

Antigen specific active immunotherapy: lessons from the first decade

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Summary

Malignant melanoma is a tumour with a steeply rising incidence and scarce therapeutic options once metastatic. Approximately 1200 new cases are reported yearly in Switzerland with roughly 220 deaths/year. An important particularity of melanoma is its immunogenicity, which has long been recognized and investigated using various clinical immunization protocols in the last fifty years. The year 1991, when the first melanoma associated antigen was molecularly characterized, represents a turning point in the quest for a melanoma immunotherapy. This opened the era of antigen specific active immunotherapy.

Many clinical centres have developed immunization strategies in an adjuvant setting for the treatment of metastatic melanoma. The molecular characterization of melanoma associated antigens

allows a fine monitoring of the elicited immune response. Certain clinical responses to these efforts have been seen and a phase of reflection is now ongoing, with refinements and further sophistication taking place in order to fully realize the potential of antigen specific active immunotherapy.

Here we provide an overview of the technologies used and of the progress reported in melanoma immunotherapy since 1991. Furthermore, we propose some research lines in basic and translational research aimed at improving our capacity to induce specific and clinically relevant immune responses against melanoma.

Key words: melanoma; immunotherapy; tumour associated antigen; recombinant virus

Introduction

The incidence of melanoma is rapidly growing throughout the world, increasing at a yearly rate of around 3–5%. Today, melanoma ranks as the fourth commonest malignancy in Western countries and the lifetime risk of melanoma in Switzerland is about 1 in 50. Nationwide, 1200 new melanoma cases are registered yearly and about 220 deaths are reported. The reasons for this increase in incidence are unknown, the reduction in the ozone layer and burst exposures to sunlight over short periods of time during leisure activities may play a role.

The majority of primary melanomas can be cured by a simple wide excision. However, when lesions are thicker (>4 mm) or regional nodal metastases are present, the prognosis is poor. Moreover, long term recurrence up to 15 years after definitive surgery is not uncommon. Once metastatic, survival is short and acknowledged therapeutic options are scarce. To date, prevention by sun avoidance, appropriate use of sunscreens and early diagnosis and excision are the best weapons against melanoma.

Nevertheless, melanoma presents a peculiar feature: around 1% of melanomas regress spontaneously and this is associated with tumour infiltrating lymphocytes and vitiligo in cases with a relatively good prognosis. The immunogenicity of melanoma was recognized early and attempts to boost the immune response against tumour cells are now entering their fifth decade [1]. A turning point in melanoma immunotherapy was the description of the first HLA restricted tumour associated antigen (TAA) in 1991 [2], which allowed tumour immunologists to specifically target cancer cells. Here, we will review the achievements of the last decade in antigen specific active immunotherapy (ASAI). We will describe the basic concepts underlying clinical ASAI, report its successes and its difficulties and try to delineate the areas where major efforts in basic and translational research are urgently needed in order to realize the potential of ASAI.

Tumour associated antigens

The molecular identification of the first TAA in 1991 [2] opened the era of ASAI and through isolation of autologous cytotoxic T lymphocytes (CTL) and tumour cell lines allowed a constant refinement of the immunotherapeutic procedures, as well as a precise monitoring of the dynamics of the immune response. CTL recognize antigens as small protein fragments of 8 to 14 residues (known as epitopes) which associate with MHC class I molecules that are expressed on the surface of antigen presenting cells (APC), such as malignant cells. As a result of the specific recognition of the T cell receptor for antigen and the epitope/MHC complex, CTL are able to kill target cells expressing tumour antigens. TAA are processed through the intracellular antigen pathway and expressed on APC in the context of a given HLA subtype, according to its restriction. Typically, immunotherapeutic approaches have focused on epitopes presented in the context of HLA A2.1 restriction, as this HLA subtype is harboured by approximately 50% of the Western population.

