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Gene expression profiling in vaccine therapy and immunotherapy for cancer

Davide Bedognetti^{1,2,3,4}, Ena Wang¹, Mario Roberto Sertoli^{2,4}, and Francesco M Marincola^{1,*}

¹Infectious Disease and Immunogenetics Section (IDIS), Department of Transfusion Medicine, Clinical Center, and Trans-NIH Center for Human Immunology (CHI), National Institutes of Health, Bethesda, MD 20892, USA

²S.C. Oncologia Medica B, Department of Medical Oncology, National Cancer Research Institute, Genoa, Italy

³Department of Internal Medicine, University of Genoa, Genoa, Italy

⁴Department of Oncology, Biology and Genetics, University of Genoa, Genoa, Italy

Abstract

The identification of tumor antigens (TA) recognized by T cells led to the design of therapeutic strategies aimed at eliciting adaptive-immune responses. The last decade experience has shown that, although active immunization can induce enhancement of anti-cancer T cell precursors (easily detectable in standard assays), most often they are unable to induce tumor regression and, consequently, have scarce impact on overall survival. Moreover, in the few occasions when tumor rejection occurs, the mechanisms determining this phenomenon remain poorly understood, and data derived from *in vivo* human observations are rare. The advent of high-throughput gene expression analysis (microarrays) has cast new lights on unrecognized mechanisms that are now deemed as central for the development of an efficient immune-mediated tumor rejection. The aim of this article is to review the data about the molecular signature associated with this process. We believe that the description of *how* the mechanism of immune-mediated tissue destruction occurs would contribute to understand *why* it happens, thereby allowing to develop more effective immune-therapeutic strategies.

Keywords

Microarray; microarrays; gene expression; cancer immunotherapy; vaccine therapy; tumor rejection; melanoma; immunologic constant

*Correspondence to: Francesco M Marincola, MD, Department of Transfusion Medicine, Clinical Center, N1-226, Bldg 10, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20892; Phone: (301) 451-4967, Fax: (301) 402-1360, FMarincola@cc.nih.gov.

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1. Introduction

The first observations about the role of the immune-system in inducing tumor regression date back to the 1700s, with the description of sporadic tumor-regression following infective episodes ⁽¹⁾.

In 1890s, William Coley, influenced by such observations, injected bacterial products (also known as Coley's toxin or Coley's vaccine) directly into tumor, achieving dramatic responses ^(2,3). This is considered the first empiric evidence of the potential of the immunotherapy for inducing tumor rejection ^(2,3,4). However, the broad acceptance of this phenomenon required several other studies and observations. In the 1980s experimental and clinical data from studies investigating the role of exogenous pro-inflammatory cytokines (interleukin-2; IL-2, and interferon alpha; IFN- α) clearly demonstrated that the immune-response contributed to tumor regression ^(5,6,7). In the early 1990s, the identification and molecular characterization in humans of tumor antigens (TA) recognized by autologous T cells gave the basis for modern vaccine anticancer therapy. In particular, van der Bruggen et al, with a landmark paper published in 1991, described MAGE-1 as the first human tumor antigen ⁽⁸⁾. This study, which was quickly followed by the description of the first human cancer-specific peptide epitope restricted by HLA-A1 ⁽⁹⁾, revolutionized the field of tumor immune biology by providing conclusive evidence that CD8+ T cells specifically recognize and kill autologous cancer cells over-expressing cancer-specific proteins. This important achievement gave molecular precision and novel distinction in this rather disregarded field and provided the opportunity to investigate with scientific accuracy the fascinating phenomenon of cancer rejection in physiologic conditions and/or in response to therapy. The subsequent identification of a myriad of TA triggered the extensive utilization of TA for the development of anti-cancer vaccines. For the first time, natural reagents (CD8+ T cells) that could selectively recognize only cancer cells were reliably generated, providing a powerful tool to analyze in molecular detail the dynamics of developing immune responses in the cancer bearing host⁽¹⁰⁾. This characteristic of high (almost absolute) specificity, confirmed also by the scarce toxicity (skin de-pigmentation), is an achievement that rarely other anticancer therapies have attained ⁽¹¹⁾. Nevertheless, although from a logical point of view TA-specific immunization reached its purpose, clinical results have been so far disappointing and, at present, no anticancer vaccine can be recommended outside clinical trials, neither in the adjuvant nor in metastatic setting. Moreover, the biological explanations for this dichotomy between immunological and clinical endpoints remain elusive ^(12,13).

Therefore, these data should encourage the optimization of immunization strategies by combining systemic immune-stimulation focusing particularly on the understanding of events downstream of TA-specific T cell generation ^(14,15).

