CMLS, Cell. Mol. Life Sci. 59 (2002) 230–240
1420-682X/02/020230-11 $ 1.50 + 0.20/0
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CMLS Cellular and Molecular Life Sciences

Review

Immunotherapy against antigenic tumors: a game with a lot of players

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Received 21 June 2001; received after revision 15 August 2001; accepted 15 August 2001

Abstract. Our understanding of how immune responses are generated and regulated drives the design of possible immunotherapies for cancer patients. For that reason, we first describe briefly the actual immunological theories and their common perspectives about cancer vaccine development. Second, we describe cancer vaccines that are able to induce tumor-specific immune responses in cancer patients. However, these responses are not always followed by tumor rejection. At the end of the review, we discuss two possible reasons that might explain this dichotomy of cancer immunology. First, the immune response generated, although detectable, may not be quantitatively sufficient to reject the tumor. Second, the tumor microenvironment may modulate tumor cell susceptibility to the systemic immune response induced by the immunization. Finally, we discuss what, in our opinion, might be the best way to improve cancer vaccine strategies and how the relationship between the tumor and its surroundings might be studied in more details.

Key words. Antigenic tumors; cancer vaccines; immune response; tumor microenvironment; microarrays.

Introduction

The goal of vaccination against tumors is to generate an immune response able to specifically attack the tumor cells without causing autoimmunity. To achieve this, an understanding is required of the mechanism(s) that regulate the fine balance between pathogen-directed immune response versus self-directed autoimmunity. The identification of numerous antigens expressed by different types of tumor has stimulated clinical trials aimed at the vaccination of cancer patients targeting such antigens. Since the majority of tumor antigens are self-molecules [1], this approach would seem unsound to tumor immunologists who initiated this research field 30 years ago, when the self-non-self model of immune recognition was predominantly accepted. According to this model, auto-reactive T cells are completely depleted in the thymus, preventing an immune response directed against self-antigens [2] and, consequently, against most tumor antigens. Since theories about how an immune response is generated and regulated should drive the design of tumor vaccination protocols, we want to briefly describe alternative contemporary models of immune regulation, before addressing different strategies used in cancer immunotherapy and their possible future directions. We briefly describe here three models, focusing on the factors that they point out as necessary for the induction and maintenance of an immune response directed against cancer cells.

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The rules of the game

The localization-dose-time model

Zinkernagel [3] suggested that to be immunogenic, antigens (independently of their self or foreign origin) have to reach the secondary lymphoid organs (spleen or lymph nodes) in minimum doses and for a sufficient period of time. Antigens distributed too rapidly, in excessive amounts, or for too long in the lymphohemopoietic compartments will instead delete all potential reactive T cells. Tumors, other than lymphomas and leukemias, are not immunogenic because they stay in peripheral tissues remaining, therefore, ignored by the immune system. An effective anti-tumor vaccination should bring tumor antigens to the secondary lymphoid organs in appropriate amounts and within a specific time range. Obviously, the difficulty consists in achieving such a fine balance in the dynamics and kinetics of antigen administration. The need for the antigen to reach the lymphoid tissues is dictated by the requirement of a second signal for T cell activation, in addition to the antigen-specific signal delivered through the T cell receptor (TCR) after recognition of a peptide in combination with major histocompatibility complex (MHC) molecules (first signal).

The pattern recognition receptors model

For Medzhitov and Janeway [4], activation of the innate immune response is necessary for the induction of the specific immune response. When cells of the innate immune system recognize through their pattern recognition receptors (PRRs) conserved molecular patterns associated with microbial pathogens, they deliver the second signal or co-stimulation. Thus, the innate immune response will be responsible for the up-regulation of co-stimulatory molecules in the antigen-presenting cells (APCs). These APCs could now trigger the adaptive immune system against the pathogen previously identified by the innate immune system. In this way, the immune system distinguishes between a non-self antigen (the pathogen structure that was recognized by the PRR of the innate system) and a self-antigen, which will not be recognized by PRRs. Therefore, self-antigens will not induce the second signal and will not generate an immune response. An effective anti-tumor vaccination according to this model could be induced only against a tumor antigen for which our innate immune system has PRRs. This model appears as a modification of the self-non-self model and, although it may not directly help in the development of specific cancer vaccines, the suggested cross-talk between innate and adaptive immune systems could bring new insights in cancer vaccine development. It also underscores, as does the localization-dose-time model, the relevance of co-stimulatory signals for the generation of an effective adaptive immune response.

