Review

A Global Approach to Tumor Immunology

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Biological and clinical advances in the understanding of tumor immunology suggest that immune responsiveness of human tumors is a complex biological phenomenon that could be best studied by a real-time comparison of tumor/host interactions in the tumor microenvironment through a high-throughput discovery-driven approach. This conclusion is derived from our recognition that too many hypotheses or, in other words, no solid single hypothesis exist, based on experimental results, to further drive experimentation in human subjects. Functional genomic studies entertained during the last few years consolidated the belief that in humans the interactions between tumor and immune cells are too complex to be approached exclusively with a hypothesis driven method. We believe that immune cells suit cancer cells in a Yin and Yang balance by opposing and yet mutually depending on each other. Indeed, immune infiltration in tumors may play a dual role modulating in different circumstances cancer cell growth or destruction through a physiological modulation of inflammation. It is reasonable to question what induces inflammation at the tumor site. We hypothesize that inflammation is primarily driven by the phenotype of tumor cells that can modulate their microenvironment through cell-to-cell interactions or the secretion of soluble factors. Thus, in analogy the observation of immune cells within tumors parallels the presence of paramedics, police and firemen at the scene of an accident, which is reactive to and not causative of the occurrence. In this review we will explore this hypothesis by reporting and summarizing most of our recent work in the frame of available literature on the subject. *Cellular & Molecular Immunology*. 2004;1(4):256-265.

Key Words: tumor immunology, cDNA arrays, genetic profiling, vaccination, melanoma

Introduction

"There are good things so in the tide pools and interesting thoughts to be generated from the seeing. Every new eye applied to the peephole which looks out at the world may fish in some new beauty and some new pattern, and the world of the human mind must be enriched by such fishing."

John Steinbeck – Foreword to the Third Edition of Ed Ricketts' "Tides".

A little more than a decade ago we had very little knowledge of the mechanism(s) regulating tumor/host interactions in humans, which could be summarized by the serendipitous and empirical observation of rare cancer regressions in response to immune manipulation. Then, the identification and characterization of tumor-antigens (TA) recognized by cytotoxic T cells (CTL) (1) gave molecular precision and novel distinction to this rather disregarded field and provided the opportunity to investigate with scientific accuracy the fascinating phenomenon of cancer rejection in natural conditions and/or in response to therapy (1). Since, most discovered TA consisted of non-mutated proteins whose expression is shared by cancers from different individuals; it became apparent that TA could serve as therapeutic targets for the treatment of broad patient populations (2). Early studies aimed at the active-specific immunization of patients with cancer using TA derivatives demonstrated that immunization could often induce CTL responses observable in the peripheral circulation and capable of recognizing autologous or Human Leukocyte Antigen (HLA) matched tumor cells (3). The success of immunization was, however, limited to the induction of clinic immune responses since the latter scarcely lead to clinical tumor regressions. We have recently argued that, in spite of its clinical ineffectiveness, the ability to induce TA-specific immune responses is a most striking achievement since, to our knowledge, it represents the only anti-neoplastic therapy characterized by absolute specificity for cancer cells (4). Yet, the induction of TA-reactive CTL responses is only the first of several steps required for the achievement of the ultimate goal of cancer rejection. Further steps may be required for effective tumor rejection that could be most fruitfully analyzed by studying the quality of the immune responses at the receiving end: the tumor site. Little is known, in fact,

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about the level of activation and differentiation of immunization-induced T cells, their ability to localize at the tumor site and maintain an active effector phenotype capable of inducing complete eradication of tumor cells (5-7). Even less is known about the characteristics of individual tumors that may modulate such responses either by increasing or dampening their effectiveness.

Therefore, future studies should focus on the identification of the algorithm responsible for tumor rejection by the host in natural and/or therapeutically-enhanced conditions. As this phenomenon occurs in autologous settings and seems based on the recognition of self proteins, this research addresses the fundamental question of target recognition by T cells broadly applicable to auto-immune and infectious diseases. However, tumor immunology is a compound field where the dynamic heterogeneity of cancer biology (8) supplements the complexity of polymorphic variation and epigenetic adaptation of human immunology (9-11). Therefore, special measures need to be taken to simultaneously catch the interactions of these unstable systems. We, therefore, have promoted and emphasized the need to develop technologies that allow a global approach to the study of tumor/host interactions by simultaneously covering genetic, transcriptional and protein analyses through an approach defined as "Integromics" (12). The utilization of integromics will empower researchers who will be capable of confronting the daunting task of inspecting with a multi-dimensional approach the complexity of tumor/host interactions (11). One might conclude that this global approach may be disproportionate to the resources available to most relatively small programs resulting in excessive dispersion of intellectual and financial resources. In contrast, we argue that a reason for the limited success of translational efforts aimed at the treatment of human disease is lack of an integrated approach to human biology (11).

