PBMC as compared to the prevaccine sample ($p<0.0001$, Figure 5&6).

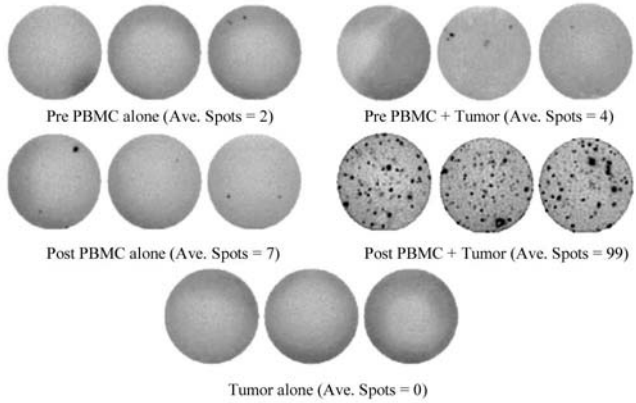

Figure 5. Representative wells of a tumor IFN-γELISPOT assay. Pre- and postvaccine PBMC (105 cells/well) were co-cultured with sCD40Lt activated autologous tumor cells (2×105 cells/well) for 48 h as described in Materials and Methods. Wells containing PBMC alone and tumor cells alone served as controls. Samples were tested blinded in triplicates. Image from the plate scan generated by the CTL Analyzer is shown. Average numbers of spots per well are indicated. Data is from a representative experiment of nine with similar results (17).

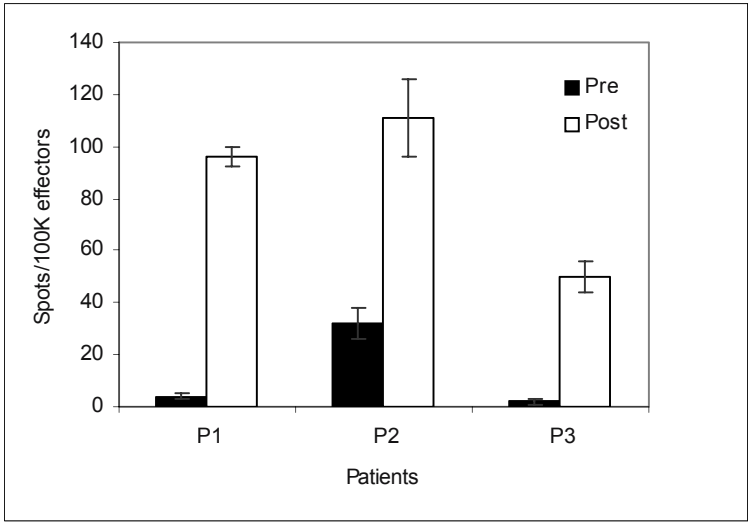

Figure 6. Cancer patients' response to vaccination. Pre- and postvaccine PBMC (105 cells/well) were co-cultured with sCD40Lt activated autologous tumor cells (2×105 cells/well) for 48 h as described in Materials and Methods. Samples were tested blinded in triplicates. Three patients (P 1-3) were tested three times each and average number of spots per 105 effectors ± SE shown. Modified from (17).

To test specificity of the T cell responses, postvaccine PBMC were co-cultured with either sCD40Lt activated autologous tumor cells or activated normal B cells in parallel. Significant number of IFN-γ spots were detected only in response to autologous tumor cells but not autologous normal B cells in all three patients that were tested ($p< 0.0001$, Figure 7).

Our data demonstrate that the ELISPOT assay could be adapted to reliably and reproducibly determine the precursor frequency of tumor-specific T cells in follicular lymphoma patients immunized with an autologous tumor-derived Id vaccine. We have also been able to use this assay to quantitate the tumor-specific T cell responses in mantle cell lymphoma patients vaccinated with Id (data not shown). Thus, this assay fulfilled our stated objectives for a clinically relevant immunological assay by demonstrating that it could functionally (IFN-γ production) and quantitatively assess the T cell responses induced by a cancer vaccine against autologous primary tumor cells.

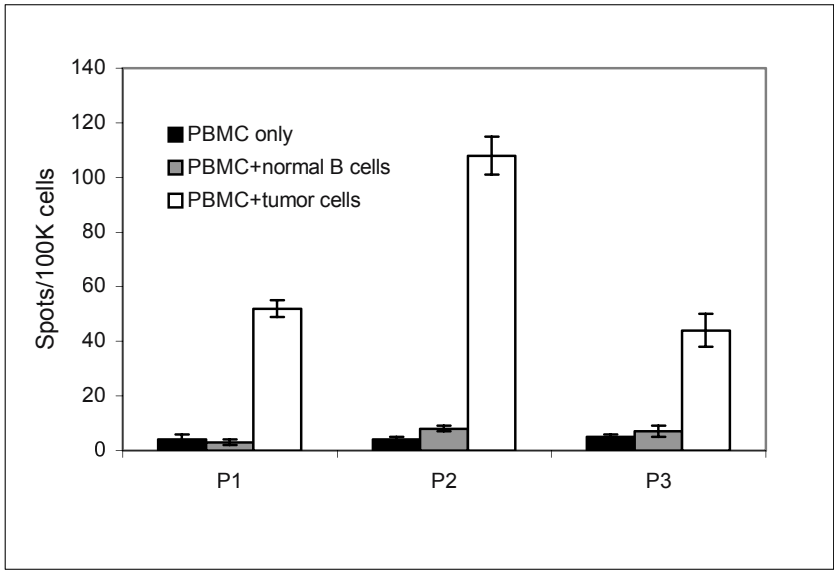

Figure 7. Specificity of the tumor-reactive T cell responses. Postvaccine PBMC samples from three patients (P 1-3) were co-cultured with either autologous sCD40Lt activated follicular lymphoma tumor cells (white columns) or activated normal B cells (gray columns) for 48 h. PBMC alone are shown in black columns. All samples were tested blinded. Average number of spots per 105 PBMC ± SE (n=3) shown (17).

As opposed to the traditional antigen-specific assays that assess immune responses against vaccine components (peptides or proteins), tumor-specific immunological assays in addition to being more clinically relevant can have

several advantages. Firstly, tumor-specific assays can potentially detect both $CD4^+$ and $CD8^+$ T cell responses since endogenous antigens are presented by both MHC class I and class II molecules (29). In contrast, antigen-specific (protein) assays detect mostly $CD4^+$ T cell responses since the soluble exogenous antigen is predominantly processed in the endosomal pathway and presented on MHC class II molecules. Secondly, tumor-specific assays can be used in patients with any HLA phenotype unlike peptide assays that are usually restricted to a single HLA phenotype (e.g. HLA-A*0201) depending on the binding affinity of the peptide. Thirdly, tumor-specific assays allow monitoring of patients when whole or lysed tumor cells are used as the immunogen and the tumor-specific antigens have not been defined.

A probable limitation for tumor recognition assays is the availability of tumor cells. While primary tumor cells are easily accessible for some cancers (e.g. lymphoma, leukemia, myeloma, melanoma), they may not be generally accessible for certain other cancers (e.g. breast cancer, renal cell cancer). When available, it may be feasible to adapt the IFN-γ ELISPOT assay for the quantitative assessment of T cell responses against primary tumor cells as a preferable alternative to antigen-specific assays.

4. CONCLUSIONS

We developed a modification of ELISPOT assay that measures GrB release from CTL. GrB ELISPOT assay is a superior alternative to the ^{51}Cr-release assay since it is significantly more sensitive and provides an estimation of cytotoxic effector cell frequency. Additionally, unlike the IFN-γ ELISPOT assay, the GrB ELISPOT directly measures the release of a cytolytic protein.

We also adapted the IFN-γ ELISPOT assay to directly measure immune responses against autologous primary tumor cells in vaccinated cancer patients. We demonstrated that the modified IFN-γ ELISPOT assay could be used to reliably and reproducibly determine the tumor-reactive T cell frequency in the PBMC of these patients.

Modifications of ELISPOT assay described in this chapter allow more comprehensive evaluation of low frequency tumor-specific CTL and their specific effector functions and can provide valuable insight with regards to immune responses in cancer vaccine trials.

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Chapter 9

INTRACELLULAR CYTOKINE STAINING

Cytokine flow cytometry for characterization of tumor-specific T cell responses

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Abstract: T cells play a central role in the defense against cancer. Assays to quantitate antigen-specific T cells have recently gained much interest especially in the field of tumor vaccination. The novel generation of sensitive T cell assays facilitates the direct *ex vivo* quantitation of specific T cell responses. Furthermore, various T cell functions can be detected by flow cytometry including the production of cytokines, proliferation, cytotoxic functions and migratory responses. The high sensitivity of modern flow cytometry allows characterizing antigen-specific T cells after stimulation with specific peptides or by combining functional flow cytometry with tetramer staining. This chapter focuses on the technique of intracellular cytokine staining for analysis of tumor-specific T cell responses.

Key words: functional cytokine staining, intracellular, interferon

1. T CELL CYTOKINE ASSAYS

Following stimulation with specific antigens, T cells are triggered to exert various functions including cytokine secretion, proliferation, migration, or cytotoxicity. The analysis of specific cytokine production is of central interest for characterizing specific T cell responses; it has been the most frequently used parameter to detect specific T cells so far. IFNγ is the cytokine most commonly analyzed to detect tumor-specific T cell responses. IFNγ production characterizes the so-called T1 subtype of T cells, including CD4+Th1 and CD8+Tc1 T cells (1). Differentiated Tc1 are typically those

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mediating cytotoxic function, while Th1 cells produce cytokines that stimulate the generation of cytotoxic T cells. T2 cells in contrast produce cytokines like IL-4, IL-5, and IL-13; and a shift of the tumor-reactive T cell repertoire to a T2-dominated phenotype was associated with tumor progression in animal models and cancer patients.

The detection of the cytokine production of a single T cell is possible with various assays including the ELISPOT assay (see chapter 7), the intracellular cytokine cytometry (ICC) and the cytokine secretion assay (CCSA, see chapter 10). Each of these assays employs the antigen-specific induction of cytokines to detect specific T cells (2-4). So far, most experience in quantitating T cell responses directly from peripheral blood has been made with the ELISPOT assay detecting the cytokines secreted by a single cell.

Considerable progress has been made recently to characterize and quantitate these T cell functions by flow cytometry-based assays. A great advantage of flow cytometry is the possibility of simultaneous phenotypic characterization of antigen-specific T cells by multicolor flow cytometry. There are two different functional approaches to detect cytokines produced by specific T cells by flow cytometry: Intracellular cytokine flow cytometry (ICC) and the so called secretion assay (4). As the majority of the cytokines produced are usually secreted it is necessary to "trap" the cytokine in order to detect it in or on the cell in flow cytometry. This can be achieved by addition of brefeldin A which blocks the secretory pathways of the cells and the cytokines are trapped intracellularly. To detect the cytokines cells are fixed and then permeabilized to allow specific anti-cytokine fluorescent antibody conjugates to pass into the cells (see Figure 1). The flow cytometric analysis of secreted IFNγ is also possible. Flow cytometric analysis of secreted IFNγ is based upon creation of an affinity matrix for the cytokine. The affinity matrix consists of Ab-Ab conjugates directed against CD45 and IFNγ to bind the secreted IFNγ on the cell surface. This relocated IFNγ can be stained with a fluorescein-conjugated specific anti-IFNγ Ab (5). The secretion assay seems to be less suited for accurate quantitation of antigen-specific T cells as the ICC due to higher background staining probably related to cytokine-trapping neighboring cells which do not produce the cytokine themselves (4). In this chapter, we focus on the analysis of specific T cells by ICC. As outlined above, IFNγ produced by Th1/Tc1 T cells is the cytokine most frequently used to track specific CD8+ T cell responses directly in peripheral blood T cells. In contrast IL-2, as well as the Th2/Tc2 cytokines IL-5 or IL-13, are produced by a much lower percentage of specific T cells often preventing their use for direct T cell analysis from peripheral blood.

When T-cell reactivity against single epitopes is identified *ex vivo*, the number of reactive T-cells is often quite low. The reliable detection of specific IFNγ -producing T cells by flow cytometry requires minimum T-cell frequencies of usually 0.05% specific T-cells in the CD3+CD8+ T-cell subpopulation, corresponding to approximately 50 - 100 specific T-cells per 1 Million total PBMC. The sensitivity of the assay is, however, dependent on the background from T cells spontaneously producing cytokines, and various procedures help to reduce background staining as outlined below. Alternatively, frequencies of antigen-specific T cells can be increased by short-term *in vitro* expansion using specific peptides, IL-2 and IL-7 (7).

2. METHODOLOGY OF INTRACELLULAR CYTOKINE STAINING

There are a several methodological variations of the ICC assay described (2, 8-11). T cells are typically stimulated with peptide epitopes for 6 to 18 hours. We usually stimulate cells with 10µg/ml of the peptide and use an irrelevant peptide as negative control, e.g., HIV in HIV-seronegative subjects, binding to the corresponding HLA-A allele. PBMC stimulated with 1µg/ml pokeweed mitogen can be used as positive control. After two hours a secretion inhibitor, mostly, brefeldin A (Sigma, Steinheim, Germany) is added for the rest of the incubation time to block the secretory pathways and to trap the cytokine intracellularly. First, extracellular antigens like CD3, CD4, CD8 are stained before cells are fixed and permeabilized for intracellular staining with anti-cytokine fluorescent antibody conjugates to detect the cytokines accumulated within the cell. Variations of the assay include the type of antigen-presenting cell (e.g. peripheral blood monocytes, dendritic cells, or tumor cell lines), and different types of cytokines analyzed. A detailed protocol used in our laboratory for the direct T cell analysis from PBMC using IFNγ is published in ref. 6. Various anti-cytokine antibodies are now available commercially. Each antibody should be titrated to determine the optimal concentration in the assay using a positive and a negative control as described below. Most frequently T cell responses are analyzed in PBMC, but also whole blood assays can be performed (2). If T cells are cyropreserved prior to analysis optimal freezing and thawing procedures are crucial for functional assays. For freezing it is recommended to use freezing containers filled with isopropanol. The advantage of freezing cells is that serial samples can be monitored in one assay, which is recommended if T cell responses during vaccination are analyzed. To reduce the background of T cells secreting IFNγ in the absence of antigen stimulation, various steps are important. These include the overnight resting of T cells after thawing, the use of non-stimulating human serum, and the

rapid and sterile preparation of cells for the assay. Activated T cells and especially those from cancer patients are highly susceptible to apoptosis. Therefore it is important to handle cells with great care and to process cells as rapid as possible.

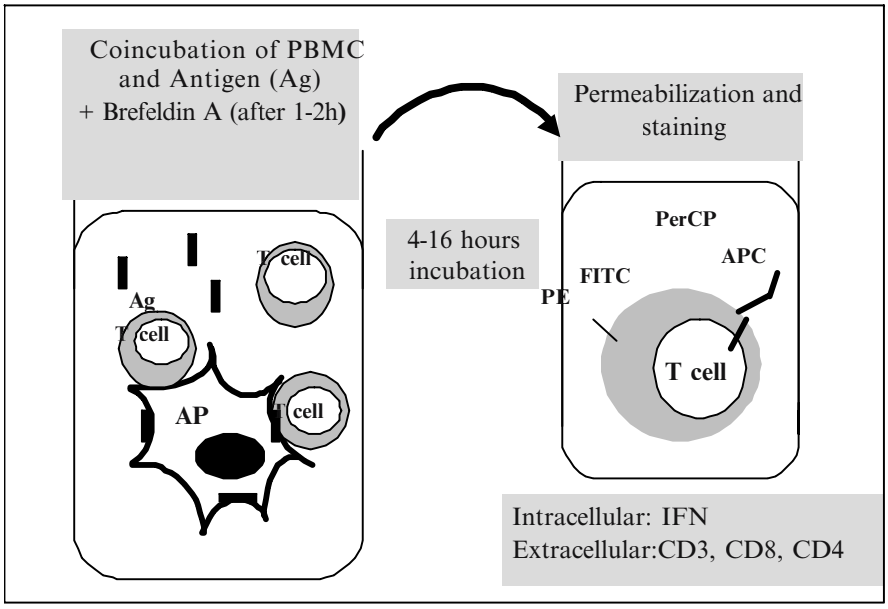

Figure 1. The principle of the ICC for detection of cytokine-producing T cells. PBMC are incubated with peptide for 8–16 hours. To avoid secretion of cytokines brefeldin A is added after 1 hour to block the secretory pathways resulting in cytokine accumulation within the cell. After fixation and permeabilization specific anti-cytokine fluorescent antibody conjugates are used to stain the intracellularly fixed cytokines. Additional markers are stained intracellularly or extracellulary using various fluorochrome dyes (FITC, PE, APC, PerCP).

Positive controls should be included to control each sample as well as the whole assay. As a positive control for the T-cell's abiltiy to produce cytokines, mitogen-stimulation is useful. We use pokeweed mitogen, which stimulates IFN-γ release in a subset of approximately 1% of CD4+ and CD8+ T-cells. Impairment of pokeweed mitogen-induced IFN-γ release usually indicates that cells have been damaged during the freezing and thawing procedure. Alternatively, the memory T cell responses to viral epitopes can be assessed as a sample control (6). An irrelevant peptide epitope like HIV should be included as a negative control. Each of these controls is, however, suboptimal, as T cells reactive to tumor antigens frequently have an effector phenotype containing high levels of granzyme B

and perforin (12) and are therefore more susceptible to undergo apoptosis than a memory T cell response to a viral antigen.

The advantage of the ICC is the possibility of simultaneous phenotypic characterization of the antigen-specific T cells by multicolor flow cytometry. For multicolor analysis a dual- or a multi-laser flow cytometry system should be available, which allows to analyze at least four colors simultaneously. Besides the identification of T cells and T cell subsets by costaining with CD3, CD8 or CD4, the staining for various other surface antigens is of great interest. Several characteristics of specific T cells are important for their ability to migrate to and kill tumor cells, and to mediate long-term protection. According to recently proposed classifications the markers CD45RA and CCR7 or CD27 and CD28 allow dividing specific T cells into naive, memory and effector T cell subsets (see below). Cytotoxic markers like GranzymeB or perforin reflect the cytotoxic potential of a cell. Finally, the functional analysis of chemokine receptor expression on specific T cells may allow evaluating their capacity to migrate to specific anatomical compartments.

3. CLINICAL APPLICATION IN CANCER PATIENTS

3.1 Spontaneous tumor immunity

It has long been a matter of debate whether tumors are spontaneously immunogenic in patients. With the availability of sensitive methods, spontaneously occurring T cells directed against tumor associated antigens (TAA) can be detected in cancer patients. There is increasing evidence that CD8+ T cells directed against TAA spontaneously occur in various malignancies including melanoma, adenocarcinomas, and leukemias (reviewed in 13). Mechanisms leading to spontaneous induction of specific T cell responses are not well understood. Whether circulating TAA-specific T cells are able to kill tumor cells *in vivo* and how they influence the clinical course of disease has remained largely unclear. Further studies are necessary for a better understanding of the role of spontaneous T cell responses against TAA.

3.2 Monitoring vaccination studies

Assays to quantitate antigen-specific T cells are crucial for the development of cancer immunotherapy. A major issue in vaccine

development is the correlation of clinical efficacy with T cell responses as surrogate markers. There is increasing evidence now from various clinical cancer vaccination trials for a relation between the detection of vaccine-induced T cells by cytokine-based assays and clinical responses (reviewed in 14). Standardization and validation of T cell assays to monitor the induction of specific T cells responses is crucial to reliable monitoring of clinical trials. Several expert workshops have been performed within the EORTC melanoma group and the International Society of Biological Therapy of Cancer (ISBTC) on T cell assay methodology and standardization (15, 16). The simultaneous use of two *ex vivo* T cell assays including a functional assay for T cell monitoring has been proposed (16). Controls for the quality of the samples as well as for the accuracy and reproducibility of the assay are a prerequiste for clinical T cell monitoring as outlined above.

An effective vaccine should elicit an effector T cell response able to mediate destruction of tumor cells as well as memory T cells providing long-term immunity. Therefore, not only quantitation but also characterization of differentiation subsets of specific T cells is of great interest. A detailed phenotypic analysis of specific T cells is possible by flow cytometric methods. The currently most frequently used classification was proposed by Sallusto et al. based on the expression of the lymph node-homing chemokine CCR7 and CD45RA classifying CD45RA+CCR7+ naive T cells, CD45RA-CCR7+ central memory T cells, CD45RA-CCR7- effector memory T cells and CD45RA+CCR7- effector T cells (17). A similar distinction of T cell subsets can be made using CD27/CD28 (18). These classifications are a very helpful tool to further characterize the type and function of TAA-specific T cell responses and induction of memory as well as effector T cells by peptide vaccination was shown in first studies (19-21). In addition, a number of further characteristics of tumor specific T cells may be important for their efficacy to attack disseminated tumor cells. These include the avidity of the TCR-antigen binding, the presence of cytotoxic granules and the type of cytokine released in response to antigen exposure, the proliferative capacity, and the expression of functional adhesion and chemokine receptors navigating T cells specifically into distinct peripheral tissues. This detailed characterisation will help to understand the nature of vaccine-induced T cell responses in much greater depth and should be valuable for rational and refined clinical development of cancer vaccines.

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Chapter 10

CYTOMETRIC CYTOKINE SECRETION ASSAY

Detection and Isolation of Antigen-Specific T Cells

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Abstract: The cell-surface affinity matrix technology, otherwise called Secretion Assay or Capture System, represents an innovative method for the analysis and enrichment of viable cells according to secreted molecules, like cytokines. The Cytokine Secretion Assay is especially useful for the detection and isolation of viable antigen-specific T cells after a short restimulation with specific antigen in vitro to induce secretion of cytokines. With direct flow cytometric analysis the Cytokine Secretion Assay allows the rapid detection of cytokine-secreting antigen-specific T cells down to frequencies of 0.01-0.1%. Combination with MACS enrichment greatly increases sensitivity of detection and allows detecting cytokine-secreting antigen-specific T cells down to frequencies of 10-6. The Cytokine Secretion Assay combined with MACS enrichment also allows the isolation of viable cytokine-secreting antigen-specific T cells for expansion and functional characterization.

Key words: Cytokine, secretion, antigen-specific, T cell, detection, isolation

1. INTRODUCTION

The cell-surface affinity matrix technology, otherwise called Secretion Assay or Capture System, represents an innovative method for the analysis and enrichment of viable cells according to secreted molecules, like antibodies or cytokines (1, 2).

Basically, the secreted product is retained on the cell surface of the secreting cell, making it accessible to the powerful technologies for detection of surface markers. The Cytokine Secretion Assay involves the following steps: (A) A cytokine specific Catch Reagent is attached to the cell surface of all cells, i.e. a cytokine-specific "catch" antibody is directly attached to the cell surface of leukocytes as a conjugate with a CD45-specific

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monoclonal antibody. (B) The cells are then incubated for 30-45 minutes at 37°C to allow cytokine secretion. The secreted cytokine binds to the cytokine-specific Catch Reagent on the secreting cells. (C) The caught cytokine is subsequently labeled with a second cytokine-specific "Detection" antibody, which is usually conjugated to a fluorochrome like phycoerythrin (PE) for sensitive analysis by flow cytometry. Optionally the caught cytokine is further magnetically labeled with specific antibody conjugated to super-paramagnetic particles for enrichment by magnetic cell sorting (MACS) (Figure 1).

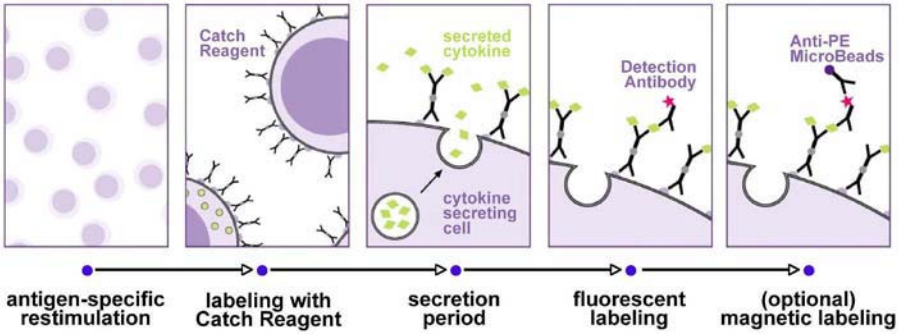

Figure 1. Principle of Cytokine Secretion Assay for labeling of cytokine-secreting cells after antigen-specific stimulation.

Analogous techniques for single-cell analysis, but not for isolation of viable cytokine secreting cells, are ELISPOT (Chapter 7) or intracelluar cytokine staining (IC-FC or ICS) (Chapter 9), which were developed in the late 1980s and early 1990s, respectively. Side-by-side comparisons or direct correlations of the different techniques show comparable results with respect to the detected frequencies of cytokine-expressing cells (3-5). The Cytokine Secretion Assay combines advantageous features and overcomes several limitations of previously used methods.

- The Cytokine Secretion Assay allows the *isolation of viable cytokine-secreting cells* for cell culture, or other downstream experiments, including adoptive transfer.
- It allows the *sensitive multiparameter analysis of viable cytokine-secreting cells* down to frequencies of 10^{-6} due to the option of enrichment of cytokine-secreting cells by magnetic cell sorting (MACS).

The Cytokine Secretion Assay is especially useful for the detection and isolation of viable antigen-specific T cells, CD4+ as well as CD8+, after a short restimulation with specific antigen in vitro to induce secretion of cytokines (6). T cells secrete cytokines only transiently upon stimulation. Therefore normally only very few T cells actually secrete cytokines in peripheral blood or other tissues. Memory/effector T cells rapidly restart to secrete cytokines after restimulation in vivo or in vitro. A large variety of different antigens can be used for stimulation e.g. peptides, proteins, cell lysates or whole cells.

Out of local areas of acute ongoing immune responses sometimes remarkable numbers of cells "spontaneously" secreting cytokines can be detected and isolated with the Cytokine Secretion Assay, most likely after recent encounter of their relevant target antigens in vivo.

With direct flow cytometric analysis the Cytokine Secretion Assay allows the rapid **detection of cytokine-secreting antigen-specific T cells** down to frequencies of 0.01-0.1%. Combination with MACS enrichment greatly increases sensitivity of detection and allows to detect cytokine-secreting antigen-specific T cells down to frequencies of 10^{-6} (0.0001%) (6).

The Cytokine Secretion Assay combined with MACS enrichment also allows the **isolation of viable cytokine-secreting antigen-specific T cells** for expansion and functional characterization.

First of all this has been used for extensive (functional, phenotypical or molecular) characterization of the cultured cells in vitro as well as in vivo, e.g. TCR clonotype analysis of HIV specific T cells (7).

In addition this might lead to improved procedures for the generation of antigen-specific CD4+ as well as CD8+ T cells for potential cellular immunotherapy applications in the treatment of infectious diseases as well as cancer.

The Cytokine Secretion Assay has been used to enrich and culture antigen-specific T cells for a large variety of antigens:

This includes the enrichment and cultivation of virus-specific memory / effector T cells for influenza virus (6), human cytomegalovirus (HCMV) (8, 9), Epstein-Barr virus (EBV) (10), human papillomavirus (HPV) (11) and human immunodeficiency virus-1 (HIV) (7, 12-15).

However it has also been used to enrich and generate T cells specific for various tumor antigens after an initial in vitro priming step with dendritic cells, e.g. for Melan-A (4, 16) CEA/colon carcinoma (17), MAGE-A (18), minor histocompatibility antigens (mHag) (19) and human epidermal growth factor receptor 2 (HER2) (20).

In addition tumor specific T cells have been enriched from tumor infiltrating lymphocytes (TIL) for renal cell carcinoma (RCC) (21).

Of course, the Cytokine Secretion Assay has also been used to analyze cytokine secretion by other cells, e.g. IFN-γ by NK cells (22) or IL-10 by dendritic cells (23).

Cytokine secreting cells have been analyzed for co-production of two different cytokines by two-color Cytokine Secretion Assays, i.e., using a cocktail of two different Cytokine Catch Reagents and Detection Reagents (see also 3.3, Figure 4).

Cytokine Secretion Assays has directly been combined with peptide-MHC tetramer staining for functional characterization of virus- as well as tumor-specific T cells (5, 24) (see also 3.2, Figure 3).

2. PRACTICAL CONSIDERATIONS

The Cytokine Secretion Assay is very useful for the detection and isolation of viable antigen-specific T cells after a short restimulation with specific antigen in vitro to induce secretion of cytokines.

2.1 Controls

A *negative control* sample, treated exactly the same as the antigen-stimulated sample but without addition of antigen (or with addition of control antigen), should always be included as a measure of spontaneous (probably in vivo induced) cytokine secretion.

(Optional) A *positive control* may be included in the experiment, e.g., using Staphylococcal Enterotoxin B at 1µg/ml for 3-16 hours. The addition of costimulatory agents like anti-CD28 mAb or anti-CD49d mAb (usually 1µg/ml) may enhance the response to the antigen. If costimulatory agents are added to the antigen sample, they also have to be included in the negative control sample.

As a *high control* to verify that all cells are labeled with the Cytokine Catch Reagent and therefore are able to capture cytokine, immediately after labeling of the cells with the Cytokine Catch Reagent a small aliquot of the cells can be incubated in recombinant cytokine (typically at a final concentration of 200-1000 ng/ml) for 10 min on ice. After washing, the captured cytokine can be detected on all cells by staining with the fluorochrome-conjugated Cytokine Detection Antibody (1-3, 25).

2.2 Kinetics and proposed time schedule

Upon stimulation with peptide, antigen-specific T cells can be analyzed for IL-2, IL-4, IL-5, IL-10 and IFN-γ secretion within approximately 3-6

hours. Upon stimulation with protein, kinetics of cytokine expression are slightly slower (due to the time required for processing) and the cells can be analyzed within approximately 6-16 hours.

For TNF-alpha kinetic of secretion is much faster than for most other cytokines, i.e. it should be analyzed within 1-4 hours upon peptide stimulation and 4-6 hours upon protein stimulation.

Human cells are typically collected and prepared for stimulation on the first day. Then antigen (protein) is either added in the late afternoon for overnight (≤ 16 hrs) stimulation or cells are stored overnight (whole blood at RT; PBMC in culture medium at 37°C, 5-7% CO_2) and antigen (peptide) is directly added in the next morning for short, 3 hrs stimulation.

Murine cells are typically collected and prepared for peptide stimulation early in the morning for short, 3 hrs peptide stimulation. For protein stimulation typically in the afternoon, murine cells are collected and prepared for overnight (≤ 16 hrs) protein stimulation.

Cytokines are usually only transiently secreted upon stimulation. Therefore the optimal time point after stimulation for analysis with the Secretion Assay has to be chosen in the individual experimental setup.

2.3 Counterstaining of cytokine secreting T cells

To identify the cells of interest counterstaining with CD4 or CD8 is required. Upon activation of T cells TCR and associated molecules, like CD3, are often downregulated and therefore are not useful markers. Exclusion of dead cells by staining with propidium iodide (PI) or 7-AAD reduces nonspecific background staining. For optimal sensitivity labeling of undesired non-T cells such as monocytes (human) or B cells (mouse) with antibodies conjugated to PerCP is recommended, e.g., using CD14.PerCP (human) or B220 (CD45R).PerCP (mouse). These cells are then be excluded together with PI stained dead cells.

Especially upon enrichment of rare antigen-specific T cells exclusion of dead cells is essential, because dead cells can severely disturb analysis of enriched cell fraction.

If fixation of cells is required, PI or 7-AAD can not be used. Then dead cells can be irreversibly labeled prior to fixation with Dead Cell Discriminator reagent.

