3. MECHANISMS OF TUMOR EVASION

MICHAEL CAMPOLI[2], SOLDANO FERRONE[2], ARNOLD H. ZEA[1], PAULO C. RODRIGUEZ[1] AND AUGUSTO C. OCHOA[1,3]

[1]Stanley S. Scott Cancer Center, and [3]Department of Pediatrics, Louisiana State University Health Sciences Center, 533 Bolivar Street, New Orleans, LA 70112, E-mail: aochoa@lsuhsc.edu

[2]Department of Immunology, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, New York 14263, E-mail: soldano.ferrone@roswellpark.org

1. INTRODUCTION

1.1. Escaping the Immune Response: A Historic Perspective

The clinical experiments of William Coley in the 1890's [1] demonstrating a therapeutic effect of the "Coley Toxins" in some patients, and the animal models of Prehn and Main [2] in the 1950's demonstrating the existence of tumor specific antigens, established an era of active research in immunotherapy as a treatment for cancer. Several studies demonstrated that tumors arising from oncogenic viruses could induce a protective immune response during the early phases of tumor development. [3–4]. However, results in some animal tumor models [5] and especially in patients with cancer, failed to demonstrate the presence of a protective immune response to the progressively growing tumor. Hersh and Oppenheim [6] instead demonstrated that Hodgkins disease (HD) patients had a decreased delayed type hypersensitivity (DTH) response to PPD and DNBC (di-nitrochlorobenzene) and a diminished in vitro response to mitogen stimulation, which persisted even in patients who had achieved a complete clinical response to chemotherapy [7]. Furthermore, Hellstrom and colleagues [8] showed a decreased cellular immune response, but a marked increase in serum immunoglobulins in patients with melanoma. Similarly observations in patients with renal cell carcinoma, prostate and bladder cancer [9], lung cancer [10] and breast cancer [11], suggested that tumors might impair the immune response. However the clinical relevance of these findings or the mechanisms causing them remained unclear.

Table 1. Mechanisms of tumor evasion

Target	Major Changes
Changes in tumor cells	Selection of tumor cells resistant to apoptosis Changes in the expression of HLA Absence of co-stimulatory molecules
Alterations in antigen presenting cells	Arrested maturation of DC Selective increase in DC2
Dysfunction of effector cells	Induction of regulatory T cells Increased apoptosis of T effector cells Alteration in T cell signal transduction

A renewed enthusiasm for immunotherapy started in the 1980's with the cloning and production of pharmaceutical grade cytokines and the isolation and purification of tumor associated antigens. However the results of clinical trials in patients failed to reproduce the undisputable therapeutic benefit shown in animal models, bringing forth the need to understand how tumors escape the immune response. Various mechanisms of tumor escape have been identified ranging from the loss of HLA markers in tumor cells making them difficult to recognize by T cells, to the gradual deterioration of the immune response with the progressive growth of the tumor. Here we will discuss some of the most recent concepts on how tumor cells may escape and/or inhibit the normal function of the immune system (Table 1).

2. CHANGES IN TUMOR CELLS

2.1. Selection of Resistant Tumor Cells

The concept of "immune surveillance" proposed by Jones and Burnet [12] in the 1970's suggested that the immune system was vigilant to destroy any malignant cells before they developed into a clinically relevant tumor. However, aside from the demonstration of the existence of natural killer cells there was little proof or understanding of how this mechanism worked. In the early 1990's work by Schreiber and colleagues [13; 14] demonstrated that early tumor growth is comprised mostly of transformed cells that undergo apoptosis when they bind IFNγ and chemokines produced by cells of the innate immune response including natural killer cells, $\gamma\delta$ T lymphocytes and macrophages. This effectively eliminates most of the tumor cells, however it also selects for a minority of malignant cells that have mutations or alterations that make them resistant to an immune induced apoptosis. The absence of one or more chains of the IFNγ receptor, or mutations in the tyrosine kinases associated with this receptor (Jak 1, Jak2 or Stat 1), prevent the triggering of the apoptosis cascade making these cells resistant to the immune surveillance mechanism. These resistant clones then develop into tumors of clinical significance unimpeded by the immune response. Therefore, the innate immune response may eliminate most transformed cells during the early stages of tumor growth, however it may also result in the selection of a resistant population of malignant cells, a process that was coined by Schreiber as cancer immunoediting [15]. Alternatively, Khong and Restifo [16]

suggested that tumors are not rejected during early stages of tumor growth because they do not cause significant tissue damage and therefore fail to send "danger signals" that could activate the immune response, a concept presented by Matzinger [17]as a means for certain normal tissues of causing immune tolerance.

2.2. Decreased HLA Antigen and Co-stimulatory Signal Expression

HLA Class I Antigen Expression

The continued growth of tumor leads to tissue destruction and the generation of "danger signals," which may trigger an adaptive immune response. The activation of tumor associated antigen (TAA)-specific T lymphocytes occurs through the recognition of two combined signals by the T cell i) peptides, derived from TAA, presented by self-HLA class I molecules (i.e. HLA class I antigen-TAA peptide complex) and ii) co-stimulatory signals such as B7.1 (CD80) or B7.2 (CD86) [18] [Fig. 1A]. This recognition results in the development of effector cytotoxic T lymphocytes (CTL) that recognize and lyse tumor cells presenting the relevant HLA class I antigen-TAA peptide complex [Fig. 1B] Therefore, tumor cells can evade hosts' immune response by being poor stimulators of T cells or being poor targets for effector CTL. Specifically, malignant cells may posses abnormalities in the expression of molecules required for effective T cell recognition, such as HLA class I antigens, costimulatory molecules and/or the TAA itself [16, 19, 20].

In the case of HLA class I antigens, a large body of evidence indicates that malignant transformation is associated with abnormalities in HLA class I antigen expression [19]. Analysis of cell lines in long term culture, through a combination of binding and immunochemical assays, has identified distinct defects in the expression of HLA class I antigens in tumor cells [19, 21] [Fig. 2]. These defects do not represent artifacts of *in vitro* cell culture, since they have also been identified in surgically removed tumors by immunohistochemical (IHC) staining with monoclonal antibodies (mAb). In fact, with the exception of liver carcinoma [22–24] and leukemia [25], IHC staining of a large number of surgically removed malignant lesions with mAb to monomorphic determinants of HLA class I antigens has identified abnormalities in the HLA class I antigen expression in 16% to 50% of all malignant lesions analyzed [19, 21, 26] [Fig. 3].