Other immune-therapeutic modalities (monoclonal antibodies, mAbs) have been effectively implemented in the last years. For instance, targeting inhibitory molecules on the surface of activated T cells (e.g. cytotoxic-lymphocyte associated-antigen (CTLA)-4), represents an emerging strategy to elicit (without specificity) the immune-response against tumor ⁽¹⁶⁾. However, most monoclonal antibodies are directed again surface molecules over-expressed in cancer cells (the majority of which are products of dominant oncogenes, e.g. grow factor receptors). Their mechanisms of action are represented by the inhibition of the targeted receptor but also by the induction of antibody-dependent cell-mediated cytotoxicity (ADCC) ⁽¹⁷⁾. For these reasons, mAbs can be taken apart from the aforementioned forms of immune- therapies; their in depth analysis is beyond our purposes and will therefore not be covered here.

2. Lessons learned from previously immunization studies and the need of a global approach to tumor immunology

Immunization strategies have included the administration of minimal epitopic determinants derived from single TA, whole TA administered as protein products or as vectors genetically engineered to encode for TA, cancer cell lysates or whole cancer cells. Comprehensive immunization approaches do not target a specific HLA/epitope combination and, as a consequence, it is very difficult to monitor the immunological effectiveness of the vaccine since it is not known which HLA/epitope combination is dominant in each individual patient⁽¹⁸⁾. In contrast, TA-specific immunization restricted to an individual epitopic determinant derived from single TA administered as human leukocyte antigen (HLA) combination offers the unique opportunity of studying in humans the dynamics of immune responses by-passing the complexity of human polymorphism and simplifying the heterogeneity of cancer biology by reducing the algorithm regulating tumor host interactions to a specific HLA/epitope interaction with the complementary T cell receptor. These simplified treatments have shown that factors other than direct T cell-tumor cell interactions need to be considered^(19,12,13). Genetic polymorphisms may affect immune responsiveness by varying the function of genes associated with antigen presentation, cytokines, killer cell immunoglobulin-like receptors and leukocyte Fc γ receptors⁽¹⁸⁾. Tumor cell biology may affect T cell function through pathways independent of HLA/epitope T cell receptor engagement^(20,15). Tumor immunology is a compound field that merges the intricacy of human immunology with the complex biology of cancer. Experimental models created to bypass such complexity by inbreeding animals and standardizing cancers miss such basic essence⁽²¹⁾. Animal models can be manipulated by enhancing or eliminating individual factors of the algorithm governing tumor growth, either in favor or against them. This exaggeration of individual components can give the false impression that each one is pivotal in determining the individual story of each human cancer. However, it is likely that in human, none of the potential mechanisms plays individually a role of the weight that can be created by over expressing or eliminating the expression of a gene in animal. Complexity, however, may provide useful insights if least common denominators required for the occurrence of a biological process could be identified. Similar biological phenomena may result from convergence of different pathways into a final outcome that, though masked by irrelevant biological variables, governs their occurrence.

Thus, the complexity of several, and redundant, molecular pathways responsible for the natural and/or treatment-induced behavior of tumor cannot be analyzed by a hypothesis-driven approach. As a matter of fact, too many hypotheses or, in other words, no solid single hypothesis exist, based on experimental results that can drive experimentation in humans. Understanding the tumor/host interaction cannot exclude a high-throughput discovery-driven hypothesis. Ideally, this strategy should cover genomic, transcriptomic and proteomic analyses, through an approach defined as 'Integromics'⁽²²⁾. Although data derived from such proposed approach are not available yet, the use of microarray technology, which can capture in real-time the physiology of disease by simultaneously monitoring the expression of thousands of human genes, has been remarkably revealing. The integration of biological findings (derived from high-throughput gene expression analyses) with clinical data (derived from immunotherapy-trials)⁽²³⁾ has allowed the formulation of intriguing new hypotheses on molecular mechanisms that govern the immune-mediated tumor rejection process. In the following paragraphs, we will discuss mostly our recent work with reference to scant information present in the current literature from other groups who investigated tumor/host interactions *ex vivo* using a similar approach.