TAA recognized by CTLs or helper T lymphocytes (HTL) were originally identified by taking advantage of a number of different methods (for a review see [3]). In malignant melanoma, at least three groups of TAA have been defined. Tissue-specific or differentiation antigens include Mart-1/Melan-A, gp100, tyrosinase, TRP-1 and TRP-2 and these are among the most frequently used in ASAI. These antigens are expressed in melanoma, but also in normal melanocytes and retina, albeit at a much lower concentration. Cancer-testis antigens

are expressed in different types of cancers (melanoma 28%, lung 20–50%, bladder 12–40%) and in spermatogonia [4]. As spermatogonia do not express MHC class I molecules for presentation of antigens to CTL, cancer-testis antigens demonstrate a high tumour specificity. Among them, Mage-1, Mage-3 and NY-ESO-1 have been used in immunotherapy trials. Finally, several mutated/unique antigens have been identified recently as tumour specific antigens, such as MUM-1 or CDK4 for melanoma. These unique antigens are tumour specific in the narrowest sense, being the product of unique mutations in the tumour. They might be suitable for immunotherapy only in the particular patient harbouring that particular unique mutation. Thus, these unique TAA are of limited clinical relevance. In other types of tumour, but not in melanoma, antigens derived from viruses potentially involved in the oncogenic process represent an additional TAA group of rising importance [5].

Increased evidence has indicated that optimal anti-tumour immunity requires participation of both CTL and HTL. TAA may display MHC class I as well as MHC class II epitopes for recognition by HTL, as this has been shown for several melanoma TAA (e.g. Mart-1/Melan-A, gp100, Mage-3 and NY-ESO-1). As for MHC class I TAA, specific immunotherapy might focus on MHC class II restriction allele borne by roughly 50% of the population, like HLA-DP4. Nevertheless, having to select patients owing to MHC classes I and II haplotypes further restricts the target population eligible for ASAI.

Immunotherapy protocols

Immunology as a science began in 1798 with the first publication of a paper on a vaccine against smallpox [6]. Today, 26 infectious diseases are preventable through vaccination, but in spite of over 200 years of vaccine research, numerous bacterial, viral and parasitic infections remain elusive to vaccination. Tumour immunotherapy has to combat billions of tumour cells in a dynamic process which involves down regulation of tumour MHC and/or TAA, selection of resistant tumour clones and other mechanisms of escape as well as local/systemic immunodepression [7]. Furthermore, melanoma TAAs used in ASAI most frequently derive from proteins also expressed in non transformed cells. This implies that the immune system is likely to have developed some degree of tolerance towards TAA. Recent experience suggests that this tolerance can be overcome by taking advantage of appropriate immunization procedures. This still represents a major difference in the use of ASAI in cancer to the conventional preventive vaccinations targeting infectious agents.

Having characterized TAA does not automatically imply an inherent capacity to generate effective immune responses following ASAI. In fact, all vaccines used in the prevention of infectious diseases, with the sole exception of BCG, are effective in that they induce the generation of an humoral antibody response [8]. These responses are incapable of targeting human TAAs that are, in a large majority, only expressed intracellularly. Most importantly, the generation of the necessary HLA class I restricted CTL responses represents a major challenge also in the context of preventive vaccinations against a number of infectious diseases such as, for instance, hepatitis C [9].

In addition, the adjuvant most frequently included in commercial vaccine preparations, alum, while excellent in promoting antigen specific humoral immune responses is unable to support CTL generation [10]. Indeed the identification and functional characterization of novel adjuvant formulations capable of enhancing CTL induction represents the main research focus of many aca-

demic and R&D departments [11]. In this context, clinical ASAI has played an ice-breaking role in the promotion of investigation on CTL generation.

