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Tumor vaccines and beyond

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Abstract

For the last two decades the immunotherapy of patients with solid and hematopoietic tumors has met with variable success. We have reviewed the field of tumor vaccines to examine what has worked and what has not, why this has been the case, how the anti-tumor responses were examined, and how we can make tumor immunity successful for the majority of individuals rather than for the exceptional patients who currently show successful immune responses against their tumors.

Keywords

cancer vaccines; gene therapy; genes; stem cell transplantation; T-cell receptor

Introduction

To combat invading pathogens the adaptive immune system mobilizes antigen-presenting cells (APC), $CD4^+$ and $CD8^+$ T cells to attack the intruder, and B cells to make antibodies. Tumors developing within the body pose a different challenge because they are usually slowly developing (1) and originate from ‘self’. Tumors arise as a consequence of acquired and inherited genetic aberrations in oncogenes and tumor suppressor genes, resulting in uncontrolled growth of what is often considered a clonal initiator population (2–4) that accumulates a concatenation of further genetic lesions during the progression from benign to malignant (5). Loss of genetic and genomic control appears to be responsible for at least some of the process of tumorigenesis (6,7). There is ample evidence that the immune system plays a prominent role in preventing tumor development: patients with immune deficiencies such as human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) have tumor types such as Kaposi sarcoma (8,9) and other malignancies (10,11) that rarely occur in healthy individuals. Tumor phenotypes are sculpted by the immune system, leading to escape from immune surveillance (12). In allogeneic stem cell transplants where leukemia patients receive T cells from human leukocyte antigen (HLA) haplotype-mismatched donors, the recipient’s leukemia may down-regulate the mismatched major histocompatibility antigen (MHC) molecules (13). A similar phenomenon has been described in a melanoma patient who developed melanoma subclones resistant to lysis by *in*

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vivo emergent autologous anti-melanoma $CD8^+$ T-cell clones (12). Sometimes patients may possess pre-existing anti-tumor immunity that is ineffective but can be amplified by T-cell growth factors (12,14). Clearly immune control of malignancies is not perfect, and various strategies are being explored to boost immunity. Vaccination of the tumor-bearing host with tumor-associated antigen (TAA)-derived peptides is one such strategy. Another approach is to target polymorphic minor histocompatibility (mH) antigens that are differentially expressed by the tumor (see below). Such antigens, originally described in the allogeneic, HLA-identical transplant setting, can exhibit both broad and restricted tissue expression, and are usually targeted by T cells specific for the polymorphic epitope. As mH-specific T cells are likely to have a high avidity to the antigen, they may represent a more powerful alternative to monomorphic tumor antigens. Excellent reviews list the large repertoire of TAA and mH antigens now identified (15–21).

We have focused on the progress made in vaccine therapies, strategies to monitor T-cell responses against the vaccine and the tumor, and the prospects for exploiting the knowledge gained from detailed studies on anti-tumor immunity to improve the efficiency of tumor vaccine therapy in the management of solid and hematopoietic malignancies.

Tumor and mH antigens

Researchers have identified a large array of tumor antigens targeted by autologous $CD8^+$ T cells derived from the peripheral blood or tumor-infiltrating lymphocytes (TIL). These epitopes can be categorized broadly into antigens that are: (a) selectively or uniquely expressed in the tumor; (b) derived from tumor-specific mutations; (c) differentiation antigens; and (d) antigens overexpressed in the tumor (20,22). We and others have shown that proteins overexpressed in myeloid malignancies, such as proteinase 3 (PRTN3), neutrophil elastase (ELA2) and Wilms tumor-1 (WT1), are targeted by T cells. The primary granule proteins human ELA2 and PRTN3 are serine proteases expressed in normal myeloid cells but overexpressed in myeloid malignancies (23,24). Both contain a nonamer (VLQELNVTV) that is presented in the context of HLA-A*0201 (23,25). Leukemic cells presenting this epitope are effectively lysed by $CD8^+$ T cells, while healthy myeloid cells are not (23–26). Under certain circumstances, such as interferon (IFN) therapy (27) and allogeneic hematopoietic stem cell transplantation (HSCT) (27,28), increased frequencies of PRTN3/ELA2-derived peptide antigen (PR1)-specific $CD8^+$ T cells have been detected in these patients. We recently demonstrated that such T cells reside predominantly in the bone marrow, and that they recognize their target antigen with high avidity (28), suggesting a contribution of PR1-specific $CD8^+$ T cells to the eradication of myeloid leukemia cells following allogeneic HSCT. Using a platform of HLA tetramers that allowed differential detection of high- and low-avidity PR1-specific $CD8^+$ T cells, we demonstrated persistence of low-avidity and loss of high-avidity TAA-specific $CD8^+$ T cells in the bone marrow prior to transplant (29), consistent with *in vitro* findings by Molldrem *et al.* (30) that suggested that leukemic cells evade immune surveillance by specifically depleting T cells with high avidity for the leukemia antigen.