2.4 Critical parameters

The most critical point within the Cytokine Secretion Assay is to prevent catching of secreted cytokine by non-secreting cells during the secretion period. Not all of the secreted cytokine is caught by the affinity matrix on the

secreting cells, i.e. some cytokine diffuses away from the secreting cell and accumulates in the culture medium. If the concentration of cytokine-secreting cells in the secretion phase is too high, and thereby the concentration of secreted and accumulated cytokine in the culture medium reaches a certain level (approximately > 0,1-1ng/ml), all cells start to catch a low amount of cytokine and subsequently are labeled with the Detection Antibody. The resulting fluorescent shift of all cells can be easily recognized in the flow cytometric analysis. This problem (which similarly can occur in ELISPOT assays) can be prevented by two different ways:

In the standard protocol the cell density in the secretion phase is adjusted according to the expected frequency of cytokine secreting cells, e.g., for <5% to $1x10^6$ cells/ml and for >5% to $1x10^5$ cells/ml. In addition all washing and incubation steps (except the secretion phase) are done in the cold (ice-cold buffer, incubations on ice, refrigerated centrifuge) to prevent cells from secretion, when they are at high density e.g. in the pellet or upon labeling with catch or detection antibody.

In some rapid protocols the assay is done without intervening washing steps on samples with different cell densities similar to the serial dilution of cells in an ELISPOT assay. In general cells should slowly be mixed in the secretion period.

3. DETECTION OF CYTOKINE-SECRETING ANTIGEN-SPECIFIC T CELLS

3.1 Detection of cytokine-secreting antigen-specific T cells

Cytokine Secretion Assays have been used to detect human as well as murine antigen-specific T cells. With direct flow cytometric analysis the Cytokine Secretion Assay allows the rapid detection of cytokine-secreting antigen-specific T cells down to frequencies of 0.01-0.1%. Combination with MACS enrichment greatly increases sensitivity of detection and allows to detect cytokine-secreting antigen-specific T cells down to frequencies of 10^{-6} (0.0001%) (6; see also 4.1 Figure 5).

The example (Figure 2) shows the direct flow cytometric detection of IFN-γ secreting HEL- and KLH-specific murine Th cells.

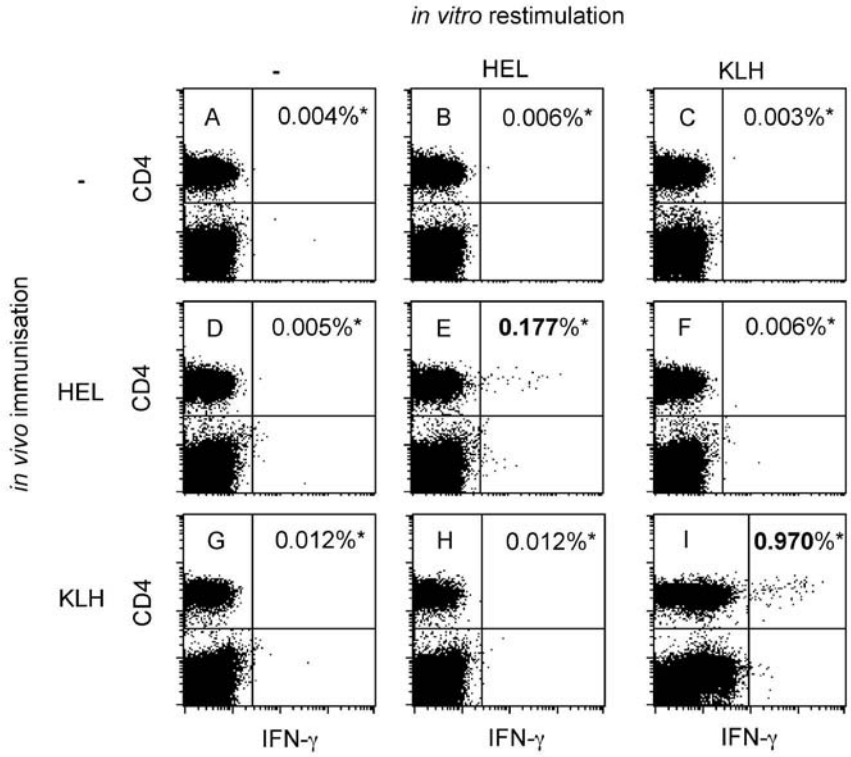

Figure 2. Detection of IFN-γ secreting HEL- and KLH-specific murine Th cells after immunization. * Frequency (%) among CD4+ cells. BALB/c mice, female, 7-10 weeks old, were intraperitoneally (i.p.) immunized with 100µg Henn eggwhite lysozyme (HEL) or Keyhole limpet hemocyanin (KLH) and 200ng Pertussis Toxin (PT) in incomplete Freund´s adjuvant (IFA), followed by an additional injection of 200ng PT in PBS 24 hours later. After 2-3 weeks spleen cells (SC) from HEL-immunized mice (D-F), KLH-immunized mice (G-I) or unimmunized control mice (A-C) were incubated in vitro for 16 hours with either 100µg/ml HEL (B, E, H), 100µg/ml KLH (C, F, I) or without antigen (A, D, G) in RPMI 1640 medium supplemented with 5% murine (heat-inactivated) serum. IFN-γ Secretion Assays were performed on all samples. Cells were stained with IFN-γ Detection Antibody.PE, CD4.FITC, CD45R(B220).PerCP and PI. For flow cytometric analysis 200.000 events were acquired. A lymphocyte gate based on forward and side scatter properties (FSC/SSC) was set and dead cells and B cells were excluded according to PI and CD45R(B220).PerCP staining in a FL2 versus FL3 plot. For analysis PE (IFN-γ) versus FITC (CD4) staining of gated (viable) lymphocytes is displayed.

3.2 Combination of Cytokine Secretion Assay with peptide-MHC tetramer staining

Cytokine Secretion Assay has directly be combined with peptide/MHC tetramer staining for functional characterization of antigen-specific T cells (5, 24) (Figure 3). Upon stimulation with antigen (or some mitogens like anti-CD3) the TCR on the specific T cells can be downregulated very rapidly. This dramatically reduces peptide-MHC tetramer staining. Therefore the peptide-MHC tetramer labeling is done prior to the stimulation with peptide. After staining with peptide/MHC tetramer cells are stimulated with the specific peptide for 2-3 hours followed by the standard secretion assay procedures.

Depending on the type of peptide-MHC tetramer used, the peptide-MHC tetramer may (a) or may not (b) stimulate cytokine secretion. In the former case (a) the control sample is stained with peptide-MHC tetramer after the incubation (without peptide or with control peptide) and the cytokine secretion assay. The example (Figure 3) shows the analysis of CMV-specific CD8+ T cells for secretion of IFN-γ and IL-2 using a combination of peptide-MHC tetramer staining with Cytokine Secretion Assay.

PBMC from a CMV seropositive, HLA-A2+ donor were stained for 1 hour at 4°C with a PE-labeled CMVpp65$_{495\text{-}503}$/HLA-A2 tetramer (A, C, D and F) or incubated without tetramer (B and E). Cells were then stimulated for additional 2 hours at 37°C with 5 µg/ml CMV peptide pp65$_{495\text{-}503}$ (B, C, E and F) or without peptide (A and D). The IFN-γ (A-C) or IL-2 (D-F) Secretion Assay were performed on the samples. Cells were stained with IFN-γ Detection Antibody (APC) (A-C) or IL-2 Detection Antibody (APC) (D-F), CD8.FITC, CD14.PerCP and PI. For flow cytometric analysis 200,000 events of each sample were aquired. A lymphocyte gate and an exclusion gate for dead cells and monocytes were set. In addition CD8+ cells were gated. For analysis APC (IFN-γ or IL-2) versus PE (tetramer) staining of gated (viable CD8+) lymphocytes is displayed (A-F).

While in this donor the majority (87%) of CMVpp65$_{495\text{-}503}$ specific CD8+ T cells secrets IFN-γ upon stimulation (Figure 3C), only 16% secrete IL-2 (Figure 3F).

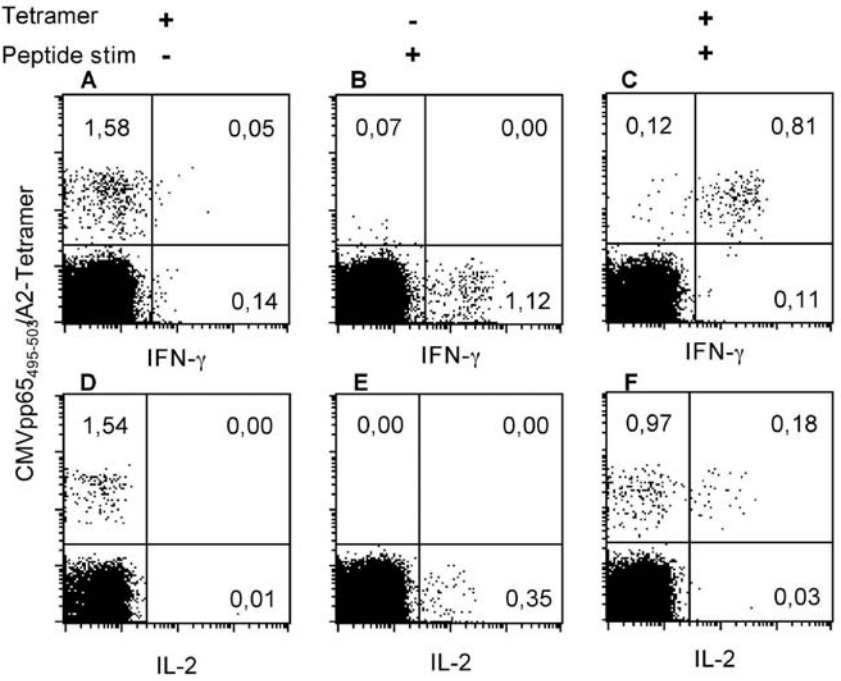

Figure 3. Analysis of expression of IFN-γ and IL-2 by CMV-specific human CD8+ cells.

3.3 Coproduction of two different cytokines

Cytokine secreting cells have been analyzed for co-production of two different cytokines by two-color Cytokine Secretion Assays, i.e., using a cocktail of two different Cytokine Catch Reagents and Detection Reagents (Figure 4).

The example shows the analysis of CMV-specific CD4+ T cells for secretion of IFN-γ and IL-10 using a combination of IFN-γ and IL-10 Secretion Assays. PBMC from a CMV seropositive donor were incubated for 16 hours at 37°C with 5 µg/ml CMV lysate (A) or without antigen (B). The IFN-γ and IL-10 Secretion Assays were performed together on both samples. Therefore cells were labeled with a premixed cocktail of IFN-γ and IL-10 Catch Reagent. After the secretion period cells were stained with IFN-γ Detection Antibody (FITC) and IL-10 Detection Antibody (PE), CD4.Cy5, CD14.PerCP and PI. For flow cytometric analysis 1,000,000 events of each sample were acquired. A lymphocyte gate and an exclusion gate for dead cells and monocytes were set. In addition CD4+ cells were gated. For analysis FITC (IFN-γ) versus PE (IL-10) staining of gated (viable CD4+) lymphocytes is displayed.

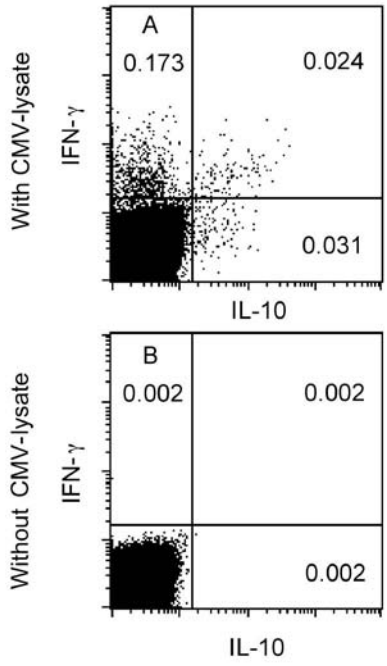

Figure 4. Co-production of IFN-γ and IL-10 by CMV-specific Th cells.

4. ISOLATION OF VIABLE CYTOKINE-SECRETING ANTIGEN-SPECIFIC T CELLS

4.1 Detection and isolation of IFN-γ secreting CMV-specific human Th cells

Combination of the Cytokine Secretion Assay with MACS enrichment greatly increases sensitivity of detection and allows to detect cytokine-secreting antigen-specific T cells down to frequencies of 10^{-6} (0.0001%) (6) as well as allows the isolation of viable cytokine-secreting antigen-specific T cells for expansion and functional characterization.

Combination of the Cytokine Secretion Assay with magnetic enrichment on the CliniMACS allows rapid, large scale enrichment and generation of antigen-specific T cells for potential immunotherapy applications (9).

The example (Figure 5) shows the detection and isolation of CMV-specific Th cells using IFN-γ Secretion Assay with MACS enrichment.

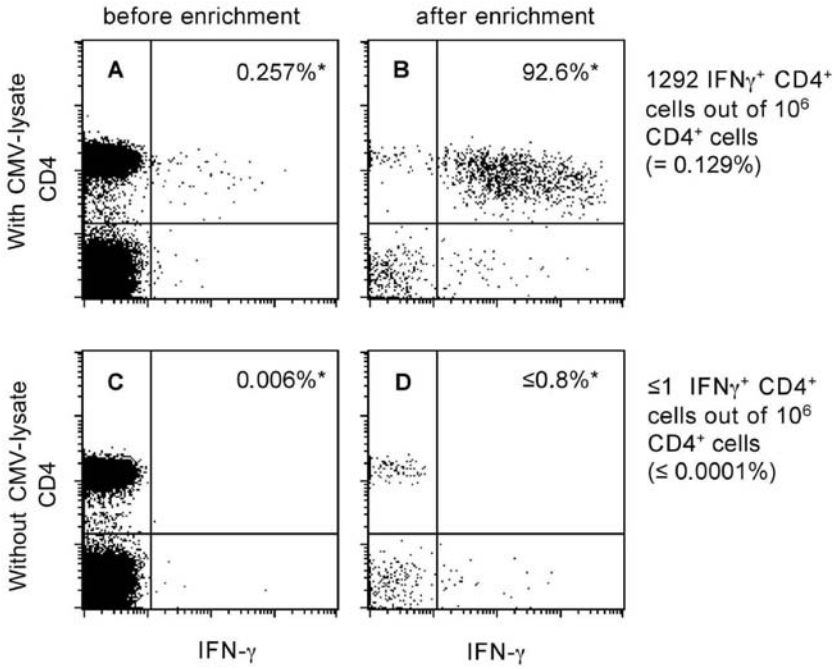

Figure 5. Detection and isolation of IFN-γ secreting CMV-specific human Th cells.* Frequency (%) among CD4+ cells. PBMC from a CMV seropositive donor were incubated for 16 hours with 5µg/ml CMV lysate (A and B) or without antigen (C and D). The IFN-γ Secretion Assay was performed with 10^7 cells on both samples including enrichment of IFN-γ-secreting cells by MACS separation. Counterstaining of T cells was performed using CD4-FITC. Monocytes were stained with CD14-PerCP and dead cells were stained with PI. Flow cytometric analysis was performed on the original fractions and the enriched fractions. At least 200.000 events of the original fractions and the complete enriched fractions were acquired. A lymphocyte gate based on forward and side scatter properties (FSC/SSC) was set and dead cells and monocytes were excluded according to PI and CD14-PerCP staining. For analysis PE (IFN-γ) versus FITC (CD4) staining of gated (viable) lymphocytes is displayed (Figure 5A-D). In the stimulated sample 1292 IFN-γ-secreting CD4+ T cells were enriched per 10^6 CD4+ T cells before separation. In the unstimulated sample no IFN-γ-secreting CD4+ T cell was enriched per 10^6 CD4+ T cells.

4.2 Expansion of cytokine secreting antigen-specific human T cells

Enriched human cytokine secreting T cells have been expanded in vitro e.g. in feeder cell based systems (8, 9). Most simple, the non-cytokine-secreting cell fraction, i.e. the negative cell fraction (flow through) of the MACS Separation, is used as a source of autologous "feeder cells" after irradiation or treatment with Mitomycin C. The cytokine-secreting cells and

the feeder cells are co-cultured at a 1:100 ratio *in vitro* in the presence of IL-2 (20-100 IU/ml) for up to two weeks. Further cultivation and expansion requires subsequent restimulations.

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Chapter 11

PEPTIDE/MHC TETRAMER ANALYSIS

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Abstract: Over the past decade, the identification of tumor-associated antigens (TAAs) has allowed a novel approach to cancer therapy aimed at the induction of systemic immune responses against cancer cells through active, specific immunization. A key step in the design of effective vaccination strategies is the ability to easily and accurately determine whether the immunogen has achieved its primary goal which, in the case of anti-cancer vaccines, is the induction of TAA-specific T cells. While several methods have been utilized for this purpose, peptide/MHC (pMHC) tetramers have become a particularly useful tool as they provide a flexible, rapid way to identify antigen-specific T cells directly ex vivo, and allow the characterization of their phenotype and viable isolation for further analysis. While this technology is certainly not restricted to the study of cancer-specific immune cells, for the purpose of this chapter, we will focus our discussion on this application.

Key words: TCR-pMHC interactions, immune monitoring

1. INTRODUCTION

Since their introduction by Altman and Davis in 1996 (1), peptide/major histocompatibility complex (pMHC) tetramers have been widely used to characterize T cell responses since they allow for accurate and rapid enumeration of antigen-specific T cells and their phenotypic characterization. In addition, pMHC tetramers have been used successfully for the viable isolation of antigen-specific T cells that could be further characterized with morphologic, functional and/or molecular methods. The utilization of pMHC tetramers spans a wide range of immune biology that

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encompasses T cell responses to viruses, cancer and autoimmune diseases. While tetramers are now available from commercial and non-commercial sources such as Beckman Coulter Immunomics or the NIAID tetramer core facility and are, therefore, widely available for use in many research and clinical settings, their utilization has not peaked as yet because many laboratories still utilize other less direct methods to assess the number and function of antigen-specific T cells.

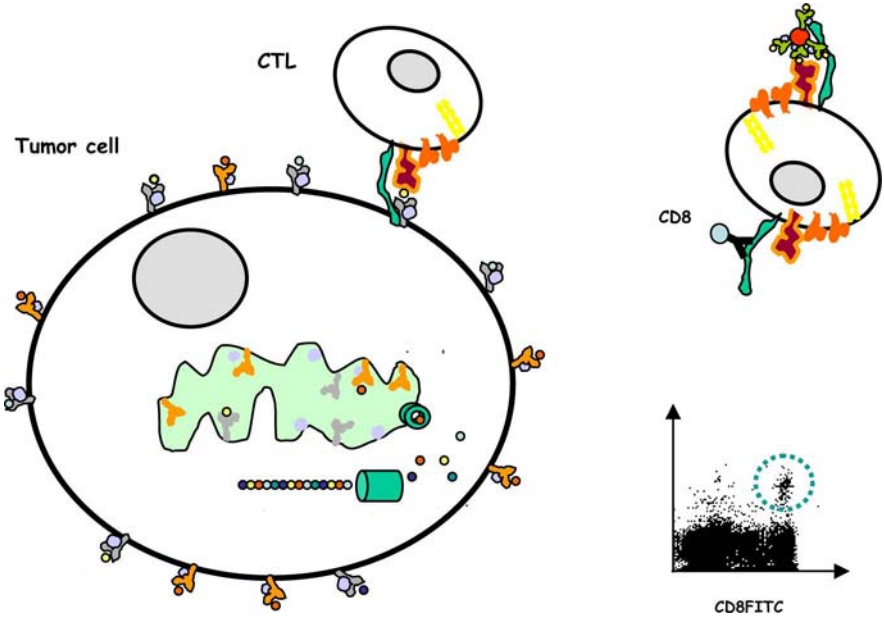

Figure 1. Cartoon of pMHC tetramer. Tetramers are a tetrahedral formation of four pMHC complexes linked via biotin to a central streptavidin. This configuration allows the simultaneous binding of three pMHC complexes to three TCRs on a T cell surface, thus enhancing avidity of binding. To facilitate flow cytometric analysis, streptavidin directly conjugated to fluorophores (such as PE or APC) are generally used. (Monsurro & Nagorsen ASHI quarterly, 2002).

This is due in part to the fact that pMHC tetramers are more difficult to use than antibodies for the staining of cell surface molecules due to significant differences in the biology of TCR binding to pMHC versus antibody-antigen interactions. Most significantly, the interactions between pMHC and TCR are characterized by orders of magnitude lower affinity than antibody-antigen interactions. Therefore, to fully take advantage of the power of tetramer analysis and utilize these reagents efficiently, a clear

understanding of a number of biological and practical issues is needed. In this chapter, we will discuss critically many of these issues. In addition, we will provide examples of novel applications of tetramer technology. Most human studies to-date have been carried in the context of MHC class I for analysis of CD8+ T cells. While human MHC class II tetramers are available, they are generally more difficult to make and use, at least in the human setting (2, 3) preventing their widespread adoption at this time. Recently, several technical problems related to the preparation of MHC class II tetramers have been solved and they are becoming more widely available and used for the assessment of CD4+ T cell responses in humans (4, 5). This has provided the opportunity to simultaneously follow CD4+ and CD8+ T cells responses in various contexts, providing a broader view of basic aspects of the cellular immune response in natural or therapeutically-modulated states. Because the use of MHC class II tetramers is still in its infancy, this chapter will focus on the use of MHC class I tetramers for the detection of CD8+ T cells in humans.

2. IMPLICATIONS OF TCR-PMHC INTERACTIONS FOR THE PREPARATION AND UTILIZATION OF TETRAMERS

The antigen specificity of individual T cells is determined by the restricted expression of a homogeneous population of T cell receptors (TCRs) which remains stable and unique for a given T cell and its progeny. The ligand of the TCR is a complex made up of a peptide presented on the groove of a MHC molecule. This interaction is normally stabilized in T cells by the engagement of the CD8 (or CD4) co-receptor with the appropriate domain of the MHC molecule as well. The affinity of the TCR-pMHC interaction in solution is much lower than that of an antibody-antigen interaction. Whereas the typical dissociation constant (Kd) of an antibody-antigen interaction is around 1 nM, TCR-pMHC interactions occur around a 10 μM range. This logarithmic difference in affinity is likely explained by the fact that antibodies are meant to bind their targets in solution, while TCRs bind their ligands through cell to cell contacts where many ligand-receptor interactions could simultaneously take place. It is becoming well known that in general cell surface molecules interact with low affinities (6), with Kd in the range of 1 – 100 μM. This has made the utilization of soluble monomeric pMHC as a ligand ineffective for the staining of antigen-specific TCRs since due to the low affinity interaction; soluble pMHC complexes bound to a T cell would fall off readily during washing. Hence, previous efforts to use soluble pMHC complexes as a staining reagent to identify

antigen-specific T cells were unsuccessful. Altman and Davis solved the problem by biotinylating pMHC complexes and multimerizing them via binding to tetravalent steptavidin molecules, resulting in 'tetramers'. Tetrameric complexes exist in a tetrahedral (pyramid) configuration, such that three pMHC molecules exist on one plane from any orientation (Figure 1). This has the potential of allowing the sterical interaction of three pMHC complexes with three TCRs simultaneously on a T cell surface resulting in strongly enhanced avidity of this interaction, which in turn stabilizes the staining of a T cell. A fluorochrome is conjugated to the tetrameric complex to allow detection. It is not clear how many interections are truly necessary, and others have shown that even dimers of pMHC complexes (linked via an immunoglobulin framework) can suffice in staining T cells (7). Direct comparisons between the two technologies suggest that pMHC tetramers provide greater signal-to-noise ratio as staining reagents. Nonetheless, dimer reagents are commercially available through Becton-Dickinson under the name DimerX; (http://www.bdbiosciences.com/product_spotlights/dimerX_presentation_tools/).

3. IMMUNE MONITORING AND PMHC TETRAMERS

Several studies have compared pMHC tetramer-based results with other methods previously used for the enumeration and/or characterization of antigen-specific T cells. These assays include limiting dilution assays (LDA), cytokine flow cytometry (CFC), and ELISPOT analyses (8-11). Typically, tetramer analyses have provided frequency estimates that exceed those detected using these alternate assay systems. The most likely explanation is that other assays are dependent upon a functional response of the T cells following cognate stimulation while tetramers reflect only TCR binding specificity. Therefore, it can be stated that in principle, tetramer staining is not a primary functional assay although it may be used for such purposes when combined with other assays. While this may be viewed as a limitation of tetramers, in fact in itself tetramer staining has the major advantage of not being dependent on the functional status of a given T cell (unless required by the experimental situation) and, therefore, can yield a highly specific and direct view of T cell phenotypes particular when samples are studied directly in ex vivo conditions. When combined with a functional readout, tetramer staining provides important additional information. Tetramers could be used to mark T cells specific for an antigen while a functional readout could be simultaneously applied to determine the fraction of the antigen-specific population responsive to stimulation. Such a

combination was used successfully to demonstrate for the first time that a tumor-specific T cell population was anergic in vivo (12). While discussion is ongoing about what may represent the ideal method to monitor antigen-specific T cell responses, each method has its own advantages and disadvantages and their selection should be tailored to the question asked by a given clinical or experimental design. In general, however, practical considerations may suggest that, in laboratories familiar with the use of pMHC tetramers, the rapidity with which information can be obtained through tetramer staining of T cells makes them preferable to more time consuming and less direct methods.

4. PRODUCTION AND VALIDATION OF TETRAMERS

The production of tetramers is a time-consuming, labor-intensive process which requires skill and experience that limited the widespread use of tetramers initially. However, tetramers are now widely available through public (NIAID Tetramer Core Facility, www.emory.edu/WHSC/TETRAMER/) and commercial (Beckman Coulter Immunomics, www.immunomics.com/) sources. The quality of tetramer reagents has also improved steadily. Excellent reagents can now be obtained, especially from commerical sources as they undergo rigorous quality control before distribution. Recently, a pentameric variant of pMHC complexes (Pro5) is being marketed by ProImmune from England. As these reagents are very new, there is little experience with their quality or how they compare to tetramers. While few individual laboratories continue to undertake the difficult task of making its own tetramers, it is worth briefly discussing the basic process involved in tetramer production.

The basic components of a pMHC complex (class I) include the peptide, MHC class I molecule, and beta-2-microglobulin (β2m). For subsequent tetramerization, the MHC class I molecule is engineered with a C-terminal substrate peptide for BirA-mediated biotinylation, biotin signal peptide (bsp). This MHC I-bsp recombinant protein (as well as β2m) is generally produced using E. coli in inclusion bodies. There are now numerous human and mouse MHC I alleles engineered with bsp in efficient E. coli expression vectors (generally BL21DE3 systems). For details of production, see the NIAID Tetramer Core Facility website (http://www.emory.edu/WHSC/TETRAMER/protocol.html) and protocols by Dr. Dirk Busch from the Technical University Munich (http://www.mikrobio.med.tu-muenchen.de/ressourcen/repository/share/busch/tetprotocol.pdf).

Briefly, recombinant MHC I-bsp, β2m, and the peptide of interest are mixed together in a folding reaction which generally goes over 48 – 72 hours. During this time, refolding of the pMHC complex occurs. This is an inefficient process in vitro, resulting in a 1 – 5% final yield. Properly folded pMHC complexes are extensively purified from unfolded materials via size exclusion columns (FPLC) and further cleaned up with anion exchange (MonoQ or ResourceQ). During this process, the biotinylation reaction is carried out via the addition of biotin-ligase (BirA enzyme), biotin, and ATP. The efficiency of the biotinylation process is generally determined via a gel-shift assay using excess streptavidin to determine the fraction of pMHC complexes which bind streptavidin, hence biotinylated, and the fraction that does not. Based on this information, a simple calculation is made to determine the amount of streptavidin to add to the purified, biotinylated pMHC complexes for a 1:4 molar ratio. Streptavidin directly conjugated to a fluorophore (generally phycoerythrin or APC) is used to facilitate their use in flow cytometry or sorting.

Upon completion of the synthesis the new tetramer is tested for specificity and titrated to optimize its utilization. In fact, newly synthesizes tetramers may not work for several reasons particularly when new peptide/MHC combinations are tested. Some peptides may have very low MHC binding affinity or low stability in solution and, therefore the refolding process into pMHC complexes may be extremely inefficient. In addition, incomplete purification or biotinylation of pMHC complexes or bad quality of streptavidin-fluorophore batches may decrease the quality of the final products in spite of good refolding efficiency. Therefore, each new batch of tetramer needs to be convincingly validated by staining a set of positive and negative controls. As a positive control, a T cell line or clone specific for the pMHC complex in the tetramer is needed. These cells are generally stained with serial 2-fold dilutions of tetramers (generally starting at 40 μg/ml), along with anti-CD8 antibodies. The data are then plotted with tetramer concentration versus median fluorescence intensity (MFI) of tetramer staining (Figure 2). In optimal conditions a sigmoidal curve results where no staining is observed below a certain threshold, followed by an exponential increase in MFI and then a plateau staining above a certain tetramer concentration. The optimal staining concentration is a level just below the plateau, where there is a maximum spread between the specific staining (signal) and background staining (noise). Above that no experimental advantage is gained and expensive material would be wasted. It cannot be overemphasized the importance of using tetramers at optimal concentrations to maximize the signal-to-noise ratio as determined via flow cytometry.

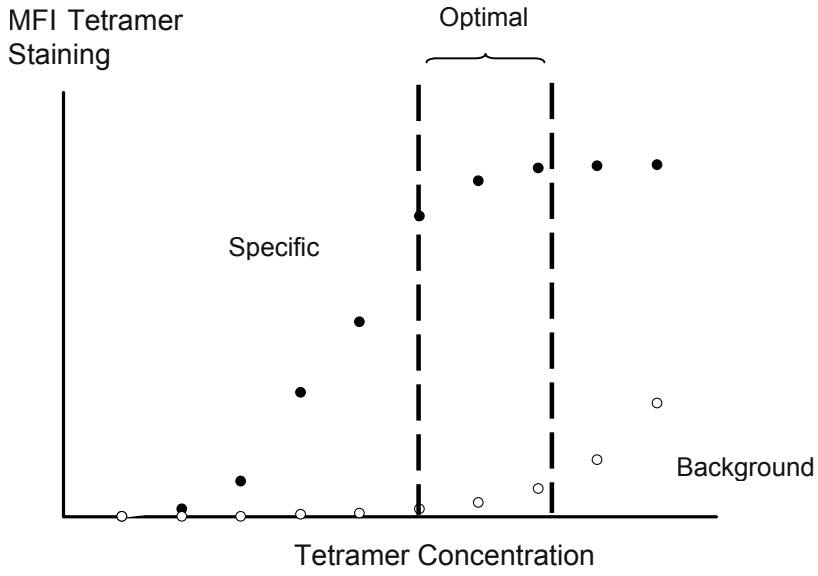

Figure 2. Titration of tetramers to determine optimal staining concentration. Varying concentrations of a tetramer (generally 1 – 40 μg/ml final) are stained against a specific T cell line or clone. The median fluorescence intensity (MFI) of tetramer staining of the antigen-specific and background populations are plotted. The optimal range is the region at which there is near plateau specific MFI staining but still minimal background MFI staining.

This is particularly important when rare T cell populations are analyzed (< 0.1%) for which the discrimination of background non-specific staining may be particularly difficult to discriminate from true specificity. Under optimal conditions, the threshold of detection for tetramer-based assays is approximately 1/8,000-1/10,000 (i.e. 0.01-.0125%) (12-14). Since pMHC tetramers are non-covalent complexes, they "degrade" at variable rates which depend in large part on the peptide-MHC affinity. Hence, a given batch of pMHC tetramers may be stable anywhere from 2 years (for many viral peptide antigens which bind MHC with high affinities) to as little as 3 months (for certain low-affinity 'self' peptides which bind MHC with low affinities). This necessitates periodic retesting of tetramers by re-titration analyses on a regular (every 6 – 8 weeks) basis depending on the peptide used. In addition, stability may be prolonged by freezing batches of pMHC tetramers.