Many different immunization approaches targeting molecularly defined antigens (table 1) have been employed in clinical trials in metastatic melanoma. A common starting point is that antigens recognized by CTL, be they TAA or viral antigens, are produced inside the cells and their peptidic fragments (= epitopes) are expressed on cell surfaces within grooves in HLA class I molecules. Thus, mimicking physiological pathways would suggest, for instance, the use of virus recombinant for TAA and capable of infecting APC to obtain effective presentation. Alternatively, APC might be exogenously loaded with synthetic epitopes, capable of binding the small percentage of HLA class I molecules that are present on cell surfaces in an empty state. However, the exogenous loading of MHC molecules by soluble epitopes is quite inefficient. Most immunization procedures

used for CTL generation in ASAI are based on these basic concepts. Accordingly, epitopes have been injected in the presence or absence of adjuvants or supportive cytokines, or loaded ex vivo on APC from patients, usually dendritic cells, before injection. Alternatively, recombinant virus encoding TAA and enabling the endogenous expression of TAA on the surface of APC has also been used, displaying more efficiency than exogenous epitope loading.

Figure 1 presents an overview of the growing number of clinical trials in metastatic melanoma, as indexed in Pubmed/Medline 1992–2004.

Immunotherapy may be divided into active and passive immunotherapy. Active immunotherapy implies the induction of antigen responses and will be the focus of the present review. Passive (adoptive) immunotherapy describes the transfer of immunity (antibodies or CTL). Table 2 summarizes current immunotherapy strategies, which will be presented below.

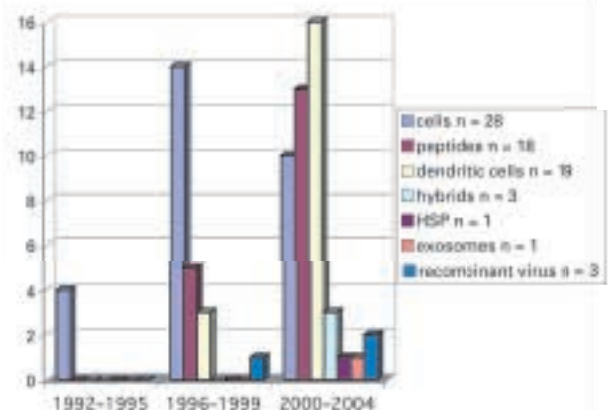
Table 1
An overview of melanoma associated antigens.

Melanoma associated antigens	Expression pattern	Immunogenicity	References
Differentiation antigens gp100, Melan-A/MART-1, tyrosinase, TRP-2 etc.	Tumour cells and non transformed melanocytes/pigmented cells: highly frequent expression in melanoma	Capacity to induce CTL and CD4 ⁺ T cell responses	3
Cancer/Testis antigens MAGE, NY-ESO-1 etc.	Spermatogonia, placenta and tumour cells of different histological origin: expressed in 10–50% of melanomas	Capacity to induce CTL and CD4 ⁺ T cell responses	2–3
Mutated/unique antigens tumour specific antigens derived from mutated genes or sequences not transcribed under physiological conditions	Limited to tumour cells of individual patients	Capacity to induce CTL and CD4 ⁺ T cell responses	5

Table 2
An overview of immunogens and treatment procedures most frequently used in immunotherapy of melanoma.

Immunogens and treatment procedures	Advantages	Disadvantages	References
1. Active immunotherapy			
Peptides with or without cytokines	Cheap, easy to produce GMP	Low immunogenicity, cytokine related side effects	19–23
Peptides with adjuvants	Cheap, easy to produce GMP	Few licensed adjuvants capable of inducing CTL, side effects related to adjuvant administration	24, 25
Dendritic cells loaded with peptides	Highly immunogenic	Difficult standardization, requirement for pre-culture of patient's own cells	32–35, 37, 38
Recombinant virus	Highly immunogenic	Safety concerns, expensive GMP production, vector specific immune responses	39–41
2. Passive immunotherapy			
Adoptive immunotherapies	Possible administration of high numbers of specific CTL	Long term culture of lymphocytes required Side effects related to immunodepletion and	47, 48
Adoptive immunotherapies with pharmacological immunodepletion	Possible administration of high numbers of specific CTL with prolonged survival	Autoimmunity after CTL administration	51

Figure 1
Overview of clinical trials in metastatic melanoma 1992–2004. Data refer to published clinical trials as reported in 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, and thus the number of trials reported here is lower than that of entries in PUBMED. Trials involving undefined antigens, e.g. tumour cells, cellular hybrids, heat shock proteins or exosomes are outside the scopes of this review.