The danger model

Matzinger [5] goes a little bit further. Damaged cells, regardless of what caused their stress or death, trigger the immune response by providing a ‘danger’ signal. The danger model is compatible with the idea that the innate immune system activates the adaptive immune system after encountering certain molecules. However, such molecules do not have to be foreign microbial molecules (as in the PRR model), but they could also be self-molecules released by damaged cells. Death cells release molecules that initiate an immune response by inducing the second signal or co-stimulation in the APCs. This model supports the existence of receptors for endogenous danger signals [6], which include the PRRs, that when triggered up-regulate the second signal in the APCs. Tumors are not immunogenic because they are not perceived as dangerous for the surrounding cells, since in most cases they consist of live, happily proliferating cells. Therefore, they do not induce the second signal necessary for immune response generation. A good cancer vaccine will be one that provides concomitantly the tumor antigen (signal one) and danger signal (signal two) to APCs. Like any cell, tumor cells could liberate danger signals when necrotic death occurs, suggesting that an optimal cancer vaccine may be stressed or dead tumor cells.

Unifying the rules of tumor immunity

The three models attempt to explain in different ways several aspects of various immunological phenomena such as autoimmunity, transplant rejection, and tumor rejection. However, if we focus our attention on the immune response against tumors, two common concepts emerge:
1) The importance of the expression of co-stimulatory molecules on APCs for the generation of an effective immune response. In fact, the difference between the models rests on how co-stimulation is induced and, in our opinion, such differences are more complementary than exclusive.
2) The tumor does not induce co-stimulation on itself and, therefore, is not immunogenic. Since most common tumor cells do not travel to lymphoid tissues, they do not bear microbial elements, and they do not release danger molecules, they will neither generate nor maintain an immune response.
These two statements should be held as keystones when designing clinical protocols involving tumor immunotherapy.

The players

A. Tumor cells

To be recognized by the immune system, tumor cells must bear antigens that can preferentially mark them as targets for the effector response of the immune system.

Therefore, a first step for clinical intervention is the identification of antigens expressed by the tumor cells and, if possible, not expressed by normal cells, in order to avoid possible autoimmune effects of the therapy. In the last 10 years, several tumor antigens have been identified. Since melanoma cells and tumor-infiltrating lymphocytes (TILs) are particularly prone to expansion from excised tumor specimens, the majority of tumor antigens have been identified in the context of this disease. However, many antigens expressed on common epithelial tumors have also been identified. Human cancer antigens have been classified into four major categories [7, 8]: (i) Melanoma-melanocyte differentiation antigens, expressed by both melanoma cells and normal melanocytes but not by other malignancies or normal tissues. Examples are MART-1/Melan-A, gp100/Pmel17, tyrosinase, TRP-1, and TRP-2. (ii) Cancer-testes antigens, found in normal cells in testes and placenta, and in various other tumor types. Examples are MAGE, BAGE, GAGE, and NY-ESO-1. (iii) Mutated antigens, which are expressed by individual patient tumors or even by individual metastases from the same patient. Examples are caspase 8, CDK-4, β-catenin, MUM-1. (iv) Overexpressed antigens, whose expression in tumor cells is higher than in normal cells. Examples are Her-2/neu, MUC-1, α-fetoprotein.

Tumor cells are characterized by extreme genetic instability, which allows loss of various tumor markers including tumor antigens. This, in addition to other factors, may allow escape from immune recognition as we previously described [9].

How did the game develop? Discoveries supporting immunotherapy as a modality of cancer treatment

The concept of tumor immunotherapy first emerged when, in contrast to what was believed previously, neoplastic formations were noted to not always be accepted as 'self' by the host organism [10]. In mouse models, tumors were mainly induced by two methods: chemically, by injection of carcinogenic substances like 3-methylcholanthrene or other aromatic derivatives, and virally, by infection with polyoma, SV40, Rous, Moloney and other viruses. Thus, tumor rejection in pre-immunized animals was considered a consequence of neo-molecule expression on the tumor cells induced by the carcinogens or viruses. In either case, the tumor cells would express new molecules recognized as foreign by the immune system [10]. However, it was not known whether the same immune response could be found in humans. Apart from the few tumors of viral origin, it was unclear whether human tumors might express 'foreign' antigens. Only if they did, they would be susceptible to recognition on the basis of the self-non-self model. Thus, the discovery of marker molecules in human tumors by Gold and Freedman [11] was the first milestone reached. The expression of these molecules was shared by carcinomas of gastrointestinal origin. The identification of a common tumor antigen expressed by several kinds of malignancies, but not by normal cells, made broad-based antigen-specific immunotherapeutics conceivable. In addition, several questions regarding immune surveillance were raised. Did the antigenicity of certain human tumors confer immunogenicity as it did in some mouse models? Could patients with cancer develop immune responses against these tumors? In 1968, Hellstrom et al. [12] observed that lymphocytes and antibodies from patients with various types of tumors had a cytotoxic effect against autologous tumor cells. Paradoxically, this immune response in patients with cancer was not sufficient to induce tumor rejection. The antigens were believed to be of viral origin or a result of somatic mutations, since an immune response directed against self-antigens was not yet conceivable. Another question regarded the kind of cells most likely to exert an effector function against cancer. With improved methods to resolve differences between lymphocytes from natural killer (NK) cells and other immune cells, T lymphocytes were identified as the effector population with the strongest anti-cancer reactivity [13]. The path was opened, now the challenge was to define a strategy to facilitate the in vivo rejection of autologous cancer cells. The most logical strategy appeared to be the ex vivo expansion and adoptive transfer of the patients' own T lymphocytes capable of killing autologous tumor cells in vitro. This strategy was called adoptive immunotherapy.