Metastatic cutaneous melanoma as a model of immune rejection in humans

The last decade yielded astounding progress in the understanding of the molecular mechanism(s) at the basis of adaptive immune responses against autologous cancer cells. In particular, the discovery of TA whose expression is shared by various tumors leads to the development of anti-cancer vaccines applicable to broad patient populations. This progress, in turn, allowed testing in humans the effectiveness of TA-specific cancer vaccines (4). Most of this progress stemmed from the study of patients with advanced cutaneous melanoma, while other cancers remained vastly unexplored. Most of the experience gathered by immunizing patients with melanoma with TA-based vaccines has been recently discussed by various groups and reported in several reviews (4, 6, 13-22). This experience can be shortly summarized as an outstanding success of vaccines in inducing cancer cell-specific cellular responses which, however, are not sufficient to lead to tumor rejection. Therefore, intense work is presently ongoing in the context of melanoma to improve strategies of vaccine production, delivery and to improve the in vivo effectiveness of immunization-induced T cell responses. We summarized our view on the subject in various review/opinion articles concluding that naturally occurring or immunization-induced TA-specific immune responses may be necessary but most often insufficient to induce cancer rejection (4-6, 23-26). In particular, we have come to the conclusion that TA-specific T cells capable of recognizing autologous cancer cells can be generated by TA-specific immunization (3, 6, 27-30), reaching the tumor site (24, 31) and interacting with autologous cancer cells (24). On the contrary, the tumor microenvironment in most cases is not conducive to the maintenance of TA-specific T cells effector function at the receiving end (6, 7). The most important practical conclusions that this work suggested are: a) tumor/host interactions are better studied in the context of active TA-specific immunization because this strategy provides a specific, time-limited assessment of the kinetics of immune responses in humans; b) these responses are better evaluated in the context of epitope-specific immunization that limits the number of immune-ological variable to be followed to one or few TA/HLA combinations; c) tumor host/interactions are not sufficiently portrayed by the analysis of circulating TA-specific T cells and, therefore, need to be complemented by the analysis of the tumor microenvironment; d) the genetic make up of individual patients should be studied in concert with their immune responses to account for genetic influences due to immune polymorphisms (4, 9).

The study of human immune responses in the context of cancer requires an open-minded, discovery-driven strategy has we have recently commented elsewhere (32). Although, this discovery-driven research has been demoted by some to a "fishing" expedition, we are of the advice that, as an alternative approach, hypothesis-driven research has failed to meet the needs of translational efforts such as the one described here because hypotheses derived from complex experimental models often simply do not translate to human pathology. Therefore, in the next section, we will address what can be learnt by applying functional genomics to the study of tumor-host interactions.

Functional genomics for the study of tumor-host interactions

"Hundred rabbits do not make a horse"
Fyodor Dostoyevsky – Crime and Punishment

We have extensively discussed the necessity of studying cancer biology and its interactions with the immune system using high-throughput strategies (11, 33-37). To this aim, a validated and refined technology for linear amplification of messenger RNA species (aRNA) allows the utilization of minimal starting material (common in clinical settings) for transcriptional profiling using cDNA-based array technology (38, 39). This methodology allows the monitoring of tumor/host interactions by introducing a temporal dimension to the study of individual lesions using repeated fine needle aspirations (FNA) (33). The application of analytical tools for the interpretation of the high-density databases generated with cDNA arrays (11) allowed to

congregate into a unified hypothesis disparate information on genes that would otherwise wander about as hundreds of playful rabbits. With this strategy, we identified predictors of immune responsiveness in patients with advanced melanoma undergoing immune therapy (40). This result suggested that some tumor lesions are pre-conditioned to respond to immunotherapy by displaying an immunologically active microenvironment (5, 40). Applying serial fine needle aspiration biopsies before and during therapy we could explore the mechanism(s) of action of systemically administered IL-2 (41) to patients with metastatic melanoma. This study revealed that, contrary to prior hypotheses, the primary effect of IL-2 administration is to induce an acute inflammatory reaction at the tumor site through up regulation of a broad panel of pro-inflammatory genes and genes associated with CTL and NK cell effector function. If correct, the interpretation of those data raises the obvious question of why some tumors may behave differently than others. Some have suggested that inflammation is beneficial and necessary for tumor growth (42, 43). This observation is only apparently contrasting with our hypothesis which suggests inflammation as a primary vector of immune rejection. It may very well be that inflammation may be helpful in promoting angiogenesis and 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 go hand in hand in response to injury. In fact, several growth factors frequently expressed by tumors have chemo-attractant and regulatory properties on immune cells (5). These molecules can induce the migration of cells 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 cells given appropriate stimulatory conditions induced by treatment such as the systemic administration of IL-2 (41). We observed that cytokine production by tumor cells may be a conditioning factor that may predispose or at least be associated with immune responsiveness (44, 45). In particular, the surprisingly high levels of IL-10 transcript (44) and protein (45) in melanoma metastases that respond to IL-2 therapy suggest a chronic conditioning of the microenvironment by this cytokine with a dual anti- and pro-inflammatory role (46). Thus, our hypothesis is that to a certain extent inflammation is beneficial to tumor growth. However, a tumor microenvironment enriched of immune cells is also more likely to be triggered by pro-inflammatory stimuli that may in the end induce tumor regression.

