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Active Antigen-specific Immunotherapy of Melanoma: from Basic Science to Clinical Investigation

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Abstract. Advanced-stage melanoma here dismal prognosis, and novel therapeutic approaches are urgently required. The possibility of taking advantage of the immune response of patients for its treatment has been an appealing concept for almost a century. Only during the last decade, however, has the molecular identification of tumor-associated antigens (TAAs) offered the possibility of vaccinating patients (e.g., active induction of TAA-specific immune responses). Active antigen-specific immunotherapy (AASIT) is currently being investigated in a number of clinical centers as a treatment option for advanced-stage melanoma. A large number of melanoma TAAs have been molecularly characterized and are being used in vaccination trials in various molecular forms and according to various immunization protocols. Here we provide a short overview on melanoma TAAs, the technologies currently in use to induce specific cytotoxic T-lymphocyte (CTL) responses in vivo, and their monitoring. We also propose a tentative AASIT agenda for the next few years, aiming at improving the capacity to induce and monitor TAA-specific immune responses and to verify their clinical effectiveness.

Melanoma is of peculiar fascination for tumor immunologists, largely because of the reported occurrence of spontaneous regressions and the association with autoimmune phenomena (vitiligo) in cases with relatively good prognosis. Furthermore, primary melanoma cell lines, easily generated from surgically excised specimens, provide reagents that allow cellular immunology studies in vitro using tumor cells and lymphocytes from the same patient. A search in public literature databases yields more than 80 entries with the keywords "melanoma and vaccinations", limited to clinical trials. The frequency of these trials is clearly increasing, albeit with partially different trends, depending on the immunization procedure (Fig. 1).

A turning point in tumor immunology occurred in 1991 with the description of the first human, HLA-restricted, tumor-associated antigen (TAA) [1]. Subsequently, the identification of a large series of TAAs [2] and the concomitant enormous increase of knowledge in basic immunology have generated high hopes regarding active antigen-specific immunotherapy (AASIT) of cancer.

Here we review basic concepts underlying clinical immunotherapy and focus on the immunization and monitoring techniques currently used in AASIT. Finally, we try to identify basic and clinical research areas where major efforts are urgently required to strengthen the AASIT rationales and to verify its clinical effectiveness.

Human Tumor-associated Antigens

The single major obstacle to application of the advances in fundamental immunology to cancer treatment has historically been the absence of suitable molecularly characterized antigens. Immunotherapists have thus been forced to use undefined "vaccines" derived from tumor cell lines or tissues or their corresponding lysates. Thus molecular identification of the first human TAAs in 1991 can righteously be considered a milestone in tumor immunology [1].

The TAAs recognized by CD8 + cytotoxic T lymphocytes (CTLs) or CD4 + T cells were originally identified by taking advantage of a number of methods [2]. For melanoma, they can be classified into at least three groups (Table 1). Differentiation antigens are typically expressed by tumor cells and by melanocytes, albeit at a lower level. Synthetic peptides deriving from these proteins, frequently involved in melanogenesis, are among the most used in the AASIT of melanoma [2–4].

Cancer/testis TAAs (C/T TAAs) are usually expressed only in spermatogonia and in tumor cells of different histologic origin. Their peculiar relevance is due to their relatively high tumor specificity. Indeed, spermatogonia do not express HLA class I, the restriction molecules for antigen recognition by CTL [1,5–7]. "Real" TAAs derive from gene products resulting from genetic alterations detectable only in tumor cells. They may include mutations, translocations, differential splicing, or the transcription and translation of intron sequences. Such TAAs are important from an immunobiological viewpoint. However, they are of limited clinical relevance in as much as their