The reason(s) for differences in the frequency of HLA class I defects is (are) not known. They are likely to reflect the time length between onset of tumor and diagnosis, since a long interval gives tumor cells more chances to mutate in the genes involved in HLA class I antigen expression and allows mutated cells to over-grow cells without abnormalities in their HLA class I phenotype in the presence of T cell selective pressure [27–29], as it will be discussed later. Figure 3 summarizes data for tumors for which at least 70 lesions have been analyzed. HLA class I antigen downregulation or loss has also been described in other tumor types. However, the number of lesions that have been analyzed is too low for one to draw definitive conclusions. These types of tumors include stomach [30], pancreatic [31], bladder [32], germ cell [33] and basal cell [34] carcinomas. It is noteworthy that HLA

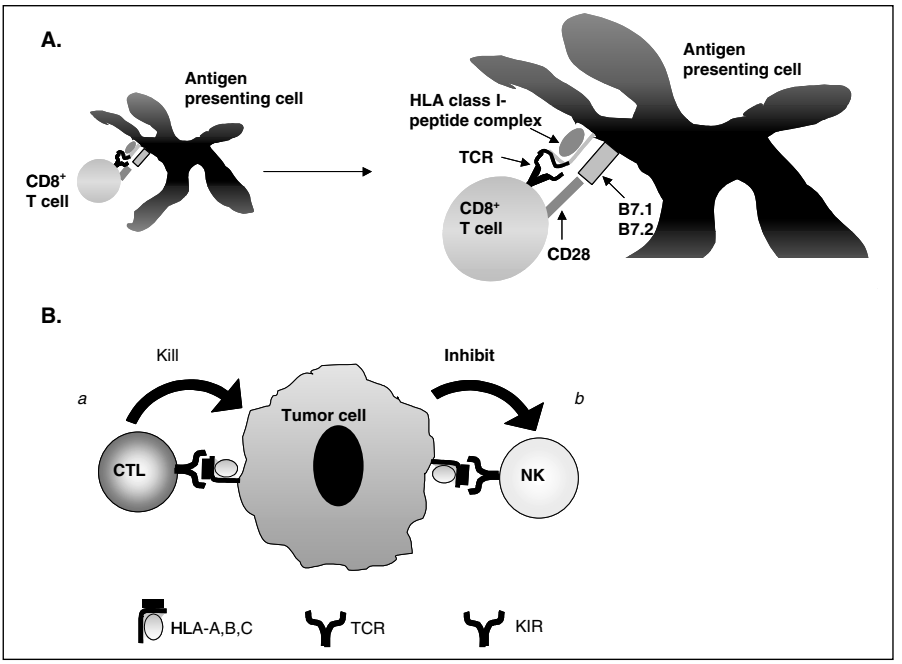

Figure 1. (A) The activation of specific T lymphocytes occurs through the recognition of two combined signals. The first signal is specific, requiring T cell receptor (TCR) recognition and binding to specific HLA class I antigen-peptide complexes presented by an antigen presenting cell. The second signal is nonspecific, resulting from the binding of B7.1 (CD80) or B7.2 (CD86) ligands on the antigen-presenting cell with its receptor, CD28, on the T cell. If both signals are provided, the T cell will proliferate and secrete cytokines. **(B)** CTL recognition of target cells occurs through the interaction of T cell receptor (TCR) with HLA class I antigens complexed to peptides generated by the antigen processing machinery. The trimeric HLA class I-β_2m-peptide complex plays a major role in the interactions between target cells and (*a*) activation of peptide-specific CTL through TCR; (*b*) inhibition of T cell subpopulations through inhibitory receptors KIR.

class I antigen loss or downregulation does not occur in all types of malignancies. In leukemia, defects in HLA class I antigen expression in malignant cells have been only occasionally identified. This finding is not likely to reflect a lack of genetic instability in leukemic cells, since like solid tumor cells, leukemic cells harbor many genetic and/or epigenetic alterations in their DNA [35]. Furthermore, in view of the role of immunoselection in the generation of malignant cell populations with HLA class I defects [27–29], lack of immune responses against leukemic cells is unlikely to be the mechanism. This possibility is supported by the higher frequency of HLA class I antigen abnormalities in sporadic diffuse large cell lymphoma than in immunodeficient and transplant-related lymphomas [36]. Therefore, it is likely that the lack of defects in HLA class I antigen expression identified in leukemia reflects the time interval between the onset of leukemia and its diagnosis, which is likely to be shorter than that of solid tumors. A short time interval between the onset of

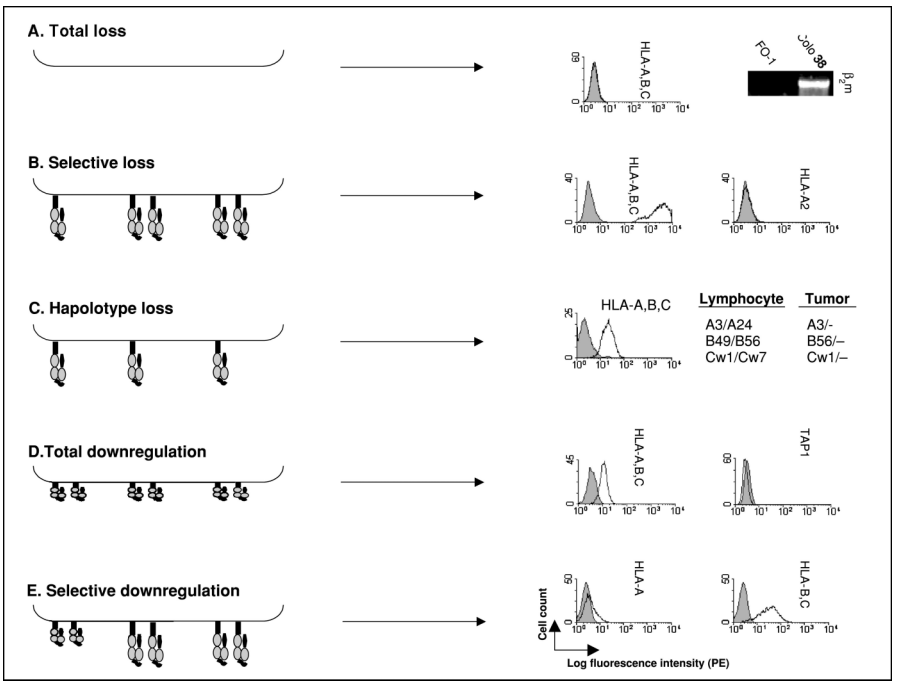

Figure 2. Abnormal HLA class I antigen phenotypes identified in malignant cells. Several molecular mechanisms can (A) Total loss of the gene products of the HLA-A, B and C loci can be caused by mutations in the β_2m gene; (B) Selective loss of one HLA class I allospecificity can be caused by loss of the gene(s) which encode the lost HLA class I allele(s) or by mutations which inhibit their transcription or translation; (C) Total loss of all HLA class I antigens encoded in one haplotype, which can be caused by LOH in of chromosome 6; (D) Total downregulation of all HLA class I antigens expressed by a cell, which can be caused by downregulation or loss of expression of the antigen processing machinery components and (E) selective downregulation of the gene products of one HLA class I locus, which can be cause be alterations in HLA class I antigen transcriptional factors.

leukemia and diagnosis may not allow sufficient time for cells to acquire mutations in the gene(s) involved in HLA class I antigen expression and for selective pressure to facilitate the expansion of malignant cells with HLA class I abnormalities. In the case of liver carcinoma, normal hepatocytes, which do not express or express very low HLA class I antigen levels [22], acquire the expression of these antigens during malignant transformation. The results obtained with liver carcinoma cell lines suggest that HLA class I antigen upregulation may result from the induction of antigen processing machinery components by cytokines secreted by immune cells infiltrating malignant lesions [22].