WT1 is a zinc finger transcription factor overexpressed in various malignancies, including leukemias (31–33). WT1 fulfills an important criterion in immunotherapy: appearing to be

essential for pathogenesis (34–36). Nevertheless the picture is not entirely clear, as WT1 mutations occur in approximately 10% of leukemias (37–39) and are associated with poor clinical prognosis (37,39–41), suggesting that wild-type WT1 might in some circumstances regulate tumor progression. Patients with WT1 mutations have significantly higher WT1 gene expression levels (42), suggesting that these patients might be especially suitable for immunotherapy using WT1-specific T cells.

Minor histocompatibility antigens were first discovered in patients who developed severe graft-versus-host-disease (GvHD) after HSCT despite a full molecular match at the MHC class I and II loci, and thus the term mH antigen was used. An mH antigen arises from a genetic difference between donor and recipient that results in the presentation of an antigenic peptide (43). Many such HLA class I- and II-restricted mH antigens have been discovered over the past 15 years, and some display tissue-restricted expression, such as HA-1(44) and −2 (45), HB-1 (46), LRH-1 (47), CD19 (48) and the more recently characterized mH antigen HEATR (49). Obviously the strengths of these antigens lie in the high avidity with which the T cells target such antigens. Until recently, mH antigens were identified in the allogeneic HSCT setting in patients who mounted an anti-leukemia response in the absence of GvHD. However, the Riddell laboratory induced and selected T-cell responses against mH antigens with hematopoiesis-restricted expression for adoptive transfer prior to the transplant (49). Although this strategy is likely to target unidentified, useful mH antigens, some characteristics of these antigens have discouraged researchers from exploiting their use in immunotherapy. First, mH antigen responses can only be elicited if the donor and patient are discordant for such polymorphisms. Thus, even if the donor and patient express a highly prevalent HLA allele, for example HLA-B*0801, and the population frequency of the mH antigen is *c.* 50%, this relatively favorable mH antigen can elicit a response in only in 5.5% of donor–patient pairs expressing this HLA allele. Second, it is unclear whether such a response is uni- or bidirectional, i.e. whether the alternative allele can also yield a peptide that is presented in the same HLA allele and may be seen by T cells in the other direction. From studies on HA-1 is has become clear that only the product from one locus is presented in HLA (44), possibly because of unfavorable binding characteristics of the non-immunogenic variant (50). Third, none of the thus far characterized mH antigens are presented in the context of HLA class I as well as class II (21), which may negatively affect the long-term persistence of CD8 T-cell responses in the absence of a CD4 component (see below). Thus, despite the high likelihood that mH antigen responses will elicit a higher quality T-cell response than the monomorphic TAA, many more mH antigens will have to be characterized to allow their targeting in a vaccine setting.

A third type of tumor antigen was recently reported by Childs *et al.* (51), where they identified an endogenous retroviral envelope-derived epitope as the target of CD8 T cells in clear cell renal cell carcinoma (RCC). About 8% of the human genome consists of retroviral elements, most of which are no longer transcriptionally or translationally active (52). In RCC, however, the human endogenous retrovirus (HERV) E is reactivated and thereby yields HLA class I-presented epitopes that are new to the immune system and targeted with high efficiency (51). These data suggest that HERV can be reactivated in some tumors and targeted by the immune system. What is unclear at this moment is whether such

HERV-specific T-cell responses can be elicited in the autologous T-cell pool of the patient via vaccination, and whether epitopes are presented in HLA class II.