3. T cell transcriptional profile after immunization

In longitudinal studies conducted in transgenic mouse models, Kaech SM *et al.* ⁽²⁴⁾ ⁽²⁵⁾ analyzed the transcriptional profile of CD8+ T cells following acute exposure to antigen and characterized distinct phenotypes at different time points from antigen exposure. These studies suggested a continuous spectrum of CD8+ T cell development from naïve → effector → memory of which classical effector and memory phenotypes represent the extremes. During the first week a rapid expansion of CD8+ T cells is observed in which CD8+ T cells are cytotoxic *ex vivo* and display a genetic profile rich of effector/activated T cell features including granzyme-A and -B, perforin and FAS ligand. In the following contraction phase a memory phenotype ensues characterized by ability to produce interferon- γ (IFN- γ) in response to cognate stimulation but lack of lytic activity and loose the expression of genes associated with T cell effector function. This model fits well with immunization-induced T cells due to the dynamics of cancer vaccine-therapy, which exposes the organism to specific antigenic stimulation in time followed by a rest period ^(26,27). We studied the functional status of circulating CD8+ vaccine-induced T cells in metastatic melanoma patients undergoing vaccination using an HLA class I restricted, modified peptide called gp100 (209-2M), derived from gp100 (a melanoma TA involved in the synthesis of melanin). Although such lymphocytes retain an effector phenotype according to canonical markers (CD27 negative, CCR7 negative, CD45RA^{high}) and can respond with IFN- γ secretion to cognate stimulation, they do not express perforin and cannot exert effector functions ⁽²⁸⁾. In a following microarray study, we better characterized a “quiescent” phenotype of immunization-induced T cells lacking direct *ex vivo* cytotoxic and proliferative potential ⁽²⁷⁾. Transcriptional profiling of quiescent circulating tumor-specific CD8+ T cells demonstrated that they lack expression of genes associated with T cell activation, proliferation and effector function (e.g. CCR5, CXCR3, perforin and granzyme A). This quiescent status may explain the observed lack of correlation between the presence of circulating immunization-induced lymphocytes and tumor regression. In fact, we had previously shown that circulating, vaccine-induced T cells can reach tumor deposits, interact with tumor cells producing IFN- γ without leading to tumor destruction ⁽²⁹⁾.

However, the lack of a proliferative and cytotoxic response of TA-specific T cells, can be recovered by *in vitro* antigen recall and IL-2, suggesting that a complete effector phenotype might be re-instated *in vivo* to fulfill the potential of anti-cancer vaccine protocols if similar conditions could be provided ^(27,14). It is likely that, at the tumor site, immunization induced T cells are exposed to antigen-recall but they are probably not exposed to the co-stimulatory drive modeled by the addition of IL-2 in *in vitro* conditions⁽³⁰⁾. Accordingly, while vaccination alone only rarely induces tumor regression⁽³¹⁾, the combined administration of immune-modulators such as IL-2 appears to enhance its clinical effectiveness suggesting that other factors are required *in vivo* for the full activation of tumor-specific T cells. In a pivotal study involved metastatic melanoma patients, only the association of gp100 (209-2M) and IL-2 obtained a considerable rate of tumor response (42%), whereas the administration of vaccine alone did not produce any clinical benefit ⁽³²⁾. The results of three independent phase II trials attributed the observed favorable outcome to the IL-2 rather than to the vaccine component ⁽³³⁾. However, a recent randomized multi center clinical trial has finally confirmed that the combination of high dose IL-2 administration with active specific immunization yields better results than either treatment alone ⁽³⁴⁾.

Understanding the effect of co-stimulation molecules, such as IL-2, in the tumor micro-environment might be the key to successful implementation of TA-specific anti-cancer therapies ^(14,12,13,35).

4. Studying the microenvironment

An emphasis has been placed on the importance of complementing the analysis of immune-responses in circulating lymphocytes, which yield the information about whether the immunotherapy had any systemic effect, with the study of tumor/host interaction directly analyzing the tumor samples ^(36,37).

The tumor microenvironment is difficult to access in humans and, therefore, immune monitoring has been limited mostly to the study of circulating immune cells, easy to access through venipuncture. Yet, we have repeatedly emphasized the need to study immune responses in the target organ where they are most relevant and implemented approaches to study tumor immune biology using high-throughput strategies ⁽³⁸⁾.

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 setting) for transcriptional profiling using microarray technology ^(39,40). We have previously shown that fine needle aspirated (FNAs), tru-cut or punch biopsies minimally perturb the tumor microenvironment ^(41,42,43,44) without affecting the outcome of therapy ⁽⁴⁵⁾. Thus, avoiding the complete excision of the lesion, it is possible to directly correlate biological signatures to treatment outcome ^(46,19). This is important since at the histological and transcriptional level synchronously biopsied metastatic lesions are quite heterogeneous ^(47,42). This heterogeneity questions the accuracy of utilizing biological information obtained from one lesion to predict the behavior of another. Thus, we proposed that a high throughout discovery-driven approach applied to the study of microenvironment at appropriate time-points (which have to include pre-treatment and post-treatment times) is critical for understanding immune-mediated tumor rejection.

Using such strategy we described the modification of the tumor microenvironment induced by IL-2 ⁽⁴¹⁾ in melanoma, monitored the effects of anti-cancer vaccination ⁽⁴²⁾ in the same disease and the effects of Toll-like receptor (TLR)-7 agonists for the treatment of basal cell cancer (BCC) ⁽⁴³⁾. Biopsies were performed in an easily accessible lesion under local anesthesia obtaining two consecutive FNA (or other non-invasive biopsies) passages according to a previously described four quadrant aspiration technique ^(46,42). The same lesion was re-biopsied, when possible, after treatment administration. The sequential approach causes some alterations in the transcriptional profiling of the lesion at the time of the second biopsy, however such changes are limited and mostly irrelevant to the biological phenomenon studied ⁽⁴⁴⁾.