B. Effector cells against autologous tumors

T cell-based cancer immunotherapies can be categorized according to two main groups. One category is adoptive therapy, which involves transfer of in vitro-generated and/or expanded lymphocytes that specifically recognize the tumor antigens. The other category consists of active therapy or vaccines, in which in vivo-generated or an expanded immune response will react specifically against the tumor antigens.

Adoptive therapy

To treat cancer patients with their own tumor-specific lymphocytes, required expanding the lymphocytes in large numbers. This solution was provided by the addition of interleukin (IL)-2 to T cell cultures. This cytokine acts as a growth factor for lymphocytes by stimulating proliferation and increasing their in vitro cytotoxic potential against autologous tumor cells [14]. IL-2 has been used to in vitro expand TILs from tumor specimens. TILs given to patients with melanoma in combination with systemic administration of IL-2 induced 34% objective responses

[15]. This therapy has the advantage of not requiring the identification of the antigens expressed by the tumor. The population of cells transferred to the patient consists of a heterogeneous mixture of CD4 and CD8 lymphocytes. However, not all patients have accessible lesions of sufficient size to provide an adequate number of T cells for *in vitro* expansion, and tumor-specific TILs can only be obtained from 50% of TIL cultures. For these reasons, after the identification of tumor antigens, two different strategies for adoptive therapy have been under consideration. The first consists of in vitro stimulation of peripheral blood mononuclear cells (PBMCs) from tumor patients with CD8 epitopes present in the tumor antigens [16]. In vitro antigenic stimulation in the presence of IL-2 will generate large numbers of tumor-reactive CD8 cells. The second strategy consists in the transfer of TCR genes from tumor-specific CD8 clones into patient peripheral blood lymphocytes (PBLs) using retroviral vectors [17]. However, none of these therapies seemed to confer additional benefits over the adoptive transfer of TILs in combination with IL-2.

Two important questions regarding adoptive therapy remain unanswered: (i) How long do tumor-specific lymphocytes remain functionally active in vivo after the transfer? (ii) Do the tumor-specific lymphocytes leave the peripheral circulation to reach the tumor site? The addition of molecular markers into transferred T cells helped track their survival in the blood and their trafficking to the tumor, showing that there was a correlation between tumor homing of the transferred TILs and tumor rejection [18]. However, the frequency of transferred T cells dropped quickly in blood days after the administration and only on few occasions did they localize at the tumor.

In contrast to this, in one patient who received adoptive transfer of MART-1-specific CD8 clones in combination with a low dose of IL-2 [19], the transferred specific CD8 cells were reported to persists in the peripheral circulation for as long as 2 weeks, and high numbers of tumor-specific CD8 cells homed to the tumor site. Although not a common finding, this individual case is noteworthy since there was tumor growth stabilization at the same time that high levels of tumor-specific CD8 cells were detected in blood and at the tumor site, suggesting that achieving and maintaining high levels of tumor-specific CD8 cells after adoptive therapy is possible. Individual cases like this one need to be studied carefully to define the differences with respect to other cases in which the number of transferred CD8 cells found were much lower.

According to the immunology models, tumors do not stimulate the immune response. Therefore, lack of persistence of the adoptively transferred lymphocytes can be partially explained. The CD8 lymphocytes transferred in terminal patients with a high tumor burden will kill some malignant cells and, without stimulation, the lymphocytes will die. To keep the anti-tumor response ongoing, specific lymphocytes should be continuously infused until the tumor disappears. In fact, it was only after the third adoptive transfer that the patient studied by Yee et al. [19] showed higher persistence in peripheral blood and homing to the tumor site of the transferred tumor-specific CD8 cells. Alternatively, patients receiving adoptive therapy could receive follow-up vaccinations to stimulate in vivo the transferred tumor-specific CD8 T cells and keep them active against the tumor. Tumor vaccines are the second immunotherapy strategy we are going to discuss here. This approach requires either a tumor specimen from the patient, or knowledge of the antigens expressed by the patient's tumor.