5. IMPORTANT PRINCIPLES FOR THE PERFORMANCE OF TETRAMER ASSAYS

5.1 Quality and numbers of cells

To ensure consistent results, roughly the same numbers of cells should be added per stain in all tetramer assays, and these cells should be in an optimum state. T cells from peripheral blood (PBMC), lymph nodes, or infiltrating tumors (TILs) may be used for tetramer analysis. Typically, one stains 1 - 2 million PBMCs per condition are used to collect as many events as possible (10^5 – 10^6) to increase the statistical power of each analysis. This is important especially when looking for rare T cell populations which may represent 0.01% of CD8+ T cells. In that case, if one collects 5 x 10^5 events, and CD8+ T cells represent 10% of PBMC, then a 0.01% antigen-specific population would represent five events. Of interest particularly for analysis of patient materials, frozen/thawed PBMC samples may be used without compromising the quality of tetramer staining data. This is important in allowing samples from a clinical trial to be collected, stored, and analyzed in batches, or shipped to a reference laboratory. This also permits testing of patient materials collected longitudinally at different times to be analyzed together in single batches, thereby limiting variability associated with inter-assay assessment. The way in which cells are frozen and thawed may significantly impact on the quality of the resultant data. To maximize cell viability and quality, thawed cells may be "rested" in complete media (containing human serum) for several hours to overnight and then enriched by discontinuous gradient centrifugation (Ficoll/Percoll) prior to staining. Of note, there is a theoretical concern that specific CD8+ T cells in cancer patients, particularly those with advanced stage disease, may undergo "spontaneous" apoptosis *ex vivo* (15) during this "resting" period and thereby not be detected in subsequent tetramer analysis. Hence, it may be advisable for each laboratory to undertake a direct comparison between fresh vs. frozen/thawed samples before routinely using one or the other. Another consideration is that certain surface markers may not stain well in frozen/thawed samples. As an example, CD62L (an important marker for the distinction between naive vs. memory T cells) contains glycosylations which may be cleaved during the freeze/thaw process (Mario Roederer, personal communication). Anti-CD62L antibodies require proper glycosylation of the protein for proper binding and may not bind CD62L+ cells from frozen/thawed samples. In such cases characterizations should depend on a preliminary validation of frozen samples that have been compared with fresh material.

5.2 Staining volume, temperature, and time

To conserve reagents, it is advisable to stain in a small volume, generally 30 – 50 µl. This is adequate for 1 – 2 million PBMCs. If staining more cells, such as for sorting, it is necessary to scale up accordingly to 100 – 200 µl and all reagents (tetramers and antibodies) should be added to a desired final concentration based on the calculated total staining volume. As an example, it may be convenient to make up a 3 x solution of tetramers and antibodies and add 10 µl of cells, 10 µl of tetramers, and 10 µl of antibodies. The temperature at which the staining occurs may significantly impact the degree of specific tetramer staining (16). Staining of T cells on ice (or at 4°C) allows for significant low avidity (frequently cross-reactive) tetramer binding, thus increasing the background. Staining at room temperature (23°C) or 37°C tends to favor higher avidity tetramer-TCR interactions and reduces background. However, staining at 37°C may induce activation of T cells and partial down-regulation of the TCR with consequent reduction in detectability. The incubation time required for optimal staining is dependent on the temperature. At room temperature (23°C) or 37°C, tetramer staining generally saturates at 15 – 30 minutes, while on ice (or at 4°C) saturation does not occur until at least 2 hours. Once staining is completed, cells should be washed and stored on ice until FACS analysis.

5.3 Tetramer concentration

As stated earlier, the concentration of tetramers used to stain cells is critical in obtaining the optimal signal-to-noise ratio and, therefore, increase accuracy. Staining with suboptimal tetramer concentrations may lead to an underestimate of antigen-specific T cell frequencies, while staining with excessive tetramer concentrations may lead to high backgrounds and overestimate of antigen-specific T cell frequencies. This is particularly important when making comparisons between samples or across multiple tetramers. For most tetramers, they are generally used at a 1 – 20 µg/ml final concentration. The optimal tetramer concentration can only be determined by careful titrations of each reagent against an antigen-specific T cell line or clone.

5.4 Anti-CD8 and other antibodies

Another important factor is the use of anti-CD8 antibodies. The MHC I molecule naturally has a low affinity for CD8 (17), such that MHC I tetramers may bind CD8+ T cells non-specifically at low temperature (4°C or on ice). The addition of anti-CD8 antibodies can block this interaction,

and several reports have suggested that tetramer staining may be affected by the concentration of anti-CD8 (or –CD3) antibodies used, and even by the particular clone of antibody (18, 19). Indeed, depending on the CD8 determinant recognized by the antibody, anti-CD8 counterstaining may block, have little impact on, or even augment the intensity of pMHC tetramer staining of T cells (16, 18, 20). In careful titration experiments, the percentage of tetramer+ events may vary depending on the concentration and type of anti-CD8 antibodies used. This could provide an additional variability when comparing data between experiments. The recent development of pMHC tetramers based on mutant class I heavy chains which do not bind CD8 should reduce such low-avidity or cross-reactive tetramer-T cell interactions based on CD8 binding and thereby further reduce background. Tetramers from Beckman Coulter Immunomics are made with such non-CD8 binding mutant MHC I molecules. In almost any bulk PBMC sample, there are cells which may non-specifically bind to staining reagents and thereby contribute to background. These are generally monocytes or dead/dying cells. The use of 'dump' antibodies to negatively gate out such non-specifically sticky cells may be quite useful in cleaning up data. For this purpose, one would use antibodies to markers not present on the cells of interest – for CD8+ T cells, useful 'dump' antibodies may include anti-CD4, 14, or 19.

5.5 Data interpretation

Lymphocytes are gated based on their forward and side-scatter, then dead and non-specifically 'sticky' cells are excluded based on propidium iodide staining and 'dump' (negative selecting) antibodies. The remaining cells are assessed for CD8 vs. tetramer staining (Figure 3).

Only cell populations that are well-clustered and display a clear separation from the CD8+, tetramer-negative T cell population are considered to be "real" events (Figure 4).

If necessary, specific tetramer staining of T cells may be confirmed via competition with unlabeled pMHC monomers or anti-CD3 antibodies (16, 19-21). As the percentage of CD8+ T cells in total PBMC can vary widely between samples, tetramer-positive events are generally normalized as a percentage of total CD8+ T cells or data may be reported as the absolute number of tetramer+, CD8+ cells per amount (i.e. µl-ml) of blood.

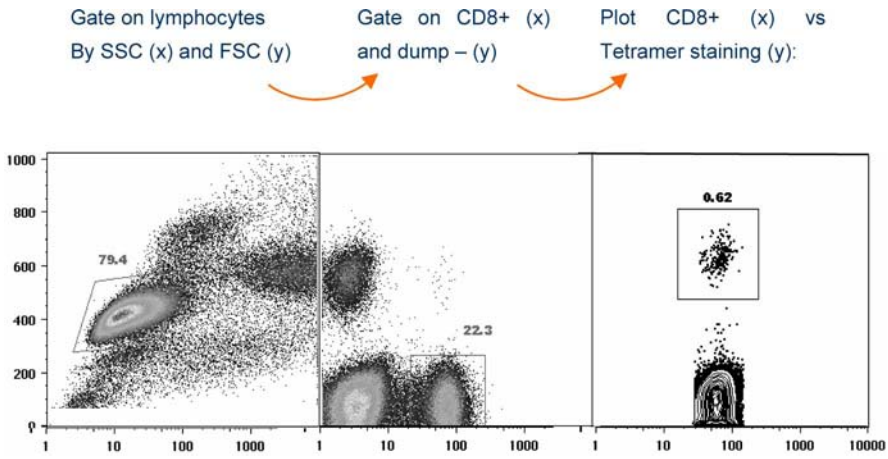

Figure 3. Typical gating strategy for analysis of tetramer+ populations, Collected PBMC data are plotted first on SSC vs. FSC with selection of the lymphocytes population. Gated cells are then plotted on CD8 vs. dump (generally CD4, 14, 19) and further gated on CD8+ dump- cells. These are then plotted on CD8 vs. tetramer staining. Tetramer+ populations are generally expressed as % of total CD8+ T cells.

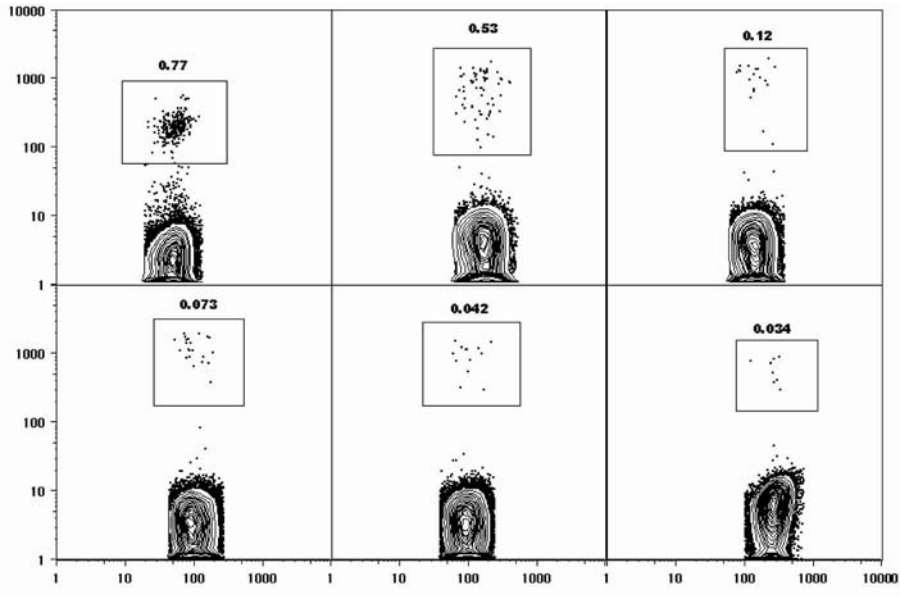

Figure 4. Examples of tetramer+ populations, With optimal staining conditions, a tetramer+ population should be well clustered and clearly separate from the bulk CD8+ population. The top three panels represent large populations (0.1 – 0.8%). However, with careful titrations of reagents, even small populations (<0.1%) may be clearly distinguishable (bottom panels).

5.6 Other uses of tetramers

The identification, enumeration and characterization of antigen-specific T cells is the primary purpose of the use of pMHC tetramers. However, tetramers have also been used to isolate antigen-specific T cells by sorting fluorescent labeled T cells. This procedure has been used to increase the specificity and efficiency of T cell cloning. In addition, complex molecular analysis can be performed in purified cells using methods that could not discriminate among different cell populations by themselves such as TCR spectratyping and microarray analyses (8, 12, 22). Finally, tetramers have been used to discern the functional status of antigen-specific T cells using multi-parameter FACS to assess surface (activation, memory, cytotoxic) markers and intracellular or secreted cytokines/chemokines (12, 14, 23).

5.7 Isolation by sorting and cloning

The viable isolation of antigen-specific T cells for cloning or further analyses is a powerful utilization of pMHC tetramers. This may be done routinely using a standard FACSort instrument (such as the BD FACS Vantage or the new Aria). For example, a tetramer+ T cell population may be isolated and directly tested ex vivo for cytolytic activity against target cells in a standard chromium release assay. It is now well established that the tetramer staining and sorting process does not impair the cytolytic activity of T cells. Care should be taken to keep the cells cold and in serum-rich media to minimize alterations of their function during sorting. Ideally, individual tetramer+ cells are sorted into individual wells of a tissue culture plate and expanded in the presence of feeder cells and cytokines to create T cell clones. Thus, precise enumeration of number of cell per well can be achieved rather than depending on the statistical assumption of limiting dilution methods. This accuracy increases the efficiency (less empty wells) and the accuracy of cloning (less well with multiple cells). Expanded cells can then be analyzed for TCR specificities and structural avidity (see below), or expanded to high numbers for adoptive cellular immunotherapy (24). More recently, sorted T cells have also been analyzed for gene expression profiles using DNA microarray technology (25).

5.8 Phenotypic analysis by multi-color flow cytometry

The advent of multi-color flow cytometry allows the extensive phenotypic characterization of antigen-specific T cells on an individual cell basis. Even with 3- or 4-color FACS, the expression of several markers by an antigen-specific T cell population of interest may be determined by

performing a few different stains. With the increasing availability of 6-, 8-, and even 10-color FACS (Table 1), large numbers of markers could be determined from only a few stains (12). This is important especially for rare populations and precious patient samples. In general, one channel is taken up by tetramers (usually PE) and another channel for anti-CD8. If cells are limiting, two channels could be devoted to two different tetramers (such as PE and APC) so that two separate antigen-specific T cell populations could be simultaneously assessed per stain. Not all antibodies work well in combination, so considerable effort must be made to optimize phenotypic panels which work well together. For example, since anti-CD3 antibodies may compete with tetramers for binding to the TCR/CD3 complex (19), it is advisable to not include this antibody in panels except for validation purposes. Panels could also be optimized for intracellular antigens (see below).

Table 1. Sample antibodies panels for phenotypic analysis.

FITC	PE	Cy5PE	APC	TR	Cy5.5 PE	Cy7 PE	Cy5.5 APC	Cy7 APC	Cascade Blue
CD11a	tHLA	CD38	CD8 beta	CD95 (Fas)	CD137 (4-1BB)	CD69	CD4	CD25	CD8
CD27	tHLA	CD45RA	CCR7	CD80 (B7-1)	CD152 (CTLA4)	ICOS	CD4	CD28	CD8
CD44	tHLA	HLA-DR	CD57	CD95L (FasL)	CD154 (CD40L)	CD134 (OX40)	CD4	CD56	CD8
CD3 zeta	tHLA	IFN-γ	IL-4	TNF-α	IL-10	perforin	CD4	IL-2	CD8

These are 10-color FACS panels incorporating PE-labelled tetramers (tHLA) for the phenotypic analysis of antigen-specific T cell populations. The top three panels involve surface stains only; the bottom panel also includes intracellular stains.

5.9 Intracellular staining

Tetramers can be combined successfully with intracellular staining to analyze expression of cytokines (e.g. IFN-γ, IL-2, IL-4, TNF-α) or cytolytic granules (perforin, granzyme A, granzyme B) by antigen-specific T cells. This is a good example of how precise functional information can be derived by combining two methodologies. After routine surface staining with tetramers and other markers, cells are stimulated with relevant or irrelevant stimulus, fixed and permeabilized, then stained with antibodies against intracellular antigens (12). As this process involves numerous washing steps, it is important to start with large numbers of cells to ensure sufficient cells

remaining at the end to detect rare populations. It is also important to note that T cells generally down-regulate the TCR complex upon activation. Since many intracellular cytokine staining protocols involve a non-specific cellular activation step with mitogens, the level of tetramer staining could go down substantially. To circumvent this problem, tetramer staining could be performed prior to cell activation such that tetramers are internalized (with the appropriate fluorophore) along with the TCR complex during the activation process. In addition, improved quality of tetramers and fluorochrome has recently decreased the significance of this problem and double stained stimulated cells can be easily detected with these assays.

5.10 Analysis of 'structural avidity'

'Functional avidity' or 'recognition efficiency' is emerging as a key factor in the effectiveness of an antigen-specific T cell response (26-30). It is a measure of the sensitivity of a T cell to different peptide concentrations for stimulation (31). Recognition efficiency of a T cell reflects the cumulative effects of the intrinsic affinity of its TCRs for their cognate peptide displayed on the target cell, expression levels of TCR, CD4/8, and adhesion molecules, as well as redistribution of these molecules on the cell membrane and their recruitment efficacy of the signaling cascade (32-34). Identification of peptide-specific T cells using pMHC tetramers, ELISPOT, or cytokine flow cytometry does not distinguish between cells of high or low recognition efficiency – at least not without careful reagent titration on each sample. While previous investigations have attempted to use the intensity of tetramer staining as a measure for binding strength (35, 36), recent evidence suggests that tetramer staining intensity alone does not directly correlate with recognition efficiency (30, 37, 38). Nonetheless, the rate of dissociation of bound pMHC tetramers from antigen-specific T cells upon addition of a competing antibody (such as anti-TCR) may be used as a relative measure of the difference in TCR affinities between T cells (39); as such, this may be a useful assay to assess the 'structural avidity' of a T cell population.

5.11 Functional analysis

Besides the combination of tetramer staining and intra-cellular cytokine measurement, a number of assays which combine tetramer staining with a functional readout have been developed. These include staining for cytolytic granules and direct ex vivo testing for cytolytic activity after sorting. A novel flow cytometric method was recently developed (26, 40) which involves the quantification of the surface mobilization of CD107 – an integral membrane protein within cytolytic granules of cytotoxic T cells – as

a marker for degranulation upon tumor stimulation. Mobilization of CD107 selectively identified T cells that were tumor-cytolytic. CD107 mobilization could be combined with peptide/MHC tetramer staining to directly correlate antigen-specificity and cytolytic ability on a single-cell level.

5.12 In situ hybridization

There is significant interest in the use of tetramers for in situ hybridization to detect antigen-specific T cells within tissue sections. While several groups have achieved success (41, 42), this is a technically challenging procedure which requires extensive optimization of staining conditions. As the TCR-pMHC interaction is of low affinity, any sample processing which may alter the conformation of TCRs on T cells – which may occur upon fixation of tissue specimens – could dramatically impact tetramer staining. Hence, most successful in situ tetramer staining have been achieved using fresh or frozen tissue sections, although some success has also been achieved with 'lightly fixed' tissue samples (41).

6. TETRAMER STAINING AND THE IMPORTANCE OF "RECOGNITION EFFICIENCY" IN THE CONTEXT OF TUMOR IMMUNOLOGY

The ability of different peptide-specific T cells to respond to different amounts of peptides is a reflection of their 'functional avidity' or 'recognition efficiency'. Recognition efficiency (RE) of a T cell reflects the cumulative effects of the intrinsic affinity of its TCRs for their cognate peptide displayed on the target cell, expression levels of TCR, CD4/8, and adhesion molecules, as well as redistribution of these molecules on the cell membrane and their recruitment efficacy of the signaling cascade (32-34). Identification of peptide-specific T cells using pMHC tetramers, ELISPOT, or CFC does not distinguish between cells of high or low recognition efficiency – at least not without careful reagent titration on each sample (Figure 5).

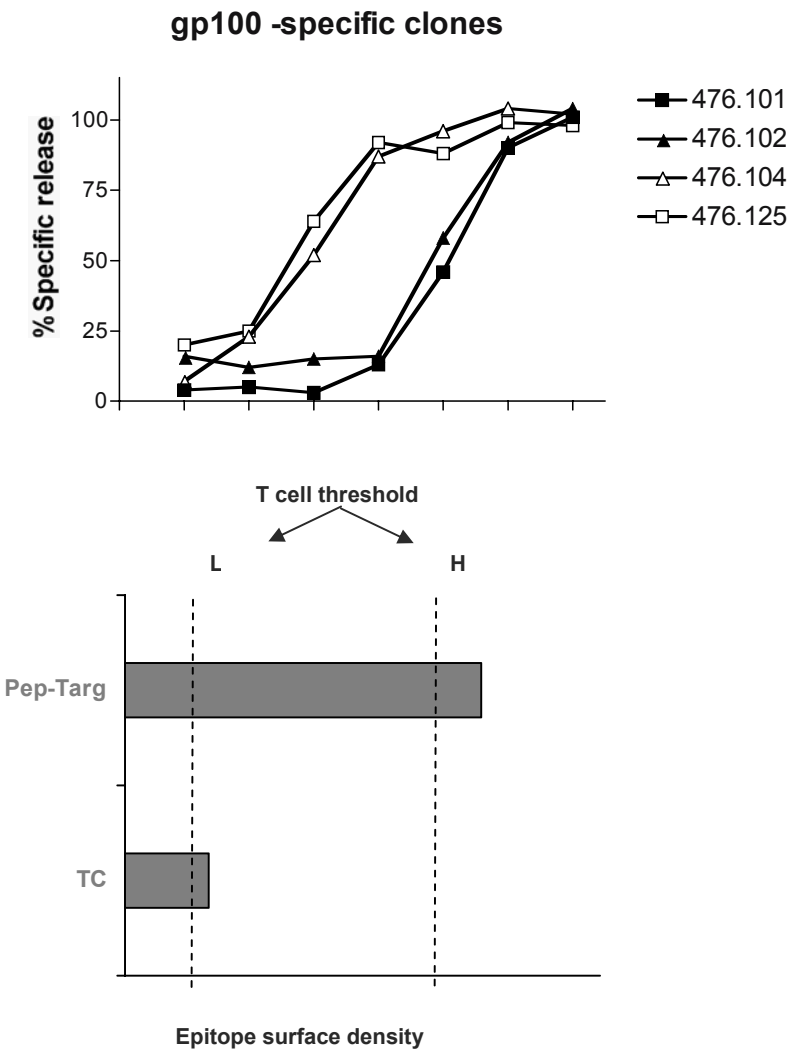

Figure 5. Significance of recognition efficiency (RE) of T cell responses. A) Four gp100-specific T cell clones (generated based on equivalent tetramer staining) were assayed against a wide titration of peptide concentrations. Two clones (476.104 and 476.125, high RE) required much lower (~100 fold) amounts of peptides for efficient lysis of T2 targets than the other two clones (476.101 and 476.102, low RE). B) As high RE T cells require much lower amounts of cognate peptide on a target cell for efficient lysis, they can lyse both peptide-pulsed and tumor targets. In contrast, low RE T cells require much higher amounts of cognate peptide on a target cell for efficient lysis, and therefore could lyse only peptide-pulsed but not tumor targets.

While previous investigations have attempted to use the intensity of tetramer staining as a measure for binding strength (35, 36), recent evidence suggests that tetramer staining intensity alone does not directly correlate with recognition efficiency (29, 30, 43). Nonetheless, the rate of dissociation of bound pMHC tetramers from antigen-specific T cells via the addition of a competing antibody (such as anti-TCR) may be used as a relative measure of the difference in TCR affinities between clones; as such, this may be a useful assay to assess the 'structural avidity' of a T cell population (39).

7. A NOVEL METHOD TO IDENTIFY TUMOR-REACTIVE T CELLS: CD107 MOBILIZATION

We developed a novel method to isolate pure, viable populations of tumor-cytolytic T cells directly ex vivo from patient blood samples using flow cytometric quantification of the surface mobilization of CD107 – an integral membrane protein within cytolytic granules – as a marker for degranulation upon tumor stimulation (26). We showed that tumor-cytolytic T cells are indeed elicited in patients post-cancer vaccination, and that tumor-reactivity is strongly correlated with recognition efficiency of the T cells for peptide-bearing targets. Combining CD107 mobilization with pMHC tetramer staining, we directly correlated antigen-specificity and cytolytic ability on a single-cell level to show that high recognition efficiency, tumor-cytolytic T cells represent only a minority of peptide-specific T cells elicited in patients after heteroclitic peptide vaccination (Figure 6). We have also shown that even high recognition efficiency, tumor-reactive T cells could be anergized in vivo – an important immune evasion mechanism for tumor cells.

A key advantage of the CD107 technique is the ability to detect tumor-reactive CD8+ T cells without knowing the peptide-MHC target. Since the assay measures T cells which degranulate in response to tumor cells, there is no *a priori* need to know the actual peptide target which would be required for most current assays. This is an important advantage since only a small number of TAAs have been identified to-date, mostly in the setting of melanoma. This technique may also be useful for immune monitoring of clinical trials involving vaccination with whole tumor cells, tumor-APC fusions, APCs pulsed with tumor lysates or transfected with tumor RNA, or other novel immunotherapeutic strategies in which the exact peptide targets are undefined. In such instances, the same cells used for vaccination could be used as stimulators in the immune monitoring assay to reveal tumor-reactive, cytolytic T cells.

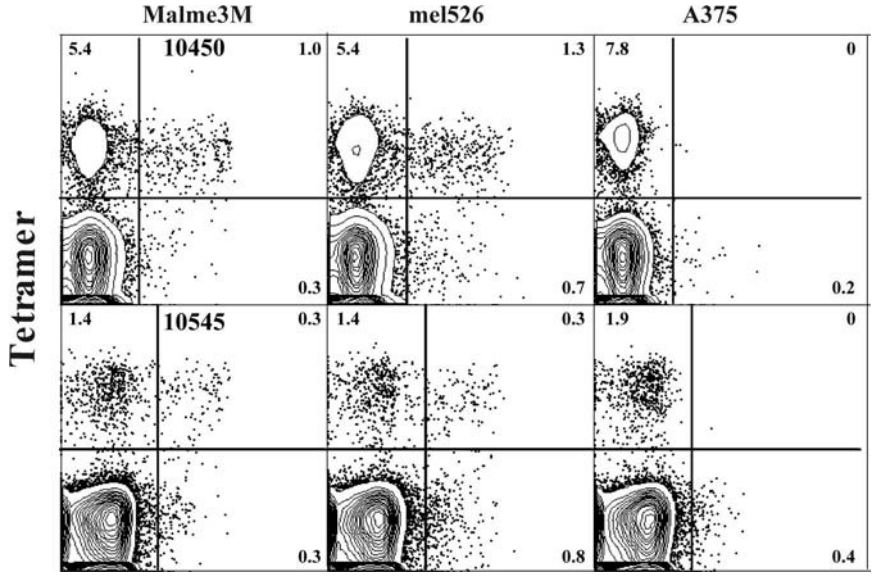

Figure 6. Tetramer staining and CD107 mobilization. Tetramer staining may be combined with CD107 mobilization to identify peptide-specific T cells which do or do not degranulate in response to tumor stimulation. Reprinted with permission from Nature Medicine.

8. USE IN CLINICAL TRIALS AND FURTURE PROSPECTS

Considerable experience has been accumulated over the past five years on the use of tetramers to analyze patient samples in clinical trials, both in viral infections (14, 44) and cancer (13, 45, 46). A good deal of knowledge has been derived on the size, kinetics, and biology of these T cell responses. However, certain hurdles prevent the widespread use of tetramers in the clinical setting. In cancer vaccination, peptide-specific T cell responses as detected by tetramers have largely not correlated with clinical outcome (11, 45, 46). However, this issue is not unique to tetramer analysis, but applies to all methods of detecting antigen-specific T cell responses, including ELISPOT and CFC. It is becoming clear that peptide-specificity does not necessarily guarantee tumor-reactivity of a T cell response – recognition efficiency of the T cells is a key factor (26). Furthermore, T cells may be rendered non-functional or anergic in vivo, either as a consequence of direct contact with tumor cells or as part of a global immunosuppressive state (12). Lastly, T cells may not home properly to tumor sites, and if so, may not function optimally due to the tumor microenvironment being hostile to

lymphocytes. These issues illustrate clearly that one cannot simply rely on enumeration of antigen-specific T cells to predict clinical outcome in clinical trials. It is critical to be able to study the biology of such cells in terms of recognition efficiency, in vivo functional status, and homing patterns. No currently available method can address all of these issues. It is likely that the immune assays in the future will involve identification, enumeration, phenotypic, functional, kinetic, and gene expression analyses of T cell responses. Tetramers will likely play a prominent role in many of these assays.

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Chapter 12

IN SITU MHC TETRAMER STAINING

In situ tetramers

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Abstract: With the onset of MHC tetramer technology came a wealth of new understanding of antigen specific $CD8^+$ T cells. This chapter discusses the application of MHC tetramer technology to stain antigen specific T cells in tissue sections. In situ tetramer staining (IST) can be used to determine the localization, abundance, and phenotype of antigen specific T cells in their native environment. IST can be used to stain essentially any antigen specific T cell in any tissue for which MHC tetramers are available. In this chapter, an overview of the technique is described including advantages and disadvantages of using thick fresh sections vs. thin frozen sections, and using direct labeling vs. indirect labeling. A summary of experimental systems that have employed IST to gain understanding of antigen specific CD8+ T cells is provided, including some interesting biology that has been revealed from these studies. Finally, the prospects for using IST to evaluate cancer specific T cells in cancer patients undergoing vaccine therapy are discussed.

Key words: In situ tetramer staining (IST), MHC class I tetramers, CD8+ T cells, vibratome, confocal microscopy

1. INRODUCTION

Antigen specific $CD8^+$ T cells are required for clearance of several cancers and most viral infections and are therefore an important cell type to understand. The use of MHC-tetramers to stain antigen specific T cells has revolutionized the study of T cells and has led to increased understanding of how these important cells work (1). A few years ago, methodologies were developed to use MHC tetramers to stain antigen specific T cells in tissue sections (2-4). This technique is called in situ tetramer staining (IST). Where flow cytometric analysis of tetramer stained cells allows for the rapid

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quantification of antigen specific cells, IST allows for the visualization of antigen specific T cells in their native environment, thus allowing the study of antigen specific T cells in 3-dimensional space and maintaining the relationship to surrounding cells within a tissue. Combining immunohistochemistry with IST allows for phenotypic characterization of antigen specific T cells and surrounding cells, including target cells, in tissues. In instances when tissue quantities are limited, such as tissue biopsies in which infiltrating cells are limited and not sufficient for flow cytometric analysis, IST is an attractive alternative. IST can be applied to essentially any tissue from any species to label any antigen specific T cell for which MHC-tetramer reagents are available. IST is a valuable tool to evaluate the localization and phenotype of cancer specific CTLs in tissues from cancer patients undergoing vaccine therapy.

2. IN SITU TETRAMER STAINING (IST)

IST involves applying MHC tetramers to fresh or frozen tissue sections (3, 5). IST with MHC class I tetramers is less effective or ineffective with fixed tissues (3, 5). IST appears to require mobility of the TCR on the surface of the T cells in order to gain avidity obtained by one MHC tetramer binding more than one TCR on the surface of the T cell. The use of peptide-loaded MHC class I molecules conjugated to dextran, referred to as MHC-multimers, has been described to stain antigen specific T cells in air dried acetone fixed tissue sections (6). Presumably MHC-multimers have enough range to bind multiple TCRs and obtain needed avidity regardless of TCR mobility.

Fresh tissue sections cut with a vibratome or scalpel offer several advantages over thin frozen sections for IST. First, the resultant tetramer and antibody stain is more crisp and has higher resolution. Second, thin sections are a single plane of one cell layer whereas thick sections contain many layers of cells. Because thick sections inherently contain more layers of cells, one can use a confocal microscope to examine antigen specific T cells deep into cellular layers and study interactions in 3-dimensional space. The third dimension is lost in thin sections. Nonetheless, frozen sections offer the great advantage over fresh tissue sections in that tissues can be collected, stored, and processed when convenient. Also, cutting tissue that is frozen is much easier than cutting fresh tissue.

IST can be performed in either a direct or indirect fashion. Direct labeling involves using tetramers coupled to a very bright fluorophore such as APC or by using MHC-dextran multimers (3, 6). Indirect labeling involves using tetramers coupled to a fluorophore such as FITC, where the

fluorophore serves as an epitope for further amplification of the signal with antibodies. In the case of FITC labeled tetramers, the signal is amplified using anti-FITC antibodies, e.g. rabbit anti-FITC, followed by additional amplification with fluorophore conjugated antibodies directed against the FITC antibodies, e.g. goat-anti-rabbit-Cy3 (5). Indirect labeling with horseradish peroxidase (HRP) labeled tetramers followed by tyramide signal amplification has also been described (7). There are advantages and disadvantages to either method. Advantages of direct IST include a simpler protocol, lower background staining, and since no antibodies are used to label the TCR, more options for co-labeling other proteins. Disadvantages of direct labeling include relatively weak intensity staining of T cells and increased costs associated with using higher concentrations of expensive MHC-tetramer reagents. Indirect labeling, in contrast, produces a very bright staining of T cells and requires smaller quantities of tetramer reagents.

Combining IST with immunohistochemistry or histochemistry allows visualization of antigen specific T cells in conjunction with other cellular markers. When using IST with anti-CD8 antibodies, care must be taken in choosing an antibody that does not interfere with tetramer binding (5, 8).

3. IST IN A NUMBER OF EXPERIMENTAL SETTINGS

IST has been used in a number of experimental systems to gain insights into antigen-specific $CD8^+$ T cells *in vivo*. The technique was developed in experimental TCR transgenic mice, and was verified by staining T cells from TCR transgenic mice adoptively transferred into non-transgenic mice[3,5]. Since this time IST has been applied to many experimental systems to gain insights into antigen specific $CD8^+$ T cell localization and function *in vivo*.