Abnormalities in HLA class I antigen expression in malignant lesions appear to have clinical significance, since they are associated with histopathological characteristics of the lesions and/or with clinical parameters in several malignant diseases [19, 37–43]. However, depending on the tumor type, HLA class I antigen defects can be associated

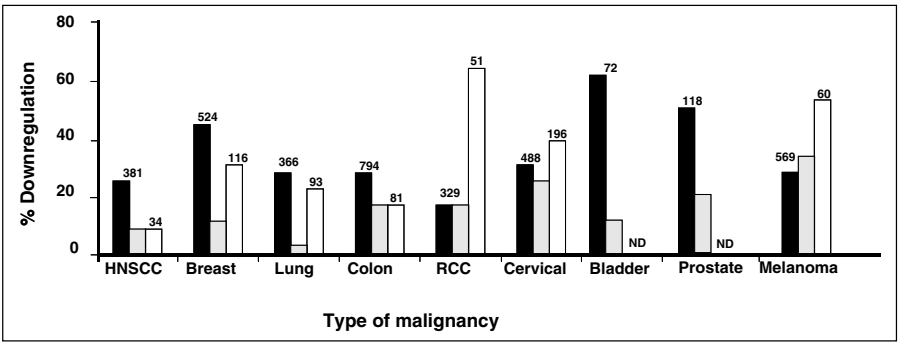

Figure 3. Frequency of HLA class I antigen and TAP1 downregulation in malignant lesions of different embryological origin. The most common types of solid tumors for which more than 70 or 30 lesions have been analyzed for HLA class I antigen or TAP1 expression, respectively, are shown. (■) Indicates total HLA class I antigen downregulation; (■) indicates selective HLA class I allospecificity loss; and (□) indicates TAP1 downregulation. Figures indicate the number of lesions analyzed. ND: not determined. Data has been adapted from

directly (head and neck squamous cell (HSCC), breast, small cell lung, prostate, bladder and cervical carcinoma and cutaneous melanoma), inversely correlated (uveal melanoma and colon carcinoma) or not associated (pulmonary adenocarcinoma, squamous cell carcinoma of the uterine cervix, cutaneous squamous cell carcinoma, large cell and large immunoblastic lymphoma and non-small cell lung carcinoma) with disease progression and/or poor clinical outcomes [36, 44–49]. The reasons for these discrepancies are not known but may reflect differences in the characteristics of the patient population, the methods of analysis and/or the system used to score HLA class I antigen expression. In addition, these findings may be attributed to differences in types of immune response elicited by tumors of different tissue or differences in routes of metastasis. An example is represented by the opposite association of HLA class I antigen downregulation with the clinical outcome in cutaneous and uveal melanomas [45]. HLA class I antigen downregulation is associated with a poor prognosis in cutaneous melanoma, where CTL are believed to control the metastatic tumor spread via the lymphatics [45]. In contrast, HLA class I antigen downregulation is associated with a favorable clinical outcome in uveal melanoma, where NK cells, which tend to kill tumor cells with a low HLA class I antigen expression [50, 51], have been suggested to limit metastasis via the blood.

The potential role of HLA class I antigen abnormalities in the clinical course of malignant disease has stimulated the characterization of the molecular mechanisms responsible for HLA class I antigen abnormalities. Through the effort of a number of investigators, characterization of cell lines originated from malignant lesions with HLA class I abnormalities has shown that distinct molecular mechanisms underlie the abnormal HLA class I phenotypes of tumor cells [Fig. 2]. The frequency of complete HLA class I antigen loss has been found to be between about 15% in primary cutaneous melanoma lesions and 50% in primary prostate carcinoma lesions [19].

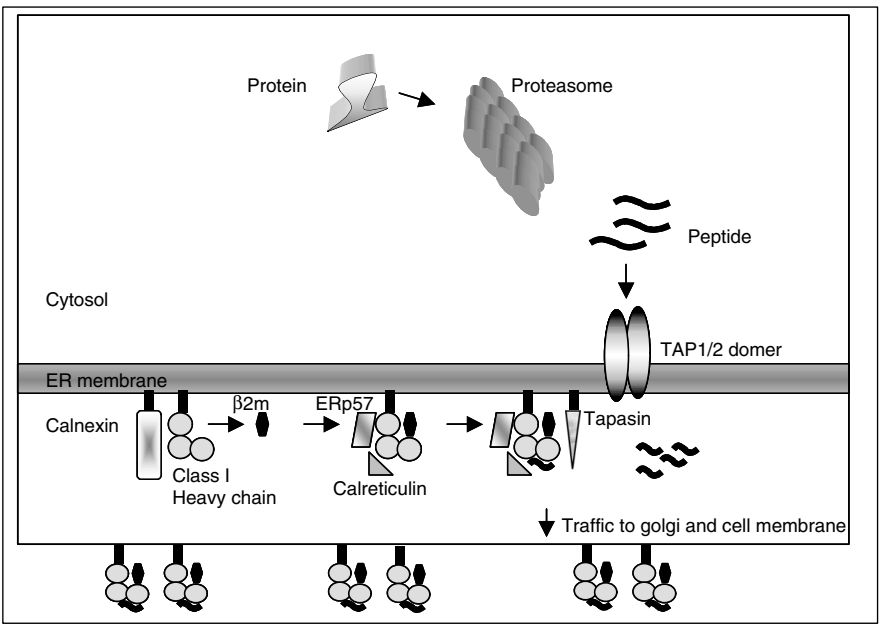

Figure 4. (A) Generation and interaction of HLA class I antigen-peptide. Intracellular protein antigens, which are mostly endogenous, are marked for ubiquitination within the cytosol and subsequently degraded into peptides by proteasomal cleavage. The constitutive proteasome subunits delta, MB1 and Z and the interferon-γ inducible immunosubunits LMP2, LMP7 and LMP10 are responsible for the catalytic activity of the proteasome. Once generated, peptides are transported into the endoplasmic reticulum through the dimeric transporter associated with antigen processing, TAP1 and TAP2. TAP is responsible for both qualitative and quantitative peptide translocation. 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-dependant reductase ERp57. Calnexin dissociation is followed by HLA class I heavy chain association with β_2m, tapasin and the chaperone calreticulin. Calnexin, calreticulin and ERp57 play a role in folding of the HLA class I heavy chain. Subsequently, tapasin brings the HLA class I heavy chain, β_2m, chaperone complex into association with TAP and plays a role in both quantitative and qualitative peptide selection. The trimeric HLA class β_2m-peptide complex is then transported to the cell membrane.