Active therapy or vaccines

CD8 epitopes

The identification of the amino acid sequence of the tumor antigen epitopes presented in association with the HLA class I molecules to the CD8 T cells allowed vaccination of cancer patients with those peptides (CD8 epitopes). The presumption is that the peptides are presented in vivo by APCs to CD8 T cells, inducing a CD8-mediated response against the antigens. When the antigen-specific CD8 cells encounter tumor cells with the same epitope on their HLA, the CD8 cells will react with their anti-tumoral mechanisms. The mechanisms consist the release of cytokines [such as interferon (IFN)-γ, granulocyte/macrophage-colony stimulation factor (GM-CSF), tumor necrosis factor (TNF)-α, IL-2] and of lytic activity through Fas ligand or perforin [20]. This therapy is convenient because it specifically targets the stimulation of CD8 populations and, therefore, simplifies the monitoring of the immune response. Several studies have exemplified the use of different assays to measure the extent of the immune response. Increased antigen-specific CD8 reactivity in patients PBMCs or TILs after peptide vaccination has been described by various groups [21–24]. However, the observed immune responses to vaccination seldom lead to clinical regression of cancer [25]. On the other hand, vaccination combined with systemic administration of cytokines seemed to have synergistic effects, as higher rates of clinical response were noted when vaccination was combined with IL-2 [26] or GM-CSF [27].

Why does peptide vaccination by itself induce clinical responses much less frequently than in association with the systemic administration of cytokines? Again, the three immunological models may suggest an answer. The problem might be that the CD8 epitope is presented to the T cells without co-stimulation. The effect might be similar to what happens when the tumor cells present the epitope: no activation of the immune response. The administration

of IL-2 or GM-CSF might add the co-stimulation signal required for optimal generation of an immune response. Thus, a good vaccine should have two components, the tumor antigen/s directing the specificity of the immune response and a second signal that induces the co-stimulatory properties of the APCs.

Dendritic cells as the brain of the immune system

Dendritic cells (DCs) were originally isolated and characterized by Steinman and Cohn [28]. They are the most potent APCs since they present high levels of MHC class I and II molecules on their cell surface and have the capacity, when activated, to present co-stimulatory molecules required for T cell activation. With the possibility of generating large numbers of mouse [29] and human [30] DCs in vitro, new interesting insights into the role of these cells in tumor immunotherapy were demonstrated. Promising results were noted in mouse models where the DCs were loaded in different ways with tumor antigens such as peptides, whole antigenic protein, antigen RNA, or were transduced with recombinant virus encoding the antigen, or fused with tumor cells. Based on the results of these experiments, several clinical trails have vaccinated patients with autologous DCs loaded with tumor antigens. The first clinical trial with DCs pulsed with peptide and tumor lysate treated 16 patients with melanoma and showed regression of metastases in five patients [31]. In another study, 11 patients were vaccinated with peptide-pulsed DCs and clinical regression was reported for 6 [32]. More recently, vaccination with DCs fused with tumor cells showed complete response in 4 out of 17 renal cell carcinoma patients [33]. The conceptual advantage of the tumor-fused DCs is significant. It loads DCs with all the antigens expressed by the tumor, with the potential capacity of generating an immune response against more than one antigen. This makes immune escape of the tumor by antigen-loss-variants more difficult. Another advantage for both tumor-fused and tumor-lysate loaded DCs is that they might present the tumor antigen in both class I and class II molecules, activating both CD8 and CD4 T cells, which might augment and sustain the immune response more efficiently. Furthermore, these strategies do not require identification of the antigens and epitopes recognized by the CD8 or CD4 T cells. One challenge is to obtain a sufficient number of DCs and tumor cells from the patients. This problem could be potentially bypassed by using allogeneic DCs [33].

The results of these three clinical trials are promising. However, we found in the literature other DC-based vaccines that did not induce as strong an anti-tumoral effect [34, 35]. These controversial results make it difficult to evaluate the real benefits of DC-based vaccines. A deeper knowledge of the different DC subsets and their capacity to generate an immune response is necessary. DCs generated in vitro represent a very heterogeneous population with different characteristics depending upon their maturation status. We still do not know all the consequences of in vitro manipulation on the in vivo immunologic activity of DCs. A review by Gallucci and Matzinger [6] analyzes the endogenous molecules that can activate DCs. DCs become the most efficient generator of an immune response in the presence of pathological cell damage that liberates endogenous danger signals such as heat shock proteins [36] and probably other yet unidentified molecules. Programmed cell death or apoptosis, a physiological cell death that happens during embryo development and throughout life, seems not to have the same stimulatory effects [37]. We apparently do not know enough about DCs to take full advantage of their co-stimulatory capacity in cancer vaccines.