The abundance and localization of influenza A virus specific $CD8^+$ T cells in lymph nodes and lung tissue was studied in influenza A infected mice [3]. IST was used to characterize LCM virus specific CTLs in peripheral lymphoid and brain tissues in mice (9), and used to characterize simian immunodeficiency virus (SIV) specific T cells in lymph nodes, spleen, vagina, cervix, and rectum, colon, ileum and jejunum in SIV infected macaques (10-13; Skinner et al., unpublished data).

IST has also been used to study CTLs in graft vs. host disease. Using a human skin explant assay, Dickinson et al., demonstrated the significance of ubiquitously expressed minor histocompatibility antigens (mHags) in graft vs. host disease and visualized the migration pattern of mHags specific T cells in skin using IST (14). In a mouse model of graft vs. host disease, Yang

et al., used IST to demonstrate chronic graft rejection associated with infiltrating T cells specific for mHags (7).

Most pertinent to readers of this book, IST has been used to study CTLs directed against cancer antigens. Using a mouse tumor model, Haanen et al. used IST to show that tumor rejection was associated with the infiltration of tumor specific T cells. In human melanoma, T cells specific for the tumor antigen survivin were readily detected in tissue sections from the primary tumor and a sentinel lymph node isolated from a melanoma patient (6). Similarly, in tissues from a breast cancer patient, survivin specific T cells were readily detectable in situ. Importantly, these cells were shown to have functional cytotoxic activity when assayed *ex vivo* (6).

Taken together, these data show that IST can be used to stain antigen specific T cells in essentially any tissue for which MHC tetramers are available. An example of MHC tetramer staining is shown in Figure 1.

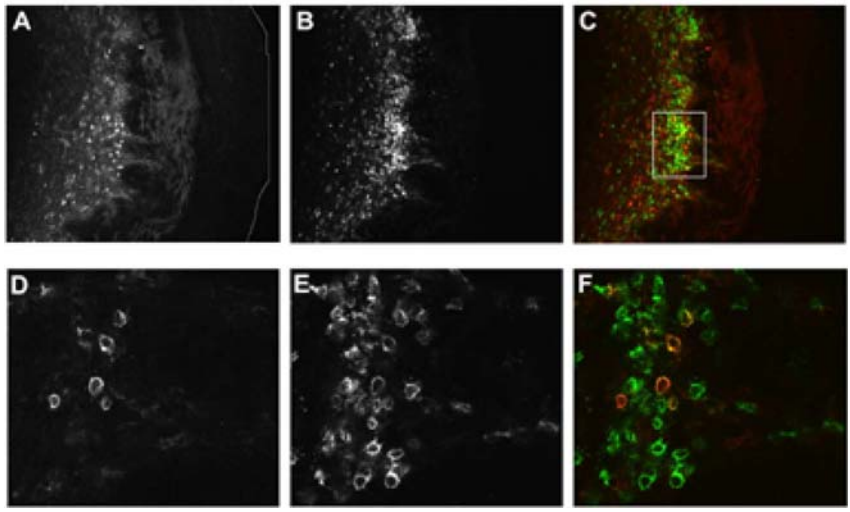

Figure 1. MHC tetramer and CD8 antibody staining of SIV specific T cells in ectocervix. A) and D) show Mamu-A*01/gag tetramer stain. B) and E) show CD8 antibody stain. C) and F) show a merged image of A) with B), and D) with E), respectively. In the merged images, tetramers are colored red and CD8 colored green. The white line drawn in panel A) delineates the epithelial surface. Please also see Color Plate Section page 305.

IST is not limited to MHC class I tetramers and multimers. Recently, Stratmann et al., made MHC class II multimeric reagents and used them in a mouse model of diabetes to stain autoreactive T cells in lymph node and pancreatic islet tissue sections (15).

When visualized with relatively high magnification, IST stained sections show a punctate staining of the TCR on the surface of T cells (3, 5). An

example of punctate membrane staining is depicted in Figure 2. When tetramer staining is performed at 37°C an increase in punctate staining and increase in overall staining intensity is observed (3). Haanen et al., suggest that the observed punctate TCR is due to multivalent MHC tetramers inducing self association of the TCRs on the T cell surface. However, it is possible that punctate staining seen from IST performed at 4° reflects a clustering of TCRs that was present at the time of staining. In fact, data from McGavern et al., indicate the when IST staining is performed at 4°, the detection of punctate TCR localization is a reflection of the innate localization of the TCR at the time of staining. They observed a rather homogenous distribution of the TCR naïve $CD8^+$ T cells and a punctate distribution of the TCR on activated $CD8^+$ T cells. They further showed that when the antigen-specific T cells were in contact with their target cell, quite frequently the tetramer stain was limited in the cell membrane to the site of interaction with the target cell membrane. It is known that after engagement of the TCR of a T cells, there is aggregation of TCR in lipid rafts and the formation of a immunological synapse (16, 17). McGavern et al., suggest that the punctate TCR staining represents cells such events. These results taken together suggest that when IST is performed at 4° it can be used to observe important biological events in tissues including the clustering of TCRs on the T cell surface, and polarization of the TCR when in contact with a target cell, but one needs to keep in mind that tetramers will effect the localization and abundance TCR when incubations are carried out at 37°.

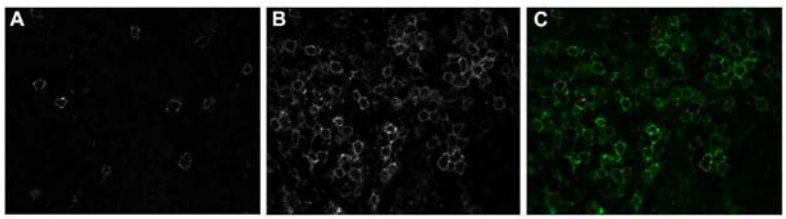

Figure 2. MHC tetramer and CD8 antibody staining of SIV specific T cells in lymph node showing a punctate tetramer staining of the T cell membrane. A) Shows Mamu A*01/gag tetramers. B) Shows CD8 antibody stain. C) Shows a merged image of A) and B) with tetramers colored red and CD8 antibodies green. See also Color Plates Section page 305.

McGavern et al. also observed individual tetramer stained cells in contact with two or three target cells. These observations suggest that an individual CTL is capable of killing multiple target cells at once. This is consistent with live cell imaging studies that showed that CTLs can kill multiple target cells in relatively short periods of time (18-20).

4. IST TO MONITOR T CELL DIRECTED VACCINE TRIALS IN CANCER PATIENTS

IST is a useful tool to evaluate the induction, localization, and phenotype of antigen specific CD8+ T cells in tissues after T cell directed vaccination of cancer patients.

Schrama et al. characterized the inoculation site of dendritic cell (DC) vaccination in patients with melanoma. They determined that antigen pulsed DC, but not unpulsed DC recruited and induced the local expansion of melanoma specific T cells at the inoculation site. They used MHC-multimers loaded with melanoma antigens MART, MAGE3, and gp100 and stained skin biopsies from vaccinated patients to demonstrate the specificity of the recruited and expanded T cells at the inoculation site.

As discussed above, T cells specific for the tumor antigen survivin taken from melanoma and breast cancer tumors were shown to be cytolytic *ex vivo* (6). In contrast, recent studies of T cells specific for the MART tumor epitope showed that the tumor specific T cells had effector function in circulating blood but were functionally tolerant in tumor lesions (21). Findings such as these demonstrate that the functional status of T cells in blood does not always reflect the functional status in tissues and stresses the importance of in situ analysis of T cells in tissues.

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Chapter 13

MHC-IG DIMERIC MOLECULES

Dimers–MHC-Ig dimeric molecules for the analysis of antigen-specific T cell responses

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Abstract: Enumeration and analysis of antigen-specific T cells play a central role in tumor immunology. Recently a number of different methods have been developed to analyze antigen-specific T cell responses. MHC-Ig dimeric molecules were developed for detection and analysis of antigen specific T cells. Using, Ig as a molecular scaffold, dimeric MHC molecules bind stably to the corresponding T cells due to their increased avidity for the T cell receptor and can be used to identify peptide specific T cells by flow cytometry. In addition, this technique can be combined with functional assays analyzing peptide specific cytokine release and functional state of the T cells. Finally MHC-Ig dimers have been used in a variety of experiments to stimulate and amplify antigen specific T cells.

Key words: Dimers, MHC molecules

1. CONSTRUCTION OF MHC-IG CHIMERIC MOLECULES

Using immunoglobulin as a molecular scaffold, MHC-Ig dimeric molecules can be formed by fusion of the extra cellular domain of the MHC molecule with the constant region of an immunoglobulin heavy chain (Figure 1) (1). The characterization and cloning of the first murine MHC-Ig molecule was described in 1993. In 1998, our group cloned the first human MHC-Ig dimer and successfully used this molecule for the analysis of peptide specific T cells (2).

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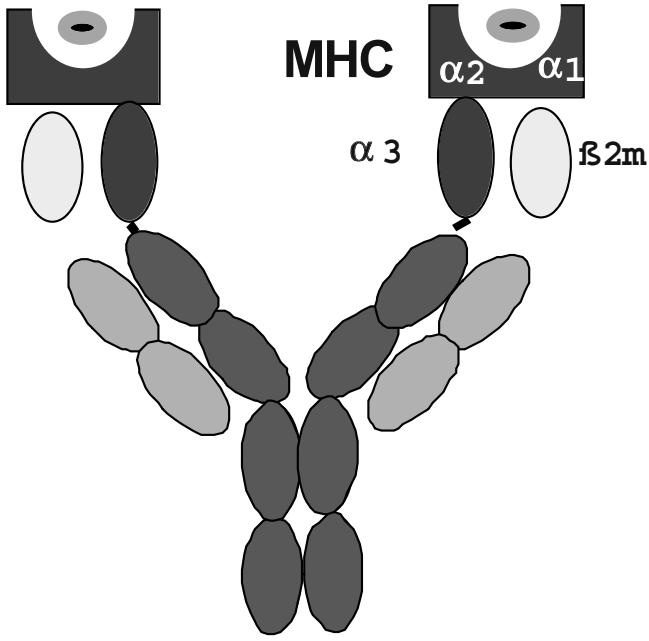

Figure 1. Scheme of MHC-Ig dimmer.

Figure 2 (see below) shows the detailed map of the expression plasmid, which is being used to produce MHC-Ig dimeric molecule. The IgG1 leader sequence is joined to the first four amino acids of the IgG1 heavy chain, followed by a His-Ser-Ala spacer and the extracellular (alpha-1 – alpha-3) domain for the MHC cDNA. The extracellular domain is joined on its 3' end to the intact variable region and the Ig constant region separated by a four amino acid linker. The plasmid coding for this sequence carries an immunoglobulin promotor, with an Ig enhancer element and the neomycin gene. This vector allows for high and stable expression of the MHC-immunoglobulin fusion construct in eukaryotic cells.

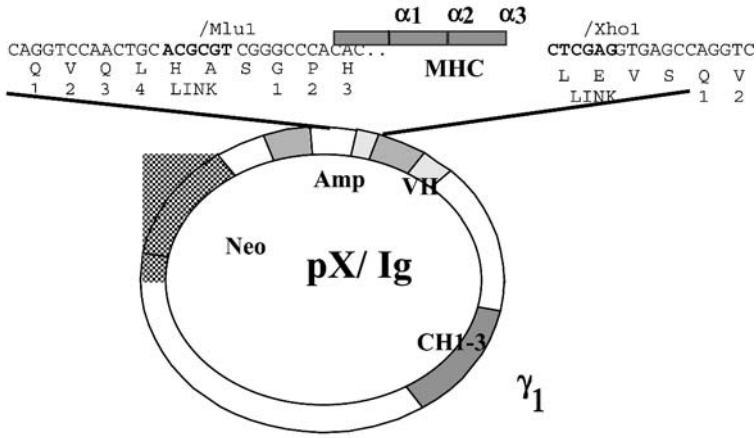

Figure 2. pXIg Expression plasmid.

2. PRODUCTION OF MHC-IG CHIMERIC MOLECULE

For production MHC-Ig chimeric molecule, the pXIg plasmid (Figure 2) is transfected into J558L cells. This cell line is derived from the murine plasmocytoma cell line J558, which is originally derived from an IgA producing plasmocytoma cell line (3). The cell line contains the complete immunoglobulin production machinery including a J chain. The special feature of J558L cells is, that this cell line does not produce an endogenous heavy chain, but a λ-light chain. Therefore, any Ig fusion protein, which is expressed in this cell line, contains a λ-light chain. This facilitates production and detection of recombinant Ig fusion proteins, which can easily be detected in a sandwich ELISA using a goat anti-mouse antibody coated to the plate and a mouse λ light chain-specific labeled antibody to detect captured dimer on the plate. MHC-Ig dimers are produced in pXIg transfected J558L cells under antibiotic selection. The MHC-Ig is secreted into the cell supernatant from which it can be purified. The producer cells are grown in complete media containing FCS, but it is also possible to use commercially available serum free media, which is routinely used for the production of monoclonal antibodies and to grow the cells in regular cell culture flasks or in roller bottles, if higher volumes of MHC-Ig containing supernatants are needed. Similar to hybridoma cells, MHC-Ig producing J558L cells can also be maintained in special hybridoma flasks such as

Integra® cell line in order to increases the MHC-Ig concentration in the cell supernatant.

Although it is possible to use the MHC-Ig dimer directly from the cell supernatant, we recommend purification of the protein first in order to enhance fluorescence intensity in flow cytometry. Different methods are available for the purification of the MHC-Ig chimeric protein. However, it should be emphasized that special attention is needed in order to keep the MHC portion of the dimeric protein in its native confirmation and intact. If the MHC-Ig molecule is produced under serum free conditions, it is possible to concentrate this supernatant using commercially available protein concentrators. However, this procedure generally leads to purities of maximum ~50%. The pXIg vector contains an immunoglobulin variable domain specific for 4-hydroxy-3-nitophenylacetyl (NP), which facilitates purification of the protein using 5-iodo-NP-sepahorse. This affinity purification procedure avoids acid or base elution, which could potentially have deleterious effects on the conformation of the intact MHC-Ig complex in contrast to purification of MHC-Ig dimeric molecules on protein A columns, the routine procedure for the purification of monoclonal antibodies from hybridoma cell lines, which also results in high purity protein preparations.

Table 1: Purification Methods.

Purification method	Advantages	Disadvantages
Concentration	Low purity	Easy, high yield
NIP-sepharose	High purity, no denaturation	Technically more difficult
Protein A/G	High purity	Low yield

Loading the dimer with the peptide of interest

MHC-Ig dimeric molecules are produced, harvested and purified from supernatant of transfected J558L cells, thus loaded with endogenous peptides. Therefore, before the dimer can be used for detection of peptide specific T cells, the endogenously loaded peptides have to be replaced by the peptides of interest in order to generate MHC-Ig dimeric molecules, which specifically bind to the T cells of interest. Different methods have been successfully developed for this process. Passive exchange using high molar excess of the peptide of interest or mild peptide elution from the MHC followed by reloading with the peptide of interest and complete renaturation of the MHC are such examples (4).

Passive loading of the peptide, which is being added in high molar excess in the presence of β2-microglobulin, is the easiest and most convenient method to load the MHC-Ig dimer with the peptide of interest. We have

noticed that this process might take up to several days, but the peptide loaded MHC-Ig dimer stays stable for at least 2 to 3 weeks when kept at 4°C. Until today, this procedure probably provides the best method if T cells specific for several different peptides restricted against the same MHC should be analyzed. Since the MHC-Ig dimer can be loaded in parallel with a number of different peptides, this clearly demonstrates the main advantage of this reagent, a scenario where T cell responses against different peptides can be analyzed in parallel. However, since it has been observed that peptides load onto the MHC with different efficiencies, alternative methods for efficient loading of peptide onto the MHC molecule should be explored.

Peptides can also be stripped from MHC molecules by mild acidic elution. We have successfully used this method to strip off endogenous peptides from MHC-Ig dimers. The MHC complex will refold in the presence of the target peptide, resulting in MHC-Ig dimer loaded with the peptide of interest. Since every MHC molecule responds differently to stripping procedures, this method has to be tested for every new MHC molecule and can differ from one MHC to another. Finally, in order to enhance loading efficiency with the peptide of interest, we have recently shown, that the peptide of interest can also be covalently bound to the MHC-Ig dimer (Figure 3) (5). While a new construct has to be cloned for every peptide-MHC-Ig dimer, this molecule has the advantage that every MHC molecule is loaded with the peptide of interest. For this purpose, the peptide is fused to the 5' end of β2-microglobulin cDNA. The β2-microglobulin cDNA is fused at its 3' end to the alpha chain of the MHC molecule, which is separated by a third spacer from the Ig heavy chain sequence. Therefore, the peptide, β2-microglobulin, the extracellular domain of HLA-A2 and the Ig heavy chain are connected to each other on one chain. This molecule does not only allow for optimal loading and therefore good staining properties, but more importantly can be used for both *in vitro* and *in vivo* applications (5, 6).

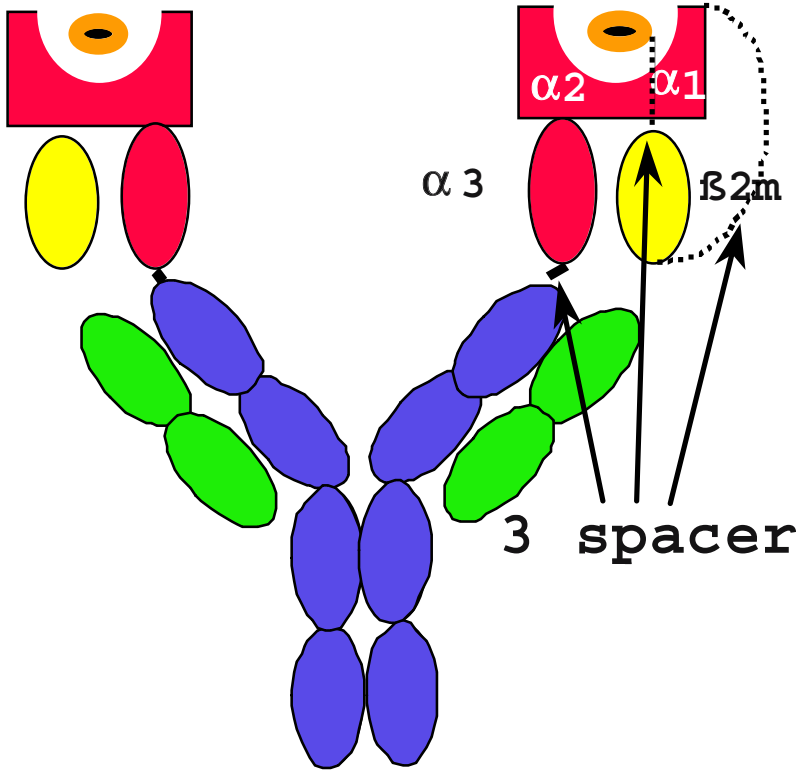

Figure 3. Scheme of Single Chain-IgG Dimer.

3. MHC CLASS II DIMERIC MOLECULES

Despite the emphasis on CD8+ T cells in immune responses, CD4+ T cells have been shown to be as critical in inducing an effective immune response against tumors, as well as in infectious and autoimmune diseases. Until today, analysis of CD4+ T cell responses using multimeric MHC molecules is hampered by the lack of suitable reagents. It is important to remember that it took more than two years from the first study showing the use of multimeric MHC molecules for detection of antigen specific T cells in 1996 until tetrameric MHC and MHC dimers became available for the analysis of T cell responses in different settings. Different groups including ours have come to realize that the technology for making tetravalent or dimeric MHC class I constructs cannot be simply transferred to the

development of multivalent MHC class II constructs. This might be due to several factors. In contrast to MHC class I molecule, MHC class II molecule consists of two polypeptides each of which have transmembrane domains. These transmembrane domains are important in facilitating pairing and hence proper folding of the α and β polypeptide chains. Therefore, MHC class II fusion constructs lacking the transmembrane domain are unstable and misfold easily. Therefore, one approach has been to introduce a leucine zipper dimerization motif, which allows the assembly and secretion of soluble MHC class II heterodimers (7). In addition, MHC class II dimers are usually expressed in bacculovirus systems (8, 9). Therefore, production yields are much lower compared with the eukaryotic J558L system. Despite the technical limitations in production of MHC class II dimers, there have been a number of studies, which have used these reagents to stimulate and stain CD4+ T cells *in vitro* (10-13). Finally, one group has recently described the production of empty MHC-class II dimers in Drosophila S2 cells. This group was able to successfully load the molecules with the peptide of interest and use them for staining of T cell by flow cytometry as well as for stimulation of T cells (14).

4. DETECTION OF ANTIGEN-SPECIFIC T CELLS

A number of studies have used MHC-Ig dimers to detect peptide-specific T cells in different clinical and experimental settings in mouse and man. We used MHC-Ig dimeric molecule for the first time to track HTLV-1 specific T cells in a viral disease setting, HTLV-1 associated myelopathy tetraspastic paresis (HAM/TSP) (2). To our surprise, we were able to detect high frequencies of peptide-specific T cells not only in peripheral blood from these patients, but also in their cerebrospinal fluid demonstrating the wide application of MHC-Ig dimers for the detection of peptide-specific T cells. Moreover, this study was also one of the first, which combined the use of multimeric MHC molecules and the simultaneous analysis of intracellular cytokine production by FACS analysis.

In subsequent studies, MHC-Ig dimers were used for the analysis of peptide-specific T cells in other viral settings as well as in cancer patients. HLA-A2 dimers were used for detection of viral specific T cells (HTLV-1, HIV, CMV) directly *ex vivo* and in *in vitro* studies (5, 15-18) as well as p53 specific T cells (19). Others and we have also been able to detect tumor specific T cells using HLA-A2 dimers. In a recent study, we have analyzed NY-ESO-1 specific T cell responses in patients with primary liver cancer. Previous studies suggested that NY-ESO-1, a member of the cancer testis antigen family, is expressed in a subset of hepatocellular carcinomas. We

decided to analyze NY-ESO-1 specific immune response in these patients in more detail using the HLA-A2 dimer among other types of analyses. After one *in vitro* stimulation of PBMC from HCC patients, we could clearly identify NY-ESO-1 specific CD8+ T cells in the peptide stimulated cell population using the HLA-A2 dimeric molecule and confirmed these results using functional assays (Korangy et al, manuscript submitted). Others have used HLA-A2 dimers to not only track but also stimulate CAP-1 or gp100 specific T cells (20-22).

Slansky and coworkers have used a murine MHC-Ig dimer, L^d-IgG to track tumor specific T cells specific for a murine colon carcinoma cell line CT26 (23). This study also demonstrated a number of other applications for the dimeric MHC molecule. L^d-Ig was loaded with the AH-1 peptide (the immunodominant peptide from CT26 murine colon carcinoma (24) as well as with a number of AH-1 peptide derivatives, which differed only by one amino acid in order to screen for peptides with higher affinity for a certain TCR. This study identified derivatives of the AH-1 peptide that clearly induced a stronger response than the original peptide in vaccinated mice. Using a similar approach, new HLA-A2 restricted CMV pp65 epitopes with an increased activity have been identified (18). Finally, the murine D^b-Ig-dimer was used in another study to stain different LCMV specific T cells after infection of mice with heterologous LCM viruses (25).

5. IN VITRO AND IN VIVO APPLICATION

Multimeric MHC molecules have opened a new field not only for the analysis of antigen-specific T cells, but also to use in other applications such as expansion and isolation of antigen-specific T cells for adoptive T cell therapy. Schneck and coworkers have recently demonstrated that MHC class I-Ig dimers coated onto beads together with anti-CD28 form very potent artificial antigen-presenting cells (26). Interestingly using MHC-Ig dimers they were able to amplify CMV and MART-1 specific T cells to cell numbers at least equal to what they obtained using dendritic cells as stimulators. These amplified T cells also lysed antigen expressing tumor cells specifically. Thus, MHC-Ig dimeric molecules have now become a powerful tool in the field of tumor immunology not only for the detection of antigen specific T cells before and after different vaccination protocols but also for adoptive T cell transfer therapy. In addition, a different study has demonstrated the use of MHC class I dimers for *in vitro* stimulation of gp100 specific T cells, when precursor frequencies are too low in peripheral blood to be detected without *in vitro* stimulation (21).

We have chosen in our laboratory a different approach to generate MHC-Ig dimeric molecules that are even more sensitive for the detection of peptide-specific T cells. We have generated peptide-β2-microglobulin-MHC-Ig single chain dimers (Figure 3), which have the advantage that every MHC is loaded with the correct peptide leading to a decrease in non-specific background staining. In addition, we have used the MHC-Ig single chain in combination with paramagnetic IgG binding beads, which only bind cells with an immunoglobulin molecule bound on their cell surface. This technology enhances the detection limit of antigen-specific T cells, allows for the enrichment of antigen-specific T cells from stimulated cultures and peripheral blood, as well as generation of T cell lines and clones (5).

Finally, MHC-Ig dimers have been used with recombinant T cell receptors bound onto plasmon resonance surface for binding studies (27). These experiments have helped in understanding peptide-MHC/T cell receptor interactions.

6. COMPARISON OF MHC-IG DIMERS WITH OTHER TECHNIQUES FOR THE DETECTION OF AG SPECIFIC T CELLS

Currently different methodologies for detection and analysis of antigen specific T cell responses are available. Functional assays depend on the secretion of different cytokines upon stimulation with the peptide of interest. Cytotoxicity assays in combination with serial limiting-dilution assays used to be the gold standard for analysis of antigen specific CD8+ T cell responses (28). However, these assays depend on the proliferation, lytic activity or cytokine secretion of the analyzed T cell population (29). In contrast, multimeric MHC molecules recognize specific T cells independently of their state of activation or ability to respond to an antigen. Functional assays can be combined with MHC-Ig dimers to determine the fraction of antigen specific T cells that respond to antigen upon stimulation. We were able to perform intracellular cytokine staining and MHC-Ig dimer analysis in parallel on PBMC from HTLV-1 infected individuals. Our results clearly demonstrated that not all dimer binding T cells respond to peptide stimulation with cytokine secretion suggesting, that these T cells might also be impaired in their *in vivo* function (2).

Currently two different multivalent MHC molecules, divalent MHC-Ig dimers and tetrameric MHC complexes are available. Others and we have used tetramers and dimers in parallel and observed similar results in flowcytometry (16, 25, 30). While some investigators have observed a slightly higher sensitivity for tetrameric MHC complexes, MHC-Ig dimers

have the advantage that they can easily be loaded with different peptides. This allows performing peptide-screening experiments, which would be more difficult using tetrameric MHC complexes, because in this case a new reagent has to be produced for every peptide tested.

7. FUTURE DIRECTIONS

Multivalent MHC molecules have originally been developed for the detection of peptide specific $CD8^+$ and $CD4^+$ T cells in different basic and clinical settings. However, recent studies suggest that these molecules can be used for a number of different applications. MHC-Ig dimers have been shown to be powerful tools for *in vitro* amplification of peptide specific T cells. These amplified T cells are now being tested for their *in vivo* function in pre clinical murine models and will hopefully soon find their way into first clinical trials. The generation of peptide-MHC-Ig single chain constructs has increased the specificity of these molecules. Future experiments will test these reagents in respect to their *in vivo* use. Initial murine *in vivo* studies using peptide loaded murine MHC-Ig dimers already suggest that these molecules can inhibit immune responses [14]. Further studies are needed to confirm these results. Finally the combination of MHC-Ig dimers with other reagents such as paramagnetic beads will enhance the sensitivity of detection. New protocols for the production of MHC class II dimers will hopefully not only allow for parallel analysis of antigen-specific CD4+ and CD8+ T cell responses, but also for amplification of antigen specific T cells *in vitro* and ultimately for their *in vivo* function.

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Chapter 14

TCR ANALYSES

T-cell receptor CDR3 analysis: Molecular fingerprinting of the T-cell receptor repertoire

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Abstract: T-cells play a crucial role in immune surveillance against transformed cells and intracellular infections; they are involved in auto-immune reactions. They recognize their targets, i.e. MHC / peptide complexes, trough the T-cell receptor. TCR usage determines the molecular interaction of the immune system with biologically relevant MHC/peptide molecules. The TCR coding genes (variable, diversity and junctional) determine the molecular composition of the TCR alpha and beta heterodimer. The random association of the VDJ genes constitutes the complementarity determining region 3 (CDR3) responsible for antigen recognition and TCR specificity. The molecular composition of a T-cell population can be objectively defined by measuring the CDR3 region. Qualitative and quantitative comparisons of the TCR composition in different anatomic compartments, or longitudinally over time, allow to asses the entire TCR repertoire. This methodology can be supplemented with functional T-cell based assays and aids to objectively describe any alteration in the T-cell pool. TCR CDR3 analysis is useful in immunomonitoring, e.g. examining patients after BMT or solid organ transplantation, patients with HAART therapy, or patients receiving molecularly defined vaccines.

Key words: TCR, CDR3

1. PURPOSE AND VALIDITY OF MOLECULAR T-CELL ANALYSIS

T-cell responses can be described by combining three categories: i) Specificity and effector functions of a T-cell population, ii) the quantity of

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T-cell responses (i.e. number of responding T-cells within the CD4/CD8 population) and iii) the 'molecular quality' of T-cells, the molecular structure defined by the T-cell receptor (TCR) structure.

Several methods to measure T-cell responses are now available including evaluation of T-cell precursors using limiting dilution, the ELISPOT assay, *ex vivo* T-cell receptor (TCR) variable (v) segment analysis determined by flow cytometry and TCR-CDR3 length analysis (spectratyping) as well as identification of peptide-specific T-cells using MHC class I or MHC class II tetramers containing appropriate peptides. T-cell responses can be molecularly defined by i) identification of monoclonal T-cell receptors which are known to bind to a defined peptide target presented by the appropriate MHC molecule, ii) gauging the T-cell population as mono-, oligo- or polyclonal for a defined T-cell receptor (TCR) family in the CD4 or CD8 T-cell population. These data aid to

1. define and compare magnitudes of CD4+ or CD8+ T-cell responses over time in individual patients (either as result of progression/regression of a certain disease, or alternatively, alterations of the T-cell pool associated with treatment)
2. compare the molecular composition of TCR αβ or TCR γδ T-cells over time or in different anatomic compartments, or
3. to compare the TCR VA/VB (or TCR VD/VG) diversity with normal healthy controls.

This 'molecular fingerprint' of the TCR-repertoire can be corrected by the qualitative assessment of individual TCR VB families. TCR fingerprinting objectively describes the TCR composition in a given sample. This purely descriptive measurement can be complemented using other functional methodologies:

1. TCR analysis in individual T-cell subpopulations defined by differentiation and homing markers, or T-cells defined by cytokine production in response to the antigen of interest.
2. T-cells with defined MHC/peptide-specificity determined by tetramer-based sorting of T-lymphocytes.

1.1 Background: TCR – nomenclature and definition

The TCR nomenclature has been adapted to the latest issue "The T cell receptor *FactsBook*" (1). Four types of genes are involved in TCR synthesis: the variable (V), diversity (D), and joining (J) genes which code for the antigen / MHC binding sites and the constant (c) genes which encode the non-variable TCR polypeptide chain.

TCR locus: A group of TCR genes that are ordered and organized in the same chromosomal location in a given (i.e. human) species. The human genome encodes four TCR loci:
TRA (14q11.2)
TRB (7q34)
TRG (7p14)
TRD (14q11.2) (embedded within the A locus, see above)

Group: A group of TCR genes that share the same gene type (i.e. V, D, J or C), a group may contain functional TCR and TCR pseudogenes.
Subgroup: Set of genes that belong to the same group and share at least 75% identity at the nucleotide level (in the germline configuration for V, D or J), e.g. the TCR VB families 7.1. or 7.2. This is also of practical relevance, since some primers implemented for TCR-CDR3 analysis may encompass several subgroups, other RT-PCR primers are needed to cover individual TCR V genes.