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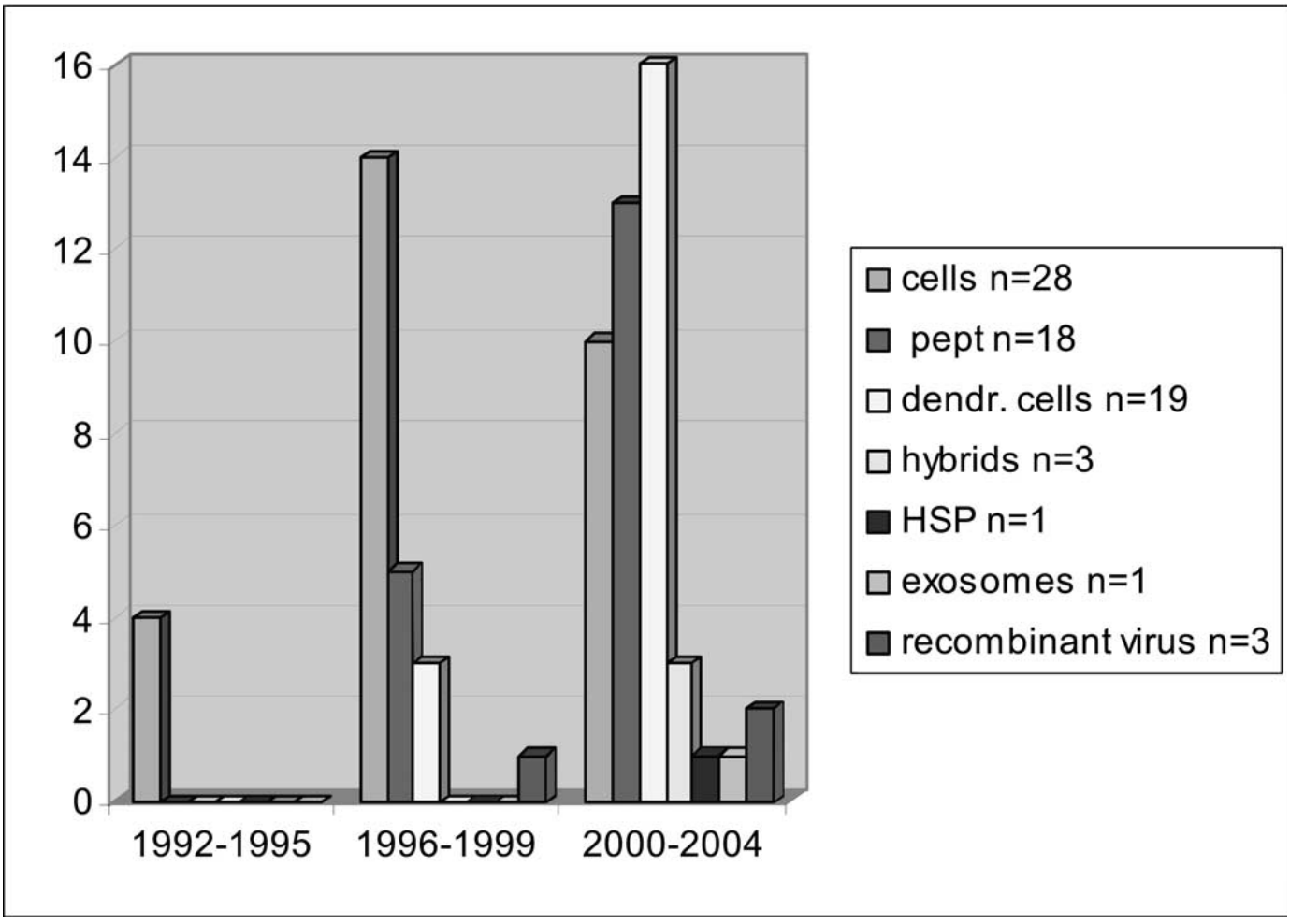

Fig. 1. Overview of clinical trials regarding metastatic melanoma 1992–2004. The data refer to published clinical trials reported in the PUBMED public literature database under the heading "melanoma and vaccination" with the "clinical trial" limit. Trials taking advantage of recombinant virus were added. Note that data from individual trials were at times reported in more than one work, so the number of trials reported here is lower than the number of entries in PUBMED. Trials taking advantage of undefined antigens (e.g., tumor cells, cellular hybrids, heat shock proteins, exosomes) are outside the scope of this review.

expression is frequently limited to tumor cells from individual patients [8, 9]. In other tumors, but not in melanoma, antigens derived from viruses potentially involved in the oncogenic process represent an additional TAA group of rising importance [10].