Genomic studies have been applied more recently to study the effect(s) of Imiquimod on basal cell carcinoma (BCC) (as later discussed) and the peritoneal cell phenotype of patients with benign or malignant ovarian tumors (Wang E, et al., manuscript submitted). This latter study, demonstrated that non-tumor bearing peritoneum of patients with malignant ovarian tumors is significantly different from that of patients with benign lesions suggesting that malignant ovarian tumors may condition the peritoneal lining through the secretion of soluble factors (47). This finding is important because it provides undisputed evidence that tumors can condition normal tissues by promoting chronic inflammation that leads to angiogenesis and tissue remodeling and yields potential information about the primary pathways involved in metastatization though modulation of the surrounding microenvironment.

Immunogenetic profiling as a complementary tool to the study of tumor heterogeneity

As previously described, our previous work suggests that in some patients, tumors may be predisposed to respond to immune therapy by inducing a microenvironment enriched of pro-inflammatory stimuli (5, 40). However, it is unclear whether the differences noted among patients are due to the heterogeneity of cancer related to the intrinsic instability of this disease (8) or rather due to genetic variation among individual patients. Indeed, the pathology of humans faces the challenge of diversity addressed in genetic terms as polymorphism. In particular, polymorphism(s) of genes associated with immune function are increasingly recognized (9). Emerging evidence suggests that the study of complex systems such as the cytokine network is complicated by inter-individual differences dictated by increasingly recognized polymorphisms. Polymorphism appears widespread among genes of the immune system possibly resulting from an evolutionary adaptation of the organism facing an ever evolving environment. We refer to this high variability of immune-related genes as immune polymorphism. Based on the possible clinical relevance that immune polymorphism may bear on the relationship between tumor and host cells we suggested a change in the approach to the study of human immunology from the targeted study of individual systems to a broader view of the organism as a whole through immunogenetic profiling (9, 48). This analysis will lead to the identification of reasons for ethnic and/or individual predisposition to disease, responsiveness to therapy or susceptibility to the toxic effects of biological agents. For this reason, we have developed a single nucleotide polymorphism (SNP) oligonucleotide-based chip that includes most of the known variants in coding and regulatory regions of cytokines as well as cytokine regulatory pathways (http://bris.ac.uk/pathandmicro/services/GAI/cytokine4.htm). The oligo chip is designed according to a novel strategy for the detection of SNP using a minimum number of oligonucleotides applicable in the clinical settings that we have recently described (49) (Figure 1).

The cytokine SNP detection chip will be utilized as part of a study in which genetic variation between different ethnic groups, pathological or physiological conditions and responsiveness to treatment will be explored using peripheral blood monocytes. Differences in transcriptional and/or protein profiles will be compared with their genome variation. We hope that this line of studies will provide a map for the interpretation of individual and/or population-based variation that could complement the analysis of tumor heterogeneity.