The significance of CD4+ T cells

WT1 (and other TAA) has been targeted by various investigators using a short synthetic HLA class I binding peptide vaccine to treat patients with leukemias (53–55). However, it is likely that such responses are not maintained in the absence of a $CD4^+$ T-cell response, and that the stimulation of $CD8^+$ T cells in the absence of helper cell engagement results in a functionally defective population often referred to as 'helpless T cells' (56–61). $CD4^+$ T cells, on the other hand, can do more than just provide help: numerous studies have demonstrated their cytotoxic potential (62–70). Additionally, it has been shown that $CD4^+$ T cells can be directly cytotoxic but also amplify a $CD8^+$ T-cell response in the patient (71). Thus, aside from the fact that $CD4^+$ T cells can regulate their own proliferation and that of $CD8^+$ T cells by autocrine stimulation via interleukin (IL)-2 production, these cells can also be very effective at eradicating target cells. Clinical evidence for a role for allogeneic $CD4^+$ T cells in tumor eradication comes from the HSCT setting, where $CD4^+$ T-cell infusions have led to achievement of full remission in a number of clinical trials (72,73).

Immunotherapy of cancer

The concept of immune therapy of cancer is well established (reviewed in 74,75). Various immunotherapy strategies have been developed: monotherapies with cytokines, antibodies, autologous and allogeneic tumor vaccines; peptides, proteins, DNA, RNA and antigen-loaded APC; adoptively transferred lymphokine-activated killer cells, T cells and natural killer (NK) cells; allogeneic HSCT with or without delayed add-back of donor lymphocytes; any combination of the above. The molecular identification of antigens recognized by autologous tumor-specific $CD8^+$ T cells since the early 1990s (19,20) has sparked an interest in using such peptides to more specifically direct the immune response against such antigens and to boost such responses *in vivo* or *in vitro*. In some studies dendritic cells (DC) have been pulsed with the antigenic peptide prior to administration to the patient, whereas in other studies a viral vector has been used to deliver the epitope to APC in the patient. A low percentage of melanoma patients vaccinated with these CD8 epitopes demonstrated tumor regression, and this regression appeared to correlate with frequencies of anti-TAA $CD8^+$ T-cell responses (76). In the course of these studies it became clear that an effective $CD8^+$ T-cell response against the tumor using peptide antigens would require help from $CD4^+$ T cells or, as an alternative, the administration of IL-2. However, the lack of CD4 help cannot be used as the sole argument for why tumor immunotherapy fails in patients, because there are a multitude of explanations. Seven are given below.

a. Tumors are hierarchically organized and originate from a dormant pool of tumor stem cells (77–79) that may give rise to rapidly cycling progeny (reviewed in 80). It has been demonstrated that such quiescent tumor cells are resistant to T-cell attack. Lymphocyte infusion from an HLA-identical donor into leukemia patients post-HSCT has been used as immunotherapy of leukemia for two decades (81,82). In one study, however, it was found that while donor T cells

isolated from the bone marrow of patients post-donor lymphocyte infusion recognized mature myeloid cells well, immature hematopoietic progenitor cells were resistant to lysis in the assays employed (83), suggesting that quiescent hematopoietic progenitor cells are not recognized by these T-cell clones. This suggests that additional strategies may be needed to revive the quiescent tumor, for example DNA demethylating agents or type I interferon (84). Alternatively, the establishment of a memory anti-tumor T-cell pool would allow the pruning of cycling tumor progeny by such cells, effectively containing the tumor within its small stem cell pool and preventing relapse.