Melanoma - Eclectic effects of IL-2 on tumor microenvironment

To understand how IL-2 administration may turn an indolent chronic inflammatory process into acute auto-immune rejection of cancer, we studied the mechanism of action of IL-2. This was done by performing FNAs of melanoma 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, contrary to what previously believed, the immediate effect of systemic IL-2 administration on the tumor microenvironment is a transcriptional activation of genes predominantly associated with monocyte function while minimal effects were noted on migration, activation and proliferation of T cells. More in detail, this study suggested that IL-2 induces inflammation at tumor site with three predominant secondary effects: activation of antigen-presenting monocytes, massive production of chemo attractants (including CXCL9 and CCL3, -4) that may recruit other T and NK cells to the tumor and activation of cytotoxic mechanisms in monocytes (calgranulin, grancalcin) and natural killer cells (NK4; natural killer receptor 4, NKG5; granulysin). These may in turn contribute to epitope spreading through killing of

cancer cells, with consequent uptake of shed antigens and presentation to adaptive immune cells. Interestingly, most of the genes identified have also been observed as markers of TA-specific T cell activation *in vitro* ⁽²⁷⁾. Moreover, IL-2 induces a cytokine storm ^(48,49), responsible for the release of a broad array of immune stimulatory cytokines by circulating mononuclear cells that can have broader immune/pro-inflammatory effects than those expected by the interaction of IL-2 with its receptor. Remarkably, the several alterations due to the effect of IL-2 therapy such as the up-regulation of classical interferon-stimulated-genes (ISGs) induced by type I IFNs, were not associated with clinical response. Nevertheless, a particular subset of genes whose expression appeared to be associated with clinical response was described. Such genes were represented by ISGs preferentially induced by *IFN7-γ/interferon-regulatory factor-1 (IRF-1)/signal transducers and activator of transcription (STAT-1)* pathway, including the expression of human HLA class I and II and genes associated with effector function: nucleolysin cytotoxic granule (TIAR), NK4 and granulysin. Although this observation was based on the analysis of a single responding lesion, it was remarkably relevant. In fact, the activation of such pathway was frequently associated to other forms of acute immune-mediated tissue destruction processes (e.g. acute allograft rejection)^(50,51,52,53,35).

Melanoma - Tumor microenvironment and effects of immunization: the switch from chronic to acute inflammation and its relation with tumor regression

Previous observations based on the analysis of cell lines or tissue preparations about which very little clinical correlation was available, suggested that cutaneous and ocular melanoma segregate into two distinct taxonomies based on global transcript analysis ⁽⁵⁴⁾. By prospectively collecting clinical information regarding lesions from which FNA samples had been serially obtained, it was possible to monitor changes in transcriptional programs of individual lesions occurring throughout time. By adding this temporal dimension to the study of cancer biology, we observed that the two melanoma subgroups did not represent two distinct disease taxonomies but rather two stages of the same rapidly evolving disease ⁽⁴²⁾. The ability to directly link genetic profiling with clinical history is a paramount effort for future studies. We demonstrated this concept by performing a supervised analysis of lesions undergoing FNA before treatment. Lesions were separated according to their clinical response to immunization combined with IL-2 administration and transcriptional profile identified gene predictors of immune responsiveness. These genes were predominantly associated with immune function suggesting that tumor deposits are pre-conditioned to respond by an immunologically active tumor microenvironment, even before treatment administration⁽⁴²⁾. In particular, the identification of interferon-regulatory factor-2 (IRF-2) over-expression as a predictor of immune responsiveness suggested that tumors likely to respond are chronically inflamed before treatment. This inflammatory process may not be sufficient to induce tumor rejection and it may be in fact beneficial for tumor growth, but it may set the stage for a conversion to an acute inflammatory process by recruiting immune cells at the tumor site ⁽³⁶⁾. Indeed, a paired analysis of FNA samples obtained before and during therapy underlined this possibility since lesions that underwent complete response were characterized by the over-expression of IRF-1, a counterpart to IRF-2, generally up-regulated during acute inflammation ^(55,56,57,58). Interestingly, non responding lesions did not demonstrate any significant changes in their transcriptional profile in response to therapy. More recently, we analyzed two melanoma metastases undergoing tumor regression after immunotherapy compared to three synchronous lesions that continued to progress in their growth ^(59,60). Transcriptional comparisons between responding and non-responding lesions identified IRF-1 as the most relevant transcription factor orchestrating the immune-concert in responding lesions (Carretero R et al, manuscript in preparation). Analysis of the over expressed genes in the predominant transcriptional network activated in the responding lesions strongly pointed to the activation of genes induced by IFN-γ as the

central modulator of rejection while IFN- α induced ISGs influenced only slightly the transcriptional profile associated with rejection.