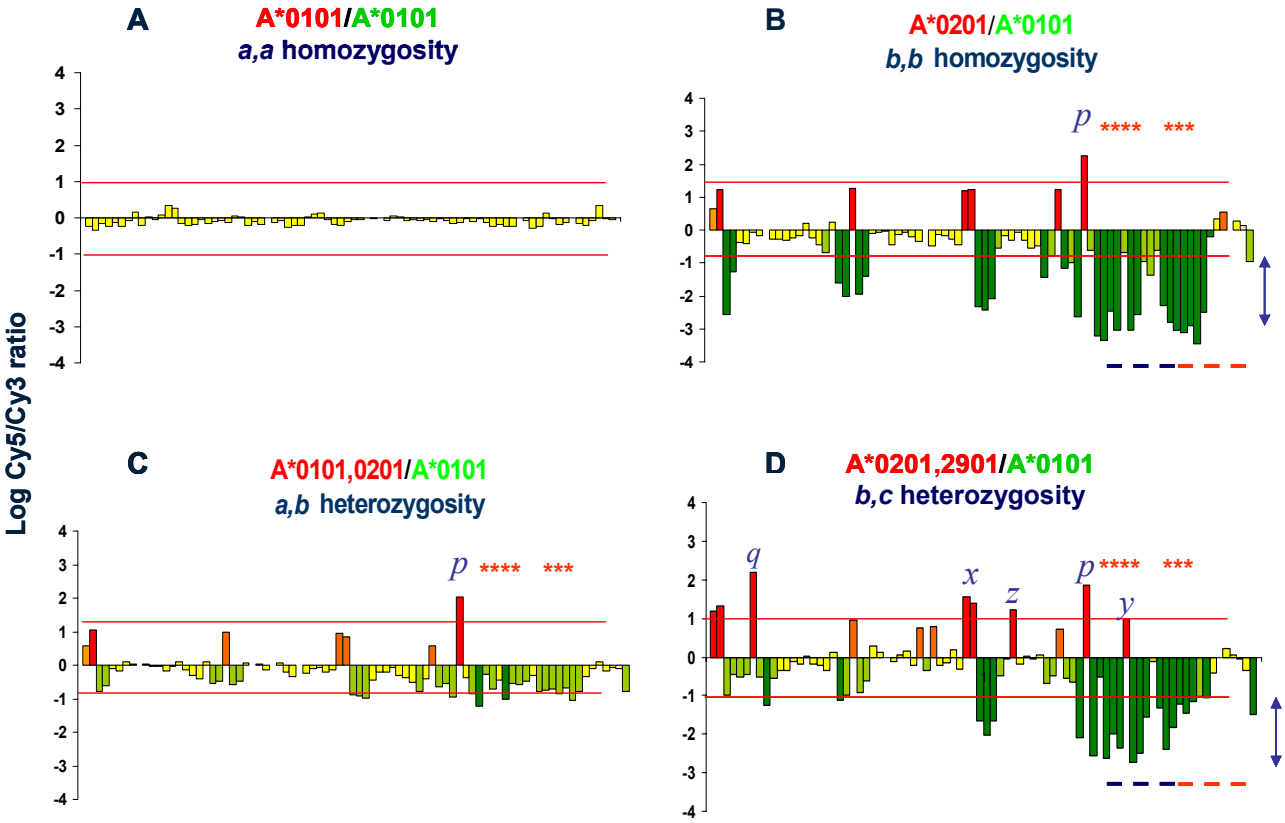

Figure 1. Profiling by proportional hybridization of HLA-A exon 2. Four patterns are described: a,a homozygosity (A); b,b homozygosity (B); a,b heterozygosity (C) and b,c heterozygosity (D). Each bar represents the Log_2Ratio for individual oligos sequentially positioned in 3' to 5' direction. Variant oligos are nested between homologous consensus oligos and appear as bright (Log_2Ratio >1) or light red (Log_2Ratio > 0.5 and ≤ 1). Log_2Ratio for any oligo between ≤ 0.5 or ≥ -0.5 are shown in yellow. Consensus oligos in various conditions of mismatch with test DNA are shown as light (Log_2Ratio < -0.5 and ≤ -1) or dark (Log_2Ratio < -1) green. The orange lines delimit Log_2Ratio between 1 and -1. The letter p points to a variant oligo with a double nucleotide variant (CA → GG at 256-257). Another double mismatch-containing oligo is pointed out by q (CA → AC at 99-100). The letter x points to a variant oligo (9-SP-A-02) spanning a region of b,c type heterozygosity (HLA-A*0201 and A*2901 differ A → G from the consensus). The letter y points to a situation where a variant oligo (14-SP-A-03, A29 at 263-280) encompasses also a "unknown" SNP present in the other allele (HLA-A*0201). z shows a variant oligo (10-SP-A-29 at 211-229) whose increased Log_2Ratio is not associated with decreased Log_2Ratio in the homologous consensus oligo. The orange asterisks and the dashed lines show a genomic region in which "unknown" HLA-A*0201 polymorphisms are associated (blue) or not associated (orange) with HLA-A*2901 polymorphic sites. Figure adapted from Wang E, et al. J Transl Med. 2003;1:4.

Combined modality therapy of melanoma with active-specific immunization and systemic immune stimulation - The most promising approach

Systemic IL-2 administration may turn an indolent and chronic inflammatory process into acute autoimmune rejection of cancer (41) and has dramatically increased the frequency of clinical regression in the context of vaccination trials (50). Although clinical responses remain relatively rare, their dramatic occurrence is characterized by a painful inflammatory process of the tumor site followed by rapid disappearance of large tumor bulks and in some instances long term disease free survival (51). The IL-2 phenomenon is of extreme biological interest and it is surprising how little effort has been devoted to the understanding of its mechanism(s) of action. It has been suggested that IL-2 facilitates the migration of TA-specific T cells from the circulation to the tumor site by increasing blood vessels permeability (50). IL-2 could induce proliferation or activate the effector functions of $CD8^+$ T cells (52). In addition, IL-2 may induce activation of intra-tumoral endothelial cells which may promote migration of TA-specific T cells within tumors (53). Finally, 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 directly dependent upon the interaction of IL-2 with its receptor (41, 54, 55). Thus, the study of the effects of IL-2 in target tissues such as cancerous lesions could yield important insights for the development of anti-cancer therapies.