1.2 Creating T-cell receptor diversity

Defining the TCR binding region: T-cell receptor fingerprinting measures the length of the complementarity determining region 3 (CDR3) (Figure 1). In most cases, rearrangement of the TCR loci results from deletional joining. The V genes are localized upstream from the J (or D and C) genes.

Two genes, the recombination activating gene 1 and 2 (RAG1 and RAG2) were identified in 1989 and 1990. RAG1 and RAG2 are transcribed in lymphocytes that show recombinase activity (i.e. B or T-cells). These genes are – with few exceptions- only active in thymic tissue pertaining to T-lymphocytes (2, 3) Junctional diversity is responsible for creation of the TCR diversity associated with antigen recognition (i.e. MHC/peptide complexes). The N-region diversity results from the deletion of nucleotides at the extremities of the coding V, D and J genes by activation of an exonuclease and the random addition of nucleotides by the terminal deoxynucleotidyl transferase (TdT). The enzyme preferentially adds G and C nucleotides at the junctions and is specifically transcribed during lymphocytic maturation. This region is determined complementarity determining region 3 (CDR3). In brief, the specificity of a T-cell resides in the CDR3 region, the length of this regions defines the diversity of the TCR repertoire. The CDR3 region in humans measures approximately 11 amino acid residues (i.e. 33 base pairs) and shows a Gauss-distribution: the most abundant transcript measures approximately 6 amino acid residues (see Figure 1).

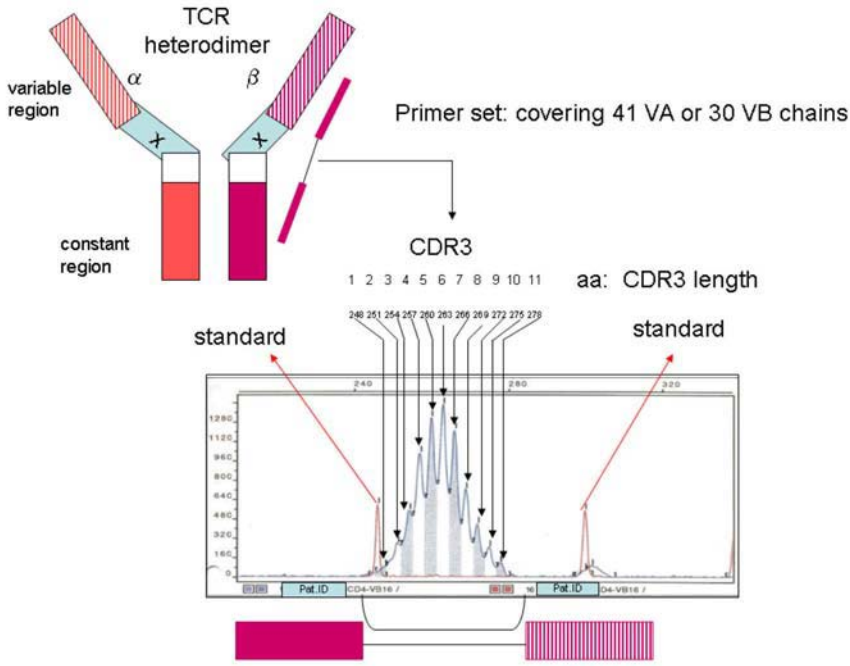

Figure 1. Determination of the TCR repertoire. A primer panel is used to amplify all possible TCR VB families using primers in the constant and in the respective variable region of the TCR. Example of the TCR CDR 3 analysis of the TCR VB16 family in CD4+ sorted T-cells. The major middle peak is 263 bp, there are 5 peaks on the left and right side respectively: a typical Gauss distribution of 11 aa coding for the CDR3 area. Note that each peak identifies 3 base pairs, coding for one amino acid residue. A single peak may suggest monoclonality. This has to be confirmed by DNA sequence analysis, since even a single peak (one amino acid residue) indicates that the length of the CDR3 region is 1 amino acid residue, however, different amino acid residues may be possible.

The CDR3 composition in a defined TCR VB "pool" may either be poly-, oligo- or monoclonal (see Figure 2). It is estimated that the theoretically possible TCR repertoire is not used in humans (4), but T-cells in human peripheral blood can carry up to 10^6 different TCR VB chains (5). Of note, most of the studies available today address the question of TCR diversity in the TCR VB chain, based on the presumption that each individual T-cell expresses only a single TCR VA chain paired with a single TCR VB chain. This is true for most T-lymphocytes in the peripheral circulation, although double TCR VB chains paired with a single TCR VA chain (and vice versa) have been reported (6, 7).

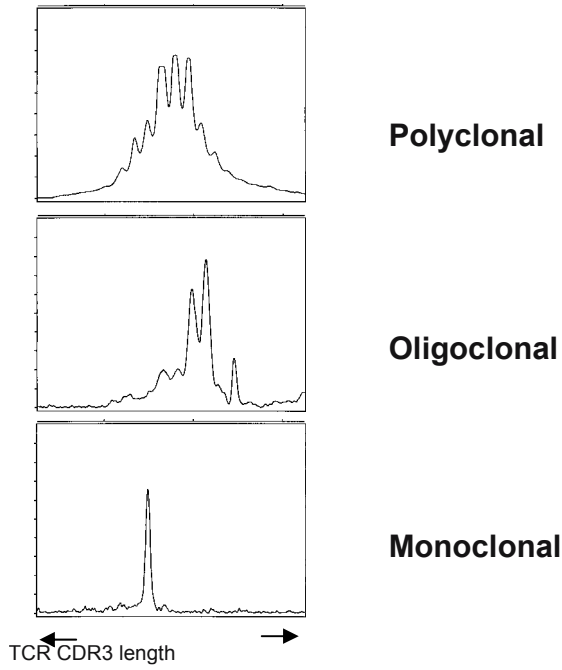

Figure 2. TCR CDR3 patterns aid to define the TCR composition.

Only a few studies addressed the molecular composition of gamma / delta T-cells which may also play a role in autoimmune disease, conferring resistance to infection or anti-tumor immune responses.

At this time, there is no indication that TCR genes can be somatically altered. TCR diversity is generated in the germline configuration and determines TCR specificity. Thus, the length of the TCR CDR3 regions describes in detail the TCR diversity identified by:

1. VB (VA/VG/VD) group or subgroup
2. Identification of the joining region
3. Measurement of the TCR CDR3 length
4. Molecular definition (sequencing) of the CDR3 region (if applicable)

2. METHODOLOGY

2.1 Determination of the TCR repertoire by DNA fragment analysis

Cells are counted (absolute cell count) and a CD4 and CD8 determination is carried out to estimate the number of CD4 and CD8- positive T-cells in the sample. Most studies are performed using entire peripheral blood T-cells (PBL), this may not allow to detect monoclonal T-cell populations in CD4+ or CD8+ T-cell subsets, since e.g. a monoclonal TCR in a single VB family in the CD4+ T-cell population may be 'masked' by a polyclonal TCR in the CD8+ T-cell population in the same sample. In addition, the 'true' number of T-cells should be determined by combining an absolute cell count in the sample combined with a flow-cytometric analysis of TCR alpha/beta – positive T-cells. Some treatments or diseases may be associated with a skewed composition of the T-cell population: In general, most applications in TCR fingerprinting measure the composition of the TCR beta chain (in TCR αβ+ T-cells). An expansion of γδ+ TCR T-cells can neither be picked up by a lymphocyte count, nor by measuring CD3+ T-cells, since both αβ TCR and γδ TCR+ cells are CD3-positive.

The determination of the cell number is crucial since i) the same cell number should be analyzed in longitudinal studies, ii) it is associated with the sensitivity of the TCR fingerprinting assay: Each laboratory which uses this methodology should run appropriate controls to ensure that each TCR V-family is identified using the appropriate primer panel. Figure 3 shows that using a suboptimal cell number leads to 'false-positive monoclonality' in individual TCR variable families. This may be associated with the primer panel used for amplification of each TCR family (sensitivity) and the number of TCR transcripts in each TCR family.

Different ways to standardize samples are possible:

1. Standardization of the cell number either in PBL or in sorted CD4 and CD8+ T-cells.
2. Standardization of mRNA transcripts in samples using 'house-keeping genes' or genes shared by all TCR families (e.g. measuring the TCR constant chain).

If T-cells are sorted into CD4 and CD8+ T-cells, at least 90% purity should be obtained for the CD8+ T-cell population, and more than 80% of the CD4+ T-cell population. CD4+ T-cells may also include CD4+ dendritic cells or macrophages. Each laboratory should assess sensitivity and specificity of the TCR – analysis: this is crucial in order to avoid false positive/negative results and to ensure a high standard in monitoring TCR transcripts longitudinally in clinical trials.

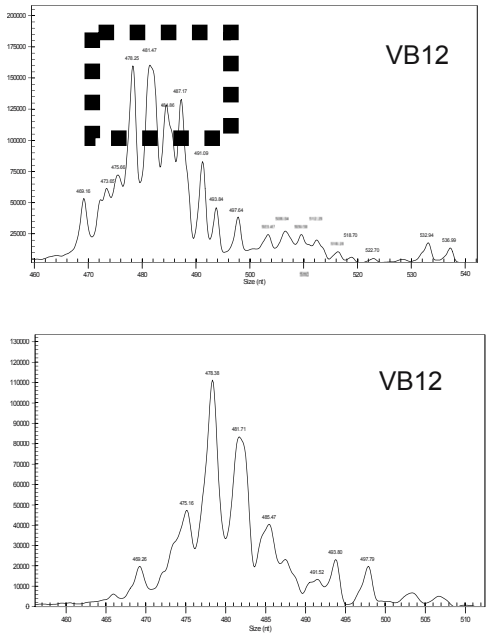

Figure 3. 'False monoclonality'. Each laboratory should assess sensitivity and specificity of the TCR CDR3 analysis. Different CD8+ T-cell numbers amplified with the same TCR VB primers (e.g. VB12) show that insufficient mRNA transcripts lead to the impression of a false 'oligo- or monoclonal' T-cell population.

2.2 Determination of VB families by flow cytometry: T-cell quantification

The TCR CDR3 analysis allows to determine the structural composition, but does not address the quantity of each TCR VB, i.e. the absolute number of T-cells examined for TCR diversity. Several ways to gauge T-cells are possible: first, protein expression of TCR determined by flow cytometry (8). Up to 80% of the entire T-cell repertoire can be covered using a panel of TCR-specific mAbs. Alternatively, mRNA transcripts can be measured for each TCR VB family (4, 9). Both methodologies are possible for cell suspensions, but are not applicable for tissue sections. Thus, if the TCR-repertoire in PBL is compared to tissues, e.g. tumor sections, or lymph nodes, the latter approach using the standardization for TCR mRNA has to be performed. In addition, TCR cell surface expression determined by flow-cytometry may provide a different result as compared to TCR mRNA analysis: Activated T-cells may downregulate their TCRs, but enhance TCR

mRNA transcription upon activation (10, 11). At this point, TCR VB families should be quantified using either flow-cytometry or by measuring TCR VB mRNA transcripts, future studies will show if a combination of both techniques will provide biologically meaningful data in the context of immunomonitoring.

2.3 Qualitative analysis of the TCR repertoire

Each individual TCR variable CDR3 profile obtained from a TCR V-family can be depicted as a function of the CDR3 length: Each peak represents three base pairs (bp) coding for one amino acid (aa) residue. In general, in human healthy subjects, 9-11 aa can be identified in each CDR3 profile. The area of the entire CDR3 analysis is estimated as 100% for each TCR VA or VB family and the area under the curve for each individual CDR3 peak is expressed as the percentage of the entire CDR3 area. Briefly, total RNA is extracted and reverse transcribed into cDNA. Each individual TCR family can be amplified using a common primer in the constant region of the TCR supplemented with a primer specific for each TCR family (Figure 1). TCR VB-specific primers have been predominantly implemented in TCR CDR3 fingerprinting, TCR VA primers have also been reported and used in clinical studies (12, 13). At this point, 41 individual TCR VA and 30 VB families have been described (1). Positive amplicons are subjected to an elongation reaction using a dye-labeled primer which covering the constant region of the TCR which allows to amplify all TCR VB (or alternatively VA) families. The exact length of each product can be determined using a DNA-sequencer with an appropriate internal bp length standard labeled with a different dye as compared to the primer used in the elongation reaction. For instance, polyclonal, oligoclonal or monoclonal TCRs can be determined (Figure 2). Monoclonality has be confirmed by DNA sequence analysis, since a single peak in a given TCR V family states only that this population shares an identical length of the CDR3 region. It may be possible that a single peak with a defined CDR3 length codes for different amino acid residues, thus ‘monoclonality’ has to be confirmed by DNA sequence analysis.

2.4 Gauging the difference

TCR-CDR3 fingerprinting allows a snapshot of the TCR composition at a given time point. However, a comparative approach is often useful in clinical contexts, e.g. for patients undergoing immunotherapy. Individual TCR VA or VB repertoires may be depicted as a two-dimensional image (Figure 4) which reports the TCR CDR3 length for each individual TCR family.

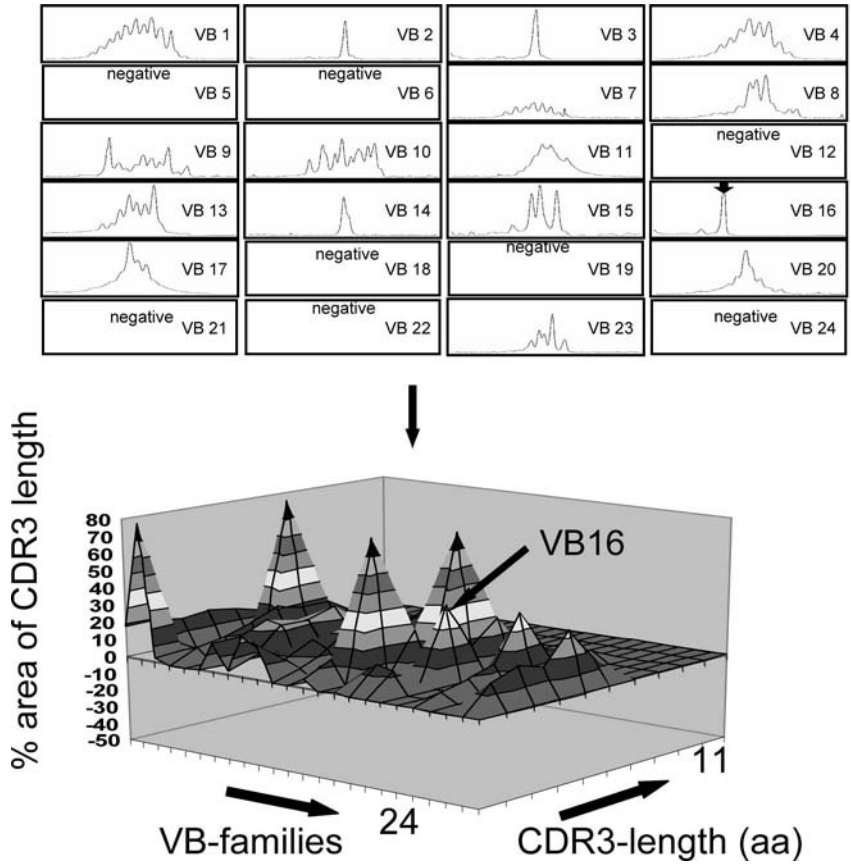

Figure 4. CDR3 length measurement. In normal healthy subjects, the CDR3 peak pattern represents a Gauss distribution. The data for each TCR family and the area under the curve for each TCR peak (i.e. 3 bp, 1 aa) are compiled. This CDR3 pattern may be depicted two-dimensionally, i.e. one picture for each TCR VA/VB family, or, alternatively, the entire TCR VA/VB repertoire may be combined in a single complex figure, thereby creating a TCR VA or TCR VB 'landscape' as a function of CDR3 length (as defined by the number of encoded aa) and the area under the curve for each individual CDR3 peak. For the sake of clarity, each 10% of the CDR3 peak is depicted in a different color. Two-dimensional (top) and three-dimensional (bottom) picture of the TCR repertoire analysis as defined by TCR VB spectratyping in a CD4+ TIL line from a patient with cervical cancer. The two-dimensional picture allows to analyze each VB family and the 'landscape' picture represents an overview of the TCR diversity. The single TCR VB16 chain (marked with the arrow) was found to be monoclonal and to recognize autologous tumor cells. See also Color Plate Section page 305.

Alternatively, TCR analysis can also be depicted as a 'landscape', i.e. as a function of the CDR3 length (z-axis), the VB family (x-axis) and the percentage of each individual CDR3 in a distinct VA or VB family (y-axis) (14, 15). This is possible using regular excel spreadsheet functions, or alternatively, using the Immunoscope® software (4, 16). This allows to describe the TCR repertoire at the time of evaluation. In order to compare either i) longitudinal differences (i.e. PBL or tissue samples collected at different time points) or ii) spatial differences (i.e. differences between a tumor sample and PBL or individual lymph node sections), the TCR CDR3 distribution pattern of individual VA or VB families can be used as a 'control' for other samples. This 'control' sample can be used from an individual patient (i.e. pre-, post-immunization, tumor/normal tissue) or a representative Gauss-distributed sample from either CD4+ or CD8+ PBL from healthy blood donors.

The difference at each CDR3 peak (for each VA or VB family) is calculated by comparing the area under the curve in the 'test' sample *versus* the control sample: Three possibilities can occur: i) the pattern is identical (a flat or 'smooth' area of the TCR landscape occurs), ii) some peaks are enhanced (resulting in 'over-representation' of certain CDR3 peaks which yields positive signals as +% perturbation (y-axis)), and iii) some peaks are diminished or ablated (resulting in 'under-representation' of CDR3 peaks, depicted as % perturbation (y-axis)). For sake of clarity, a certain percentage, (e.g. 10%) of over- or under-representation of the TCR CDR3 area (i.e. comparison of two samples) is depicted in a different color (Figure 5).

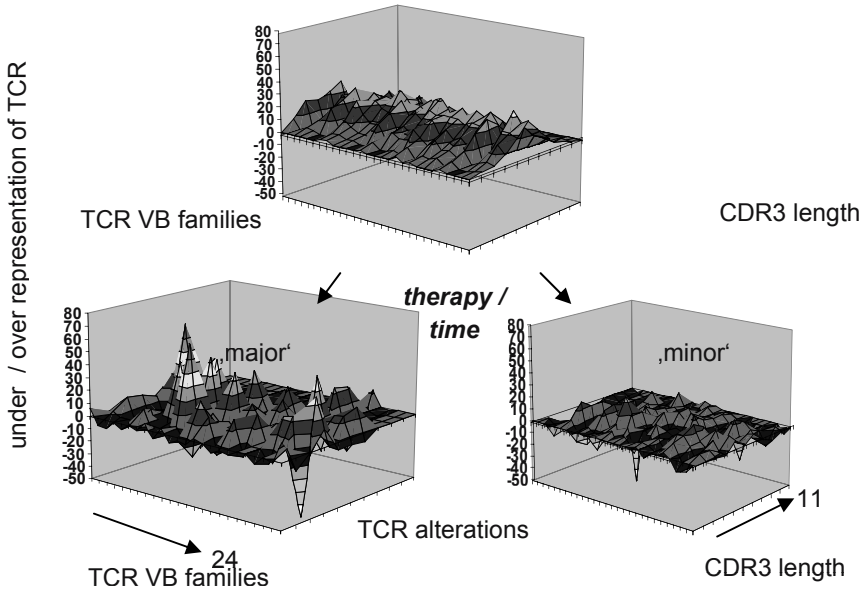

Figure 5. Visualizing the difference. TCR CDR3 patterns can be subtracted, i.e. each area under the CDR3 peak (e.g. CD4+ T-cells from PBL, top) is compared to the respective CDR3 peak areas of each individual TCR VA/VB family in the control sample. This results in over- or under-representation of individual TCR CDR3 peaks in each VA/VB family. 10% of difference are differentially colored. 'Major alterations' of the TCR repertoire (left, bottom) or a smooth surface (in blue) indicates no difference to the control sample (right, bottom). Differences can be time (longitudinal sampling of blood in an individual patient), spatial (e.g. tumor versus LN or PBL), or control samples from 'healthy subjects'.

This comparison allows the detailed description and comparison of the entire VA/VB repertoire. However, if the frequency of individual VB families is also available, this information can also be implemented in the comparison of the TCR VB chains, which would in this case include the comparative analysis of the quality (CDR3 length) and quantity (% frequency) of the TCR repertoire (Figure 6). Again, alterations in the quantitative composition of the TCR repertoire can be measured using flow-cytometry (8) or the RNA of each TCR family (9) using real-time PCR based quantification of individual TCR VB mRNA transcripts. The topology of TCR VB mRNA distribution displayed as a three-dimensional T-cell transcriptome has been coined TcLandscape (14, 17).

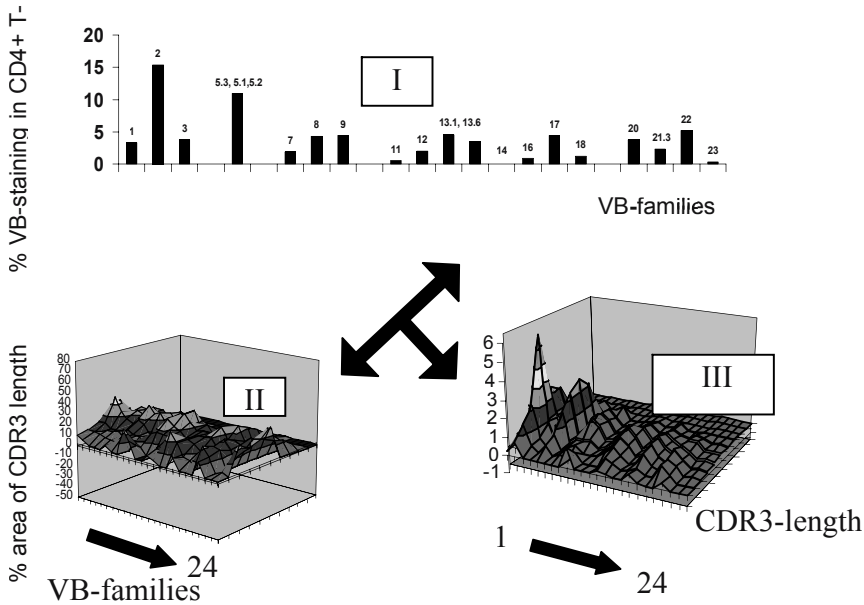

Figure 6. Gauging T-cell expansion. Qualitative and quantitative assessment of T-cells. Flow-cytometric determination of individual TCR VB families (I). TCR-VB-CDR3 analysis was performed (II). This landscape picture can be 'corrected' by implementing TCR VB frequencies of individual VB families as defined by flow cytometry (III). Note the difference between the 'quality' (II) and the 'quality-quantity' analysis (III). T-cells represent freshly harvested CD4+ TIL.

3. DATA INTERPRETATION

3.1 Emergence of TCR clonality and preferential usage of TCR chains

In general, healthy, 'antigen-experienced' individuals may show clonally altered, or even monoclonal TCRs. These 'private' alterations in the TCR repertoire have been reported in a number of apparently healthy individuals and identified in the CD8+ T-cell population, preferentially in the CD57+ T-

cell subset (18). Monoclonal or oligoclonal TCR VB families may occur in EBV or CMV-specific T-cell responses and reflect the immunological memory of previous encounters with antigens (19, 20). Some alterations can also be identified in CD4+ T-cells, presumably reacting to common infectious agents, e.g. CMV (21). Due to structural constraints of the TCR interaction with its nominal MHC/peptide ligand, some T-cell responses are characterized by a common usage of a TCR VB family, but not with a common CDR3 motif. For instance, the HLA-A2 restricted CD8+ T-cell response targeting the influenza matrix peptide M1 (aa 58-66) shows a preferential usage of the TCR VB17 family (22) which is polyclonal in nature. Other, preferential usage of certain TCR variable families may also be present in the human population. This is difficult to assess, since these studies depend either on the generation of *bona fide* T-cell clones directed against a single MHC/peptide complex, or alternatively on tetramer-sorted T-cells. For instance, the TCR VA2.1 chain appears to be frequently utilized in CD8+ T-cells recognizing the human HLA-A2 restricted melanoma – associated antigen Melan-A/MART-1 as determined by tetramer-sorting and T-cell cloning analysis (23, 24).

3.2 Clonal tracking of T-cells

Monoclonal expansion of T-cells may be indicative for antigen-driven process either in blood or in tissue. Since each monoclonal TCR can be molecularly defined (see above), a molecular probe specific for the respective CDR3 region can be designed and the individual T-cell clone can either be traced in different compartments or longitudinally over time in a patient. This methodology has been used to tag clonal T-cell populations in autoimmune myositis (25), or in patients with multiple sclerosis (MS) suggesting that expansion of certain TCR VB families may be associated with disease onset or progression (26, 27). Similarly, if a monoclonal T-cell response can be linked to MHC peptide recognition, it allows to visualize monoclonal TCRs with defined specificity *in situ*, e.g. in a patient with melanoma responding to peptide vaccination: An TCR VB16+ Melan-A/MART-1 reactive T-cell clone could be demonstrated in a vitiligo lesion associated with destruction of melanin-positive cells (i.e. melanoma cells and normal melanocytes) (28). Similar studies have been performed to track HPV-specific T-cells in patients with cervical cancer (29). Specific T-cells can be determined in tissue by using fluorochrome-labeled probes and real-time PCR (30) using the unique composition of the CDR3 region of a T-cell clone.

3.3 Clonal expansion and link to T-cell function

Antigen specific T-cells undergo a selection process driving T-cell proliferation. In patients with cancer, clonal expansions may be associated with tumor-surveillance. This has been demonstrated in patients with melanoma undergoing peptide vaccination: molecularly defined TCRs, present in PBL and in blood, were able to recognize autologous tumor cells (9). Indeed, some T-cell responses involved in tumor containment may be mediated by a few effective T-cell clones or even a single clonotypic TCR (31).

3.4 TCR perturbation and link to therapy

TCR CDR3 analysis has been determined in patients with severe combined immunodeficiency syndrome (SCID). Of interest, the appearance of CD45RO + T-cells has been associated with a skewed TCR repertoire, and low TREC levels (T-cell receptor excision circles). In contrast, the appearance of CD45RA+ T-cells was associated with high TREC levels, a marker for thymic output and a broadened TCR repertoire (32). Particularly in patients with HIV infection, the advent of highly active retroviral therapy (HAART) has changed the prognosis and outcome of HIV infection. Progression to AIDS, the clinical presentation with opportunistic infections is associated with perturbation of the TCR repertoire. HAART is able to restore the TCR repertoire. Thus, CDR3 analysis may represent a valuable marker to compare efficacy of novel treatment protocols, together with other markers, e.g. CD4 counts and viral load determination (16, 33).

HAART drastically reduces viral load and also anti-HIV directed CD8+ T-cell responses. In order to boost anti-HIV directed T-cell responses, HAART therapy may be interrupted to stimulate CD8+ anti-HIV specific T-cells. The monitoring of this critical state is crucial for clinical management and to determine the time point to restart HAART therapy. TCR CDR3 analysis may present an objective marker to study the TCR ‘perturbation’ in the structured stop (and start) of HAART therapy.

3.5 Transplantation

Allograft rejection is mediated by T-lymphocytes, the appreciation of the T-cell infiltrate into the transplant may represent an interesting prognostic marker, or alternatively, represent a surrogate marker for testing novel drugs preventing graft rejection. TCR CDR3 analysis has been studied in the context of allograft rejection and tolerance induction, e.g. in the setting of heart transplants (15) or in the context of allogeneic hematopoietic stem cell

transplantation (34), the detailed analysis of the TCR usage may also be helpful in the molecular definition of the effector cell population mediating either graft-versus-leukemia and graft-versus-host reactions (35). More recent studies suggest that TCR CDR3 analysis may represent a valuable tool to monitor GVHD after allogeneic stem cell transplantation (36).

3.6 Link to vaccination and infection

Dramatic clonal expansion of T-cells in human healthy subjects has been reported, most of them are possibly linked to common viral pathogens, e.g. EBV or CMV. Similarly to infection with influenza A, leading to expansion of the TCR VB17 family in HLA-A2 positive individuals, the anti-EBV response is apparently driving the TCR VB6 family if the infected individual carries the HLA-B8 allele. Recognition can be linked to the HLA-B8 restricted EBV-derived peptide FLRGRAYGL (37). Tetramer complexes and TCR CDR3 analysis may also be combined to determine it the TCR repertoire changes over time as a result of therapy or vaccination. For instance, if T-cells responding to a defined antigen exist prior to (therapeutic) vaccination, a tetramer – based analysis is not able to answer the question if antigen-specific T-cells induced by the vaccine are similar or different as compared to the pre-existing T-cells. Tetramer-guided sorting, followed by TCR CDR3 analysis is able to identify molecular differences in the TCR repertoire reacting to the nominal antigens used in the vaccine (38).

3.7 In search of a culprit

The pathogenesis of a number of diseases is still not very well understood. The exact mechanisms of containment of either transformed or virally infected cells have not been determined. In general, effective adaptive T-cell responses are desirable in these diseases. The flip side of the coin – in the context of cellular immune responses – is a strong T-cell response which mediates auto-immune disorders. Many parameters exist to measure disease activity in autoimmune diseases, but the magnitude of a cellular immune response is hard to assess, particularly if no molecular targets have been identified. Since TCR CDR3 analysis visualizes objectively every alteration in the TCR composition, it may be helpful to define new markers of disease activity in autoimmune diseases; it may also present a potential matrix to gauge immuno-suppressive effects of novel drugs. For instance, TCR diversity has been suggested as a readout in PBL from patient suffering from SLE (39), or in the synovial fluid from patients with RA (40) and TCR CDR3 analysis suggested that the T-cell infiltrates in patients with Crohn's disease are indicative of an antigen-driven process (41). Recently, TCR

CDR3 analysis supported the notion that T-cells play a major role in mediating inflammation in patients with MS (42, 43) or in patients with Guillain-Barre and Fisher syndrome (44) and shed light on the association of psoriatic lesions and joint inflammation (45) as well as a certain form of polymyositis and HTLV-infection (46). In addition, TCR CDR3 analysis may be helpful to appreciate the TCR repertoire of individuals with exposure to defined pathogens. For instance, TCR CDR3 analysis in HIV+ and HIV negative men, but with 'high-risk- exposure to the pathogen showed that – in general – high antigenic exposure is associated with a restricted T-cell repertoire (47). This underlines the need to select appropriate control populations if the TCR repertoire is compared to 'healthy controls'.

4. REPORTS

Comprehensive reports may include (for CD4 / CD8-sorted T-cells) the following data:
Data acquisition and data analysis.
Absolute cell count, CD4/CD8 ratio.
Sorting: CD4/CD8 absolute count after sorting.
After mRNA isolation and cDNA transcription:
Quality control of mRNA / cDNA.
TCR variable gene analysis (for individual VA, VB, VG or VD genes as requested).
TCR CDR3 analysis:
Synopsis of each TCR V family and potentially a three dimensional data report.
Quantification of individual TCR families either by flow-cytometry (see Figure 6) and/or measuring individual TCR mRNA transcripts.
A quality control report of the laboratory which addresses the sensitivity and specificity of the TCR CDR 3 analysis, including data for inter- and intra-assay variation.
Monoclonal TCRs should be confirmed by DNA sequence analysis. More than 2 monoclonal TCRs in a healthy individual: Repeat the entire panel, including flow-cytometry, if applicable. The presence or absence of each TCR variable family should be noted. The process of TCR CDR3 analysis is compiled in Figure 7.