The frequency of this phenotype varies significantly between different malignancies. As indicated above, it is likely that these differences reflect the time length between onset of tumor and diagnosis. Complete HLA class I antigen loss can be caused by defects in β_2-microglobulin (β_2m) which is required for the formation of the HLA class I heavy chain-β_2m-peptide complex and its transport to the cell membrane [20], epigenetic changes in the DNA or alterations in the antigen processing machinery components [52–56] [Fig. 2A]. The latter play a crucial role in the assembly of functional HLA class I antigen-peptide complexes and in their expression on the cell membrane [57] [Fig. 4]. Inactivation of the β_2m genes completely abrogates HLA class I antigen expression at the cell surface and has marked effects on peptide presentation. β_2m defects result from two events: loss of one copy of the $\beta_2 m$ gene

at chromosome #15, which carries the $\beta_2 m$ gene in humans [58], and mutations in the other copy the $\beta_2 m$ gene which inhibits its transcription in a few cases and its translation in most cases. It is not known which of these two events occurs first in malignant cells. The mutations identified thus far in β_2m genes range from large to single nucleotide deletions; in most cases they inhibit the translation of β_2m mRNA [20, 59–62]. Although the mutations are distributed randomly in β_2m genes, a mutation hotspot has been suggested to be located in the CT repeat region in exon 1 of the β_2m gene. Mutations in this region have been identified in more than 75% of tumor cells with total HLA class I antigen loss [63] and have been found to parallel the mutator phenotype in tumor cells [64], reflecting the increased genetic instability of this region during malignant transformation of cells [64]. It is noteworthy, that for some tumors such as head and neck squamous cell, laryngeal, breast, colorectal, renal and bladder carcinoma, β_2m gene mutations are not responsible for complete HLA class I antigen loss [52–56, 65]. These observations suggest that genetic mutations in the β_2m gene may not be the predominant molecular mechanism underlying total HLA class I antigen loss and suggest that other mechanisms may be involved in total HLA class I antigen loss. In this regard, post-transcriptional regulation of the β_2m gene expression has been suggested as a possible mechanism for total HLA class I antigen loss [52–56, 65]. In addition, epigenetic changes that cause total HLA class I antigen loss have been observed. Hypermethylation of three HLA class I antigen loci has been observed in neoplastic cells to selectively switch off HLA class I antigen gene expression. The characteristics of these tumors are the significant reduction in or complete absence of mRNA from the heavy chain gene and normal expression of β_2m and antigen processing machinery components. DNA hypermethylation has been implicated as a major mechanism for transcriptional inactivation of HLA class I antigen genes in esophageal squamous cell carcinomas and is also responsible for the total HLA class I antigen loss in melanoma [53, 66, 67].

Selective HLA class I allospecificity loss, e.g. HLA-A2 loss, is caused by loss of the gene(s) encoding the lost HLA class I heavy chain(s) or by mutations which inhibit their transcription or translation [63] [Fig. 2B]. It is noteworthy, that selective HLA class I antigen loss results from only one mutational event in a heterozygous allelic background. This may explain why, in most malignancies, the frequency of selective HLA class I antigen losses is higher than that of total HLA class I antigen losses [19]. As in the case of the β_2m gene, the mutations found in HLA class I heavy chains range from large deletions to single base deletions [68–72]. The mutations appear to occur randomly. Whether a mutation hotspot in the genes encoding HLA class I heavy chains exists remains to be determined.

Loss of one HLA class I haplotype, e.g. HLA-A24, -B56, -Cw7, appears to be frequently caused by loss of segments of the short arm of chromosome 6 where HLA class I genes reside [73], however in some instances it can be caused by the loss of specific transcription factors that specifically bind to HLA-A or HLA-B promoters [74] [Fig. 2C]. This phenotype is often identified by HLA class I genotyping and LOH analysis of chromosome 6. LOH at chromosome 6 appears to represent a frequent mechanism contributing to selective HLA haplotype loss in tumors [75].

This finding may reflect the frequent genetic recombination events at the human *MHC* located at chromosome 6p21.3, which carries the highest density of genes among all gene loci in human chromosomes [76].

Total HLA class I downregulation can be caused by multiple mechanisms. First, transcriptional activity of HLA class I heavy chain genes can be suppressed by the presence of silencer located at the distal promoter [77] or by epigenetic mechanisms such as hypermethylation and/or altered chromatin structure of the HLA class I heavy chain gene promoters [66, 78, 79]. Second, the restoration or enhancement of HLA class I antigen expression in malignant cells by IFN-γ suggests that altered regulation of non-mutated genes may play a part in defects in HLA class I antigen expression [80]. Lastly, the level of HLA class I antigens expressed on cells can be reduced by downregulation or loss of antigen processing machinery components [37] [Fig. 2D]. Defects in antigen processing machinery components may effect the generation of peptides from antigens, their transport into the endoplasmic reticulum (ER), their loading on HLA class I antigens and/or the repertoire of peptides presented by HLA class I antigens. It is noteworthy that in the majority of cases, antigen processing machinery component loss or downregulation can be corrected by treating cells with cytokines, e.g. IFN-γ, indicating that these abnormalities are usually caused by regulatory and not structural defects [19, 81]. This mechanism may explain why the frequency of downregulation of one or multiple antigen processing machinery components in malignant lesions is high, in spite of the codominant expression of the two genes encoding each antigen processing machinery component. An alternative, although not exclusive, mechanism is represented by the downregulation, by IL-10, of antigen processing machinery components, which leads to reduced HLA class I antigen cell surface expression [82]. This finding may be of clinical relevance,since a large number of human tumors secrete IL-10 [83]. Therefore, these patients, at variance with those with structural defects in HLA class I antigen-encoding genes, are likely to benefit by combining T cell-based immunotherapy with administration of IFN-γ and/or anti-IL-10 antibodies.