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. This was done by performing FNA of melanoma

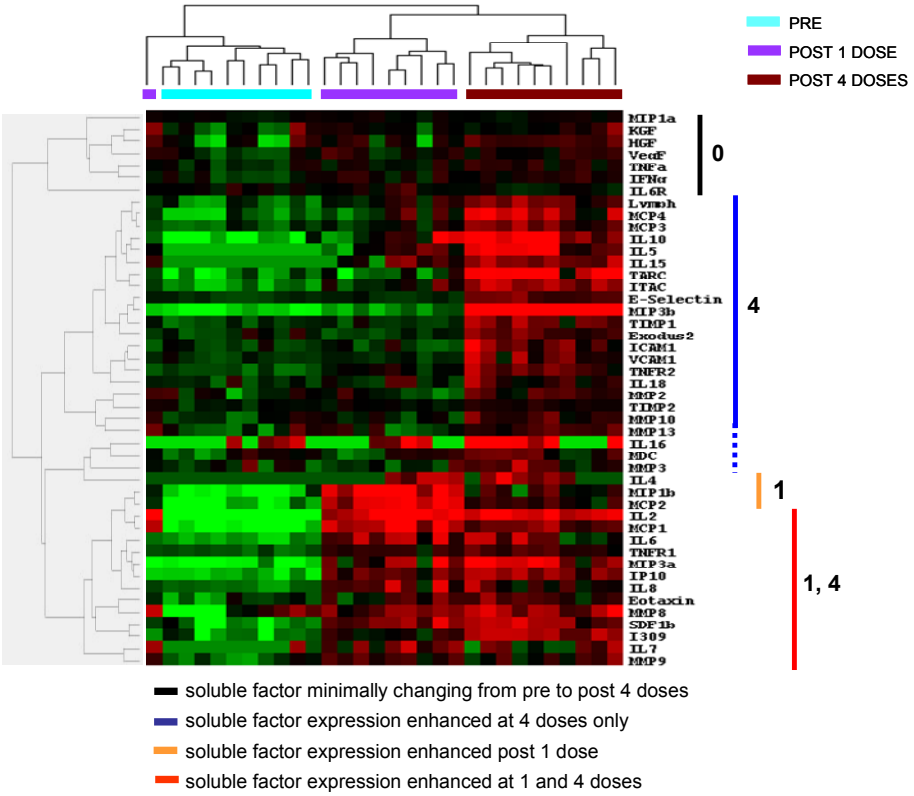

Figure 2. Unsupervised Hierarchical clustering (Kendall's Tau) of serum samples from RCC patients obtained before, after 1 and 4 doses of IL-2 (720,000IU/kg). Hierarchical clustering was applied to the data set encompassing 46 cytokines significantly expressed (excluding IL-2) between before and after 4 doses of IL-2 across 10 serum samples (P2,3,4,5,9,12,13,14,15,16) obtained before, after 1 and after 4 doses of IL-2 (720,000IU/kg) **.** Values corresponding to soluble factors concentration in pg/ml were transformed in natural log (LN) values, average corrected across experimental samples and displayed according to the central method for display using a normalization factor as recommended by Ross. Patients' serum samples clustered according to the three time points of IL-2 administration. Expression of soluble factors segregated in 4 distinct kinetic profiling. Black bar = soluble factor minimally changing from before to after 4 doses; blue bar = soluble factor expression enhanced at 4 doses only; orange bar = soluble factor expression enhanced after 1 dose; red bar = soluble factor expression enhanced at 1 and 4 doses. Figure adapted from Panelli, et al. J Transl Med. 2004;2:17.

metastases in patients undergoing systemic IL-2 administration before therapy and three hours after the first and fourth dose of treatment (41). The results of this study surprisingly suggested that the immediate effect of systemic IL-2 administration on the tumor micro-environment 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 turns a chronic into an acute inflammatory process at the 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 and activation of cytotoxic mechanisms in monocytes (calgranulin, grancalcin) and natural killer cells (NKG5, NK4). These primary reactions 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 recent work suggesting that immunization-induced, TA-specific T cells rest in a "quiescent" status 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 (6, 7).