In support of the hypothesis that lesions undergoing regression upon immune therapy are subjected to a powerful acute inflammatory switch, is the clinical observation that during IL-2 therapy these lesions become tender and swell before disappearing. Similarly, ulceration and inflammation occur in basal cell carcinoma, during treatment with TLR-7 agonist, sparing the surrounding skin.

It is likely that the antigen-specific interaction of activated T-cells with their target not only activates killer mechanisms but, importantly, induces the secretion of pro-inflammatory cytokines, which in turn amplify the effector cascade that leads to a tissue destruction. Thus, TA-specific T cells could be considered a vehicle to deliver general pro-inflammatory signals in a highly specific manner. However, in tumor/host biology, this phenomena occurs only rarely spontaneously. Triggering this switch may represent a key to improve the efficacy of anti-cancer therapy.

The observation that lesions likely to respond to therapy are pre-conditioned by an immunologically active microenvironment 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 ^{(61,62) (63)}. This observation is only apparently contrasting our observation. It may very well be that inflammation is helpful in promoting angiogenesis and acts as a direct stimulus to tumor growth as many factors released during tissue remodeling and repair have in fact stimulatory effects on tumor cell growth. Thus, growth factors 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 have chemo-attractant and regulatory properties on immune cells. 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 anti-cancer cells given appropriate stimulatory conditions that may be induced by treatment such as the systemic administration of IL-2 ^(41,48).

BCC - Microenvironment and effect of TLR-7 agonist

Imiquimod belongs to a family of synthetic small nucleotide-like molecule with potent pro-inflammatory activity mediated through TLR-7 signaling. This drug targets predominantly TLR-7 expressing plasmacytoid dendritic cells (pDC). The toxicity associated with systemic toxicity limits its use to topic application. It was proved to enhance the tumor activity of cancer vaccine in animal models ⁽⁶⁴⁾. A randomized phase II trial, formally comparing its efficacy with that of the traditional incomplete Freund's adjuvant for the vaccine therapy in resected high risk melanoma patients, has been recently completed but data are still not available. However, Imiquimod, in monotherapy, is currently approved to treatment of basal cell carcinoma. Although Imiquimod function seems particularly associated to IFN- α stimulated genes ⁽⁶⁵⁾, it is not clear whether this pathway is solely responsible for all the downstream effects ultimately results in tumor clearance. The known high rate of tumor response associated to Imiquimod and the easily accessibility of BCC lesions yield this topical administration an outstanding model for the study of the mechanism of immune-mediated tumor rejection ⁽⁴³⁾. This model emphasizes the quantitative aspects of immunotherapy suggesting that the high concentrations of immune stimulator that could be achieved with a topical treatment could shift the balance between host and cancer cell interactions in favor of the host by local manipulations of the microenvironment that cannot easily and specifically achieved through systemic routes. Thus, we conducted a prospective,

randomized, placebo-controlled double-blinded trial comparing the gene expression profile of paired punch biopsies pre and post treatment (approximately 1 day after the last dose administered). It must be specified that, according to protocol, tumor regression did not represent an end point and tumors were removed at the end of the study. However, 41% (9/22) and 7% (1/14) of samples were devoid of cancer cells in imiquimod and placebo arm, respectively ($p = 0.05$). This data confirm the role of Imiquimod (rather than artifact due to vehicle administration or surgical trauma) in inducing tumor clearance. The comparison with a placebo rules out the possibility that the signatures associated to Imiquimod-treatment are induced by artifacts related to the biopsies themselves or to the eccipients used for the delivery of the active component. The result of this analysis demonstrated that the eradication of BCC is a complex multi factorial phenomenon. Of 637 genes specifically induced by Imiquimod, only a minority (98 genes) was canonical type I IFN-induced ISGs⁽⁴³⁾ while the rest portrayed additional immunological functions predominantly involving innate and adaptive immune effector mechanisms. Thus, even in this model, ISGs appeared to be necessary but not uniquely responsible for tissue specific immune rejection. However, how indicated by quantitative protein chain reaction (qPCR), IFN- γ transcription was more prevalent than IFN- α transcription. The abundance of IFN- γ suggests that pDCs trigger other immune functions through the production of IFN- α , which in turn activates T and NK cells, selective producers of IFN- γ . This finding was perhaps in line with the evidence of the activation NK and CD8+ T cells cytotoxic mechanisms (granzymes, perforin, granulysin, NK4 and IL-32). Moreover, the up-regulation of cytokine and corresponding receptors within the common γ chain receptor (IL-15 and IL-15 receptor α chain, the IL-2/IL-15 receptor β chain and common γ chain itself) suggest early activation of NK and CD8+ T cells within the tumor microenvironment. Other relevant IFN- γ stimulated genes were class I and class II HLAs, C1QA (complement component 1a) and STAT-1. CXCR3 ligands (CXCL9 and -10) and CCR5 ligands (CCL3 and -4), were also over expressed. These chemokines represent the major chemotactic factor for T helper -1 (Th1) cells, activated CD8+ and NK cells. It is noteworthy that most of the genes over expressed in this study were found unregulated also during acute allograft rejection episodes^(51,52). Similarly to the two aforementioned melanoma studies^(41,42) these data suggest that immune-mediate tumor-rejection may share several immunological properties of this apparently unrelated, immunological phenomena.