Recombinant viral vaccines: another way to deliver antigen with co-stimulation

Infections by some viruses or bacteria can up-regulate co-stimulation, and thus vaccines incorporating these viruses may provide another type of active therapy. Viral vectors genetically engineered to carry tumor antigens can be injected in tumor-bearing hosts, infecting any kind of cells, and inducing expression of the recombinant tumor antigen. Since viral infections cause necrosis, and travel to secondary lymphoid organs, this strategy is supported by both danger and localization-dose-time models. Since we do not have PRRs for viruses but we do for bacteria, recombinant bacteria vaccines are also supported by the PRR model. In fact, *Listeria monocytogenes* [38] and *Salmonella typhimurium* [39] have been successfully used in mouse models as cancer vaccines. Vaccinia virus [40] was the first recombinant virus to show its capability as a vector for antigenic molecules inducing a specific immune response. Later, DCs, transduced with the entire sequence of a tumor antigen, were demonstrated to generate a specific response against several CD8 epitopes [41] and to stimulate in vitro both CD8- and CD4-specific responses against the same antigen [42]. However, although viral vaccines have been successful in mouse models [43–45], no significant response rate has been achieved in clinical trials [46, 47].

After following the rules we don't score.
Why doesn't the generated specific immune response clear the tumor?

The strategies described above have occasionally been successful in generating an immune response against autologous tumors (table 1). The concept of giving tumor antigens and co-stimulation by DCs at the same time is promising; however, even this strategy has not provided a panacea. Patients with cancer still die in the large majority of cases. Thus, we are still confronting the paradox al-

Table 1. Immune and/or clinical response in patients enrolled in different clinical protocols.

Treatment	Immune response[a]	Clinical response[b]	Reference
None	Y (ex vivo)	N	12
TIL+IL-2	Y[c]	Y	15
MART-1-specific CTL clones	Y (ex vivo)	Y[d]	19
MART-1 CD8 epitope in IFA	Y (in vitro)	N	21
NY-ESO-1 CD8 epitope in 33% DMSO + GM-CSF	Y (in vitro)	N[e]	22
Modified gp100 CD8 epitope in IFA	Y (ex vivo)	N	23
Modified gp100 CD8 epitope in IFA	Y (ex vivo and in vitro)	N	24
MAGE-3 CD8 epitope	N	Y	25
Modified gp100 CD8 epitope in IFA+IL-2	Y (in vitro)	Y	26
MART-1, gp100 and tyrosinase CD8 epitopes + GM-CSF	Y (in vitro)	Y	27
Tumor lysate or peptide-pulsed DCs + KLH	Y (in vitro)	Y	31
MAGE-3 CD8 epitope-pulsed DCs + tetanus toxoid or tuberculin	Y (ex vivo and in vitro)	Y	32
Tumor cell-DC hybrids	Y (ex vivo and in vitro)	Y	33
MAGE-1 CD8 epitope-pulsed DCs	Y (in vitro)	N	48
None	Y (ex vivo)	N	49
MART-1 and modified gp100 CD8 epitope-pulsed DC	Y (in vitro)	N	34
Modified tyrosinase and gp100 CD8 epitope-pulsed DC	N (ex vivo), Y (in vitro)	N	35
Modified gp 100 CD8 epitope in IFA	Y (ex vivo)	N	51

[a] Detected by in vitro and/or ex vivo methods.
[b] In those patients where an immune response was detected.
[c] There was a strong correlation between TIL lytic activity against autologous tumor before the transfer and tumor rejection.
[d] When the highest percentage of tetramer-positive CTLs was found.
[e] Regression of some individual metastases was observed.
CTL, cytotoxic lymphocyte; DMSO, dimethylsulfoxide; IFA, incomplete Freund's adjuvant; KLH, keyhole limpet hemocyanin.