Tumour lysates

Early attempts to immunize melanoma patients took advantage of irradiated tumour lysates or whole tumour cells mixed with non specific adjuvants. Autologous or allogeneic cell preparations were used, necessitating labour intensive steps to harvest, grow, engineer and re-administrate such vaccines under aseptic conditions. Moreover, concerns about possible transmission of infective pathogens through allogeneic preparations cloud this approach. Many of these attempts were supported through industrial developments and were

tested in phase I/II clinical trials. Overall response rates around 20% (CancerVax [12], Melacine [13]) were observed. Another formulation based on live vaccinia virus infected allogeneic polyvalent melanoma cell lysate (vaccinia melanoma oncolysate) was first successfully tested in a phase I/II trial. These encouraging results were not confirmed in a phase III multicentre trial treating 250 locally advanced, resected UICC stage II melanoma patients [14].

Antibody based immunotherapy

The generation of anti-idiotypic monoclonal antibodies (anti-Id) is the major antibody based ASAI approach. Anti-Id mimic the natural TAA. Immunization begins with the endocytosis and processing of the anti-Id by APC. Subsequently, TAA epitopes are presented along with MHC class II and I molecules to HTL and CTL, respectively. Specifically activated HTL stimulate T helper II (antibody driven) and T helper I (CTL driven) immune responses, resulting in production of TAA specific antibodies (anti-anti-Id) by B lymphocytes, as well as proliferation and activation of TAA specific CTL.

Passive immunotherapy with humanized mouse anti-Id generated an inflammatory response with specific CTL, but was hampered by the rapid development of human anti-mouse antibodies [15]. In fact, a series of sequential protocols suggest that combination with the adjuvants KLH and QS21 might be the best strategy to induce a potent specific humoral response. Phase I/II trials supported by the pharmaceutical industry (Melimmune, TriGem) with various adjuvants (alum, GM-CSF, QS21, interferon alpha) have suggested an improved clinical outcome. A planned phase III adjuvant trial should compare high-dose interferon- α alone or in combination with anti-Id and alum.

Gangliosides are transmembrane glycoprotein that contribute significantly to the antigenic profile of cells. Malignancies of neuroectodermal origin (melanoma, neuroblastoma, astrocytoma, soft tissue sarcoma and small cell lung cancer) display a high level of ganglioside expression, the most commonly expressed in melanoma being GD3, GD2 and GM2.

ASAI with purified gangliosides injected alone did not induce an antibody response. Over the years, many studies with purified or modified gangliosides demonstrated induction of moderate to high titres of TAA specific IgM, but failed to generate high titres of TAA specific IgG. The induction of an IgG antibody response is desirable, because IgGs exhibit better affinity to the TAA, penetrate tissues more easily and are able to mediate antibody dependent cellular cytotoxicity. Clinically, the extensively studied GM2-KLH/QS21 (KLH and QS21 are adjuvants) vaccine has been compared to high-dose interferon alpha in patients with resected stage IIb-III melanoma in a large prospective randomized trial. While antibody response to GM2 was associated with a trend towards improved relapse-free and overall survival, the study was stopped after a planned interim analysis demonstrated superiority of the interferon- α arm [16].

Soluble peptide epitopes as vaccines

Manufacturing synthetic peptide epitopes derived from TAA is a simple and cost-effective way to produce immunotherapeutic reagents. Direct administration of soluble epitopes [17, 18], alone [19] or together with supportive cytokine, including GM-CSF aimed at mobilization of endogenous antigen presenting cells (APC) or IL-2 to promote the expansion of TAA specific CTL has been assessed by several groups [20-23]. Notably, the administration of soluble epitopes without adjuvant has been associated with CTL tolerance/an-

ergy. Thus, adjuvants of experimental use, including incomplete Freund's adjuvant [24, 25] have also been widely used. Moreover, peptides frequently represent poor immunogens largely due to their fast hydrolysis by serum or cell associated peptidases [26, 27]. To circumvent this difficulty, peptide analogues resistant to enzymatic digestion have been designed for a number of epitopes [28, 29], as well as epitope carrier formulations like liposomes [30], which create a protective depot effect and are preferentially endocytosed by APC.