Thus, melanoma TAAs used in clinical AASIT most frequently derive from proteins also expressed in some type of nontumoral cells. This implies that the immune system is likely to have developed some degree of tolerance toward them. Recent experience suggests that this tolerance can be overcome by taking advantage of appropriate immunization procedures. However, this represents a major difference if one compares AASIT of cancer to conventional preventive vaccinations targeting infectious agents.

Vaccination Protocols

Having characterized the TAAs does not automatically imply the capacity to generate effective immune responses following vaccination. Actually, all vaccines used in the prevention of infectious diseases, with the sole exception of bacille Calmette-Guérin (BCG), are effective in that they induce the generation of humoral antibody responses [11]. These responses are unfit to target human TAAs which are mostly expressed only intracellularly. Notably, the generation of HLA class I restricted CTL responses represents a major challenge also in preventive vaccinations against a number of infectious diseases, such as hepatitis C [12].

In addition, the adjuvant most frequently included in commercial vaccine preparations, alum, is excellent for promoting antigen-specific antibody responses but is unable to support CTL generation [13]. Indeed, the identification and functional characterization of novel adjuvant formulations capable of enhancing CTL induction represents the main research focus of many academic centers and research and development departments of companies [14]. In this context, clinical AASTI has played an ice-breaking role in the promoting investigations on CTL generation.

Table 1. Overview of melanoma-associated antigens.

Tumor-associated antigens in melanoma	Expression pattern	Immunogenicity	Selected references
Differentiation antigens: gp100, Melan-A/MART-1, tyrosinase, TRP-2, among others	Tumor cells and nontransformed melanocytes/pigmented cells: highly frequent expression in melanoma	Capacity to induce CTL and CD4+ T-cell responses	2–5
Cancer/testis tumor-associated antigens: MAGE, NY-ESO-1, among others	Spermatogonia, placenta, and tumor cells of different histologic origin: expressed in 10–50% of melanomas	Capacity to induce CTL and CD4+ T-cell responses	1, 2, 5–7
Tumor-specific antigens derived from mutated genes or sequences not transcribed under physiologic conditions	Limited to tumor cells of individual patients	Capacity to induce CTL and CD4+ T-cell responses	8, 9

CTL: cytotoxic T lymphocyte.

Table 2. Overview of immunogens and treatment procedures most frequently used for active antigen specific immunotherapy of melanoma.

Immunogens/treatment procedures	Advantages	Disadvantages	Selected references
Peptides with or without cytokines	Inexpensive, easy to produce GMP	Low immunogenicity, cytokine-related side effects	15–19
Peptides with adjuvants	In expensive, easy to produce GMP	Few licensed adjuvants capable of inducing CTLs, side effects related to adjuvant administration	20, 21
Dendritic cells loaded with peptides	Highly immunogenic	Difficult standardization, requirement for preculture of patient's own cells	22–27
Recombinant virus	Highly immunogenic	Safety concerns, expensive GMP production, vector-specific immune responses	28–30
Adoptive immunotherapies	Possible administration of high numbers of specific CTLs	Long-term culture of lymphocytes required	31, 32
Adoptive immunotherapies with pharmacological immunodepletion	Possible administration of high numbers of specific CTLs with prolonged survival	Side effects related to immunodepletion and autoimmunity after CTL administration	33