Information in the literature regarding antigen processing machinery component expression in various types of malignancies is scanty. Only a few components have been analyzed and only in a limited number of lesions. It is also noteworthy to point out that no information is available as to what constitutes normal or abnormal expression profiles of antigen processing machinery components in cells, since to the best of our knowledge no study has quantitated the level of antigen processing machinery component expression in normal cells of different embryological origin. The paucity of the available information reflects the limited or lack of availability of antibodies and methodology to quantitate antigen processing machinery components. Therefore, one must exercise caution in interpreting studies that analyze antigen processing machinery component expression in malignant cells, since the phenotype of the normal counterparts is not known in many cases. Among the antigen processing machinery components, TAP1 has been most extensively investigated. TAP1 downregulation and/or loss has been found in HNSCC, in carcinomas of the breast, small cell lung (SCLC), colon, kidney, cervix and prostate and in cutaneous

melanoma with a frequency ranging from 10–84% [37, 84] [Fig. 3]. A few studies have investigated TAP2 expression in malignant cells and the frequency of TAP2 downregulation tends to correlate with that of TAP1 [19]. TAP1 downregulation or loss is likely to be caused by abnormalities in regulatory mechanisms, since in some instances they can be corrected by *in vitro* administration of cytokines, such as IFN-γ and TNF-α, and is accompanied by an increase in HLA class I antigen expression [85, 86]. The increase in HLA class I antigen expression following induction of TAP1 expression is correlated with an increased susceptibility to TAA-specific CTL lysis, in most but not all cases [87–89]. In addition, it is expected that the frequency of TAP downregulation is higher than that of total HLA class I antigen losses, due to the distinct mechanisms underlying these two phenotypes. While two mutational events are required for total HLA class I antigen loss, TAP downregulation appears to be primarily due to abnormalities in regulatory mechanisms. To the best of our knowledge, structural defects in TAP1 as a result of mutations have been observed only in two human tumor cell lines [90, 91].

Only recently has tapasin expression been analyzed in a few types of tumors. Abnormalities in tapasin expression can lead to reduced HLA class I antigen expression, alterations in the repertoire of peptides presented by HLA class I antigens and resistance of malignant cells to CTL [57]. Heterogeneous and reduced levels of tapasin mRNA has been observed in HNSCC, SCLC, hepatoma, RCC, colon carcinoma, pancreatic carcinoma, neuroblastoma and cutaneous melanoma cell lines [92, 93]. In the majority of cases, *in vitro* incubation of cells with cytokines such as IFN-α, IFN-γ, TNF-α and IL-4 has resulted in tapasin transcriptional upregulation [92, 93]. However, in the melanoma cell line COPA159 we have identified a single-base deletion at position 684 in exon 3 of the *tapasin* gene resulting in a reading frameshift of the mRNA with a subsequent introduction of a premature stop codon at positions 698–700. This cell line demonstrates reduced HLA class I antigen expression, which can be restored upon transfection with the wild-type *tapasin* allele [Chang et al., unpublished data]. To a limited extent, tapasin expression has been investigated in surgically removed malignant lesions. In these studies tapasin has been found to be downregulated in both RCC and HNSCC lesions [50, 94, 95]. In the latter malignancy, this downregulation is associated with poor prognosis [95]. If this is a cause-effect relationship, it is likely to reflect the reduced susceptibility of tumor cells to CTL-mediated lysis because of HLA class I antigen downregulation and alterations in the HLA class I antigen peptide repertoire in cells with reduced tapasin expression.

Selective downregulation of the gene products of one HLA class I locus can be caused by alterations in the transcription factors for genes encoding HLA class I heavy chains [96, 97] [Fig. 2E]. However, there is limited information regarding selective downregulation of the gene products of one HLA class I locus, since the expression of some HLA class I allospecificities in malignant lesions has not been assessed because of the lack of appropriate probes.

The major role played by the HLA class I-TAA peptide complex in the recognition of tumor cells by CTL can be further illustrated by the association found between abnormalities in the expression and/or function of antigen processing machinery

components and poor clinical course of the disease in some malignancies [19]. This association most likely reflects the importance of these components in the generation of functional HLA class I-TAA peptide complexes. Notably, TAP1 downregulation has been reported to associate with tumor staging and reduction in patients' survival in breast carcinoma, SCLC, cervical cancer and cutaneous melanoma [19]. An increased frequency of TAP1 downregulation in metastatic lesions when compared to primary lesions has also been reported in breast carcinoma, cervical carcinoma and cutaneous melanoma [19]. Most recently, the role of tapasin in the clinical course of malignant diseases has been suggested by Ogino et al. who reported that tapasin downregulation in conjunction with HLA class I antigen downregulation was associated with reduced survival in patients with maxillary sinus squamous cell carcinoma [94]. It remains to be determined whether this finding applies to other types of tumors. Nevertheless, all of these findings are likely to reflect the crucial role of TAP1 and tapasin in the generation of HLA class I antigen-TAA peptide complexes and suggest that alterations in the repertoire of peptides presented by HLA class I antigens may provide an alternate route of immune escape for malignant cells. This possibility highlights the need to monitor specific HLA class I antigen-TAA derived peptide complex expression in malignant lesions. To this end, we have begun to develop probes capable of recognizing allospecific HLA class I antigen-TAA derived peptide complex expression on malignant cells (manuscript in preparation).

Generation of cells with HLA class I antigen defects results from mutations in the gene(s) which are involved in the expression of HLA class I antigens. It is likely that these mutations occur randomly due to increased epigenetic changes and genomic instability in the early stages of tumor development [63]. In general the frequency of HLA class I antigen defects in metastatic lesions is higher than that in primary and premalignant lesions [19]. It is also noteworthy to point out that especially in malignant cells isolated from patients with advanced disease the presence of multiple defects affecting different antigen processing machinery components and HLA class I subunits appears to be the rule more than the exception [63]. Moreover, an increase frequency of HLA class I antigen loss variants have been found in recurrent metastatic lesions in patients who had experienced clinical responses following T cell-based immunotherapy [28]. Therefore, one important question to ask is which mechanism(s) play(s) a role in the expansion of cells with HLA class I defects in malignant lesions. In view of the continuous exposure of tumor cells to the host's immune response [21, 15], one might ask whether immune selective pressure plays a major role in the expansion of cells with HLA class I antigen defects so that they become the major population in a lesion. One can envision two possible scenarios: (i) if immune selective pressure plays a major role, then tumor cells with HLA class I defects expand because of escape from host's immune response which targets tumor cells without HLA class I antigen defects; (ii) if on the other hand, immune selective pressure does not play a role, then the expansion of tumor cells with HLA class I antigen defects is independent of the development of an immune response in the host. The available evidence derived from studies in animal model systems and in patients treated with T cell-based immunotherapy argues in favor of a major role

played by immune selective pressure in the generation of malignant lesions with HLA class I antigen defects [27–29]. From a practical viewpoint, the possible role played by immune selective pressure in the generation of malignant lesions with HLA class I antigen defects suggests that the use of T cell-based immunotherapy for the treatment of malignant diseases may only be successful in a limited number of cases.