Antigen loaded dendritic cells

Dendritic cell (DC) based vaccines are the newest development in clinical cancer immunotherapy [31]. DCs are possibly the most efficient antigen presenting cells and so have a key role in activating antigen specific immune responses on both the humoral and the cytotoxic arms. Clinical protocols were originally initiated using immature DC, generated upon culture of CD14⁺ peripheral blood monocytes or bone marrow derived CD34⁺ cells in the presence of IL-4 and GM-CSF [32–34] and later shifted to DC matured in the presence of cytokines when their superior capacity to boost CTL responses emerged from experimental studies [35]. DCs can be loaded with TAA, either synthetic or through co-incubation with tumour lysates and can be transduced with tumour DNA or tumour RNA to express TAA. Further attempts have taken advantage of

hybrid cell vaccines fusing DC with tumour cells, either autologous or allogenic [36]. Both intranodal and subcutaneous routes of administration have been explored, in the presence or absence of additional antigens capable of eliciting strong CD4⁺ T cell responses [37, 38]. As with whole tumour-based vaccines and tumour lysates, impressive results have been shown in preventing tumours in animal models, but this has only translated into anecdotal clinical responses in the therapy of advanced disease patients. Major disadvantages of DC based immunotherapy are the cumbersome *ex vivo* manipulations of patient tissues needed, which preclude a broad clinical application, owing to pitfalls linked to prolonged cell cultures, pathogen contamination, inherent difficulties in standardizing protocols [31] and regulatory issues.

Recombinant viruses

Recombinant viruses mimic the physiological antigen presentation pathways for HLA class I restricted epitopes, thus representing an attractive alternative to exogenous peptide loading. Clinical trials taking advantage of different viruses have been published [39–41]. In particular, our group has developed and tested in clinical trials a recombinant vaccinia virus including the melanoma TAA Melan-A/MART-1, gp100 and tyrosinase genes limited to sequences encoding immunodominant HLA-A0201 restricted epitopes, as well as the genes encoding CD80 and CD86 co-stimulatory molecules to enhance the generation of specific

CTL [40, 41]. Recombinant viruses rank among the most effective immunogens. However, replication inactivation of the viruses, recommended to improve the safety of these reagents, may result in low expression of recombinant genes and virus specific immune responses may limit their efficacy. Early promoter expression of the recombinant transgenes may allow for efficient transcription and evidence is accumulating for persistent transgene expression despite repeated administration and rising antibody titres against the recombinant virus (unpublished observation from our ASAI clinical trials).

DNA vaccines

The direct inoculation of plasmid coding for TAA may result in long lasting immune responses through prolonged expression of the encoded TAA. Comparison of various methods of inoculation of naked DNA for their relative efficiency identified the intramuscular route as the best transfection route, whereas inoculation of DNA-coated gold particles (gene gun) requires significantly lower doses of DNA [42]. The probability of tumour-promotion by plasmid DNA integra-

tion was calculated to be below the lifetime probability of occurrence of a corresponding spontaneous mutation [43]. DNA vaccines are relatively cheap and simple to produce, are not associated with a vector specific immune response but display a low, but long-lasting expression of the encoded antigens. Nevertheless, clinical trials taking advantage of this approach are still disappointing [44, 45] and further optimization is warranted in order to realize the potential of DNA vaccines.

Heterologous immunization protocols

To capitalize on the advantages of the immunization protocols detailed above, while limiting their drawbacks, heterologous procedures (prime/boost strategy) have also been developed. Antigens are sequentially administered in different molecu-

lar forms, e.g. as soluble peptides or encoded within recombinant virus. Considering that TAA specific immunization mostly requires multiple boosts, these technologies are also useful in reducing the extent of vector specific responses.