T cell function in the context of anti-cancer vaccines

The utilization of TA in active-specific immunization trials to induce TA-specific T cells which left clinicians and researchers perplexed by the paradoxical observation that immunization-induced T cells can recognize tumor cells in standard assays but cannot induce tumor regression. A closer look at T cell physiology and tumor biology suggests that this observation is not so surprising. Indeed, in a human melanoma model of tumor-antigen (TA)-based immunization, we tested the functional status of TA-specific $CD8^+$ T cells. A "quiescent" phenotype lacking direct *ex vivo* cytotoxic and proliferative potential was identified that was further characterized by comparing its transcriptional profile to that of *in vitro* sensitized (IVS) TA-specific T cells. Quiescent circulating tumor-specific $CD8^+$ T cells were deficient in expression of genes

associated with T cell activation, proliferation and effector function. This quiescent status may explain the observed lack of correlation between the frequency of circulating immunization-induced lymphocytes and tumor regression. In addition, the activation by *in vitro* antigen recall and interleukin-2 suggests that a complete effector phenotype might be re-instated *in vivo* to fulfill the potential of anti-cancer vaccine protocols (56). This work completes a series of analyses aimed at the enumeration and characterization of immunization-induced T cells (27-30). The quiescent phenotype identified by the recent study may explain the paradoxical co-existence of TA-specific $CD8^+$ T cells in the tumor bearing patient following immunization and closely approximates the linear model of T cell activation *in vivo* proposed by Ahmed's group (57, 58).

Other unexplored cancer models

It could be also suggested that by studying other immune responsive cancers more could be understood about the requirements necessary for tumor rejection. Here, we shall briefly discuss the scientific need to study cancers other than metastatic melanoma. It could be argued that similar biological phenomena may result from convergence of different pathways into a final outcome represented by a least common denominator that, though masked by irrelevant biological variables, governs their occurrence. Thus, the immune responsiveness of melanoma could be compared for example to that of renal cell cancer (RCC) or basal cell carcinoma (BCC) which are also quite responsive to immune manipulation in spite of distinct biological profiles (59). In contrast, nasopharyngeal carcinoma (NPC) represents a good example of a cancer with minimal propensity to respond to immune manipulation in spite of obviously intense interactions between cancer and immune cells (60). Our hypothesis is that by identifying commonalities and diversities among various cancers it will be possible to sieve those patterns that may be most relevant to immune responsiveness and/or lack of. Indeed, some human cancers are clearly affected by interactions with immune cells, which when activated can in some circumstances mediate the rejection of autologous tumors while in most cases they seem to peacefully coexists and perhaps foster tumor cell growth. Yet, the mechanism(s) responsible for this phenomenon remains unknown. In melanoma, it is believed that adaptive immune responses play a key role for the simple reason that they can be easily demonstrated. However, other cancers such as RCC and BCC are similarly responsive to immune manipulation but TA-specific immune responses are elusive. Thus, it is possible that adaptive immune responses may simply represent an upstream even that could facilitate cancer rejection without being the central effector mechanism (5). According to our observation, TA-specific immune responses could selectively bring inflammation to the tumor microenvironment by specifically releasing pro-inflammatory cytokines such as interferon-γ, tumor necrosis factor-α, granulocyte-monocyte colony-stimulating factor and, in some circumstances, interleukin-2. This pro-inflammmatory effect may facilitate the occurrence of immune responses without being unique as the final effector mechanisms responsible for tumor rejection may be activated by other mechanisms.

Renal cell cancer (RCC) and interleukin-2

We have extensively analyzed in the past the immune response in the context of active-specific immunization against melanoma in combination with systemic IL-2 therapy (61). This work leads to the hypothesis that IL-2, a potent biological agent clinically used to treat advanced melanoma and renal cell carcinoma, acts through the secondary induction of a large array of cytokines in PBMC (41, 55). In addition, based on a small sample study, we made the preliminary observation that *in vitro* stimulation of PBMC with IL-2 segregates individuals in high and low producers of secondary cytokines (55). This difference, if substantiated by a larger sample analysis could be explained by genetic polymorphism of the IL-2 receptor or down-stream modulators along the signaling pathway induced by IL-2. Therefore, we have recently completed a small pilot study in which the effects of IL-2 were studied in patients with metastatic RCC who received systemic high-dose IL-2 administration. Serum samples were obtained before and after 4 doses of IL-2 treatment and tested for variability in secondary cytokine release using protein arrays. Analysis of the data demonstrated that the down-stream effects of systemic IL-2 administration are rapidly amplified by the production of soluble factors that included growth factors, cytokines, chemokines, metallo-proteinases and soluble forms of adhesion molecules (62). Each of these soluble factors could have powerful effects against tumor cell growth as well as induce the toxic response to this therapy. This study, therefore, emphasized the need for a global approach to the analysis of therapeutic mechanism(s) and prompted the organization of a larger study in which biochemical and genetic data will be compared to clinical information regarding toxicity of treatment and clinical outcome in the context of RCC (Figure 2).