HLA class I antigen downregulation may provide malignant cells with a mechanism to escape CTL recognition and destruction. This possibility has raised the question of why HLA class I antigen downregulation does not increase the sensitivity of malignant cells to NK cell-mediated cytotoxicity. The latter phenomenon has been convincingly shown in mice where MHC class I downregulation is correlated with increased target cells' susceptibility to NK cells (missing-self hypothesis). The mechanisms by which NK cells recognize and kill target cells have been poorly understood only until recently [98, 99]. NK cell recognition and killing mechanisms are now believed to be governed by a balance between activating and inhibitory signals received by the NK cells. These signals are generated by specific target cell ligand-NK cell receptor interactions. To date, there is evidence that the non-classical HLA class I antigens HLA-E, F, G may serve as inhibitory NK cell ligands [100], while the MHC class I related chain A and B (MICA and MICB) [98, 99] and the UL16-binding protein 1, 2 and 3 (ULBP1, ULBP2 and ULBP3) [98, 99] may act as activating NK cell ligands. In this regard, tumors can express stress induced ligands MICA and MICB, [52, 101–103], which inhibit NK cytotoxic function and IFN-γ production when released in soluble form [104].

Co-stimulatory Molecule Expression

To achieve activation, T cells require a minimum of two signals provided by antigen and co-stimulatory membrane proteins such as CD80 and CD86 [105]. Stimulation of T cells in the absence of costimulatory signals leads to anergy of T cells and eventually to T cell apoptosis [18]. A decreased expression of costimulatory signals CD80 (B7-1), CD86 (B7-2) and CD45 has been demonstrated in B cell malignancies, lung and colon cancer, making them not only poor stimulators of a T cell response, but also potential inducers of T cell apoptosis [106–110]. In concert with these observations, in vitro experiments showed that transfection of tumor cells with the CD80 and CD86 genes, increased their immunogenicity. Although this led to the rejection of B7 transfected tumor in murine models, it did not always lead to the regression of the non-transfected malignant cells [111]. Tumor cells can also evade recognition by T cells by decreasing the expression of tumor antigens through mechanisms that remain unclear, but appear to be independent of HLA expression. The loss of gp100, MART 1 and tyrosinase in melanoma have been associated with tumor progression and resistance to immunotherapy [112, 113].

3. CHANGES IN CELL MEDIATED IMMUNE RESPONSE IN CANCER

During the early 1980's North and colleagues [114–117] developed animal models where they carefully studied T cell function during progressive tumor growth.

An initial protective T cell response could be readily demonstrated during the first days after tumor implantation, followed by a rapid decline in the response with the appearance of Ly1+ suppressor T cells. This suppressor function could be transferred into naive animals and was eliminated with low doses of cyclophosphamide, re-establishing a therapeutic anti-tumor response. These findings provided an insight into a dynamic interaction between the tumor and the immune system that could be manipulated to the benefit of the host. An alternative explanation to the presence of suppressor cells came from studies on the function of cytokines produced by CD4+ helper clones. Mossman and colleagues classified T helper cells according to the type of cytokines they produced and the response elicited. Th1 cells mainly produced IL2, IFNγ, and TNFα, promoting cellular responses, while Th2 cells mainly secreted IL4, IL13 and IL10, promoting antibody production [118; 119]. It was therefore possible that the progressive growth of tumor induced a loss of Th1 activity and an increased Th2 function, leading to a diminished cellular response and an enhanced antibody production. Most of these concepts remained as interesting research observations, but with minor relevance in the treatment of patients.

The advent of immunotherapy in the 1980's using the adoptive transfer of tumor-infiltrating lymphocytes (TIL) revealed to a greater extent the degree of T cell dysfunction in patients with cancer. In vitro testing of freshly isolated TIL demonstrated that these cells had a markedly decreased proliferation when stimulated with mitogens or tumor cells and had a significantly diminished clonogenic potential [120–122]. This T cell dysfunction was however not limited to TIL cells, but was also seen in peripheral blood T cells or splenic T lymphocytes in tumor bearing mice. Furthermore this cellular dysfunction appeared to have a major detrimental effect on the therapeutic success of immunotherapy. Loeffler and colleagues [123] studying an immunotherapy model of adoptive transfer of T lymphocytes, demonstrated that T cells from mice bearing tumors for >21 days had a markedly diminished anti-tumor effect when used to treat tumor-bearing recipients. In contrast, T cells from mice bearing tumors for <14 days had a high therapeutic efficacy when transferred into tumor bearing recipients. In vitro tests demonstrated a diminished cytotoxic activity in the T cells from long-term tumor bearing mice, which could in part be explained by a diminished expression of the perforin gene [124]. Sondak et al. [125] also confirmed the diminished cytotoxic function of T cells from tumor bearing mice, which was more significant in mice bearing visceral metastases as compared to those with subcutaneous tumors. Therefore animal models not only reproduced the T cell dysfunction seen in cancer patients, but also suggested that these alterations could have an important impact on the outcome of cancer immunotherapy. These observations sparked an increased research effort to elucidate the mechanisms of tumor escape that started in the 1990's and continues today.

Major advances in understanding the fundamental mechanisms of antigen processing and presentation, costimulatory signals and T cell activation, as well as the molecular basis for T cell signal transduction, provided important tools to start exploring the intricate interactions between tumors and the immune system.

3.1. Changes in Antigen Presenting Cells

Antigen presenting cells (APC) in the form of macrophages or dendritic cells (DC) process and present antigens to T lymphocytes. Gabrilovich and colleagues [126, 127] first described a selective increase in the number of immature myeloid DC in the circulation of tumor bearing mice and cancer patients. Surgical removal of the tumor resulted in a decrease in the immature DC cells and a recovery of T cell responses. In tumor-bearing mice, the immature myeloid cells are represented by a population of Gr-1, CD11b and MHC class I positive cells. Gr-1 (+) cells do not impair T cell responses to mitogens such as Con A, but completely block T cell responses *in vitro* and *in vivo* to peptides presented by MHC class I. Therefore, immature DC preferentially inhibit CD8-mediated antigen-specific T cell responses [128]. The increased immature DC cells appear to be result of VEGF produced by tumor cells, which arrests DC maturation by suppressing the activation of the transcription factor NFκB. In fact, there is a high degree of association between increased serum levels of VEGF and a high numbers of immature DC in patients with gastric, lung and head and neck cancer [128]. In addition to arresting dendritic cell maturation, tumors can also induce a selective increase in the number of DC2 cells or regulatory dendritic cells, which can induce T cell anergy [129]. Stromal derived factor–1 (SDF-1) produced by ovarian carcinoma cells selectively recruits plasmacytoid dendritic cells and modulates their function [130]. These in turn appear to preferentially activate regulatory T lymphocytes that express CD25.

Tumors may also impair the cytotoxic function of macrophages by blocking nitric oxide production. Nitric oxide is an important component of the cytotoxic mechanism displayed by macrophages, endothelial cells and neurons. Several studies have found that macrophages from patients with cancer or tumor bearing mice have a decreased production of nitric oxide when compared to normal individuals. However, these studies did not find a decreased expression of iNOS, suggesting that other mechanisms, such as the depletion of the nitric oxide substrate, arginine, may be the mechanism for the inhibition in nitric oxide production[131, 132].