b. Vaccine induction of suppressor T cells (85–90): Francois *et al.* (87) vaccinated melanoma patients with MAGE-A3 HLA-DP4-binding peptides and found that a significant proportion of clonally expanded antigen-specific CD4 T-cell clones suppressed the proliferation of MAGE-A3-specific $CD4^+$ T-cell clones. Recently, Jandus *et al.* (88) identified FOXP3-expressing $CD4^+$ T cells following vaccination of patients with a Melan-A vaccine using HLA-DQ tetramers, suggesting that the vaccine induced both effector and regulatory T cells. Increases in TAA-specific regulatory T cells have also been described in other settings (89). Bonertz *et al.* (90) analyzed the antigen specificity of some tumor-infiltrating regulatory T cells in patients with colorectal cancer. By priming regulatory T cells with synthetic peptides and incubating these cells with polyclonally stimulated control cells, they demonstrated that regulatory T cells primed with some tumor antigens inhibited the proliferation of non-specifically activated regulatory T-cell depleted peripheral blood mononuclear cells (PBMC). The authors showed furthermore that the regulatory T cells recognized fewer antigens than the effector T cells. Given that regulatory T cells suppress a T-cell response non-specifically, the data suggest that, even though the tumor microenvironment contains regulatory T cells with fewer antigen specificities, they can still suppress effector T cells via a bystander mechanism.

c. Rather than eradicating the tumor, TAA-specific T cells may instead support tumor growth via the production of cytokines (65,91). The T-cell clones recognize the tumor but instead of destroying tumor cells they provide help to the malignancy (91).

d. It has been shown that T cells infiltrating the tumor microenvironment are functionally inert or anergic, a state that can be reversed by the administration of IL-2 (92–94).

e. The tumor may express indoleamine 2,3-dioxygenase (IDO), which catabolizes tryptophan along the kynurenine pathway. IDO is expressed constitutively by certain tumors or induced by IFN-γ (95,96) or reverse signaling through cytotoxic T-lymphocyte antigen-4 (CTLA-4) and CD40 ligands (97,98). T-cell proliferation is inhibited by IDO-expressing cells (99) via either depletion of local tryptophan levels (100) or the apoptosis-inducing effect of its metabolites (101). Clinical evidence for the relevance of IDO-expressing DC comes from melanoma, where the presence of IDO-expressing DC in tumor draining lymph

nodes is associated with a poor clinical outcome (102), thereby effectively depleting an essential amino acid from the milieu and suppressing a T-cell response by amino acid starvation. IDO has been demonstrated to be critical for maintenance of maternal tolerance to the fetus (103) and inhibiting anti-tumor responses (104). Furthermore, IDO gene and protein expression as well as functional activity occurs in acute myeloid leukemia (105).

f. A variety of myeloid cells has been identified in the tumor environment and thought to play a role in tumorigenesis (106). One prominent yet heterogeneous group of myeloid cells is the myeloid-derived suppressor cells (MDSC), which have been detected in patients with various malignancies but not found in healthy donors (107). MDSC are the progeny of bone marrow-resident progenitor cells and express CD11b, CD33, CD34 and CD15 (106,108), have low or no expression of HLA-DR (109) and in some instances have been reported to express CD14 (110,111) and intracellular arginase I (112–114). MDSC have been found in patients with tumors such as RCC (109,112–114), prostate cancer (115), hepatocellular carcinoma (110), colon carcinoma (116), non-small cell lung cancer (117) and melanoma (107,116,118), all with slightly distinct phenotypes but similar functions, i.e. the suppression of T cells (118) and NK cells (111). How these cells suppress immune responses has not been fully elucidated, but may involve the secretion of transforming growth factor (TGF)-β (107) and arginase (110) by MDSC, which suppresses proliferation (113,116,118), cytokine production (113,118), CD3ζ chain expression level (117) and direct effector functions of lymphocytes (111,118).

g. T cells responding to antigen stimulation will do so via the ligation of their antigen receptor with peptide-major histocompatibility complex (pMHC) complexes, and CD28 with CD80/CD86 on the target cells. However, during the response negative regulatory mechanisms are activated that significantly dampen the response, which include the alternative CD80/CD86 ligand CTLA-4 (119) and the PD-L1/PD-L2 ligand PD-1 (120). Some tumors (121–125) are known to overexpress these ligands, which hampers the success of tumor immunity. It therefore makes good sense to target the various inhibitory pathways exploited by malignancies using pharmacologic inhibitors (126), or blocking antibodies (127) in combination with immunotherapy (114,128–130).