GMP: good manufacturing practice

Many immunization approaches targeting molecularly defined antigens (Table 2) have been employed in clinical trials on metastatic melanoma. A common starting point is that antigens recognized by CTLs, be they TAAs or viral antigens, are usually produced inside the cells, with their peptidic fragments (epitopes) expressed on cell surfaces inside HLA class I molecules' grooves. Thus mimicking physiologic pathways would recommend, for instance, the use of virus recombinant for TAAs, capable of infecting antigen-presenting cells (APCs). Alternatively, APCs might be exogenously loaded with synthetic epitopes (peptides), binding the small percentage of HLA class I molecules present on cell surfaces in an empty state. Most immunization procedures used for CTL generation in AASTI are based on these basic concepts. Peptides have been injected directly, in the presence or absence of adjuvants or supportive cytokines, or loaded ex vivo on APCs (usually dendritic cells) prior to reinjection. Alternatively, recombinant virus encoding TAAs in different forms have also been used.

Soluble Peptides as Vaccines

Several groups have proposed direct administration of peptides [34, 35], alone [15] or together with supportive cytokine treatments. The latter include granulocyte/macrophage-colony-stimulating factor (GM-CSF), aiming at in vivo mobilization of endogenous (APCs), interleukins 2 or 12 (IL-2 or IL-12) to promote the expansion of TAA-specific CTLs [16–19]. Furthermore, adjuvants in experimental use, including incomplete Freund's adjuvant [20, 21], have also been widely used. Although inexpensive and easy to produce under sterile, good manufacturing practice (GMP) conditions, peptides are frequently poor immunogens largely owing to their fast hydrolysis by serum or cell-associated peptidases [36, 37]. To circumvent this difficulty, peptides modified to improve their resistance to enzymatic digestion have been designed for a number of epitopes [38, 39].

Antigen-loaded Dendritic Cells

The wealth of information accumulated over the last years on the immunobiology of dendritic cells (DCs), possibly the most efficient APC identified so far, has readily resulted in their clinical application in AASIT [40]. Initially, immature DCs, generated upon culture of CD14+ peripheral blood monocytes or bone marrow-derived CD34+ cells in the presence of IL-4 and GM-CSF [22–24] were used.

At present, most studies take advantage of DCs matured in the presence of cytokines because of their superior capacity to induce CTL responses [25]. Early protocols relied on culture media that included foreign proteins (fetal calf serum) [41], whereas nowa-

Table 3. Overview of technologies currently in use for monitoring active antigen-specific immunotherapy of melanoma.

Procedure	Advantages	Disadvantages	Selected references
Phenotypic assays			
Tetramer staining on fresh or cultured cells	Fast and highly specific; possible on fresh blood samples	Low sensitivity; does not provide functional information	47, 48
Functional assays			
Cytotoxic activities of bulk cultures or limiting dilution analysis of CTL precursor frequency	Provide informations about the capacity of CTLs to kill tumor cells	Labor-intensive; requires relatively long cultures	29, 30
Elispot, mainly, but not exclusively, for IFNγ	Fast and specific; requires minimal culture times and provides functional informations	Further characterization of specific cells difficult	49
Intracellular cytokine staining of tetramer positive cells	Fast and specific; requires minimal culture times and provides combined phenotypic and functional informations	Low sensitivity	50
Gene expression assays			
Antigen-stimulated cytokine gene expression	Fast, specific, requiring minimal culture times	Requires quantitative PCR equipment	51–53
In vivo assays			
DTH	In vivo antigen-specific reactivity	Low sensitivity, difficult quantitation	22

IFNγ: interferon-γ; DTH: delayed type hypersensitivity; PCR: polymerase chain reaction.

days media containing serum autologous to the patient or serum-free media are more frequently employed. Furthermore, both intranodal and subcutaneous routes of administration have been explored, at times in the presence of additional antigens capable of eliciting strong CD4+ T-cell responses [26, 27]. The major disadvantages of this approach are the cumbersome ex vivo culture of large numbers of monocytes or CD34+ bone marrow cells from patients to generate DCs and the difficulty of standardizing culture protocols [40].