Signatures associated with response to immunotherapy

Recently, during the *'ISBTc-FDA-NCI Workshop on Prognostic and Predictive Immunologic Biomarkers in Cancer'*, the need to design adequate clinical prospective randomized trial, which allow to obtain prospectively appropriate material for identification of predictor biomarkers, has been underlined⁽³⁷⁾. Biological markers predictive of favorable outcome in anticancer therapy have been also reviewed. So far, however, only few studies addressed the subject of predictive biomarkers for response to immunotherapy and validation studies are lacking.

Recently, using an high-throughput proteomic approach, Sabatino et al⁽⁶⁶⁾ identified high level of serum vascular endothelial grow factor (VEGF) and fibronectin as predictor factors of IL-2 therapy in metastatic melanoma patients. This data fit with recent observations, which suggest that, beyond the angiogenic activity, VEGF can act as immune suppressant by blocking maturation of dendritic cells⁽⁶⁷⁾ or by inhibiting effective priming of T cell response⁽⁶⁸⁾.

As for IFN- α , a recent study suggested that IFN signaling is disrupted in patients with solid tumors^(69,70) and high ratio of phosphorylated STAT1 (pSTAT1)/phosphorylated STAT3 (pSTAT3) in tumor before treatment appeared predictive of prolonged survival (in neo adjuvant setting) in melanoma patients treated with IFN⁽⁷¹⁾. Moreover, a study conducted

by using a high-throughput assay technology, evaluated the impact of several cytokines, chemokines, angiogenic growth factors, and soluble receptors, in predicting the outcome in melanoma patients treated with IFN- α . The authors found that pretreatment levels of pro inflammatory cytokine IL-1- β , IL-1 α , IL-6, tumor necrosis factor- α (TNF- α), and CCR5 ligands (CCL3, -4) were found to be significantly higher in the serum of patients with longer relapse free survival ⁽⁷²⁾. These data are in line with our observations that inflamed melanoma lesions are more likely to respond to immunotherapy based on the assumptions that the circulating factors may represent a reverberation of an immunologically active tumor microenvironment ^(42,73). Preliminary data from a MAGEA3-based vaccination against melanoma EORTC (European Organization for Research and Treatment of Cancer) trial monitored by high throughput gene expression profiling, suggest that a combination of genes with immune-related function including CCL11, CCR5 ligands (CCL5), IFN- γ , inducible T-cell co-stimulator (ICOS) and CD20 expression, could predict response to therapy ⁽³⁷⁾. A similar approach has been successfully used to identify immunologic signatures predictive of response to adjuvant MAGEA3 in non-small cell lung cancer, but detailed results are still not available. Accordingly, a similar pattern was observed experimentally in a melanoma xenograft mouse model in which endogenously produced chemokines of the CXCR3 and CCR5 ligand families induced cancer regression by recruitment of CD8-expressing T cells ⁽⁷³⁾. Finally, confirming the key role of such chemokines in modulating the immune-response against cancer, is the recent finding that the presence of the CCR5Delta32 polymorphism, which encodes for a non functional protein, results in a decreased survival following immunotherapy in patients with metastatic melanoma ⁽⁷⁴⁾.

5. Gene expression profiling in vaccinia virus oncolytic therapy

The ability of some viruses to mediate tumor rejection was supposed in the early twentieth century, when some cancer patients were observed to experience tumor regression after systemic viral infections ^(75,76). As in the aforementioned case of the Coley's toxin, it was hypothesized that a specific immune-stimulus induced by viral infection could elicit an immune response against cancer.

In the last years, an increasing number of pre-clinical and clinical trials have been carried out using tumor-tropic, oncolytic viruses ^{(77) (78)}. Although this approach is believed to work by a direct virally-induced oncolytic process, experimental data suggest that it might operate with the "assistance" of the host's immune system.

Vaccinia virus (VACV) has been a promising candidate for oncolytic therapy due the extensive experience gathered in humans because of its worldwide use as an anti smallpox vaccine. The innate immune response initially stimulated by the virally infected cells and/or the VACV itself is directed automatically against the infected tumor cells and we suggested that this is part of the mechanism leading to tissue destruction by oncolytic therapy.