3.2. Induction of Regulatory T Cells

The recently described subset of regulatory T cells comprises a subset of mostly CD4+, CD25+ T cells, which constitute approximately 5–10% of the total CD4+ cells and appear to control key aspects of tolerance to self antigens [133]. Depletion of this T cell subset can induce an autoimmune response against endocrine organs in mice. Patients with melanoma, colon and head and neck cancer have an increased percentage of regulatory T cells (CD4+/CD25+) in their circulation. The depletion of these cells in tumor bearing mice increases the response to tumor associate antigen [134]. However, depletion of regulatory T cells alone is not enough to treat established tumors in mice [135].

3.3. Apoptosis of Effector T Cells

Elimination of T cells responding to autologous antigens through the binding of Fas ligand (FasL) to the Fas receptor is a well-established mechanism for the induction of

apoptosis and tolerance to normal tissue antigens. A high expression of FasL has been reported in tumor cells from lung carcinoma, melanoma, colon carcinoma and liver carcinoma [136–138]. Therefore tumors that express Fas-ligand, or shed Fas-ligand into the serum could induce apoptosis in T cells infiltrating the site of tumor or in circulating T cells, effectively escaping the effector arm of the immune response [139]. Several reports have recently suggested an increased percentage in apoptosis of T cells in the peripheral blood of patients with head and neck cancer [140]. Tumor cells have also been shown to lose the expression of Fas, developing resistance to apoptosis induced by FasL expressed by effector cells of the immune system.

3.4. Changes in T Cell Signal Transduction

In the mid 1980's major advances in T cell biology provided the basis to understand the molecular events that lead to T cell activation. Among these were the elucidation of the elements that form the T cell antigen receptor (TCR) and the mechanisms of T cell signal transduction after antigen stimulation [141–143]. Briefly two polymorphic chains, the α and β chains confer antigen specificity to the T cell and form the antigen-binding site. These are covalently linked to the CD3 complex formed by the invariant chains $\gamma\delta\varepsilon$ and ζ The latter forms homodimers $\zeta\zeta$ (CD3ζ) or heterodimers ($\zeta\eta$). Two Src family members of tyrosine kinases are critical in the signal transduction of this structure, namely $p56^{lck}$ that is associated with CD4 or CD8, and $p59^{fyn}$, associated with CD3ζ. The binding of antigen to the $\alpha\beta$ TCR complex triggers the mobilization of calcium from intracellular stores and the hydrolysis of IP3 to IP2 freeing high-energy phosphates used by kinases in the phosphorylation of several signal transduction proteins. In parallel, HLA molecules and CD80 and CD86 bind to their receptors, activating various tyrosine kinases including $p59^{fyn}$ and ZAP-70, which activate nuclear transcription factors such as NFκB that translocate into the nucleus and activate or repress various genes [144, 145].

Major advances were also made in understanding the molecular changes that accompany T cell unresponsiveness or anergy. Quill and colleagues [146, 147] and Jenkins and Schwartz [148] demonstrated that T cells stimulated by antigens presented on fixed antigen presenting cells (APC) were anergic, i.e., unresponsive to repeated antigenic stimuli and unable to produce IL2. Furthermore, stimulation of T cells with streptococcus superantigen produced a state of T cell anergy and resulted in a decreased expression of $p56^{lck}$ and $p59^{fyn}$[149, 150]. Anergic T cells also had several molecular changes including the inability to phosphorylate p21 Ras [151] and a decreased ability to activate nuclear transcription factors NFκB and AP-1, important in regulating cytokine production [152].

In the early 1990's Mizoguchi and colleagues [153] studying the dysfunctional T cells from long-term tumor bearing mice demonstrated a marked decrease in the expression of CD3ζ chain, $p56^{lck}$ and $p59^{fyn}$ tyrosine kinases. These changes were accompanied by a decreased tyrosine kinase phosphorylation and a diminished Ca++ flux. These findings provided for the first time a molecular basis to explain T cell dysfunction in cancer patients. Li [154] and Ghosh [155] later showed that T cells from some patients with renal cell carcinoma and from long-term tumor bearing mice were unable to translocate NFκBp65 nuclear transcription factor, resulting in a

Table 2. Most frequent T cell signal transduction abnormalities reported in cancer patients

Target	Effect
CD3ζ	Decreased expression
$p56^{lck}$	Decreased expression
JAK-3	Decreased expression
Calcium signaling	Decreased mobilization
NFκBp65	Inability to translocate
IL2 production	Decreased production

predominance of NFκBp50/50 homodimer known to act as a repressor of the IFNγ gene [156]. In fact, cytokine production during the progressive growth of tumors in mice demonstrated a Th1 response (IL2 and IFNγ) early after tumor implantation, followed by an increased production of Th2 cytokines (IL4 and IL10) after three weeks [157].

Results in cancer patients confirmed the initial observations in murine models. T cells and NK cells from approximately half of the patients with renal cell carcinoma, colon carcinoma, ovarian carcinoma, gastric cancer, breast cancer, prostate cancer, Hodgkins disease, acute myelocytic leukemia and other tumors showed a decreased expression of CD3ζ chain and a decreased in vitro response to antigens or mitogens [158–162]. In addition, T cells from renal cell carcinoma patients also had a diminished ability to translocate NFκBp65. However, changes in signal transduction molecules were not limited to those associated with the T cell receptor (Table 2). 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 renal cell carcinoma patients [163]. Initial work in colon carcinoma [164] and renal cell carcinoma [165] suggested that patients with more advanced stages of the disease had a higher frequency of T cell signal transduction alterations. However, in cervical carcinoma [166] some patients with carcinoma *in situ* already showed a diminished expression of CD3ζ, suggesting that T cell signal transduction alterations could occur early in the disease and were not an exclusive characteristic of advanced stages of cancer. Other reports have also suggested an association between the expression of CD3ζ and survival. Patients with muetastatic melanoma (Stage IV) treated with IL2 + anti-CD3 monoclonal antibody and patients with head and neck cancer that had normal levels of CD3ζ chain at the initiation of treatment had a significantly longer survival compared to those who had undetectable levels [167, 168].

The expression of CD3 ζ changes with treatment. Patients with non-Hodgkins lymphoma and patients with Hodgkins disease [169, 170] who responded to chemotherapy showed a re-expression of normal levels of ζ chain, which decreased again in patients who had a recurrence of the disease. Limited data from clinical trials in ovarian carcinoma, melanoma, renal cell carcinoma and colon carcinoma showed that patients receiving IL2 based therapies could recover CD3ζ expression

[171]. However, this did not always coincide with a full recovery of T cell function since tyrosine kinase activity was not always fully restored.