Recombinant Viruses

Recombinant viruses are likely to mimick the physiological antigen presentation pathways for HLA class I restricted epitopes, thus being attractive alternatives to exogenous peptide loading. Clinical trials taking advantage of various vectors have been published [28–30]. In particular, our group has developed and tested in a clinical trial a vaccinia virus-based vector including melanoma differentiation antigens Melan-A/MART1, gp 100, and tyrosinase genes limited to sequences encoding HLA-A0201-restricted epitopes. Furthermore, genes encoding CD80 and CD86 co-stimulatory molecules were added to the vector to enhance the generation of specific CTL [29, 30]. Recombinant viruses rank among the most effective immunogens. However, inactivation of the replicative capacity of the vectors, recommended to improve the safety of these reagents may result in low expression of recombinant genes. In addition, vector-specific immune responses may limit their efficacy.

Heterologous Immunization Protocols

To capitalize on the advantages of the immunization protocols detailed above while limiting their drawbacks, "heterologous" procedures have also been developed [42]. Antigens are sequentially administered in various molecular forms (e.g., as soluble peptides and encoded within recombinant virus). Considering that TAA-specific immunization mostly requires multiple boosts, these technologies are useful for reducing the extent of vector-specific responses when recombinant viruses are also used.

Adoptive Immunotherapies

Adoptive immunotherapies are characterized by "active" antigen specific expansion of CTLs obtained in vitro. Conventional AASIT has usually been applied at relatively late stages of neoplastic diseases, such as in the presence of high numbers of tumor cells (1 cm^3 of solid tumor mass contains approximately10^9 neoplastic cells). Correspondingly high numbers of specific CTLs might then be required. Thus in addition to the difficulties inherent in CTL generation, in common with virus-specific responses, the long-term maintenance of large numbers of specific CTLs might be of critical clinical relevance [43] in tumor immunotherapy. To address this issue, adoptive immunotherapy has been used to reinfuse large numbers of TAA-specific CTLs generated in vitro under controlled conditions [31, 32].

Under physiological conditions, the number of lymphocytes is homeostatically controlled, and long-lasting major expansions of given T-cell populations are considered possible only in lymphopenic hosts [44, 45]. Capitalizing on this notion, pharmacological immunodepletion followed by adoptive transfer of polyclonal T-cell populations recognizing TAA accompanied by systemic recombinant IL-2 (rIL-2) treatment has been proposed for metastatic melanoma [33]. This trial has resulted in measurable clinical responses in 6 of 13 patients. However, autoimmunity inclusive of vitiligo and uveitis and high-dose IL-2-related toxicity was observed. Furthermore, the labor-intensive culture of specific T cells to achieve high numbers severely limits wide applicability of this technology.

Monitoring AASIT Responses

A critical issue in AASIT is the possibility of providing direct links between the immune responses induced and the clinical responses. This raises the problem of adequate monitoring of CTLs induced by immunization. Again, the large body of knowledge about preventive vaccinations is of limited use. In most cases, their monitoring has mainly focused on the humoral response, with titers of the antibodies specific for the targeted bacteria/viruses the recognized gold standard. Cellular monitoring of vaccinations against infectious diseases in humans is rare [46]. It is

remarkable that the development of active AASIT has provided a decisive impetus for the development of cellular monitoring techniques (Table 3) as close as possible to the in vivo situation [47].

Limiting dilution analysis (LDA) of CTL precursor frequencies is a classic method for evaluating specific T-cell responsiveness quantitatively. Using this technology, we were able to demonstrate increases in TAA-specific CTLs following in vivo administration of a recombinant vaccinia virus encoding specific epitopes [29, 30]. However, the detection of CD8+ effector cells in LDA, usually performed using cytotoxicity assays, requires their expansion in >10-day cultures typically supplemented with exogenous cytokines. This raises the question of the in vivo relevance of the data obtained.