Transcriptional analysis of mouse xenografts using a mouse-specific platform to identify the host's response genes revealed the activation of innate immune mechanisms in regressing breast cancer compared to non-infected control tumors ⁽⁷⁹⁾. Up-regulation of pro-inflammatory chemokines such as CCL2, -9, -12, CXCL9, -10, and 12 was seen together with an increase of interleukins (IL-18) and interleukin and chemokine receptors (IL13R and CCR2) transcripts. Such significant activation of ISGs was observed in association with increased STAT-1. This strongly suggests that type I and/or type II IFNs are critically involved in the process. Immunohistochemistry of VACV-infected, regressing xenografts showed an intense peri- and intra-tumoral infiltration of mononuclear cells, which confirmed the up-regulation of CD69, CD48, CD52, and CD53 seen on the host's gene expression

arrays. These markers are expressed on activated T-cells, NK cells, macrophages, granulocytes and DCs, and are associated with leukocyte activation and NK cytolytic function ⁽⁷⁹⁾.

To better dissect the mechanisms associated with VACV-driven tumor destruction, we compared VACV-infected GI-101A xenografts sensitive to oncolytic therapy to GI-101A xenografts from non-infected animals, and to HT-29 colon cancer xenografts that do not respond to oncolytic therapy in spite of VACV colonization. Nude mice were used for this purpose. We evaluated gene expression profiles of the oncolytic interaction by adopting organism-specific microarray platforms: 36k whole genome human arrays to test for alterations in the human cancer cells; 36k whole genome mouse arrays to examine the host's infiltrating stromal cells and lastly; custom-made 1K VACV arrays to characterize changes in viral transcription patterns.

Human transcript analysis revealed no differences in non-responding, infected HT-29 tumors compared to control tumors and only a limited set of genes which was altered after GLV-1h68 inoculation in regressing GI-101A xenografts; most transcriptional changes were observed in the infected responding tumors at a time when cell death had not occurred yet and revealed profound down-regulation of genes associated with cellular metabolic processes reflecting the shutdown of cancer cell metabolism due to VACV infection. Analysis of mouse expression arrays representing the host's infiltrating cells demonstrated that infected, non-responsive HT-29 tumor were not affected by the viral presence in cancer cells similarly to HT-29 tumors from non-infected control animals. On the contrary, a large number of genes were up-regulated in GI-101A tumors after VACV delivery compared to non-infected GI-101 xenografts. Further analysis discovered a significant enrichment of immune-related genes; among those, ISGs and other IFN signaling genes represented the most up-regulated canonical pathways. These signatures strictly resembled those previously observed in human BCC treated with TLR7 agonists ⁽⁴³⁾. Among chemokines, CXCR3 ligands (CXCL 9, 10, and 11) were strongly expressed in regressing GI-101A xenografts together with CCR5 ligands (CCL5).

Since these mice lack both T and B cells, this immune-mediate tissue destruction is supposed to be induced by innate immune effectors such as NK cells and activated macrophages. This study suggests that, at least in this model, innate immunity can be an independent effector of tissue-specific destruction not requiring the adaptive immunity.

6. Expert commentary & five-years view

Complex problems do not necessarily require complex solutions ^(80,53). Identifying the common, downstream, mechanisms that lead to the immune-mediated tissue destruction in different conditions may allow the development of novel target treatments without requiring the understanding of the individual phenomenologies.

In 1969, in a seminal manuscript entitled 'Immunological paradoxes: theoretical consideration in the rejection or retention of graft, tumors and normal tissue', Jonas Salk proposed that chronic infections, allograft rejections, autoimmune disorders and cancers belong to a common phenomenon that he named the "delayed allergy reaction" ⁽⁸¹⁾. This outstanding observation stated almost half a century ago, seems to have found today its molecular explanation.

Although mechanism triggering tissue-specific destruction (TSD) differ among distinct immune pathologies, we proposed that TSD follows a common pathway which we termed the "immunologic constant of rejection (ICR)" ⁽⁵³⁾. We formulated four axioms that summarize the phenomenon: 1) TSD does not necessarily occur because of non-self

recognition but also occurs against self or quasi-self; 2) the requirements for the induction of a cognate immune response differ from those necessary for the activation of an effector one; 3) although the prompts leading to TSD vary in distinct pathologic states, the effector immune response converges into a single mechanism; and 4) adaptive immunity participates as a tissue-specific trigger, but it is not always sufficient or necessary ⁽⁵³⁾.