Intriguingly, despite the many failed attempts to control the tumor via vaccine therapies, some patients do respond to this treatment by mounting either a T-cell response against the vaccine or against the antigens expressed by the tumor. Careful observational studies such as those reported by Coulie,Van der Bruggen (20) and others at the Ludwig Institute for Cancer Research (LICR; Brussels, Belgium) may help to extend successful anti-tumor immunity to patients who are currently unresponsive. Furthermore, the implementation of large-scale mH antigen discovery technologies (131) will greatly expand the repertoire of such antigens for vaccination and other forms of immunotherapies. Central to improving the efficacy of immunotherapy is the implementation of immune and tumor monitoring as described below.

Monitoring anti-tumor responses: how and when

Depending on the frequencies of responder cells, availability of reagents and antigen used to stimulate the responder T cells, prior *in vitro* amplification of the anti-tumor response may be necessary to allow the characterization of such responses. This has been the strategy used most often by laboratories such as the LICR. The LICR has mainly generated T-cell clones from peripheral blood or from metastases and tested them for tumor reactivity in classic cytotoxicT lymphocyte (CTL) assays and by cytokine production (132,133), or by culture of PBMC with peptide under limiting dilution conditions, followed by tetramer-guided sorting of clones (134–136). This approach gives a frequency of anti-TAA responses based on very limited $CD8^+$ or $CD4^+$ T-cell numbers capable of sustained *in vitro* proliferation and may not be truly representative of the *in vivo* situation. However, the advantage of this methodology is that it yields T-cell clones that can be examined in greater detail than otherwise possible, for example the analysis of antigen recognition, functional avidity and composition of the T-cell receptor (TCR); recognition of tumor cells from the same or different types; design of clonotype-specific polymerase chain reaction (PCR) primers for the *ex vivo* analysis of localization and frequency prior to and following immunotherapy; correlations with effective versus failing immune control; behavior in tumor metastases, etc.

The peripheral blood is most often sampled to examine the anti-tumor response; however, tumor-reactive T cells may accumulate at or near to the tumor site. Our studies have demonstrated that in patients following allogeneic HSCT from their related HLA-matched donors, anti-TAA $CD8^+$ T-cell responses can be identified almost exclusively in the bone marrow and the site of the malignancy (28), suggesting that the monitoring of an anti-tumor response may miss the anti-tumor response if the wrong compartment is sampled. Similarly higher frequencies of anti-tumor T cells were found within the melanoma by Mazzocchi *et al.* (137) and Carrasco *et al.* (133). Carrasco *et al.* (133) identified anti- melanoma antigen (MAGE) $CD4^+$ and $CD8^+$ T cells in the peripheral blood and slightly enriched in slowly progressing metastases. The most interesting finding was that a T-cell response against a non-vaccine antigen, MAGE C2, was detected following vaccination and enriched by over three logs in metastases relative to blood, suggesting that effective anti-tumor responses preferentially localized to the tumor. Mazzocchi *et al.* (137) similarly demonstrated a higher preponderance of anti-tumor T cells at the site of the tumor than in the peripheral blood. This phenomenon of determinant spreading, possibly mediated via the generation of an inflammatory environment by some tumor-reactive T cells, which induces apoptosis of tumor cells followed by cross-presentation of tumor-derived antigens, has also been observed in other vaccine settings (138–140) and may be an important mechanism of vaccine-associated anti-tumor immunity. Thus the question why the same vaccine works in some patients and not in others could be related to whether or not the patient can mount a T-cell response to antigens presented by the same tumor (12). Therefore, in the monitoring of vaccine therapies, ideally the response of a patient's T cells against the vaccine but also the tumor itself should be analyzed.