Tetramers are multivalent fluorochrome-labeled reagents consisting of entire HLA class I dimers in soluble form containing specific antigenic peptides in their binding grooves. Therefore tetramer staining allows fluorescence labeling of T cells expressing antigen receptors specific for defined peptides in the context of defined HLA restrictions. The development of tetramers [48] has represented a revolution in CTL monitoring in both basic immunology and clinical trials [47]. Importantly, these tetramers can be used on fresh uncultured cells. The main pitfalls concern the sensitivity of this technology, allowing reliable detection by flow cytometry of frequencies of CTLs in the 1: 2000 to 1/10,000 range. This is often insufficient for direct ex vivo evaluation. Furthermore, this technology, a current standard, falls short of providing functional data about antigen-specific T cells. To circumvent this difficulty, the intracellular staining of tetramer-positive cells with cytokine-specific monoclonal antibodies following short incubations (4–6 hours) in the presence of specific antigens has been proposed [50].

Elispot technology also represents a standard in tumor vaccination protocols. It is based on the notion that upon encountering the specific antigen activated T cells produce cytokines. Their detection as "spots" on the bottom of culture plates by immunoenzymatic methods allows accurate, sensitive quantification of the number of T cells responding to a given TAA. The culture time required usually does not exceed 24 hours. A drawback is that it is impossible to study "positive cells" further for additional functional and phenotypic characterization [49].

More recently, monitoring techniques based on the detection of cytokine gene expression following short-term exposure of T cells to antigens by quantitative reverse transcription-polymerase chain reaction TaqMan technologies have also been developed [51, 52].

A main question in the monitoring of AASIT regards the nature of the cells to be monitored. Usually, accessible T cells are from peripheral blood. However, because activated cells are known to extravasate, some groups advocate monitoring satellite lymph nodes or tumor tissues, with the material collected by fine-needle aspiration [53].

Toxicity

Most of the AASIT protocols described above have been shown to be devoid of significant toxicity, as indicated by their safe application in relatively large numbers of patients. Most frequently detected side effects range from autoimmunity of varying extent to effects attributable to the supporting cytokines or adjuvants or specific vaccine formulations. They range from skin rash and flu-like symptoms to life-threatening leak syndromes.

In melanoma AASIT, vitiligo, which results from targeting of melanocytes by CTLs specific for differentiation antigens [54], is usually localized and frequently considered a favorable prognostic marker. Other pigmented tissues, however, including the retina and substantia nigra, might also be recognized by these T cells. It could be speculated that the usual absence of ocular or neurologic symptoms in patients vaccinated with melanoma differentiation antigens may be related to poor immunization rather than to immune privileges of defined anatomic districts.

Indeed, it has been shown that functional blockade of CTLA-4, an antagonist of T-cell activation, may result in severe autoimmunity, affecting multiple organs [55]. Clearly, improvements in the effectiveness of vaccination strategies call for careful investigations regarding the fine line between therapeutic efficacy and undesired autoimmunity.

Adjuvants may also cause severe toxic side effects [56]. Regarding cytokines, IL-2 and IL-12 toxicities have widely been reported [57, 58]. Furthermore, GM-CSF, which is frequently used as a supportive cytokine during vaccination protocols, has been shown to cause somewhat milder side effects, including skin rashes [59, 60].

Clinical Effects

Despite years of clinical investigations with a wealth of clinical trials, we are still unable to provide solid data regarding the clinical effectiveness of AASIT in melanoma. Indeed, spontaneous regression of melanomas have been reported in the past [61, 62]. Evidence of tumor regression of varying extent has ranged between 10% and > 40% of the patients enrolled in clinical AASIT trials (Table 2). Can this be attributed merely to spontaneous tumor regression? On the other hand small-scale nonrandomized clinical trials are frequently subject to subtle bias during the patients' recruitment, the impact of which is difficult to quantify. Thus evidence of clinical effects still must to be considered anecdotal in the absence of randomized control groups or Phase III adjuvant studies in high risk patients [63]. Clearly, organizational and financial problems play a major role. Other issues, however, should also be considered. First, there is no standard treatment for metastatic melanoma against which to compare the outcome of immunization procedures. Interferon (IFN) type I is widely used. However, IFN administration protocols largely differ in timing and dosage; and considering its inherent toxicity, many oncologists choose not to use it.