The limited work performed by our group so far studying in real-time the events occurring before and during therapy in the tumor microenvironment suggests that immune rejection is associated with the activation of ISGs accompanied by the activation of genes that are expressed naturally by NK cells and by CD8 T cells upon activation. Among them we observed that NK and CD8+ T cells effector function genes (e.g. perforin, granzymes and granulysin) seem to predominate during the switch to acute rejection. Interestingly, accordingly with other human studies investigating different processes, the activation of such 'NK like' mechanism appears to be a convergent molecular mechanism of several forms of immune-mediated process. We recently summarized the common functional units that, when TSD destruction occurs, are activated in a coordinate fashion:

1. the STAT-1/IRF-1/T-bet/IFN- γ , IL-15 path
2. the Granzyme A/B, TIA-1 pathway
3. the CXCR3 ligand chemokine pathway
4. The CCR5 ligand chemokine pathway

We observed, in different disease models, their presence; studies in humans have identified these signatures to be associated with improved survival of patients with colon, lung and ovarian cancer or melanoma ^(82,83,84,85,86,87,73); the same patterns were observed in neoplastic lesions responsive to immunotherapy both in humans ^(42,41,43,37) and in experimental models ⁽⁸⁸⁾. In transplantology, several studies have reported the activation of the same pathway during the occurrence of acute allograft rejection ^(50,89,90,52,51,91). In particular Saint-Mezard et al. ⁽⁵¹⁾, by comparing three independent microarray data sets of kidney biopsies, identified IRF-1 as the main transcription factors that regulated the 70 genes consistently represented during acute allograft rejection episode. Imanguli et al ⁽⁹²⁾, observed similar patterns by studying biopsies of tissues suffering chronic graft versus host disease and similar patterns where observed in the liver during clearance of HCV infection ^(93,94,95,96,97). Recently similar signatures were observed in the destructive phases of acute cardiovascular events ^(98,99), chronic obstructive pulmonary disease ⁽¹⁰⁰⁾ and placental villitis ⁽¹⁰¹⁾.

We believe that decrypting the codes that govern the balance between tolerance and rejection, as well as the events that can suddenly induce the switch from an indolent process to a destructive one, may allow to identify a key molecular process, the targeting of which could represent the rational for the development of a new-generation cancer therapy.

Tools are available nowadays to study biological processes in their globality. The study of individual genetic predisposition to disease and response to treatment could be combined with that of epigenetic changes during life and disease progression and that of real-time adaptation of the transcriptional profile of biological samples in relevant conditions. The problem resides in the availability of relevant samples to study. In particular, functional genomics studies rely on the measurement of messenger RNA levels that are very susceptible to metabolism and degradation. Thus, only carefully and prospectively collected samples are usually worth studying. The understanding of the biology of cancer cells, their relationship with the host and their response/adaptation to therapy would be an achievable goal if clinical studies were designed to answer these questions and not only to test the potency of a given treatment ⁽²¹⁾. With the purpose of co-coordinating future clinical efforts

in this line several issues will need to be considered beyond genetic profiling to acquire a more global sophistication in the design and conduct of clinical trials in the future ⁽³⁷⁾.

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Key issues

1. In the field of anticancer vaccine therapy, the immunologic endpoint (evaluation of TA-specific T cells) is not a surrogate of clinical end point (response rate or overall survival). Clinical trials have definitely shown that highly specific T-cell responses can be generated. Nevertheless, clinical results are disappointing. These data indicate that immune-mediated tumor rejection requires more than simple T cell/target interaction.
2. The biological explanations for the dichotomy between immunological and clinical endpoints remain elusive.
3. Tumor immunology is a compound field that merges the intricacy of human immunology with the complex biology of cancer. Experimental models created to bypass such complexity by inbreeding animals and standardizing cancers miss such basic essence. Understanding the phenomena of tumor immune-response cannot avoid a high-throughput discovery-driven hypothesis performed analyzing human tumor samples comparing serial time points to delineate the dynamic association with the inflammatory switch.
4. Through this approach the following concepts emerge: 1- lesions likely to respond to therapy are pre-conditioned by an immunologically active microenvironment; 2- tumor regression is associated with switch from chronic to acute inflammation; 3- IL-2 does not promote migration of cytotoxic T cells to the tumor microenvironment nor their activation or proliferation but rather induces a cytokine storm responsible for the release of a broad array of immune stimulatory cytokines by circulating immune cells; 4- beyond the direct cytotoxic mechanism, mediated by interaction of specific T cells with their target cells, T cells could be considered a vehicle to deliver general pro-inflammatory signal in a selective manner; 5- molecular mechanisms activated during immune-mediated tissue destruction are shared by other, apparently unrelated, immune-mediated tissue destruction process (e.g. allograft rejection, viral clearance and graft versus host disease).
5. We proposed a convergent molecular mechanism associated with immune-mediated tissue destruction that we named 'immunological constant of rejection'. This constant include the coordinate activation of the interferon stimulated genes and the immune-effector functions.
6. We summarized the functional units of the ICR: a) STAT-1/IRF-1/T-bet/IFN- γ , IL-15 path b) Granzyme A/B, TIA-1 pathway c) the CXCR3 ligand chemokine pathway d) The CCR5 ligand chemokine pathways.
7. Identifying the clinical mechanisms that lead to this final common pathway in individual tumors may define a better rationale for targeted therapies that may take advantage of each individual cancer's biology.