Imiquimod and the rejection of basal cell cancer (BCC)

Imiquimod is a small molecule developed by 3-M (St Paul, MN) as an anti-viral agent but soon recognized to possess anti-cancer activity through stimulation of innate immune mechanism(s) (63, 64). This drug is too toxic for systemic administration and it is predominantly used for the local treatment of skin cancers such as squamous cell carcinoma and BCC. In the case of BCC, Imiquimod induces an acute inflammatory process followed in the large majority of cases by complete regression of BCC. Like interleukin-2, Imiquimod has no direct anti-cancer activity and its mechanism(s) of action appears to be related to the induction of acute inflammation associated with massive infiltration of macrophages and dendritic cells (64). It is, therefore, reasonable to postulate that this treatment model could yield useful information about the postulated least common denominator responsible for tumor rejection. A double blind randomized protocol sponsored by 3-M is presently ongoing designed to treat 48 patients with BCC

with Imiquimod or placebo. Punch biopsies will be obtained before and during therapy for immune histochemical and transcriptional analysis (cDNA arrays and quantitative real-time qRT-PCR) using a strategy previously described for metastatic melanoma (33) and adapted for the processing of skin lesions. Results of this clinical trial may yield useful information about the role that innate immunity may play in cancer regression.

Nasopharyngeal cancer (NPC) as an immuno-genetics and immunotherapy model

Nasopharyngeal carcinoma (NPC) is an Epstein-Barr virus (EBV)-associated disease. EBV is a γ-herpes virus that infects at least 90% of the human population throughout the World. After the acute infection is over, the virus establishes a lifelong latent infection of B-lymphocytes. This is associated with the expression of an array of EBV proteins that include the Epstein-Barr Nuclear Antigens (EBNA) 1, 2, 3A, 3B and 3C, leader protein (LP) and the latent membrane protein (LMP) 1and 2 (65). The same proteins are consistently expressed in EBV-transformed lymphoblastoid cell lines (LCL) (65). In the carrier state the latent virus induces several neoplastic disorders that can be grouped into two categories: a) lymphoproliferative disorders associated with immune suppression; b) neoplastic disorders occurring in immune competent individuals.

Withdrawal or reduction of immune suppression is widely accepted as an effective treatment of post-transplant lymphoproliferative disorders that express the full range of viral proteins (66). In addition, complete regression of post-transplant lymphoproliferative disorders can be mediated by adoptive transfer of HLA-matched EBV virus-specific cytotoxic T cells (CTL) (67-69) and prophylactic administration of CTL can prevent their insurgence (70). These observations suggest that, at least in this setting CTL play a major role in regulating tumor growth. In contrast with the "LCL-like", lymphoproliferative disorders in immune compromised patients, a second group of EBV-associated tumors occurs in immune competent individuals in which the expression of EBV proteins is more restricted (71). These neoplastic disorders include Burkitt's lymphoma, Hodgkin's disease and NPC. The restricted expression of EBV proteins in this latter group may explain the lower effectiveness of adoptive transfer of EBV-specific CTL in the context of Hodgkin's disease (71) and NPC (72).

Among cancers induced by EBV in immune competent individuals, NPC is of particular interest to tumor immunologists and more broadly to the immunogeneticists for several reasons: 1) NPC is potentially associated with genetic predisposition; 2) It is tightly associated with the expression of specific Human Leukocyte Antigen (HLA) class I alleles; 3) It consistently expresses at least one EBV-related protein: LMP 2; 4) patients with NPC manifest a spontaneous although reduced CTL response toward LMP 2. For this reason, NPC may represent a perfect model for the understanding of tumor/host interactions in the context of genetic polymorphism as a hallmark of human immune pathology and cancer heterogeneity (variability of HLA and antigen expression, aberrant antigen processing, differentially activated tumor micro-environment etc.).