ready described by Hellstrom et al. in 1968 [12]: the coexistence of a tumor-specific immune response and tumor cells in the same patient. There are at least three explanations. First, antigen-specific CD8+ T cells might be unable to leave the peripheral circulation and reach the tumor. Second, vaccine-elicited CD8+ T cells might be functionally inactive at the tumor site. Third, the immune responses elicited by the vaccine might be qualitatively inappropriate or quantitatively insufficient. The first two possibilities are unlikely, as functional vaccine-induced CD8+ T cells have been documented at tumor sites [24; 48]. The third explanation is more likely. Although tumor-reactive T cells have been documented ex vivo in vaccine-naive patients with cancer [49] and in response to vaccination [50], these immune responses might be below a required quantitative threshold for immune rejection of tumors. This possibility has not been addressed, largely due to a lack of direct methods able to assess accurately the magnitude of functional immune responses ex vivo. Recent work has shown that direct ex vivo methods for enumeration of immune responses usually detect vaccine-induced immune reactivity less frequently than methods that include in vitro expansion with arbitrary doses of epitope and immune-proliferative stimuli such as IL-2 [23, 24]. In addition, the heterogeneity of tumor cells accounts for the difficulty in studying cancer patients. The heterogeneity includes variability in HLA and antigen expression by tumor cells, variability in growth rate, different susceptibility to the immune effector mechanisms (such as cytokine secretion and lysis), and different interactions with the environment. In a mouse model where tumor and host variability can be eliminated, we found that there is a correlation between the extent of the systemic immune response generated by a viral vaccine, measured ex vivo, and tumor rejection [unpublished observations]. This work supports the idea that strong immune responses have to be generated to eliminate large tumor masses. This seems also to indicate that the responses detected in cancer patients may not be sufficient to induce tumor rejection. Increasing the frequency or number of vaccinations may generate greater levels of immunization [5]. We have shown [51] how the number of tetramer-positive, as well as IFN-γ-producing cells increased in cancer patients after receiving more boosts of a CD8 epitope vaccine (fig. 1). This highlights out the necessity in clinical trials of quantifying ex vivo the immune response generated after vaccination, while attempting to optimize the best administration schedule for each vaccine.

C. Tumor microenvironment

In humans, strong immune responses seem to be necessary for tumor rejection, but in some cases they may not be sufficient. In addition, mixed responses, in which

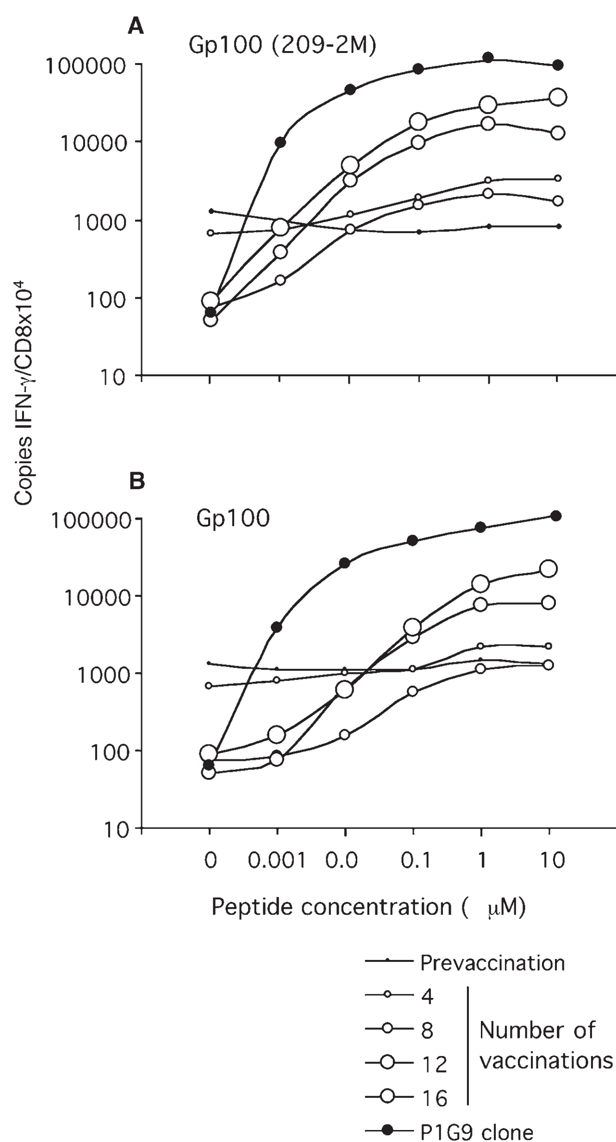

Figure 1. Ex vivo detection of antigen-specific IFN-γ production by PBMCs from a melanoma patient vaccinated 16 times with gp100-modified CD8 epitope in IFA. PBMCs were obtained after 4, 8, 12, and 16 vaccinations, rested overnight in medium without additional cytokines and tested by quantitative real-time PCR for IFN-γ transcript production in response to 2 h of stimulation with increasing concentrations of the modified gp100 CD8 epitope, gp100 (209-2M) (*A*), or the native gp100 CD8 epitope (*B*). Pretreatment PBMCs and CD8 clone P1G9 (specific for gp100 (209-2M)) were tested in the same experiment. Transcript abundance is expressed as the number of IFN-γ mRNA copies/10^4 CD8 mRNA copies.