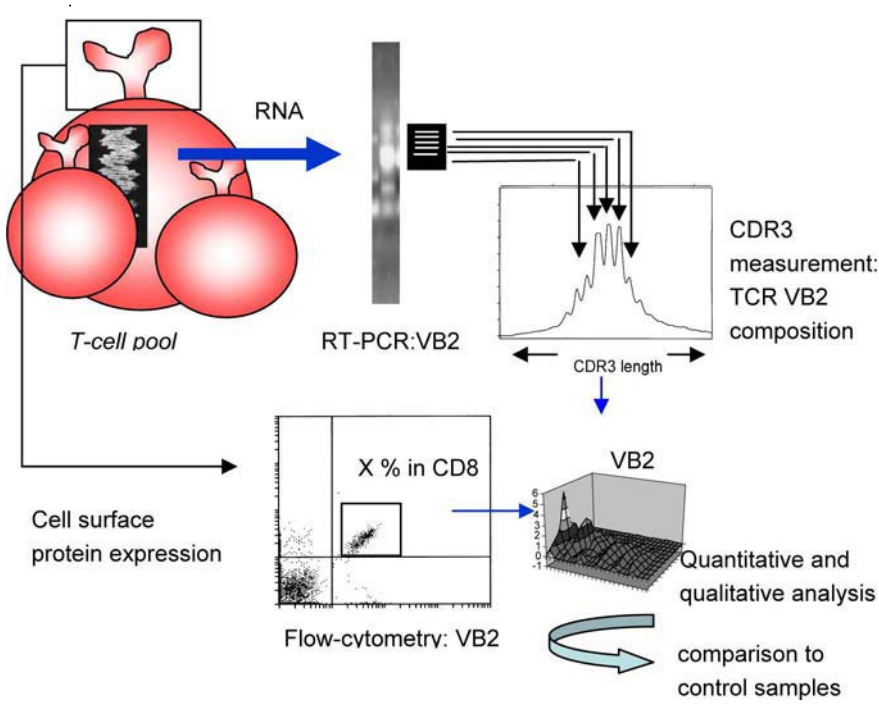

Figure 7. Compilation of TCR CDR3 fingerprinting: Amplification of each individual TCR V-family (e.g. here TCR VB2), run-off reaction and measuring the CDR3 length. T-cells may be quantified either by measuring the TCR VB mRNA content, or by flow-cytometry. Comparative analysis will aid to visualize differences in the T-cell pool, e.g. as response to therapy or any intervention affecting the cellular immune system.

If monoclonal TCR sequences are reported: report of the variable family, the joining region, D-region (for VB families) and the exact nucleotide and amino acid sequence of the CDR3 region responsible for TCR specificity.

If flow-cytometric analysis has been implemented to gauge the TCR repertoire:

List of each individual TCR VB family.

Differences in regard of quality (molecular composition) and quantity of each TCR family are reported as percent over- or under-representation as compared to

1. a control sample determined by the investigator in individual patients (i.e. prior or after therapy),
2. different anatomic compartments (e.g. TIL versus PBL), or
3. data from a normal healthy control population.

Selected reasons for false positive results (e.g.'false' monoclonality):
Insufficient number of T-cells may indicate monoclonality: higher cell 'input' will reveal the real picture (Figure 3).

False 'negative results:
Insufficient sorting/purity of T-cells, e.g. a monoclonal TCR in the CD8 population (e.g. in the TCR VB1 family) may be 'masked' by a polyclonal TCR population in the contaminating CD4+ T-cell population.
Poor mRNA isolation and quality in the target population / tissue of interest.
TCR mRNA 'turnover' may be associated with T-cell activation or resting state.

Specimens suitable for evaluation:
- Blood
- Fresh frozen tissue (not paraffin-embedded tissue, although CDR3 analysis has been performed using paraffin-embedded tissues (48)
- Biopsies
- Synovial fluid
- Cerebrospinal fluid
- Material from bronchoalveolar fluid

5. ASSESSMENT AND OUTLOOK

TCR CDR 3 analysis is a descriptive method to visualize the composition of the entire T-cell repertoire, which can be combined with other, functional readout assays of the cellular immune system. At this point, it represents a highly reproducible and high-throughput approach to gauge the T-cell repertoire in any tissue. Comparative analysis aids to compare the TCR diversity over time or in different anatomic compartments and aids to establish novel markers in the context of immunotherapy or disorders of the immune system. TCR analysis has been predominantly employed in human diseases. TCR CDR3 analysis can also be performed in murine disease models, or in non-human primates (49). The determination of the TCR repertoire may also be helpful in pre-clinical settings, i.e. in testing differences in protein or peptide composition, or vaccine formulations which affect the cellular immune response. Since T-cells are able to sense a single amino acid residue difference in antigenic peptides, the impact of subtle alterations can be reflected by differential expansion of TCR families (50). Potential indications for implementing TCR CDR3 analysis are listed in Table 1.

Table 1. Indications for TCR CDR3 fingerprinting.

Analysis	Advantage/comment(s)
Definition of the CD4+/CD8+ T-cell population in patients suffering from cancer, viral infection, or undergoing epitope-based vaccination.	This may be useful if no tetramer reagents are available, or if the entire CD4/CD8 T-cell population is examined for TCR diversity, particularly in studies addressing T-cell homeostasis.
Identification of T-cell malignancies.	Detection of monoclonal TCR transcripts and expansion of clonal T-cells.
Determination of superantigen effects on T-cells.	Expansion of certain polyclonal VB families.
Identification of individual expanded VB families in PBL or freshly harvested tumor infiltrating T-cells (TIL)	Individual VB families may be present in sufficient numbers to allow flow sorting. Such T-cells may represent effector cells directed against cancer cells or virus-associated antigens.
Longitudinal analysis of the quality and quantity of peripheral T-cell in patients undergoing T-cell 'reconstitution', e.g. during highly active anti-retroviral therapy (HAART), patients after bone marrow transplantation or patients with solid organ transplantation.	Gauging restoration of the CD4/CD8 T-cell compartment(s): Effective HAART-treatment is associated with a broad TCR-repertoire, graft monitoring after BMT, differentiation between graft versus tumor and graft versus host effects. Prognosis and assessment of tolerance in solid organ recipients.
Comparing TCR diversity within an individual patient in different anatomic compartments.	In order to compare the T-cell infiltrate in tumor (or any inflammatory) lesion, the T-cell infiltrate should be separated after CD4+/CD8+ staining in order to allow comparison with the TCR diversity within the CD4+ or CD8+ T-cell population.
Experimental: Identification of monoclonal TCRs in any tissue / anatomic compartment e.g. by laser-microdissection.	Identification of monoclonal TCRs, subsequent cloning into appropriate vector systems allows to generate TCR-transgenic T-cells which can be utilized to identify the target antigen of interest.
Experimental: Identification and tracking of clinically relevant T-cell subsets, e.g. suppressor (CD25+) T-cells, or memory T-cells mediating immune-surveillance.	'Tagging' T-cells allows following clinical relevant T-cell subsets associated with response/non-responsiveness to therapy defined by molecular marker analysis.
Pre-clinical analysis: Assessing differences in 'immunogenicity' of proteins, peptides or different antigen formulations	Alterations of the TCR repertoire in vitro may be helpful to gauge differences of antigens used for therapy. TCR CDR3 alterations may be combined with functional assays of the cellular immune system.

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Chapter 15

PEPTIDE/HLA-GFP COMPLEXES

Detection of Antigen-Specific T Cells by Acquisition of Peptide/Hla-Gfp Complexes

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Abstract: Antigen-specific T cell responses are essential in host immune defense in health and disease. For many years, it was believed that the immune system was effective only in combating infectious diseases caused by invading agents such as bacteria and viruses. More recently, however, the immune system has been shown to play a central role in protection and recovery against cancer. This latter role is not well understood, but there are numerous reports that the immune system slows down the growth and spread of tumors in cancer patients. Many clinical trials for cancer immunotherapy are in progress and use anti-tumor vaccinations that have been designed to elicit antigen-specific T cell responses. Therefore, in defining anti-tumor immune responses that may be used for immunotherapy trials, the detection and quantitative analysis of antigen-specific T cell populations has been an important step toward understanding the cellular immune response in patients. In this chapter, we introduce a newly established system for the analysis of antigen-specific T cells, which is based on the technology using artificial antigen presenting cell expressing human leukocyte antigen (HLA)-A*201 coupled to the enhanced green fluorescent protein (GFP) (HmyA2GFP cells). Antigen-specific CD8+ T cells have been demonstrated to acquire peptide-major histocompatibility complex (MHC) clusters through T cell receptor-mediated endocytosis upon specific antigen stimulation. We generated an antigen-presenting cell expressing HLA-A*201 coupled to the GFP which when pulsed with antigenic peptide will deliver the GFP to an antigen-specific T cell. We demonstrated the quantitative identification of human T-cell lymphotropic virus type I (HTLV-I) Tax11-19 peptide-specific T cell populations in peripheral blood mononuclear cells from patients with HTLV-I associated neurologic disease and defined a novel CD8+ T cell epitope in the HTLV-I Envelope region.

Key words: green fluorescent protein, HLA, endocytosis, HTLV

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1. INTRODUCTION

The immune system plays an important role in protection and recovery against cancer. With the discovery of tumor-associated antigens more than a decade ago, the application of immunotherapy against tumors, which is focused intensively on anti-tumor vaccination, has been challenging. Although vaccines have revolutionized public health by preventing the development of many important infectious diseases, to date, it has been much more difficult to develop effective vaccines to prevent cancer, or in the treatment of patients with existing disease. In spite of this, significant interest has been generated by recent advances in the areas of immunology and cancer biology, which have led to more sophisticated and promising vaccine strategies than those previously available. Cancer vaccines typically consist of a source of cancer-associated material (antigen), along with other components, to further stimulate the immune response against the antigen. The challenge has been to find better antigens, as well as to package the antigen in such a way as to enhance the patient's immune system to fight tumor cells expressing the appropriate antigen. Therefore, efficient screening and useful monitoring systems for analyzing antigen-specific T cells is important toward discovering novel disease-associated antigens, understanding cellular immune responses against these tumor-specific molecules, and establishing effective anti-tumor vaccination.

In this chapter, we introduce a newly established system for the analysis of antigen-specific T cells, which is based on the technology using artificial antigen presenting cell expressing human leukocyte antigen (HLA)-A*201 coupled to the enhanced green fluorescent protein (GFP) (HmyA2GFP cells) (1). This system is related to the cellular immune response between antigen presenting cells (APC) and CD8+ T cells, and thought to be an advanced method to monitor T cell directed vaccine trials in cancer patients as well as to analyze cellular immune responses against tumor-associated antigens. During the process of antigen recognition by T cells, antigen-specific T cells recognize peptides of defined length that are bound to major histocompatibility complex (MHC) molecules on APC, and after antigen recognition, the peptide-MHC clusters are acquired by CD8+ T cells and internalized through T cell receptor (TCR) (2, 3). Because this internalization is based on the specificity of peptide-MHC-TCR recognition, we have postulated that the acquisition of peptide/MHC complexes by T cells can be used for the quantitative detection of antigen-specific T cell populations, identification of antigen-specific T cell epitopes, and for analysis of T cell responses after antigen-stimulation. In the following sections, we demonstrate the methods for the generation of artificial APC and the acquisition of peptide/HLA complexes by T cell clones. We utilize

this system to quantitate antigen-specific T cell populations from bulk peripheral blood mononuclear cells (PBMC) of patients. Moreover, we extend this system to define novel virus-specific CD8+ T cell immunodominant epitopes.

2. ACQUISITION OF PEPTIDE/HLA COMPLEXES BY T CELLS

2.1 Generation of HLA-A*201-GFP Expressing Antigen Presenting Cells

The full-length HLA-A*201 cDNA construct was obtained from RSV/HLA-A2 vector (4). The HLA-A2-GFP expression vector was generated by insertion of the HLA-A*201 cDNA with a stop codon mutated into the pEGFP-N3 vector (Clontech, Palo Alto, CA) (Figure 1a). HLA-A and B locus defective immortalized B cell line (Hmy2.CIR) was transfected with the HLA-A2-GFP vector using Trans-IT (Mirus, Madison, WI), according to the manufacture's instruction. The cells were incubated for 48 hours at 37 ˚C, and subsequently replaced in selection medium {D-MEM supplemented with 10% fetal bovine serum, 2mM l-glutamine, 40 U/ml penicillin, 40 μg/ml streptomycin (Gibco-BRL, Grand Island, NY) and 400 μg/ml of G418 Sulfate (Cellgro, Herndon, VA)} to establish a stable cell line expressing HLA-A*201-GFP fusion protein (HmyA2GFP) (Figure 1b).

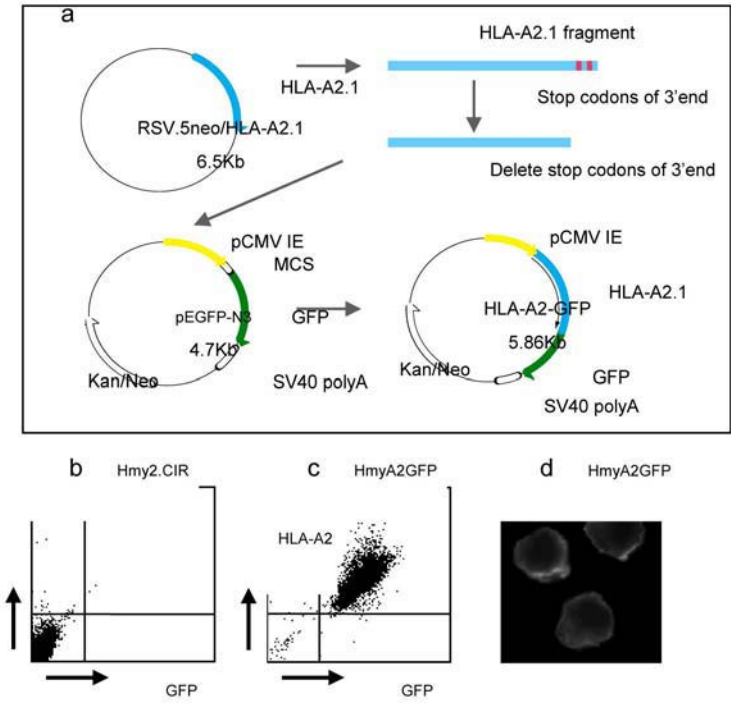

Figure 1. Generation of artificial antigen presenting cells, HLA-A*201-GFP expressing APC. Generation of HLA-A*201-GFP expressing vector. (b,c) Expression analysis of HLA-A*201 molecule and GFP signal on Hmy2.CIR cells and HmyA2GFP cells. The expression of HLA-A*201 molecule and GFP signal were double positive on HmyA2GFP cells (c) but negative on Hmy2.CIR cells (b). (c) GFP signal on the HmyA2GFP cells were also visualized under fluorescence microscope.

2.2 Demonstration of Peptide-HLA Acquisition by CD8+ T cells

In this study, antigen-specific acquisition of HLA-GFP complexes by T cells was assessed by analyzing human T-cell lymphotropic virus type I (HTLV-I)-specific CD8+ T cell recognition of the immunodominant human T-cell lymphotropic virus type I (HTLV-I) Tax11-19 peptide. The HTLV-I Tax11-19 peptide is one of the most stable binders for HLA-A*201 (5) and the HLA-A2/Tax11-19 peptide/TCR complex has been extensively characterized at the crystallographic structural level (6). HmyA2GFP cells

were pulsed with Tax11-19 peptide or control peptide, human immunodeficiency virus (HIV) Gag77-85 peptide at a concentration of 10uM, and incubated in a round bottom 96 well culture plate for 30 min at 37 ˚C. The HmyA2GFP cells were washed twice to remove any free peptide and mixed with HTLV-I Tax 11-19-specific cytotoxic T-lymphocyte (CTL) clones (HmyA2GFP cells / CTL = 5 / 1) in a round bottom 96 well culture plate. Then cell mixture was centrifuged at 1000 rpm for a few seconds to provide immediate contact of the cells, and incubated for 30 min at 37˚C. To detect HLA-GFP molecules in CD8+ CTL clones, cells were stained with phycoerythrin (PE)-labeled monoclonal antibody to CD8 (Caltag, Burlingame, CA), and the acquisition of HLA-GFP by T cells was assessed by flow cytometry. As shown in Figure 2, after 30 minutes, most HTLV-I-specific CTL clones were positive for HLA-GFP but negative with the control HIV Gag77-85 peptide. We also demonstrated peptide-specific acquisition of HLA-GFP molecules by T cells using cytomegalovirus (CMV) pp65 peptide (495-503)-specific CTL clones.

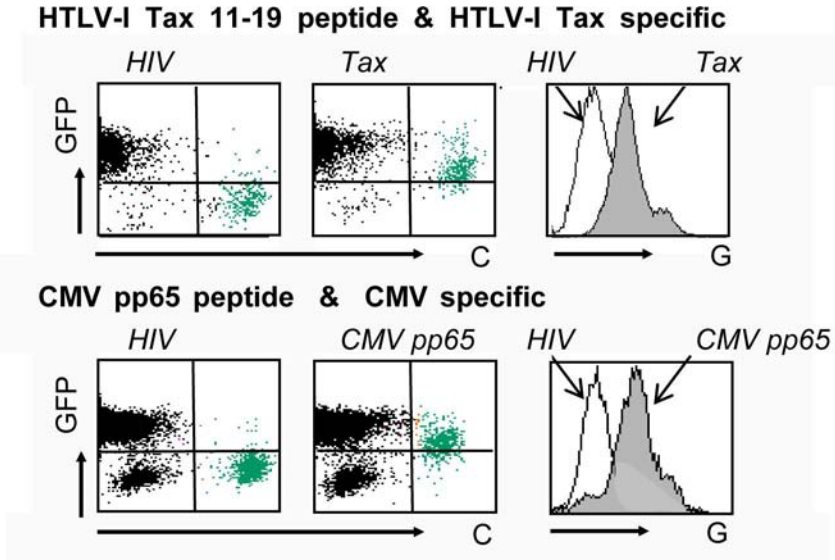

Figure 2. Flow cytometric analysis of peptide-specific acquisition of peptide/HLA-GFP complexes by antigen-specific CTL clones. HmyA2GFP cells were pulsed with HIV Gag77-85 peptide, HTLV-I Tax11-19 peptide and CMV pp65 peptide respectively, and cultured with a HLA-A*201 restricted HTLV-I Tax11-19-specific CD8+ T cell clone, or a HLA-A*201 restricted CMV pp65-specific CD8+ T cell clone for 30 min. The acquisition of HLA-GFP by those CTL clones was analyzed directly by flow cytometry.

To assess the time-dependent kinetics of peptide/HLA-GFP acquisition by T cells, Tax11-19-specific CTL clones were incubated with Tax11-19 peptide-pulsed HmyA2GFP cells for various time points (Figure 3a). After 30 minutes, the majority of the Tax11-19-specific CTLs were positive for HLA-GFP when the fluorescence level plateaued for 2 hours and then declined. Examples of images of peptide/HLA-GFP acquisition by CD8+ T cell clones are shown in Figure 3b. After incubation of peptide-pulsed HmyA2GFP cells with CD8+T cell clones, within 5 minutes, HLA-GFP molecules formed dense clusters at the T cell-APC contact site. After 30 minutes, small aggregates of HLA-GFP appeared within HTLV-I -specific CTL clones. A dose-dependent titration of HTLV-I Tax11-19 peptide pulsed-HmyA2GFP cells demonstrated a lower limit of detection of 100 pM of peptide (Figure 4a), and as shown in Figure 4b, the acquisition of peptide/HLA-GFP complexes by CD8+ T cells plateaued at a 1:5 effector-target (CTL/HmyA2GFP) ratio.

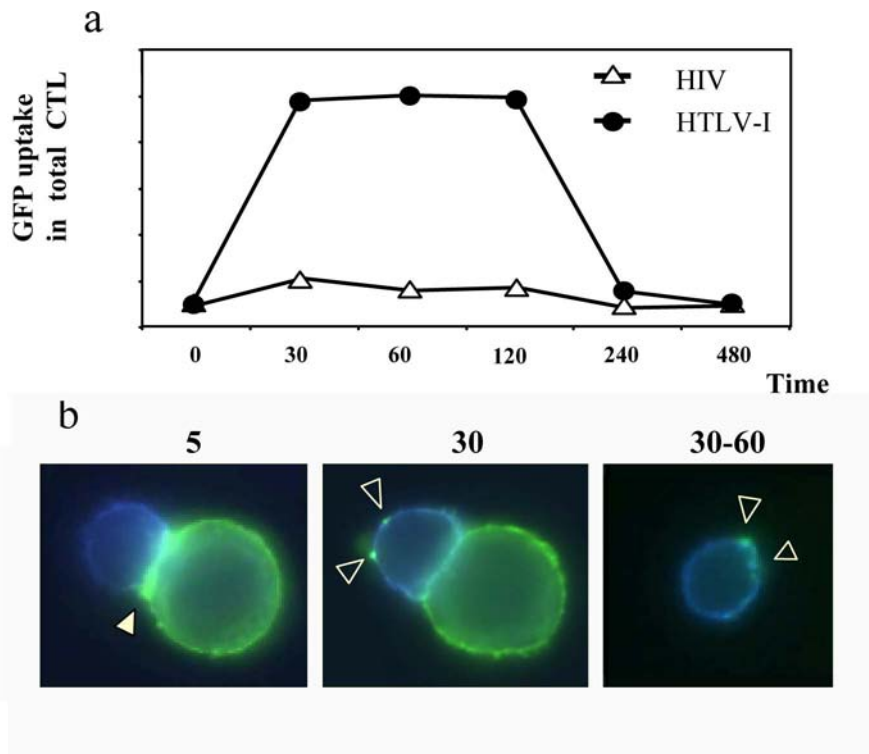

Figure 3. Time-dependent acquisition of peptide/HLA-GFP complexes by T cells. (a) HTLV-I Tax11-19-specific CTL clone was incubated for the indicated time with HmyA2GFP cells pulsed by Tax11-19 peptide or HIV Gag peptide. HLA-GFP acquisition by CD8+ CTL population was directly analyzed by flow cytometry. The levels of HLA-GFP acquisition are expressed by mean fluorescence intensity (MFI). (b) Visualized images of peptide/HLA-GFP

complexes acquisition by HLA-A*201 restricted HTLV-I-specific CTL clone. Within 5 minutes, HLA-GFP molecules formed dense clusters that could be readily visualized at the T cell-APC contact site (solid white arrow). After 30 minutes, small aggregates of HLA-GFP appeared within the Tax11-19 peptide-specific CTL clone (open arrow). See also Color Plates Section page 305.

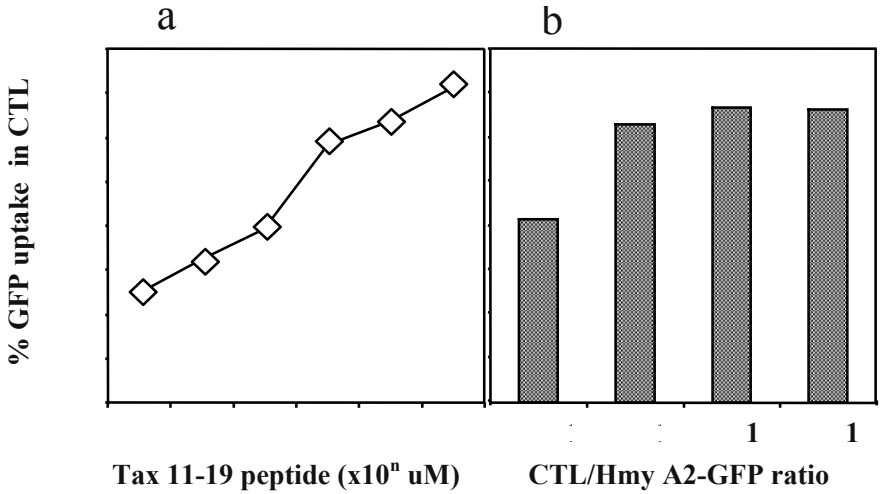

Figure 4. Peptide dose-dependent and effector/target ratio-dependent acquisition of peptide/HLA-GFP complexes by T cell clones. HmyA2GFP cells were incubated with indicated doses of peptide, and the acquisition of HLA-GFP was directly analyzed by flow cytometry. (b) HmyA2GFP cells were pulsed with HTLV-I Tax11-19 peptide at a concentration of 10uM, and incubated with HTLV-I-specific T cell clones in indicated effector/target ratio.

3. DETECTION OF VIRUS-SPECIFIC T CELLS BY ACQUISITION OF PEPTIDE/HLA-GFP COMPLEXES FROM BULK PBMC

In section 2, we have demonstrated the acquisition of peptide/HLA complexes by CD8+ T cell clones. In this section, we will show that by using the HmyA2GFP system, we were able to identify and quantitate antigen-specific T cell populations from bulk PBMC. To extend our system for ex vivo PBMC, we used PBMC from HTLV-I-infected patients with an inflammatory disease of the central nervous system termed HTLV-I-associated myelopathy / tropical spastic paraparesis (HAM/TSP). HTLV-I is a human retrovirus that is associated with an aggressive mature T-cell

malignancy termed adult T-cell leukemia (ATL) (7) as well as HAM/TSP (8) (9). In HAM/TSP patients, a high frequency of HTLV-I-specific CD8+ CTL, most of which recognize the HTLV-I Tax11-19 peptide, has been demonstrated in HLA-A*201 patients (10-12). In addition, it has been suggested that these HTLV-I-specific CTL play an important role in the pathogenesis of this disease (11, 13). Therefore, PBMC from HAM/TSP patients is an ideal system to analyze antigen-specific T cell responses in a human disease. HmyA2GFP cells were pulsed with Tax11-19 peptide or control HIV Gag77-85 peptide at a concentration of 10uM, and incubated in a round bottom 96 well culture plate for 30 min at 37 ˚C. The HmyA2GFP cells were washed twice to remove any free peptide and were mixed with PBMC from HAM/TSP patients (HmyA2GFP / PBMC = 1 / 1) in a round bottom 96 well culture plate, then centrifuged at 1000 rpm for a few seconds to provide immediate contact of the cells, and incubated for 30 min at 37˚C. Cells were then stained with TC-labeled monoclonal antibody to CD8 (Caltag) and PE-conjugated Tax11-19 peptide loaded HLA-A*201 tetramer (provided by National Institute of Health AIDS Research and Reference Reagent Program). The percentage of GFP-positive CD8-positive cells from total PBMC as measured by flow cytometry would define the HTLV-I Tax11-19-specific T cell population. As shown in Figure 5a, from a representative HAM/TSP patient (HAM #6), 25.13% of CD8+ cells were GFP-positive when incubated with HmyA2GFP cells pulsed with Tax11-19 peptide relative to the control peptide. This frequency of Tax11-19-specific T cells in PBMC from this patient was comparable to the frequency measured by HTLV-I Tax11-19/HLA-A*201 tetramers (28.21%). We further analyzed the frequencies of HTLV-I-specific T cells in PBMC in 6 HLA-A*201-positive HAM/TSP patients, 3 HLA-A*201-negative HAM/TSP patients, and 3 HLA-A*201-positive healthy donors, and as shown in Figure 5d, HTLV-I Tax11-19-specific T cells were detected in PBMC from all patients at a frequency comparable with tetramer, but not in HLA-A*201 negative patients nor in healthy donors. Moreover, we were able to utilize this A2GFP system for enumerating CMV-specific T cells in HAM/TSP patient at a frequency comparable with that measured by CMVpp65/HLA-A*201 tetramer (data not shown).

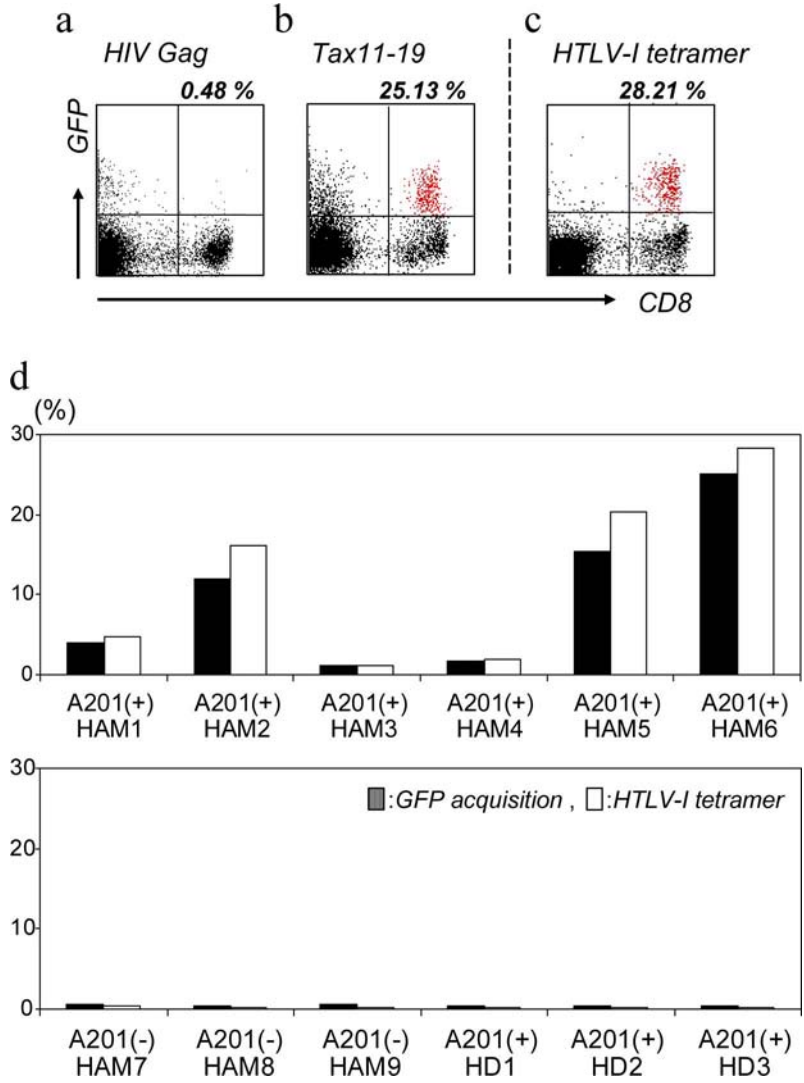

Figure 5. The quantitative detection of virus-specific CD8+ T cells from bulk PBMC of HLA-A*201 HAM/TSP patients. (a, b) HmyA2GFP cells were pulsed with HIV Gag77-85 peptide or HTLV-I Tax11-19 peptide, and cultured with PBMC from HAM/TSP patient #6 for 30 minutes, respectively. Cells were stained with anti-CD8 monoclonal antibody (Caltag, Burlingame), and the acquisition of peptide/HLA-GFP complexes by CD8+ T cells was analyzed by flow cytometry. The percentages of HLA-GFP positive CD8+ T cells in total CD8+ cells are shown in the upper right quadrant. (c) The percentage of HTLV-I Tax11-19/HLA-A*201 tetramer reactive cells in total CD8+ PBMC from HAM/TSP patient #6 is shown in the upper right quadrant. (d) Frequency of HTLV-I Tax11-19 peptide specific HLA-GFP acquiring CD8+ T cells and Tax11-19/HLA-A*201 tetramer positive CD8+ T cells in HAM/TSP patients and healthy donors.