Immunization and monitoring protocols show wide variations and are difficult to standardize. The numbers of patients required to provide statistically significant results are high and require the development of multicenter trials. Also, the unwillingness of patients bearing advanced tumors to participate to randomized studies should not be underestimated.

Although formal proof of the clinical effectiveness of AASIT is missing, preliminary explanations of its failures can now be proposed. As detailed above, not only CTL generation remains a challenge, but the mere "self" nature of most TAAs provides additional hurdles because of existing tolerance. Furthermore, current trials mostly recruit patients in advanced stages of the disease who are possibly immunodepressed. Large tumor burdens are unlikely targets of CTLs because of the unfavorable ratio

between the numbers of specific T cells and tumor cells. Last, but perhaps most importantly, tumor cells have been shown to have developed a number of strategies to escape recognition by the immune system. Such mechanisms range from down-regulation of TAA or HLA molecule expression to actively killing CTLs [64].

Outlook

Where should we go from here? In our opinion, the main advantage of AASIT compared to other immunotherapy strategies may rely on the possibility of establishing a solid relation between immune responses to molecularly defined antigens and clinical responsiveness. Currently, this is still a major unfulfilled goal of our translational research.

Admittedly, the TAAs identified so far do not represent ideal targets inasmuch as they are not uniquely tumor-specific. Furthermore, because their expression is not linked with oncogenesis or tumor cell survival, they are dispensable for cancer growth. Therefore, despite the large number of TAAs described so far, there is still wide room for research in this area.

A clinical AASIT agenda urgently requires the performance of rigorously controlled randomized trials and Phase III adjuvant studies for high risk melanoma. Such investigations, by definition, imply the engagement of a number of clinical centers and considerable financial efforts. Notably, the funds necessary are usually too high for academic institutions or public research agencies, and the interest of pharmaceutical companies in this area is still limited.

Most importantly, however, performing multicenter trials mandates the choice of easily standardizable immunogens and immunization techniques. Monitoring technologies should also be refined and standardized, possibly avoiding in vitro culture steps without jeopardizing their sensitivity. Patients with a high risk of recurrence or who have been surgically rendered tumor-free should be specifically considered.

On the other hand, the data obtained so far underline, at best, the incomplete nature of our understanding of the rules governing the generation and maintenance of CTL immune responses and urge renewed basic research efforts.

Indeed, the best investigated experimental models of CTL response to viral challenges provide arguments for reflection. The kinetics of CTL responses in these studies are classically characterized by a CTL expansion phase upon infection or vaccination, followed within weeks by a contraction phase [65] with the persistence of low numbers of memory cells, characterized by a slow renewal.

Consistent with these investigations, the few published AASIT clinical trials reporting the kinetics of immune responses eventually induced [25, 29, 30, 39] indicate that responsiveness is mostly short-lived and rarely sustained. These ephemeral responses might be capable of facing viral infections and might result in clearance of the aggressor. However, tumor-specific therapeutic vaccination shares the same tools as virus-specific immune responses (e.g., class I restricted CTLs) but presents peculiar features. In the presence of substantial tumor burdens, clearance of neoplastic cells might be an unlikely outcome even following successful immunization. Developing clinically applicable protocols that allow maintenance of large numbers of specific memory CTLs is an important challenge for tumor immunologists.

Conclusions

Immunotherapy is slowly finding its place into antitumor weaponry. In a field typically characterized by waves of enthusiasm and disappointment, we are presently witnessing an unusual reflective phase. Past the era of molecularly undefined reagents, we are now able to use the science of immunology for the good of our patients. The complexity of this science could justifiably lead to skepticism [66]. However, the continuous refinements to the art of induction and maintenance of cellular immune responses might provide a reasoned, low-key optimism for the future.

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