some of the patient's lesions are rejected while others continue to grow during therapy, are often noted in clinical trials. In those cases, the different clinical outcome of the lesions can probably be attributed to the heterogeneity of the tumor and/or its environment, since the systemic immune response is the constant. A suitable strategy for understanding the different behavior of various lesions in response to immune therapy is to sort out their differences at the molecular level. In an effort to address this question, our group has taken advantage of microarray technology [52]. The ex vivo analysis of tissue samples using cDNA microarray technology, however, has been limited by the amount of RNA necessary for conventional cDNA microarrays. Our group has developed a technique that enriches of limiting amounts of RNA 10^5-fold [53]. The amplified cDNA collection still retains the relative proportions of the original messenger RNA populations and can then be used in cDNA microarrays. Thus, analyzing global gene expression profiles in samples from fine-needle aspirates of tumors during treatment is now feasible [54] (fig. 2). With this strategy, we have been able to study the gene expression profile of several samples, from responding and non-responding lesions, pre- and post-immunotherapy [unpublished observations]. Although the results need further confirmation, surprisingly, we identified main differences in gene expression in pre-treatment samples correlated with the subsequent response or not to immunotherapy. This might indicate that some lesions were predisposed to be responsive targets to immune responses induced by the vaccination. The differences noted were specific for some immune regulatory genes such as TIA-1 (a molecule associated with cytotoxic function) and EBI-3 (a molecule associated with DC maturation). Although interesting, these findings did not explain the reasons for this predisposition.

We hypothesized, therefore, that some tumors might produce factors that may have immune-regulatory activity. In particular, we noted that the tumor microenvironment is capable of expressing a large number of lymphokines, chemokines, growth factors, and metalloproteinases [unpublished observation]. These have strong immune regulatory properties that may influence the extent of immune responses to the point of predisposing some lesions to immune-mediated rejection.

Experiments performed in mice are extremely relevant to understanding the factors that might account for the difficulty of an ongoing immune response to reject a tumor. In mouse tumor models, a dichotomy can be found between immune response and tumor rejection when tumors become 'old' and 'big'. With these terms we refer to tumors that are established for 7 or more days before the mouse is treated with immune regulatory measures. 'Old' refers to the idea that by that time, the tumors may have developed relationships with their environment. This situation contrasts with 'young' tumors, or tumors that receive the hit of the immune response before they have time to develop relationships with their environment. The latter situation is the case of tumor models where the vaccine is given before or in the first days after the tumor challenge. 'Big' refers to the idea that an old tumor mass, at this point, is composed of a large number of cells, in contrast to 'young' tumors, which have fewer cells. Nu-