4. DETECTION OF NOVEL CD8+T CELL EPITOPES BY ACQUISITION OF PEPTIDE/HLA-GFP COMPLEXES

In addition to detecting known peptide-specific T cells, we have extended this system to detect heretofore-unidentified antigen-specific CD8+ T cells by pulsing HmyA2GFP cells with a panel of novel peptides. To test this possibility, we searched for HTLV-I Envelope (Env)-specific CD8+ T cells from PBMC of HAM/TSP patients. HmyA2GFP cells were pulsed with a series of 26 overlapping peptides from the HTLV-I Env gp46 region (Figure 6a) (14) at a concentration of 10uM, washed twice, and incubated with HLA-A*201 HAM/TSP patient PBMC (HAM#1) for 30 minutes, and HLA-GFP acquisition by CD8+ T cells was analyzed using flow cytometry. As shown in Figure 6b, significant HLA-GFP acquisition by bulk CD8+ T cells was observed with the Env291-305 peptide, suggesting there was significant HTLV-I Env291-305-specific T cell population in the PBMC of this HAM/TSP patient. To confirm this observation, CD8+ cells that had acquired HLA-GFP from Env291-305 peptide pulsed HmyA2GFP cells (putative Env291-305 peptide-specific T cell population) were sorted by flow cytometry and assessed for cytotoxicity using a variety of target cells. As shown in Figure 6c, HTLV-I Env291-305 pulsed targets were significantly lysed compared to other control HTLV-I Env peptide. Moreover, target cells infected with a Vaccinia-Env construct or an autologous HTLV-I infected CD4+ T cell line (RSCD4) known to express HTLV-I (15) were also lysed by the Env291-305 sorted CD8+ T cells. No cytolytic activity was observed on control target cells. These results clearly showed that the sorted Env291-305-specific CD8+ T cells detected by the A2GFP system were peptide-specific and were functional cytolytic. Importantly, these sorted CD8+ T cells recognized endogenously processed HTLV-I Env protein since Vaccinia-Env infected targets and a long term T cell line known to express HTLV-I Env were also lysed. These results suggest that the Env291-305 region of HTLV-I may contain an immunodominant, HLA-A*201-specific CTL epitope. Of interest is the observation that this Env291-305 region contained a relatively strong HLA-A*201 binding motif (5) based on an estimation of the dissociation rate of the HLA-peptide complex (http://bimas.dcrt.nih.gov/molbio/hla_bind/). Thus, this novel A2GFP system enables the isolation and characterization of viable antigen-specific T cells and the detection of novel CD8+ T cell epitopes.

a

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1   MGKFL ATLIL FFQFC PLIFG DYSPS CCTLT IGVSS YHSKP CNPAQ PVCSW   50
51  TLDLL ALSAD QALQP PCPNL VSYSS YHATY SLYLF PHWIK KPNRN GGGYY  100
101 SASYS DPCSL KCPYL GCQSW TCPYT GAVSS PYWKF QHDVN FTQEV SRLNI  150
151 NLHFS KCGFP FSLLV DAPGY DPIWF LNTEP SQLPP TAPPL LPHSN LDHIL  200
201 EPSIP WKSKL LTLVQ LTLQS TNYTC IVCID RASLS TWHVL YSPNV SVPSS  250
251 SSTPL LYPSL ALPAP HLTLP FNWTH CFDPQ IQAIV SSPCH NSLIL PPFSL  300
                gp46 ←——  ——→ gp21
301 SPVPT LGSRS R / RAVP VAVWL VSALA MGAGV AGGIT GSMSL ASGKS LLHEV  350
351 DKDIS QLTQA IVXNH KNLLK IAQYA AQNRR GLDLL FWEQG GLCKA LQEQC  400
401 RFPNI TNSHV PILQE RPPLE NRVLT GWGLN WDLGL SQWAR EALQT GITLV  450
451 ALLLL VILAG PCILR QLRHL PSRVR YPHYS LIKPE SSL  488
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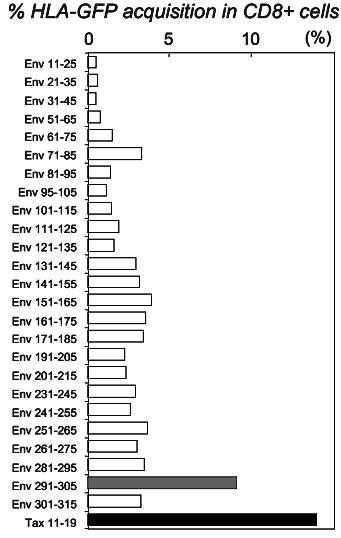
b



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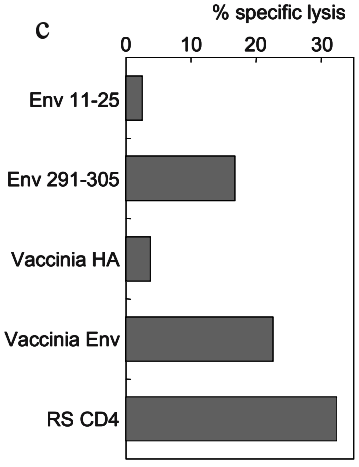

Figure 6. Detection of a novel CD8+ T cell epitope from the envelope region of HTLV-I. HmyA2GFP cells were pulsed with a series of 26 overlapping peptides from HTLV-I Envelope gp46 region and cultured with PBMC from HAM #1 patient for 30 minutes. Cells were stained with anti-CD8 monoclonal antibody and the frequency of HLA-GFP acquiring CD8+ T cells was analyzed by flow cytometry. (a) Each peptide is designated by the position of its amino acid sequence versus the frequency of HLA-GFP positive/CD8+ cells. (b) HmyA2GFP cells were pulsed with Env291-305 peptide and cultured with PBMC from HLA-A*201 HAM patient #1 for 30 minutes. Cells were stained with anti-CD8 monoclonal antibody and HLA-GFP acquiring CD8+ T cells were sorted by flow cytometry. (c) Sorted cells were examined for their functional capacity to lyse HTLV-I Env expressing target cells in a 4 hour Europium CTL assay as described previously (17). Target cells consisted of HLA-A*201 transfected human B cell line pulsed with Env11-25 and Env291-305 at a peptide concentration of 100nM for 1hour; HLA-A*201 transfected human B cell line infected with vaccinia recombinant viruses expressing HTLV-I Env or the control vaccinia expressing influenza virus hemagglutinin (HA) protein as described previously (10, 18); An autologous HTLV-I infected CD4+ T cell line known to express HTLV-I (RSCD4) (15). All targets were used at a concentration of 2000 cells per well and effectors were added at 3:1 effector-target ratio.

5. CONCLUSIONS AND PROPOSED APPLICATIONS FOR A NOVEL PEPTIDE/HLA-GFP COMPLEX ACQUISITION SYSTEM

Antigen-specific T cell interactions are important components of cellular immunity to microbial agents, self-proteins, and tumor antigens. In defining these interactions, the detection and quantitative analysis of antigen-specific T cell populations has been an important step toward understanding the cellular immune response in health and disease. In this chapter, we introduce newly established system for the analyzing antigen-specific T cells, an artificial APC system which express HLA-A*201 coupled to GFP (A2GFP system) (1). A2GFP system is based on the cellular immune response between APC and CD8+ T cells. During peptide/MHC recognition by T cells, membrane components of APC containing MHC class I molecules are acquired by CD8+ T cells through the TCR (2, 3). Since the acquisition of HLA-GFP/peptide complexes by CD8+ T cells is antigen-specific, and readily visualized by fluorescence microscopy and quantified by flow cytometry, we have exploited peptide/HLA acquisition by T cells for quantitative identification of antigen-specific T cell populations from bulk PBMC. Using A2GFP system, antigen-specific CD8+ T cells could be detected from bulk PBMC of patients with chronic viral infection at levels and sensitivities comparable to detection by tetramer. Moreover, this system could be used for the screening of unknown CD8+ T cell epitopes. We also suggest that our system may be applied to analyze antigen-specific T cell population in anti-cancer vaccination trials as well as to detect new CD8+ T cell epitopes that relate to tumor antigens.

Although quantitative detection of T cell populations by such peptide-MHC tetramers has been demonstrated to be useful for monitoring antigen-specific T cell immunity in laboratory and clinical settings (16), the construction, isolation, and purification of tetramers are typically beyond the technical capacities of most laboratories. Moreover, an essential requirement to generate these MHC/peptide tetramers is the need to *a priori* identify an immunodominant peptide known to bind the appropriate MHC. In contrast to tetramers, this artificial APC system is easy and economical to establish, and can be used for the detection of unknown T cell epitopes. The significant advantages of A2GFP system as a screening method for antigen-specific T cells are its simplicity and applicability. Since the HmyA2GFP cells stably express HLA-A*201-GFP, any peptide can be easily pulsed to HmyA2GFP cells in a short 30 minute incubation process. This method is also applicable for other HLA alleles as the construction of the HLA-GFP stable transfected cells are straightforward. We think this approach will be useful in the

analysis of other infectious agents, tumors, and autoantigens and will further enhance our understanding of antigen-host immunological interactions.

Additionally, the A2GFP system may afford us the opportunity to assess antigen-specific T cell responses after antigen recognition. The acquisition of peptide/HLA complexes by T cells is correlated with the TCR recognition and subsequent formation of the immunological synapse between APC and T cells. In Figure 3b, this can be seen at the interface between the CD8+ HTLV-I Tax peptide-specific T cell clone and the HmyA2GFP Tax peptide pulsed APC. The formation of immunological synapse is important in providing sustained TCR signaling required for T-cell function, including killing of target cells, cytokine production, and T cell proliferation. Therefore, the acquisition of peptide/HLA complexes by T cells may relate to these T cell function, and may become a useful tool for an indicator of T cell function in health and disease.

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Chapter 16

QRT-PCR

Quantitative RT-PCR for the Analysis of T cell Responses in Immunized Cancer Patients

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Abstract: The field of immune monitoring is evolving as the knowledge of tumor immunology grows and the application of immune therapies becomes more prevalent. Currently, there is no single assay that is satisfying from both an immunologic and clinical perspective. The paradox of monitored immune reactivity in the setting of tumor progression may be a function of inaccurate assays or insufficient assays. Certainly, the complex interaction of a tumor, its host, and delivered immune therapies may be difficult to measure with any single available modality. Thus, the development and application of novel technology is essential to the further understanding of these interactions. Tools such as real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) can provide the ability to measure subtle and direct molecular changes in patient derived samples. In this review we describe the use of qRT-PCR for the analysis of T cell responses in immunized cancer patients. The development, application, and precautions of this technique are highlighted.

Key words: cytokine, qRT-PCR, molecular T cell analysis

1. INTRODUCTION

The development of sensitive methods for the detection of *immune responses* against tumor antigens is essential for the rational development of cancer immunotherapy in humans. Perhaps, more essential, is the development of modalities that correlate and/or predict *clinical responses* to these novel immunotherapies. Presently, there is no single ideal methodology, but rather multiple assays that each demonstrates a component of what tumor immunologists and clinical oncologists believe to be important in the monitoring of immune responses to treatment.

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Unfortunately, none of these assays has been shown to reliably correlate with clinical outcome. The current dichotomy between detected immune responses and observed clinical responses has made the field of immune monitoring of cancer patients confusing and quite controversial. Epitomizing this observation has been the development and monitoring of active immunotherapies for metastatic melanoma. With the identification and characterization of melanoma-associated tumor antigens (TA), the logical strategy to immunize patients with derivatives of these TAs has been adopted (1-4). For example, in prominent pilot clinical trials, immunization with peptides derived from TAs have been shown by *in vitro* assays to mediate an increase in anti-peptide and anti-tumor T cell precursors circulating in peripheral blood (2, 5-8). The assays capable of detecting these reactivities depend on *in vitro* sensitization (IVS) (6, 7, 9-11). This process requires *ex vivo* incubation and expansion of peripheral blood mononuclear cells (PBMC) with the peptide used for *in vivo* immunization and subsequent testing of the cultured PBMCs for either cytokine release or lysis of antigen expressing target cells. Despite this type of evidence for immunization, none of these patients showed objective clinical responses. These findings have prompted the debate of whether these patients are truly immunized against these tumor antigens, and if so, whether these immunizations are sufficient to result in clinical tumor regression. As a consequence, current monitoring assays have been criticized for the lack of quantitative measurement of the immune response, the lack of assessment of the tumor target, and the extensive *in vitro* manipulation of the host's immune cells to detect reactivity. Up until recently, the low frequency of anti-tumor precursors has precluded the routine ability to detect these reactivities in lymphocytes obtained directly from peripheral blood or from the tumor site. Thus, analysis of the anergic or tolerant state of circulating PBMC to tumor antigens has been difficult because of the reversible nature of the functional state of the lymphocytes following prolonged *in vitro* incubation. Current approaches to directly measure the anti-tumor immune response from peripheral blood included the use of HLA/peptide tetramers (12), ELISPOT assays (13, 14), limiting dilution assays (15), and intracellular cytokine FACS (16). The advantages and limitations of these techniques are described in other chapters.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) represents an additional unique methodology that may shed light on the current paradox in immune monitoring. The ability to measure subtle differential gene expression from limited starting populations of cells during treatment or during variable immune states provides a powerful tool to examine patient derived samples. Direct studies of peripheral blood from immunized patients (17, 18) indicate this sensitive analysis may be

performed with minimal in vitro manipulation, thus, theoretically, providing a more accurate and quantitative representation of the endogenous immune status of these cells when compared to less sensitive assays that require in vitro expansion and stimulation. Further, small populations of cells that are obtained sequentially from biopsies of tumor sites or other relevant biologic compartments may finally be analyzed. Perhaps, the most significant aspect of qRT-PCR analysis is the ability to assess virtually limitless candidate genes that may be of significance. Given the potential of this technology, qRT-PCR represents a promising tool that will likely play a role in the future of immune monitoring. The development, application, and precautions of this methodology are described.

2. PRINCIPLES AND PRECAUTIONS OF QUANTITATIVE REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (QRT-PCR)

There are currently a variety of chemistries that have been validated for qRT-PCR. We have had the most experience with the TaqMan probe based chemistry developed by Applied Biosystems (19). In addition to gene specific primers, this system utilizes an additional oligonucleotide probe which is labeled at the 5'end with a reporter dye molecule such as FAM (6-carboxy-fluorescein; emission λ_{max}=518 nm) and at the 3' end with the quencher dye molecule TAMARA (6-carboxytetramethyl-rhodamine; emission λ_{max}=582 nm). Upon amplification, probes annealed to the template are cleaved by the 5'-nuclease activity of *taq polymerase*. This process separates the fluorescent label from the quencher and allows release of 1 U of fluorescence for each cycle of amplification. By monitoring the real-time accumulation of fluorescence with respect to cycle number, an amplification plot for the PCR reaction may be created. At any given cycle within the exponential phase of amplification, defined as the threshold cycle (C_T), the amount of PCR product is proportional to the initial template copy. Thus, quantitation of gene products in the starting material may be calculated. By utilizing known dilutions (or copy number) of the target gene, a standard curve may be generated and the amount of gene specific transcript in a cDNA sample can be extrapolated. This quantitative assay demonstrates high accuracy and efficiency if certain parameters are maintained. Primers and probes are designed to span exon-intron junctions to prevent amplification of genomic DNA and, also, to result in amplicons (amplification products) that are <150 base pairs to enhance efficiency of PCR amplification. The addition of a fluorescent labeled probe, rather than nonspecific DNA binding dyes, enhances specificity of gene amplification.

Standardized protocols and reagents commonly result in reliable reproducibility, both inter-assay and intra-assay, for a given sample.

Distinct advantages of qRT-PCR stem from the availability of gene sequence data that allows investigation of limitless targets when compared to traditional antibody detection approaches. Typically RNA is isolated from patient material and stored as cDNA which is very stable and may be used for a variety of gene expression studies now or in the future. With the availability of RNA amplification techniques (20), extended profiling may be performed with even a limited amount of starting material. Further, qRT-PCR can be used for the validation of microarray profiling studies (21).

Inconsistencies amongst qRT-PCR assays appear from the choice of housekeeping gene and the interpretation of results. Quantitation of a housekeeping gene allows standardization (or normalization) that arises from variability in the number of cells in samples and variability in reverse transcriptase efficiency during cDNA preparation. Typically, the quantity of the gene of interest is divided by the quantity of the housekeeping gene and the samples are reported as the ratio. Ideally, the housekeeping gene should be expressed in stable amounts in the target tissue and should have negligible perturbation with experimental conditions. With the growing experience with qRT-PCR, several traditional reference genes, such as β-actin and GAPDH, have been found to be inadequate for certain analyses (22,23). Especially important in the quantitation of T cell gene expression is the variability in housekeeping gene expression that results with their stimulation. For example, β-actin is rapidly upregulated in response to stimulation of T cells with mitogens or cytokines, such as IL-2 (personal communication from F. Marincola). Another aspect of housekeeping gene choice involves the analysis of mixed populations of cells. This difficult situation presents the complexity of identifying a reference gene that is stably expressed amongst all of the cellular components. Possible solutions include separation of cell populations with sorting or micro dissection techniques (24) or utilizing highly specific marker genes for certain cells in a mixed population. As an example, in a CD8 T cell stimulation assay from bulk PBMC (described in this chapter), cytokine mRNA production resulting from antigen specific stimulation was normalized by the amount of CD8 gene expression. This choice was appropriate given that the stimulus was a highly specific MHC class I peptide that could only stimulate CD8+ T cells. Thus, any changes in cytokine expression could generally be ascribed to the function of this particular population of cells. This was validated by the observation that CD8 mRNA levels remain stable, despite mitogenic or epitope specific stimulation. As a precaution, qRT-PCR cannot discern the ability of a few cells have very high expression of a gene versus many cells having a moderate expression of a gene. The results must be considered an

average over the population. Therefore, the selection of the housekeeping gene should be tailored to the experimental conditions and validated prior to analysis of specimens.

Interpretation of results and the definition of what constitutes a "positive result" are still not standardized amongst researchers. We have adopted the definition that a 2 fold difference from the mean expression of test genes in a control population is *statistically* positive (17). This value was found to be more than five standard deviations above the mean expression in control specimens. Further interpretation of results will need to be individualized for the particular assay and validated with independent assays examining a similar outcome. Reproducibility of an outcome is also essential. Clinical correlation, the ultimate validation of the assay, has yet to be found.

3. DIRECT ASSESSMENT OF T-CELL RESPONSES AGAINST TUMOR-ASSOCIATED ANTIGENS UTILIZING A QRT-PCR ASSAY

An immunization monitoring assay that could detect CTL (cytotoxic T lymphocytes) reactivity directly from peripheral blood, rather than after prolonged in vitro proliferation, was developed in the context of a clinical trial in which melanoma patients were vaccinated with modified peptide derivatives from the gp100 tumor antigen (17). PBMC were obtained by leukapheresis from patients before and after two cycles of peptide vaccination. Initial optimization experiments were conducted by simply exposing bulk PBMC *ex vivo* to the immunizing and native peptides or melanoma tumor cells. No prior *in vitro* sensitization or culturing of the PBMC was performed, nor were exogenous cytokines added to the cells. Because of the low overall frequency of tumor reactive CTL in bulk PBMC after immunization, changes in cytokine release after peptide elicitation were typically below the sensitivity of standard antibody detection (ELISA) assays. Therefore, real-time qRT-PCR for cytokine gene expression was performed on mRNA, which was isolated from these bulk PBMC specimens at times ranging from 2-10 hours after peptide exposure. Pre-immunized PBMC subjected to peptide and tumor exposure, demonstrated no detectable changes in cytokine production. However, the post-immunized PBMC, within a 2-hour peptide exposure, demonstrated significant increases in mRNA for the CD69 CTL activation marker, the IL-2α receptor (CD25), and the cytokines IFN-γ, TNF-α, GM-CSF, and IL-2. Peptide exposure did not result in changes in the gene expression for IL-1α, IL-1β, IL-4, IL-5, IL-8, IL-12, or IL-15. The cytokine kinetics of IFN-γ, GM-CSF, and IL-2 showed strikingly similar characteristics, however the quantitative

expression of IFN-γ mRNA was several fold higher than that of the other two genes. The favorable signal to noise ratio for IFN-γ mRNA expression made this the most suitable single gene to follow as a highly sensitive and specific marker of reactivity in our subsequent studies. Reproducible results further delineated that IFN-γ mRNA expression peaked between 2-3 hours after peptide exposure and experiments conducted with peptide concentrations ranging from 0.01 to 10 μM found that 1 μM of peptide provides optimal stimulation. We consistently noted that post-immunized PBMC demonstrated greater induction of gene expression after exposure to the peptides modified to enhance MHC binding when compared to the native peptides.

To account for potential variability in the number of CD8+ T cells in the samples and variability in reverse transcriptase efficiency during cDNA preparation, normalization of IFN-γ transcripts was performed by dividing by CD8 mRNA copies. CD8 mRNA expression was stable during experiments when compared to traditional housekeeping genes such as β-actin, GAPDH, and ribosomal RNA. To define the approximate sensitivity limit of this molecular assay, *in vitro* cultured peptide-reactive CTL clones were spiked into non-reactive autologous PBMC. Significant peptide reactivity (compared to response to an irrelevant melanoma antigen epitope) could be seen at a spiked dilution of 1 CTL clone in 50,000 PBMC. To determine whether the observed peptide reactivity was associated with tumor reactivity, bulk PBMC were exposed directly to a panel of melanoma cell lines. Response from post-immunized PBMC was found against HLA-matched melanomas that expressed gp100, but neither against HLA-matched melanomas that did not express gp100 nor HLA-mismatched melanomas that expressed gp100. Thus, our cumulative observations of peripheral lymphocytes demonstrated that peptide immunization could result in a significant increase in circulating CTL with highly specific activity directed against a tumor antigen target. Further, these findings were evident by this assay in cells which were obtained directly from patients without any prior in vitro manipulation.

4. APPLICATION OF QRT-PCR IN IMMUNE MONITORING OF PATIENTS

The original application of qRT-PCR for the measurement of specific T cell responses to tumor antigens was described in a pilot study examining patients immunized with peptides derived from a melanoma tumor antigen (17). This study demonstrated tumor specific CTL directly in the peripheral blood of patients who had undergone immunization and further validated the

conclusion that peptide vaccination can result in specific reactive CTL *in vivo*. The findings from the molecular assay were verified in a subsequent study and were highly correlated with the results of the standard labor intensive *in vitro* sensitization assay (18), as well as, tetrameric HLA analysis and intracellular cytokine FACS analysis (25,26). Since then, other investigators have validated the technique for the assessment of T cell reactivities in a variety of cancers treated with varying immunization protocols including HLA matched peptides, whole proteins, and whole tumor cells (27-32).

An important additional application of qRT-PCR is in the monitoring of *in vivo* tumors during therapy. Previously, macroscopic, microscopic and molecular changes in targeted tumor sites required resection and analysis. This approach would therefore eliminate the ability to sequentially follow an individual lesion prospectively during treatment. We have developed techniques to perform serial fine needle aspirates (FNA) of accessible tumor sites before and during immunotherapy. This proved to be atraumatic to the tumor, while leaving it *in situ* for clinical observation and immunologic monitoring. Initially these FNAs were analyzed with histologic techniques, such as immunohistochemistry. The limitations were the availability of specific antibodies and the ability to maximally gain information from such a small amount of tissue. The use of highly sensitive and specific gene expression analysis of FNAs represented a new and powerful tool. Concomitant with the analysis of PBMC in immunized melanoma patients, we conducted serial analysis of FNAs from tumors in these same patients (17). Using qRT-PCR we were able to demonstrate significant increases in IFN-γ mRNA in FNAs of tumor metastases after immunization with peptide vaccination. This was not seen in patients receiving non-peptide therapy. Further, we quantitated tumor antigen gene expression of the targeted protein in these same lesions. Interestingly, there was a strong correlation between IFN-γ mRNA and tumor antigen expression in lesions of post-immunized patients. An additional use of qRT-PCR is to perform *in vivo* tracking studies of T cells that are given in adoptive cell transfer protocols. By analyzing specific T cell receptor (TCR) gene expression for the variable region of the TCR β chain, a T cell clone can be followed, localized and quantitated at the tumor site or in peripheral blood. Parallel analysis of peripheral circulating lymphocytes and aspects of the tumor microenvironment may represent a more immunologically relevant approach to monitoring.

5. CONCLUSIONS

The field of immune monitoring is evolving as the knowledge of tumor immunology grows and the application of immune therapies becomes more prevalent. There is no single assay that is satisfying from both an immunologic and clinical perspective. The lack of clinical correlation between current monitoring and patient response is still a puzzle. Perhaps, analysis of a single biologic compartment with a single assay will not describe the whole story of a tumor and its complex host interactions. In an effort to develop more accurate forms of evaluation, newer technology will be important. Tools such as qRT-PCR represent a powerful adjunct to this analysis. Utilizing this technique, we and others have shown the ability to measure subtle and direct molecular changes in patient derived samples. This seems essential to the understanding of treatment effects. As with any new technology, the use of qRT-PCR needs to follow strict and validated guidelines to provide relevant information. In the appropriate context, this type of analysis represents a great potential.

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Chapter 17

MICROARRAYS

Gene expression profiling approaches for the monitoring of anti-cancer immune responses

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Abstract: Modulation of cancer growth by the immune system is a natural phenomenon that can be enhanced by immune manipulation. The complexity of this biological event has only partially explored. Conventional monitoring of immune responses has extensively focused on specific interactions between immune and cancer cells based on limited number of well defined molecules. The discovery of additional co-factors and multiple components involved in a variety of signal transduction and other regulatory pathways of immune recognition has broadened the horizons of conventional immunology studies. As the understanding of the network of interactions between individual molecules associated with immune function increases, it is becoming apparent that no single mechanism or hypothesis can in itself explain complex phenomena such as immunologically-mediated tumor rejection. As described in depth in previous chapters, the components of the innate and adaptive immune response that may be involved in successful tumor rejection are far more complex than it could be described with a hypothesis centered approach as least at the time of this writings. Several components of the immune response may be genetically pre-programmed, epigenetically modified and variably recruited or regulated within the tumor microenvironment by factors with immune modulatory properties secreted by tumor and/or bystander cells. Such complexity can only be appreciated and resolved with the help of high throughput tools capable of providing a global view of biological processes as they occur. A dynamic snap shot at the global transcript level could provide a whole insight of tumor host interaction and lead to a better understanding of the mechanisms of tumor rejection. In this chapter, we will select examples of how high-throughput gene expression profiling may contribute to the understanding of anti-cancer immune responses and strategies which could be applied in immune monitor during cancer therapy.

Key words: microarray, gene expression profile, antigen-specific, T cell

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1. INTRODUCTION

It has been extensively documented that tumor infiltrating lymphocytes (TIL) expanded *in vitro* in the presence of T cell growth factors such as interleukin-2 (IL-2) could recognize and lyse autologous and HLA matched cancer cells (1;2). Based on this observation, efforts have been made to enhance the specific immune recognition of cancer cells by immunological manipulation of patients with cancer, in particular melanoma and renal cancer which lead to dramatic tumor regression in a small but reproducible proportion of patients (3;4). The study of circulating CD8+ T cells from a long term cancer survivor demonstrated that these cells specifically killed autologous cancer cells through the recognition of a non-mutated protein over-expressed in cancer tissues (5). These T cells were used for the identification of the first tumor antigen (TA) recognized by T cells. This strategy was rapidly adopted and proved extremely efficient in the identification of an ever growing number of cancer-associated molecular targets of T cell recognition (see chapter 1).

The identification of TA not only provided a powerful tool to deepen our understanding of the complex biology of tumor host interactions but also brought a therapeutic opportunity for the enhancement of immunological treatment of cancer. In addition to T cells, autologous antibodies can also recognize proteins preferentially expressed by cancer cells (6) providing further evidence that adaptive anti-cancer immune responses are a natural phenomenon. However, while the biological significance of this observation is undeniable (6), the relevance that the humoral immune response has in the recognition and control of cancer remains unclear since most TA recognized by antibodies are intra-cellular proteins that cannot be reached by them. Therefore, TA recognized by T cells have received proportionally more attention for the development of anti-cancer vaccines since they are readily accessible to T cell recognition (7;8). As a consequence, a large number of anti-cancer trials are aimed at enhancing cellular immune responses using active-specific immunotherapy have become the centre of various clinical trials in the last decade particularly in the context of melanoma (9). These clinical studies have provided us with the unprecedented opportunity to study at the molecular level the dynamics of a developing immune response in the cancer bearing host and lead to an in depth understanding of the complexity of anti-cancer immune response.

The prospective of using vaccination with TA and TA associated epitopes restricted to specific HLA alleles attracted great enthusiasm among immunologists at the beginning. In most clinic trials, HLA-associated nine to ten amino acid peptides representing CD8+ T cell epitopes were used often in combination with adjuvant emulsions for the immunization of patients

with cancer. Their effectiveness in inducing systemic enhancement of TA-specific immune responses was easily validated by us and many others through the identification of increased epitope-specific circulating lymphocytes following immunization (10). However, in spite of the induction of tumor-specific circulating CD8+ T cells, in general, vaccination alone could not, or only rarely induced, tumor regression. It was only when the systemic administration of high dose IL-2 was combined to immunization that clinical responses were observed with a frequency that appeared higher than that observed when IL-2 alone had been used for treatment of historical controls (11). The low frequency of objective tumor regression contrasting with the high frequency of detection of vaccine-specific T cell responses provoked a reactive pessimism as it was felt that immunization had failed the expectations and lest perplexed tumor immunologist. As a consequence, the enthusiasm for the adoption of epitope-specific immunization gradually faded and alternative approaches are being explored aimed at increasing the intensity of the immune responses while counteracting factors that might impede their full implementation in patients.

From a strictly immunological point of view, however, TA-specific immunization has been extremely successful since it reproducibly achieves its only biological goal which is to induce T cells that can recognize TA. Extensive evidence supports this conclusion as systemic TA-T cell responses can be readily detected by *ex vivo* after immunization (9). Therefore, for the first time we are capable of enhancing cancer-specific immune responses using natural reagents. In addition, epitope-specific immunization offers the unique opportunity of studying in humans the dynamics of immune responses by-passing the complexity of human polymorphism and simplifying the heterogeneity of cancer biology. In this clinical model, the algorithm regulating tumor host interactions is simplified by reducing to a least common denominator a single human leukocyte antigen (HLA) interaction with a tumor-specific epitope and the complementary T cell receptor (TCR). In this fashion, the number and phenotype of T cells induced by immunization can be easily tracked using epitope-specific assays (see chapter of ELISPOT, tetramer and peptide-HLA-GFP complexes). In addition, the evolving nature of cancer cell phenotypes whether dictated by the intrinsic genetic instability of the disease (12) or by immune editing by the host (13) can be accurately followed by serially verifying their expression of the relevant HLA allele and/or TA.

The contradictory observation of low frequency of clinic cancer regression in the presence of easily detectable systemic immune responses could be studied in these simplified models and has suggested that factors beyond the direct T cell-tumor cell interactions are responsible for lack of

clinical effectiveness of active immunization trials (14;15). In fact, immune responsiveness may very likely be modulated at the receiving end, the tumor microenvironment by factors secreted by cancer or normal bystander cells. Biologically, cancer is a systemic disease characterized by uncontrolled cell proliferation due to genetic changes that affect control of cell growth and differentiation. In addition, the impaired DNA repair, accumulation of genetic and epigenetic changes, constant tissue remodeling and homeostatic feedback from the host organism make cancer an irreversible, unstable and a dynamic changing process. This dynamic state determines not only the establishment of cancer but also its natural history such as local invasiveness and metastatic spread. Thus, the natural history of individual cancers is a multi-factorial phenomenon resulting from complex interfaces between the biological characteristics of individual tumors determined by the genetic instability of malignant cells and the polymorphic response of the host. In this process, it is likely that cancer cells develop phenotypes that can modify to their benefit the surrounding microenvironment by increasing factors that may provide for their growth and decreasing the effectiveness of those that may pose a barrier to their growth. Therefore, the understanding of mechanisms involved in tumor recognition and rejection by host should be sought at a broader dimension which takes into consideration system-system interactions, cell-cell interactions, molecule-cell interactions, gene-molecule regulation and molecule-molecule regulation. Hence, the complexity of the tumor/host interaction network can only be appreciated with tools that allow a comprehensive global and dynamic view of their interactions. As immune responsiveness may be related to individuals' propensity to respond to immune stimulation, we will first start considering genetic variation in individual genomic make up as a possible arbiter of immune responsiveness.