3.5. Mechanisms Leading to Alterations in T Cell Signal Transduction

Otsuji et al. [172] and Kono et al. [173] demonstrated in a series of elegant in vitro experiments that H_2O_2 from macrophages induced the loss of CD3ζ chain in naive T cells, a phenomenon that could be blocked by the depletion of macrophages or the addition of oxygen radical scavengers. A similar effect was seen with H_2O_2 produced by neutrophils in patients with pancreatic and breast cancer [174]. Other macrophage products also appear to be able to alter the expression of T cell signal transduction proteins. Kolenko and colleagues [175] showed that PGE2 in combination with substances that increase cAMP can diminish the expression of Jak-3 in naive T lymphocytes, effectively blocking signal transduction through the IL2 receptor. A second mechanism leading to loss of CD3ζ chain was found while studying Fas-FasL induced T cell apoptosis [176, 177]. T cells undergoing apoptosis lose the expression of CD3ζ as one of the early changes in this process. Gangliosides expressed on the membrane of different tumors have also been shown to be powerful immunosuppressors of T cells. Uzzo et al. showed that gangliosides from renal cell carcinoma cells can suppress nuclear transcription factor NFκBp65 in T cells and induce apoptosis [178–180]. Therefore the diminished expression of CD3ζ chain seen in cancer patients could in part be explained by an increased frequency of apoptotic cells in peripheral blood.

3.6. Modulation of T Cell Function and CD3ζ Expression by Amino-Acid Availability

Recent observations have demonstrated the important role of amino-acids in regulating T cell function. Among these tryptophan and arginine appear to play an important role in cancer. Dunn and colleagues demonstrated that macrophages producing Indoleamine 2, 3-dioxygenase (IDO) can deplete the essential amino-acid tryptophan and sensitizes activated T cell to apoptosis. Preliminary data in tumor bearing animals suggests that tumor cells may express IDO [181, 182]. The use of an IDO inhibitor, 1-methyl-tryptophan (1-MT) is currently being studied in animal models as a means reversing the inhibitory effects of this metabolic pathway.

Non-essential amino acids can also cause severe T cell dysfunction. Taheri et al. and Rodriguez and colleagues recently demonstrated that T cells cultured in the absence of arginine lose the expression of CD3ζ, have a decreased proliferation and a decreased production of IFNγ [183, 184]. Arginine levels can be regulated in vivo by the enzymes nitric oxide synthase (iNOS) and arginase I produced by macrophages or tumor cells. Arginase I production in macrophages is increased by Th2 cytokines and is able to deplete the extracellular levels of arginine, causing a down-regulation of CD3ζ. In addition, cells cultured in the absence of arginine show a decreased translocation of NFκBp65 similar to these observed in T cells from cancer patients. The CD3ζ down-regulation in the absence of arginine is caused by post-transcriptional mechanisms leading to a decrease in CD3ζ mRNA stability.

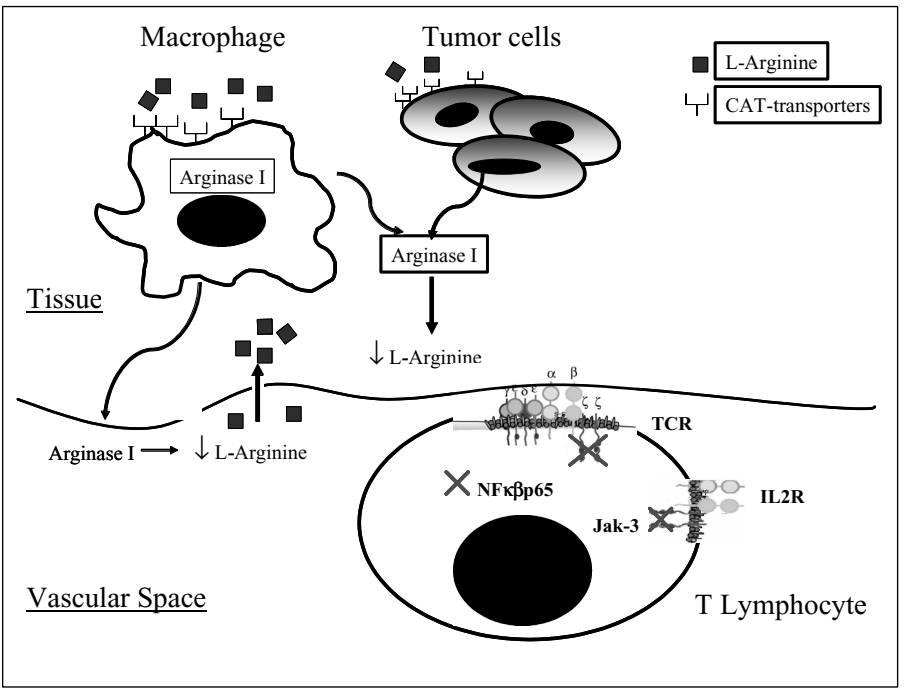

Figure 5. Regulation of T cell signal transduction can occur by limiting the availability of the amino acid arginine. Arginine is avidly taken up by activated macrophages and tumor cells through the CAT transporters. In addition, arginase produced by tumor cells or macrophages depletes arginine in the tissues (tumor microenvironment) or in the vascular space, inducing a decreased expression of CD3ζ, Jak-3 and NFκBp65 in activated T lymphocytes, effectively impairing the effector arm of the adaptive immune response.

These recent observations have led us to postulate the following scenario for the induction of T cell dysfunction in cancer [Fig. 5]. Arginase I produced by tumor cells or macrophages depletes arginine, causing the loss of CD3ζ and inhibiting cytokine production by T cells. This results in the inability of T cells to develop an anti tumor response and may impair the efficacy of immunotherapies.

4. SUMMARY

The results from in vitro immunological experiments, murine tumor models and patients with cancer clearly demonstrate that tumors have multiple mechanisms to evade the immune response. During the early stages of tumor development malignant cells can be poor stimulators, present poor targets or become resistant to the innate immune response, while at later stages, progressively growing tumors impair the adaptive immune response by blocking the maturation and function of antigen presenting cells and causing alterations in T cell signal transduction and function. Preliminary results also suggest a correlation between some of these changes

and an increased metastatic potential of the tumor cells, a diminished response to immunotherapy, and poor prognosis. Carefully coordinated basic research studies and clinical immunotherapy trials will be required to fully determine the impact on the outcome of the disease and the response to treatment. However, understanding the mechanisms used by tumor cells to evade the immune system could result in new therapeutic approaches for preventing and/or reversing these immune alterations and have the potential of improving the current results of immunotherapy trials.

ACKNOWLEDGEMENTS

This work was supported by PHS grant RO1 CA67108 awarded by the National Cancer Institute, DHHS (SF) and RO1-CA88885, RO1-CA82689 (AO).

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