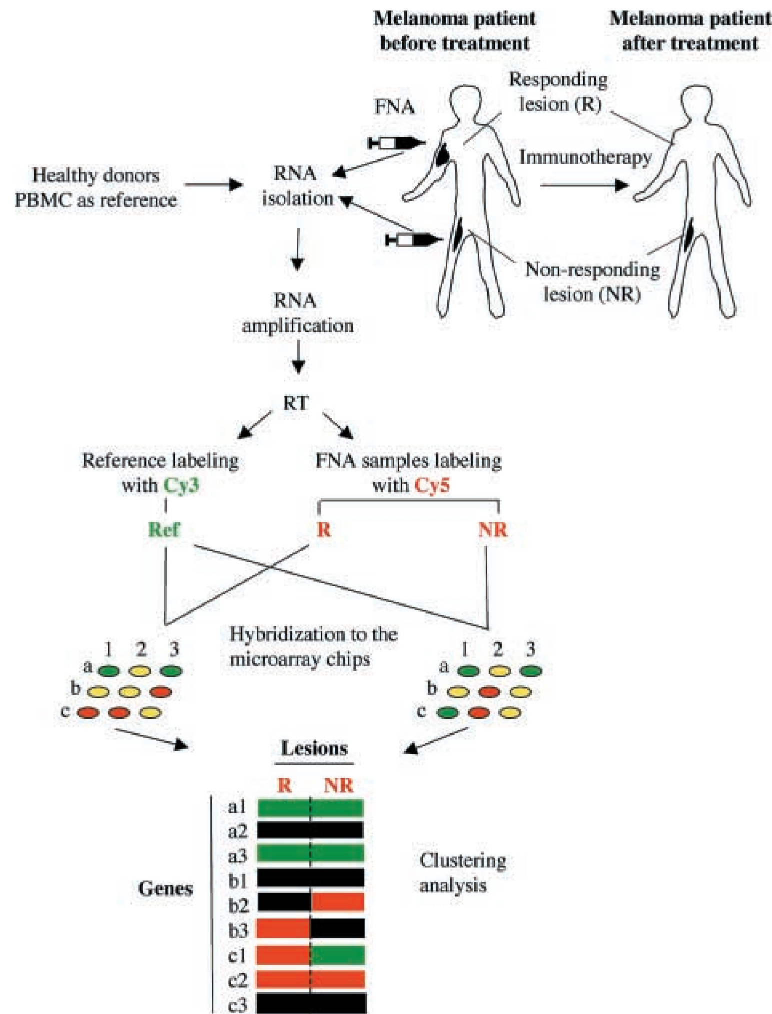

Figure 2. Molecular analysis of lesions from melanoma patients before immunotherapy. Fine needle aspirates (FNA) are taken from melanoma lesions. After RNA isolation and two rounds of RNA amplification, their mRNA is labeled with the fluorochrome Cy5 (red color). In the same way, RNA from PBMCs of healthy donors is amplified and labeled with Cy3 (green color). Samples from responding (R) or non-responding (NR) lesions are hybridized with the reference sample (PBMCs) in a microarray chip. Overexpressed genes in a melanoma sample versus PBMCs will appear red (as gene b3 in R or gene c2 in R and NR lesions). Genes expressed at similar levels in melanoma samples versus PBMCs will appear yellow (as genes a2 or b1 in both R and NR lesions) and, finally, genes expressed at lower levels in melanoma lesions versus PBMCs will appear green (as gene a1 in both R and NR lesions). The relative gene expression levels between the different melanoma lesions (R and NR) are compared in a clustering analysis. In this case, gene b2 is up-regulated in NR versus R lesions and genes b3 and c1 are up-regulated in R versus NR lesions.

merous vaccines in mouse models are good at treating 'young' tumors, in either protection [43, 55] or treatment [56, 57] experiments. However, the same vaccines that are effective against 'young' tumors are unable to reject 'old' tumors [58] even when both tumors are in the same mouse [59]. This excludes the possibility that the large tumor might have induced systemic tolerance or immune suppression. In our opinion, both factors, cell number and tumor environment, may account for the difficulty rejecting 'old/big' tumors in mouse models. Since some of the models discussed have utilized xenogeneic antigens that may be more immunogenic, some of the conclusions might need to be validated by models using naturally expressed tumor antigens. Regarding tumor cell number, the experimental observations suggest that immunotherapy should be preferentially proposed for patients with minimal residual disease or after excision of large tumors. On the other hand, an established relationship between the tumor and its environment may also account for the difficulty in treating solid tumors. In this case, more study is necessary to identify, at the molecular level, the possible factors that 'protect' the tumor from the attack of the immune response generated by the vaccine.

Endothelial cells, fibroblasts, and other normal cells that are part of the environment of an established tumor have been somewhat disregarded by tumor immunologists.

However, two unrelated studies, in our opinion, opened new insights about the possible cross-talk between endothelial cells and the immune system. St Croix et al. [60] observed genes differentially expressed between endothelial cells surrounding normal versus tumor cells. One of the genes overexpressed in endothelial cells from tumor lesions was metalloproteinase-2 (MMP-2 or gelatinase-A). A different group [61] found that MMP-2 has a catalytic domain that cleaves the monocyte chemoattractant protein-3 (MCP-3). MCP-3 function is to attract monocytes [62] and activated lymphocytes [63] at inflammation sites. When MCP-3 is cleaved, it still binds to its receptors but it does not induce the cascade signals. Therefore, it acts more like a chemokine antagonist that dampens inflammation. These findings might point to a role of the tumor environment in camouflaging the danger of the tumor. The endothelial cells are seeing the tumor cells as normal, growing, tissue that does not have to be rejected by the immune response. On this regard it is important to note in which other tissues MMP-2 is expressed. It can be found in the granulation tissue of healing wounds [60] and in human endometrial stromal cells [64], places where we do not need or we want to avoid an immune response.

Concluding remarks

Following the rules established by immunology models, an immune response can be induced in patients against their tumors. However, to improve the effectiveness of vaccines in tumor immunotherapy, we need to increase our understanding of the mechanisms that regulate the effector phase of the immune response, as well as the relationship between the tumor cells and their environment. Recent work from our laboratory [unpublished observations] suggests that some melanoma metastases are predetermined to respond to immunological treatment by secreting a large array of bioactive molecules such as growth factors, angiogenic factors, and metalloproteinases. Several of these factors may have additional immune stimulatory and/or inflammatory properties sufficient to synergise with the immune response generated by adoptive or active immunotherapy. More detailed study of the gene expression profile in samples that, under the same systemic immune response, behave differently with respect to rejection, may give important information about possible future adjuvants for the actual tumor vaccines.

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