CTL responses against EBV lytic proteins in NPC patient are predominant in long-term carriers although strong responses are identifiable also against latent proteins [73]. Among the latter, the strongest CTL response in normal volunteers and patients with NPC is directed against EBNA 3A, 3B and 3C whereas responses to LMP 1 and 2, though frequent, display a subdominant character (74-76). Importantly, many Chinese healthy donors mount detectable CTL responses to LMP 2 that can kill NPC tumor cells expressing LMP 2 epitopes (77). Although LMP-2-specific immune responses are of a subdominant character, it may be possible to boost LMP 2-specific responses using active immunization strategies involving whole proteins or known epitopic determinants. No comprehensive epitope mapping of LMP proteins has been so far reported. Most studies have been performed in Caucasians (78). Not surprisingly, these studies identified predominantly LMP epitopes associated with HLA-A *0201, an allele characteristically prevalent in Caucasians (78) and relatively rare among Asians. A comparative analysis of CTL responses in Chinese (n = 4) *vs* Caucasian (n = 9) subjects was performed by stimulating peripheral lymphocytes with autologous LCL transformed with the standard type 1 EBV B95.8 (79). This limited study identified 5 CTL epitopes that could be recognized in association with HLA alleles common in both ethnic groups. However, the subjects in both groups displayed HLA phenotypes disproportionably overlapping compared to the expected prevalence of HLA alleles. This was particularly the case for HLA-A2 alleles whose poly-morphism plays a limiting role in the cross-recognition of epitopes. In fact, most of the HLA-A*0201 associated-epitopes could not be recognized when presented in the context of LCL expressing HLA-A*0203, -A*0206 or -A*0207 common in the Asian/Chinese population (80). This finding is in line with our experience derived from testing epitope recognition across related HLA alleles (81). Thus, in the absence of supporting evidence that the HLA-A*0201-associated epitopes so far discovered are immunogenic in association with other HLA-A*02 alleles, we believe that HLA-A*02 is not an appropriate immunization target in non-Caucasian, most frequently Chinese, patients.

HLA-A*1101 and HLA-A*2402 are other alleles expressed with high frequency (50% and 30% respectively) in the Southern Chinese population. Thus, they may represent a suitable target for the immunization of a broad patient population. LMP-2 derived cytotoxic T cell (CTL) epitopes have been identified for both alleles. LMP-2: 340-349 (SSCSSCPLSK) and LMP-2: 419-427 (TYGP-VFMCL) are epitopes that induce CTL reactivity in association with HLA-A*1101 and -A*2402 respectively in EBV experienced individuals as well as patients with NPC (74, 78, 79).

Peptide-based immunization and other immunologic approaches have been tested only in a limited number of patients with NPC (60, 72, 82). Therefore, in contrast with melanoma, there is very limited information available to allow judgment about the justification for an immunologic

approach to this disease. In the context of melanoma, our group has devoted particular attention to the study of the kinetics of immunization with minimal epitopic determinants administered as an emulsion in Montanide ISA-51 (3, 5, 24, 26-30, 83, 84). Although in humans a direct relationship between induction by the immunization of tumor-antigen specific CTL could not be established, it appeared that such correlation could be easily demonstrated (85) in mouse models in which tumor heterogeneity could be prevented by utilizing a stable tumor cell phenotype. Thus, we postulate that one of the main goals of immunization is the induction of as high a CTL precursor frequency as possible. This frequency is directly proportional in the melanoma model to the number of immunizations administered (29).

Thus we are stating an immunization protocol aimed at the induction of LMP-2 directed cellular immune responses. This is the first of a series of clinical protocols aimed at the study of immunization against NPC. Since the natural immune response to NPC as determined for instance by the specificity of tumor infiltrating lymphocytes is not known, we use LMP-2 as a "surrogate" TA since this viral protein is constitutively expressed by NPC cells and cytotoxic T cell epitopes have been identified in the context of HLA phenotypes common in Chinese (HLA-A*1101 and HLA-A*2402). The efficacy of known LPM-2 epitopes in inducing $CD8^+$ T cell responses in patients with high risk of recurrence on NPC will be assessed by weekly injections according to a well establish immunization scheme that we have extensively utilized in the context of metastatic melanoma (50). This study will verify the usefulness of these epitopes as immunogen to be used for future clinical trials in which they will be combined with systemic immune stimulators such as IL-2 for the treatment of patients with advanced NPC. Our hypothesis is, in fact, that immunization will not lead to regression of established tumor unless associated with systemic immune stimulation (6, 7).

Conclusions

In this review, we summarized the present knowledge derived from the analysis of immunization-induced T cell responses against metastatic melanoma. In particular, we emphasized the need and feasibility to study directly in humans adopting a global approach the interaction between cancer and immune cells during immune activation. We then suggested, that although cutaneous melanoma remains an hallmark for the study of tumor immune biology in humans, other neoplastic disorders may represent interesting models remaining largely unexplored and should be considered with particular interest in the future as a complement to the study of melanoma biology. We believe that the characterization of similarities and diversities among these diverse models could simplify the detection of common pathways leading to immune- mediated cancer rejection. Tools presently available to study directly in human at a multidimensional level immune activation have been summarized. Several of the issues regarding the application of these tools and strategies used for the study of tumor/host interaction have been raised by this review. We encourage future researchers to consider prospective accrual of materials during the conduct of clinical trials suitable for this type of analyses.

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