In situations where the frequency of tumor-reactive T cells is higher (above 0.1%), other methodologies for direct *ex vivo* analysis that are less time consuming and more informative can be employed. The days where we would enumerate antigen-specific helper

and cytotoxic precursor frequencies using limiting dilution assays have largely been replaced by polychromatic flow cytometry assays that quantify the frequencies, phenotypes and an array of functions of antigen-responsive T cells (28,141). With the introduction of soluble HLA class I (142) and class II (143) multimers, antigen-specific T cells can be examined directly and isolated for further analyzes, for example molecular analysis of the T-cell repertoire (144–146). Obviously, the flow cytometry-based functional and phenotypic characterization of anti-tumor responses is preferable above other methodologies for the *ex vivo* monitoring of pre-existing anti-tumor responses and the effect vaccination and HSCT have on the quality and quantity of anti-TAA and virus responses (28,147), but the molecular characterization of TAA-specific $CD4^+$ and $CD8^+$ T cells could bring us one step closer to a better understanding of T-cell clones that may have contributed to TAA recognition (88,148–150) and tumor eradication. An example where clonotype analysis has provided insight into features of protective immunity has recently been uncovered in a non-human primate model of human HIV infection, where the expansion of $CD8^+$ T cells with a particular TCR-β sequence appeared to correlate with protection; these findings where then confirmed in a vaccine setting in the same model (151).

How can we generalize the success of tumor vaccine therapy?

Currently in any immunotherapy study only a few patients show a response. However, detailed study of responders may open the way to generalizing treatment strategies to make them effective for the majority of patients. Coulie *et al.* (12) described three patients vaccinated with autologous melanoma clones who had unusually favorable clinical courses that may have been the result of either the antigenicity of their tumors, their susceptibility to lysis by their autologous T-cell clones, or specifics of the responder population that made their efficacy so high. It seems unlikely that the antigenicity could have explained the favorable clinical progression, as the antigens identified in these three patients were expressed by a large number of melanomas. Rather, the immune response seems to have displayed qualities that allowed the eradication of initial and subsequent melanoma clones: all three patients had CTL targeting at least five distinct antigens. Thus the molecular characterization of the TCR of these cells could hint at a strategy that might provide a novel treatment modality in other patients harboring melanomas with similar HLA constitution but less efficient induction of a T-cell response against their autologous tumors. Rather than having to rely on their immune efficacy, one could simply target their malignancies with a multitude of distinct TCR.

To exploit fully the success of immunotherapy, mH antigen and TAA-specific T-cell clones will have to be generated to identify the TCR α- and β-chains and, via gene transfer studies, define their role in tumor eradication (see below). Antigen-specific T cells can be isolated from peripheral blood, bone marrow, tumors and other compartments by electronic sorting of T cells with defined specificities using HLA class I or II tetramers or, if the antigen is unknown, via the isolation of T cells that up-regulate CD40 ligand (152,153), CD107a (154) and CD137 (155) upon antigen recognition. Such cells can be sorted as lines and expanded by one stimulation with the selecting antigen source. The T-cell lines could then be examined for clonotype composition, and cloned to allow detailed functional analyzes and characterization of the TCRαβ pairs per clone. Clonotype analysis may identify T-

cell clones with potential clinical relevance. With the rapid improvements in the ectopic expression of TCR (156–161), this antigen specificity can be transferred to non-expressing cells, thereby conferring the therapeutic potential of the original T-cell clone onto new cells (162,163). Pre-clinical studies in animal models could help identify TCRαβ that could control and eradicate human tumor tissue. Such studies could help select TCR with the greatest potential, which could subsequently be used in phase I trials.

Conclusion

Studies on the interaction between tumors and the immune system have led to the identification of many TAA in the autologous setting and mH antigens in the HLA-matched sibling transplant settings. These studies have not only proved that various classes of tumor antigens are targeted by the immune system, but also that tumors escape by changing their phenotype (clonal evolution) and modifying the tumor environment. The molecular characterization of TAA brought the promise that antigens could be used in vaccine trials to boost an existing immune response to the malignancy. Tumor immunotherapy still has far to go to be universally successful, but we are making progress. Vaccines have great appeal in their relative simplicity; however, this approach depends mainly on how well a patient can mount an affective immune response to the vaccine or other tumor antigens. Through detailed immunologic studies we can define the criteria for a successful T-cell response. TCR gene transfer now provides the opportunity to exploit these molecularly characterized successful anti-tumor T-cell clones in the treatment of other patients.

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