2. GENETIC POLYMORPHISMS AND CANCER HETEROGENEITY

Genetic polymorphism is the genesis of the diversity of human beings. Polymorphisms are particularly frequent in genes associated with immune function possibly resulting from an evolutionary adaptation of the organism facing an ever changing environment (16). Although, several genes associated with immune function may bear relevance to tumor/host interaction(s) (17), most studies attempting to link human polymorphism to immune responsiveness have been focused on the associations between the Human Leukocyte Antigen (HLA) complex and disease occurrence or response to therapy. However, an extensive analysis of patients with metastatic melanoma treated with systemic interleukin-2 administration

failed to convincingly identify a correlation between HLA phenotype, immune responsiveness and toxicity (18). Extended studies based on polymorphism of lymphokines, chemokines and their receptors have demonstrated an association in individual susceptibility to immune pathology, survival of transplanted organs or predisposition to cancer (19;20) (21-23) (16). The vast majority of polymorphism found in cytokine genes and their receptors are located in the promoter, intronic and 3' untranslated regions. The sequence variances occurring in untranslated regions of the gene can, however, still affect gene expression and function. In addition, promoter polymorphism may disrupt or abolish binding of regulatory elements such as NF-*k*B, Jak, STAT and other molecules involved in signal transduction pathways. Furthermore, some of this molecules central to cytokine expression levels are polymorphic themselves. Finally, intronic variation may affect enhancer/silencer sequences and certain polymorphisms may alter the structure of transcription factor binding elements (24). Thus, in the future more focus should be put in the understanding of genetic traits that may affect the ability of individuals to mount effective immune responses against their cancers.

3. GENETIC VARIATION AS A FUNCTIONAL PARAMETER OF A COMPLEX BIOLOGICAL EVENT

Biological processes, similarly to weather forecasts, are based on patterns that follow non-linear mathematics (25-28). With modern technology, it is now possible to confront biological processes as a whole by identifying genetic, epigenetic changes in a genome wide fashion and at the same time follow transcriptional activation of all or almost all genes simultaneously (28;29). This approach yields global information comparable to pictures broadcasted by satellites about weather patterns around the globe. Unfortunately, while the technological tools are available for the global study of the dynamics of tumor/host interactions, very little material of clinical relevance is available to apply these new powerful technologies.

In fact, although this approach is nowadays feasible, a challenge to the study of human disease is the difficulty in controlling the amount and quality of clinical material available to study. Human samples should be valued as the fruit of a unique and un-repeatable experiment to be tested for commonalities and diversities with other similar events. Thus special strategies should be implemented to optimize the information obtainable from precious samples of blood, tumor or other tissues. In addition, as tissues and tumors cannot be as easily removed for experimental purposes

from humans as in animals, strategies need to be applied that allow serial sampling of relevant material at different time points without compromising the patient's status. We have recently summarized our thoughts on the subject by suggesting for instance fine needle aspirates (FNA) as a tool to follow the natural history of cancer or its response to therapy (30). In this fashion, tumor biology can be followed serially during a given treatment to test its mechanism of action. In addition, samples can be obtained before a given treatment is administered while leaving the tumor deposit in place to directly compare the biological profile of individual lesions with its own outcome.

4. DISSECTING T CELL FUNCTION BY GLOBAL TRANSCRIPT ANALYSIS

Although several groups have observed that epitope-specific immunization induces TA-specific, tumor-reactive CD8+ T cells (15;31-37), it is clear that with few exceptions (36), these immune responses are not associated with tumor regression. There are many reasons why cytotoxic, CD8+ T cells may not exert effector function at the desired site. Their number may not be sufficient, their status of differentiation or activation may not be suitable or they may not localize in the target organ. Experimental work by Kaech SM *et al*. (38;39) dramatically illustrates this point. These authors followed the transcriptional profile of CD8+ T cells following acute exposure to antigen in a transgenic mouse model. P14 transgenic mice that harbor P14 CD8+ T cells expressing a TCR that recognizes the GP33-41 epitope of the LCMV protein were exposed to LCMV infection and the antigen-specific CD8+ T cells harvested 8 and 40 days after. This model seems to apply very well to epitope-specific immunization which also provides time-limited exposure to TA at the time of vaccination. This immune stimulation is then usually followed by a rest interval ranging generally between two to four weeks. In the mouse model, global transcript analysis of antigen-specific CD8+ positive T cells identified distinct phenotypes at different time points from antigen exposure that could be correlated to functional parameters (39). During the rapid expansion of CD8+ T cells in the first week after antigen exposure an effector phase is observed that peaks at around 8 days. At this time point CD8+ T cells are cytotoxic *ex vivo*, can respond to cognate stimulation with production of interferon (IFN)-γ and have a genetic profile rich of effector/activated T cell features including granzyme-A and -B, perforin and FAS ligand. In the following days, the immune response goes through a contraction phase that lands into a memory phenotype approximately 40 days after antigen

exposure. At this point, CD8+ T cells specific for the immunogen can still respond to cognate stimulation with IFN-γ production but they lost the ability to kill target and lost the expression of a variety of genes associated with T cell effector function.

This linear model contrasts with previous models in which a switch from naïve to memory cells is followed by a gradual transformation into an effector phenotype characterized by $CD45RA^{high}$, perforin and IFN-γ expressing T cells (40). However, this model of T cell activation/differentiation seems to better explain experimental observations related to immunization-induced T cells (38). Although these cells seem to retain an effector phenotype according to common markers (CD27 negative, CCR7 negative, $CD45RA^{high}$) and can respond with IFN-γ secretion to cognate stimulation, they do not express perforin and cannot exert effector functions (41). Analysis of their extended phenotype is presently ongoing in our laboratory: we could successfully separate using magnetic beads vaccine-specific CD8+ T cells according to tetrameric HLA/epitope complex (tHLA) staining and amplify their messenger RNA for micro-array analysis (15;42;43). Preliminary work suggests that the transcriptional profile of circulating CD8+ T cells approximates that of memory cells with a relative down-regulation of the expression associated with cytotoxic and other effector functions (15). Therefore, immunization T cells display a phenotype closer to classic memory cells. Therefore, it may not be that surprising to observe that the frequency of tHLA staining, epitope-specific T cells observed in the peripheral circulation may not represent a direct correlate of tumor rejection as these T cells may be depleted of true effector function (14).

5. THE INTERACTIONS BETWEEN TUMOR CELLS AND TA-SPECIFIC T CELLS

The hypothesis that circulating, vaccine-induced T cells may be capable to reach the tumor site and interact with tumor cells is substantiated by previous work from our laboratory. We measured by quantitative real-time polymerase chain reaction (qRT-PCR) the messenger RNA level of various cytokines supposedly produced by T cells before and during immunization. The genetic profile of individual lesions was followed by serial FNA. This study demonstrated that presence of immunization-induced T cells in the circulation correlated with increments in cytokine transcription (in particular IFN-γ) during immunization compared with before. In addition, increased messenger RNA levels correlated with expression of the antigen targeted by the immunization and the localization of tHLA-staining, immunization-

specific T cells intra-tumorally (44). It appeared, however, that this interaction between immunization-induced T cells and tumor cells that could induce the expression of IFN-γ was not sufficient to induce tumor regression as the growth of all the lesions studied progressed during treatment.

These findings was in agreement with earlier studies done at the National Cancer Institute, NIH in which intra-lesional localization of TA-specific T cells was noted to be necessary although not sufficient for tumor regression. Obviously, the tumor infiltrating lymphocyte phenomenon in itself provides the best evidence that the presence of TA-specific CD8+ T cells within a tumor is not sufficient to cause its regression (1). As we also previously discussed, increments in the frequency of TA-specific T cells can be demonstrated during vaccination which also does not lead necessarily to tumor regression (45). Most dramatically, the adoptive transfer of *in vitro* expanded TIL labeled with radioactive ^{111}In demonstrates that their localization is necessary but not sufficient for tumor regression since TIL home in all the lesion that responded to therapy but not all the lesions in which TIL localize respond to treatment (46). Thus factors other than tumor/T cell interaction at the tumor site are responsible for immune responsiveness. It could be hypothesized that TA-specific T cells naturally or during therapy may reach the tumor micro environment but in most cases they are not exposed to sufficient stimulation for their activation into full effector cells. Tumor cells may lack sufficient antigen presentation or the tumor micro-environment may lack sufficient co-stimulatory properties (47). We favor the second hypothesis since we have never been able to accumulate evidence that lack of antigen expression is a primary reason for tumor unresponsiveness and, as previously discussed we have observed in several cases direct interactions between TA-specific T cells and tumor cells within the tumor microenvironment that did not produce clinical response. A recent study in which the transcriptional profile of tumor lesions was assessed before and during therapy demonstrated that when lesions to not response to therapy, no changes can be identified in the expression of the TA targeted by the vaccine (48). In addition, the level of expression of the TA targeted by the vaccine is not a predictor of responsiveness (48). Since this study was done in lesions that expressed the HLA antigen associated with the immunization, HLA loss could also not be deemed responsible for clinical outcome. In addition, lack of response was associated with a “silent” genetic profile characterized by no significant differences in global gene expression between treatment and pre-treatment samples (49). This finding was also corroborated by an independent analysis based on global transcript analysis (49). Interestingly, while TA expression was not predictive at response, loss of TA expression was consistently observed during therapy in lesions destined to regress while no changes were noted in lesions that did

not respond (48). Loss of TA expression preceded clearance of tumor cell during response as the expression of other TA irrelevant to the immunization remained stable and cytological analysis confirmed the presence of tumor cells. Thus, it is likely that TA-specific T cells induced by immunization reach the tumor site, interact with tumor cells and are exposed to antigen recall but a secondary co-stimulation is lacking to further expand their number *in vivo* and activate their effector function (14). It is presently unclear what could act as a stimulator of T cells in those rare instances in which tumor regression occurs.

6. THE TUMOR MICROENVIRONMENT MAY CONTRIBUTE TO THE IMMUNE RESPONSE

The tumor microenvironment is complex, heterogeneous and ever changing in adaptation to immune pressure, response to therapy or simply as a consequence of the genetic instability of cancer cells that can rapidly adapt to their environment (14). This concept, however, has received little attention by immunotherapists involved in the monitoring of anti-cancer immune responses mostly because the analysis of the tumor microenvironment is difficult to perform in humans because requires in most cases surgical intervention. Thus, immune monitoring has been in most cases limited to the study of immune responses in circulating lymphocytes that are easy to access through venipuncture. We have, however, extensively emphasized how tools are nowadays available to study in real-time adaptation of the tumor microenvironment to immune manipulation through the serial sampling of tumor samples using fine needles aspirates (30). For instance, immune responses can be predicted by sampling tumor lesions before the beginning of therapy and documenting their clinical outcome prospectively (49). This strategy allowed direct correlation of experimental observations with clinical parameters. In addition, the same approach can be used to study the mechanism of actions of therapeutic agents like systemically administered IL-2; this can be achieved through the comparison of the expression profile of tumor lesions biopsied before and during therapy (50).Thus, it is obvious that, unless serious efforts will be spent trying to understand tumor/host interactions in their target tissue no major conceptual or practical progress will be made.

7. THE IMPACT OF GENETIC VARIATION AND CANCER HETEROGENEITY ON IMMUNE RESPONSIVENESS

It is obvious that prediction of immune responsiveness is very beneficial because it can spare the pain and unnecessary therapy to a patient with an expectedly short life span. In addition, it may add the conceptual value of approaching the understanding of the biological process responsible for immune response which in turn may lead to more focused therapies.

As we previously discussed, genetic background may influence immune responsiveness. However, the genetic background of patients has not been extensively scrutinized as a predictor of immune responsiveness (16). Obvious genetic markers that may affect immune responsiveness are the HLA complex that codes for molecules responsible for antigen presentation to T cells (51). However, correlates between HLA phenotype and treatment outcome or survival failed to provide conclusive evidence that individual variability in antigen presentation may determine immune responsiveness in the context of anti-cancer immune therapy (18;52;53). Others have pointed to other polymorphisms as harbingers of immune responsiveness. For instance polymorphisms of the IL-10 gene appears to be responsible for differential levels of expression of this cytokine in various conditions. Interestingly, individuals with a phenotype associated with low IL-2 production appear to bear an increased incidence of melanoma and prostate cancer suggesting a surprisingly protective role of this cytokine against cancer growth (23;54). More generally, this finding suggests that a genetic predisposition may modulate cancer growth through the action of immune regulatory molecules. However, there is no information about the relevance of various genetic factors associated with immune function and response to therapy. Indeed, awareness to this aspect of immune polymorphism only recently has been appreciated as a significant factor that may significantly modulate immune pathology (16;54) and only recently techniques suitable for the screening at a genome wide level of polymorphism(s) in clinical settings have been described (16;55).

8. DYNAMIC MONITORING OF ANTI-CANCER IMMUNE RESPONSES

Although genetic background may be responsible for immune responsiveness, it is also possible that the unstable nature of cancer cell phenotypes can strongly influence the susceptibility of cancerous lesions to immune attack. In fact very little is known about the algorithm that may

determine the occurrence of immune-induced cancer regression in humans (56). The introduction of gene profiling arrays is particularly suited to circumstances when little is known about a biological event to conceive plausible hypotheses. This is clearly the case of immune-mediate cancer rejection. We tested whether global transcript analysis could segregate lesions likely to respond to immunotherapy by obtaining FNA from subcutaneous melanoma metastases prior to immunotherapy (49). This work was based on a previous observation suggesting that cutaneous melanomas can be segregated into two distinct taxonomies based on global transcript analysis (57). Such observation stimulated the question of whether two disease pathologically defined as melanomas had a different biology and consequently, perhaps, different predisposition to respond to immune therapy. However, the original observation was based on the analysis of cell lines or tissue preparations that has been collected a long time before and for which very little information about the clinical outcome of the patient from which they were obtained was available. By prospectively collecting clinical information on those lesions from which FNA samples had been obtained, it was possible to link directly their gene expression profile to their response to therapy. In addition, since FNA allows serial sampling, it was possible to monitor the changes in the transcriptional profile occurring with time and/or in response to therapy in individual lesions. The results of this study underlined the importance of introducing a temporal dimension to the study of cancer biology. By studying the transcriptional changes of individual lesions with time whether or not in relation to treatment it was possible to understand that the two melanoma subgroups did not represent two distinct disease taxonomies but rather two stages of the same disease rapidly evolving because of its intrinsic instability (12). Indeed, it appeared that the two subclasses of melanoma represented two different stages of differentiation with one displaying a transcriptional profile close to normal melanocytes and the second cluster including later samples with an undifferentiated phenotype characteristic of a more advanced stage of disease (49). Although categorization of melanomas failed to predict immune responsiveness, it was possible to identify more subtle predictors of immune responsiveness by separating lesions that regressed from those that did not in response to therapy. In fact, a supervised analysis of lesions according that did or did not respond to active-specific immunization combined with IL-2 administration identified several genes whose level of expression could predict of immune responsiveness. Analysis of functional annotations revealed that these genes were predominantly associated with immune function suggesting that analysis of tumor deposits before therapy is informative as it demonstrates that some tumors are pre-conditioned to respond perhaps because their tumor microenvironment is more

immunologically active before treatment administration (49). Several genes associated of immune responsiveness were of particular interest. For instance, interferon-regulatory factor-2 (IRF-2) was found to be over-expressed in lesions predisposed to respond to therapy. Since this gene up regulation is often seen in chronic inflammation this finding suggests that tumors likely to respond are chronically inflamed before treatment and this chronic inflammatory process may be beneficial or favorable to the outcome of immune therapy. Obviously, the inflammatory process is not sufficient in itself to induce tumor rejection without therapy and it may be beneficial for tumor growth, but it may set the stage for a conversion to an acute inflammatory process by recruiting immune cells at the tumor site that can be, in turn activated, by exogenous immune stimulation (58). A paired analysis of FNA samples obtained before and during therapy underlined this possibility since lesions that underwent complete response over-expressed of IRF-1 during therapy. IRF-1 acts as a counterpart to IRF-2 and its expression is up regulated during the development of an acute inflammatory process (59). Interestingly, lesions that did not undergo regression did not demonstrate any significant changes in their transcriptional profile in response to therapy (49).

It remains unclear why some tumors may behave differently than others and why some are more likely to be triggered into an acute inflammatory process. Some have suggested that inflammation is beneficial and necessary for tumor growth (60;61). This observation is not contrasting with our observation that acute inflammation may be necessary to induce cancer regression. Moderate inflammation may be helpful for the promotion of angiogenesis or may act as a direct stimulus to tumor growth as many factors released during tissue remodeling and repair have stimulatory effects on tumor cell growth. Thus, growth factor produced by tumor cells for the selfish purpose of survival may mimic the normal response of the organism to injury that promotes repair. This beneficial biological process may at the same time act on immune cells as inflammation and repair collaborate in response to injury. In fact, several growth factors have chemo-attractant and regulatory properties on immune cells. These molecules can induce the migration of cell of the innate and adaptive immune system within the tumor microenvironment. Such cells are probably not capable by themselves to exert anti-cancer properties but could rapidly turn into powerful effector anti-cancer cells given appropriate stimulatory conditions that may be induced by treatment such as the systemic administration of IL-2 (50).

In an effort to identify what conditions are most likely to turn and indolent chronic inflammatory process within the tumor site into an acute auto-immune rejection of cancer we studies the mechanism of action of IL-2. This cytokine is a powerful anti-cancer agent that has not direct effects of

cancer cells growth IL-2 seems to increase the chances that tumor lesions are conditioned toward a switch from chronic to acute inflammation. In fact, although clinical responses are relatively rare, their dramatic occurrence is characterized by a rapid disappearance of large tumor bulks and in some instances long term disease free survival (3).Thus, IL-2 is the best gauge we have presently to study the mechanism(s) responsible for immune responsiveness in humans.

The effects of IL-2 are, independently of their therapeutic effects, are of extreme biological interest and it is surprising how little it is spent trying to understand its mechanism(s) of action. Some have suggested that IL-2 acts by facilitating the passage of tumor-specific T cells from the circulation to the tumor site by increasing blood vessels permeability (11). In addition, IL-2 has been considered a growth factor or activator of CD8+ T cells (62). Others postulated that IL-2 may induce activation of intra-tumoral endothelial cells which may in turn promote migration of TA-specific T cells within tumors (63). In addition, IL-2 induces a secondary production of an extensive array of cytokines through stimulation of circulating mononuclear cells that could have broader immune/pro-inflammatory effects than those expected by the interaction of IL-2 with its receptor (50;64;65). In a recent study we compared the early changes in the transcriptional profile of circulating mononuclear cells with those occurring within the tumor microenvironment of melanoma metastases following systemic IL-2 administration (50). The results of this study surprisingly suggested that the immediate effect of systemic IL-2 administration on the tumor microenvironment is a transcriptional activation of genes predominantly associated with monocyte function while minimal effects were noted on migration, activation and proliferation of T cells. Thus, this study suggested that IL-2 induces inflammation at tumor site with three predominant secondary effects: activation of antigen-presenting monocytes, massive production of chemo attractants that may recruit other immune cells to the tumor (including MIG and PARC) and activation of cytotoxic mechanisms in monocytes (calgranulin, grancalcin) and natural killer cells (NKG5, NK4) that may in turn contribute to epitope spreading through killing of cancer cells, uptake of shed antigens and presentation to adaptive immune cells.

This information is important in view of some recent unpublished work suggesting that circulating immunization-induced, TA-specific T cells are in a “quiescent” status of activation that requires for full activation the combination of antigen recall (readily available at tumor site) with a second signal possibly provided by pro-inflammatory cytokines or co-stimulatory molecules induced by the effects of IL-2 on the tumor microenvironment (66). Thus, it is obvious that the relationship between T cell and tumor cells within the tumor microenvironment should be the focus of future research

since the determinism of immune responsiveness will be locked and submerged by factors that modulate the function of T cells in their target organs.

9. CONCLUSIONS

Tools are available nowadays to study biological processes in their globality (29). The study of individual genetic predisposition to disease and response to treatment (16;55) can be combined with that of epigenetic changes during life and disease progression (29) and to real-time analyses of the adaptation of the transcriptional profile of biological samples in various clinical conditions (29). The combination of these tools should be able to provide an understanding of human diseases and their treatments rapidly and this is a preliminary step for the design of truly rational therapies. This may particularly apply to cancer immune therapy that is compounded by the chaotic essence of cancer biology, the complexity of the immune system and the polymorphic nature of our species all of them limiting the relevance of animal models (16). High-throughput technologies (29) allow the efficient screening in humans of theoretical models generated from animal experimentation, from in vitro studies or from mere speculation. With these new tools it is reasonable to hope for a rapid identification of the algorithm required for cancer rejection by the immune system.

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CONCLUDING REMARK

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More than a dozen methods have thus far been developed for monitoring and analyzing T cell responses to tumor-associated and viral antigens. ELISPOT, IC-FC, and tetramers, have emerged as standard assays for first-line monitoring. All other techniques described in this book play a special role in advancing the analysis of T cell responses. Several clinical trials aimed at inducing antigen-specific T cell responses hold out hope that cellular immunotherapy will join chemotherapy, surgery, radiation, and hormone therapy as the fifth pillar of cancer treatment. It has already been pointed out that measuring a specific immunotherapy-induced T cell response is not a surrogate marker for a clinical response. However, monitoring T cell behavior during immunological treatment approaches like vaccination will promote the development of more efficient therapeutic strategies. While immunological assays now dominate T cell monitoring, it will be interesting to see how particularly molecular/transcriptional methods and more functional assays will contribute to the development of cancer immunotherapy. Particular attention will also have to be paid to the direct tumor-host interaction within the tumor microenvironment, the role of regulatory and helper T cells, and the contribution of mononuclear phagocytes, i.e. antigen-presenting cells, at different stages of development.

We thank all those who have contributed to this book. We are especially grateful to the authors from four continents for their state-of-the-art survey of T cell analysis.

Berlin and Bethesda, March 2005
Dirk Nagorsen & Francesco M. Marincola

COLOR PLATES

Chapter 3

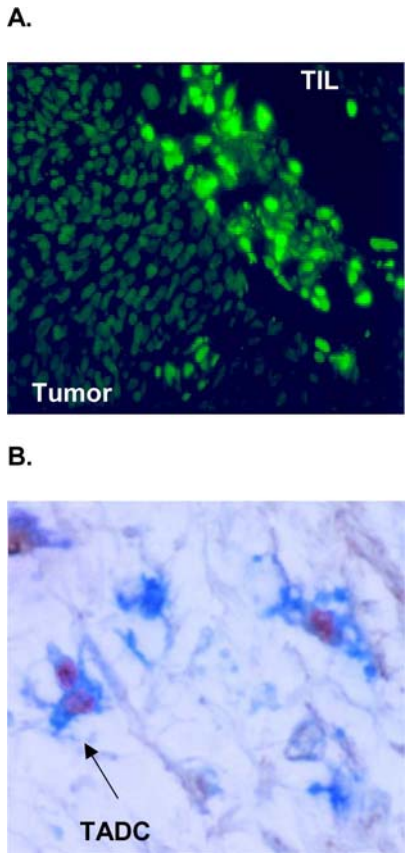

Figure 4. Apoptosis of lymphocytes and dendritic cells in the tumor microenvironment. Surgically removed sections of (A) human oral carcinoma and (B) prostate carcinoma in situ were analyzed for the presence of (A) lymphocyte apoptosis by TUNEL staining and (B) dendritic cell apoptosis by determination of caspase activity. TIL=tumor infiltrating lymphocytes; TADC=tumor associated dendritic cells. Black arrow denotes caspase+ (red) DC (blue = CD83+).

Chapter 12

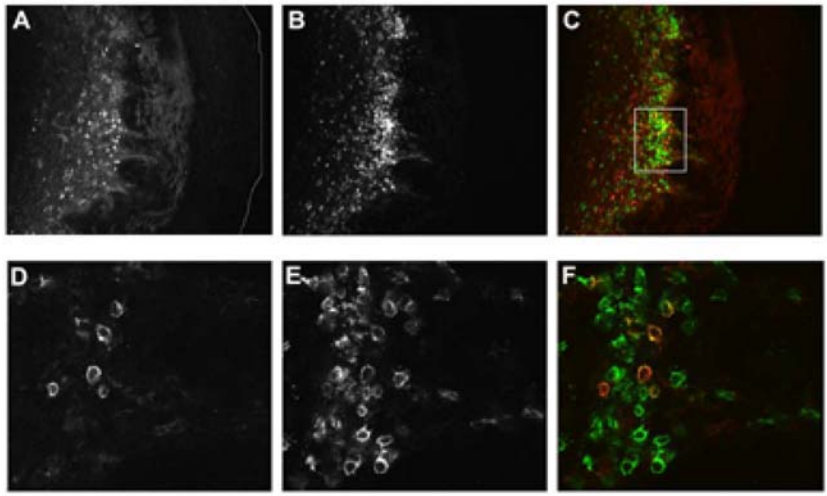

Figure 1. MHC tetramer and CD8 antibody staining of SIV specific T cells in ectocervix. A) and D) show Mamu-A*01/gag tetramer stain. B) and E) show CD8 antibody stain. C) and F) show a merged image of A) with B), and D) with E), respectively. In the merged images, tetramers are colored red and CD8 colored green. The white line drawn in panel A) delineates the epithelial surface.

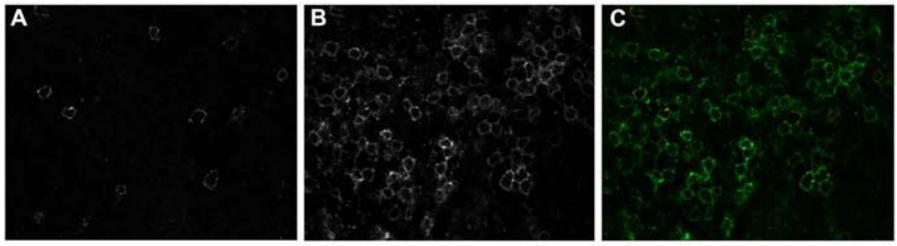

Figure 2. MHC tetramer and CD8 antibody staining of SIV specific T cells in lymph node showing a punctate tetramer staining of the T cell membrane. A) Shows Mamu A*01/gag tetramers. B) Shows CD8 antibody stain. C) Shows a merged image of A) and B) with tetramers colored red and CD8 antibodies green.

Chapter 14

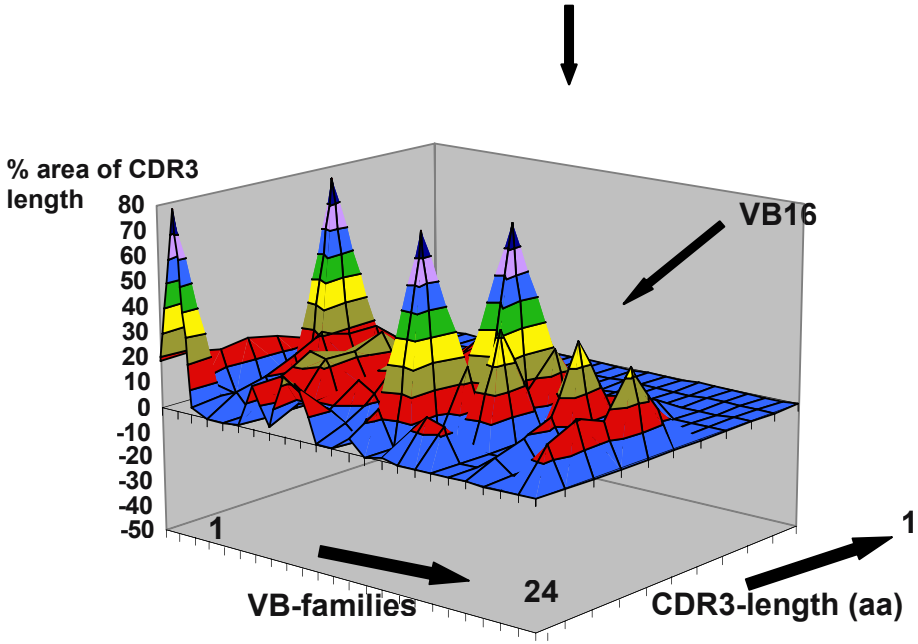

Figure 4. CDR3 length measurement. In normal healthy subjects, the CDR3 peak pattern represents a Gauss distribution. The data for each TCR family and the area under the curve for each TCR peak (i.e. 3 bp, 1 aa) are compiled. This CDR3 pattern may be depicted two-dimensionally, i.e. one picture for each TCR VA/VB family, or, alternatively, the entire TCR VA/VB repertoire may be combined in a single complex figure, thereby creating a TCR VA or TCR VB 'landscape' as a function of CDR3 length (as defined by the number of encoded aa) and the area under the curve for each individual CDR3 peak. For the sake of clarity, each 10% of the CDR3 peak is depicted in a different color. Two-dimensional (top) and three-dimensional (bottom) picture of the TCR repertoire analysis as defined by TCR VB spectratyping in a CD4+ TIL line from a patient with cervical cancer. The two-dimensional picture allows to analyze each VB family and the 'landscape' picture represents an overview of the TCR diversity. The single TCR VB16 chain (marked with the arrow) was found to be monoclonal and to recognize autologous tumor cells.

Chapter 15

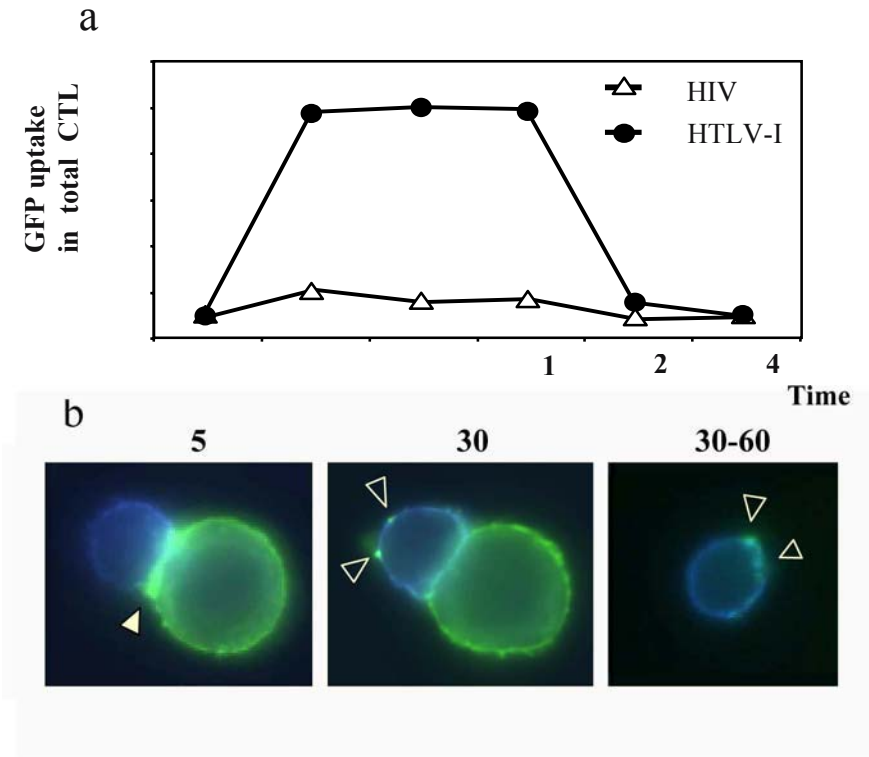

Figure 3. Time-dependent acquisition of peptide/HLA-GFP complexes by T cells. (a) HTLV-I Tax11-19-specific CTL clone was incubated for the indicated time with HmyA2GFP cells pulsed by Tax11-19 peptide or HIV Gag peptide. HLA-GFP acquisition by CD8+ CTL population was directly analyzed by flow cytometry. The levels of HLA-GFP acquisition are expressed by mean fluorescence intensity (MFI). (b) Visualized images of peptide/HLA-GFP complexes acquisition by HLA-A*201 restricted HTLV-I-specific CTL clone. Within 5 minutes, HLA-GFP molecules formed dense clusters that could be readily visualized at the T cell-APC contact site (solid white arrow). After 30 minutes, small aggregates of HLA-GFP appeared within the Tax11-19 peptide-specific CTL clone (open arrow).

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