Adoptive immunotherapy

Adoptive transfer of tumour infiltrating lymphocytes into autologous melanoma patients along with interleukin-2 (IL-2) has resulted in objective regression of tumour in 23% of patients [46], suggesting that T lymphocytes play a critical role in tumour regression. Large numbers of patient derived, TAA specific CTL can be generated *in vitro* and reinfused into the donor patient [47, 48]. However, the maintenance of large pools of antigen specific CTL is tightly regulated under physiological conditions, a contraction/memory phase following shortly after the antigen specific expansion phase [49, 50].

Only lymphopenic hosts are considered to be able to maintain a long lasting population of antigen specific CTL. A clinical trial [51] has attempted the adoptive transfer of expanded polyclonal TAA specific CTL along with IL-2, after lymphodepletion of the patients. Measurable clinical responses were observed in 6/13 metastatic patients, at the expense of autoimmunity inclusive of vitiligo and uveitis and high dose IL-2 related toxicity. Furthermore, the labour intensive expansion of specific CTL in culture severely limits a wide application of this technology.

Monitoring of ASAI

A critical issue in ASAI is the possibility of providing direct links between the immune responses eventually induced and clinical responses. This raises the problem of adequate monitoring of immunization induced CTL. Again, the large body of knowledge on preventive vaccinations is of limited use. In most cases, their monitoring has focused on humoral response, with titres of antibodies specific for the targeted bacteria/viruses as the recognized gold standard. Cellular monitoring of vaccination against infectious diseases in humans is rather rare [52]. It is remarkable that the development of active ASAI has provided a decisive impetus for the development of cellular monitoring techniques as close as possible to the *in vivo* situation [53].

Limiting dilution analysis (LDA) of CTL precursor frequencies represents a classical method to evaluate quantitatively specific T cell responsiveness. By using this technology, we were able to demonstrate increases in TAA specific CTL following the *in vivo* administration of a recombinant vaccinia virus encoding specific epitopes [40, 41]. However, the detection of effector cells in LDA

requires their expansion in >10 days cultures, typically supplemented with exogenous cytokines, thus raising the question of the *in vivo* relevance of the data obtained.

Tetramers are multivalent fluorochrome labelled reagents consisting of HLA class I dimers in soluble form, containing specific antigenic peptides in their binding grooves. Therefore, tetramer staining allows the fluorescent labelling of T cells expressing antigen receptors specific for defined peptides in the context of defined HLA restrictions. The development of tetramers [54] has represented a revolution in CTL monitoring, both in basic immunology and in clinical trials [53]. Importantly, they can be used on fresh uncultured cells. Main pitfalls concern the sensitivity of this technology, allowing the reliable detection, by flow-cytometry of frequencies of CTL in the 1/2000–1/10.000 range, often insufficient for direct “*ex vivo*” evaluation. Furthermore, this technology, a current standard, falls short of providing functional data on antigen specific T cells. To circumvent this difficulty methods have been devel-

Table 3

An overview of technologies currently in use in the monitoring of active antigen specific immunotherapy of melanoma.

	Advantages	Disadvantages	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	53, 54
Functional assays			
Cytotoxic activities of bulk cultures or limiting dilution analysis of CTL precursor frequency	Provide information about the capacity of CTL to kill tumour cells	Labour intensive; requires relatively long cultures	40, 41
Elispot, mainly, but not exclusively, for interferon- γ	Fast and specific, requires minimal culture times and provides functional information	Further characterization of specific cells difficult	56
Intracellular cytokine staining of tetramer positive cells	Fast and specific, requires minimal culture times and provides combined phenotypic and functional information	Low sensitivity	55
Gene expression assays			
Antigen stimulated cytokine gene expression	Fast, specific, requiring minimal culture times	Requires quantitative PCR equipment	57–60
In vivo assays			
DTH	“ <i>In vivo</i> ” antigen specific reactivity	Low sensitivity, difficult quantification	32

oped allowing intracellular staining of tetramer positive cells with cytokine specific monoclonal antibodies following short (4–6 hours) incubations in the presence of specific antigens [55].

Elispot technology also represents a standard in tumour 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 ELISA methods allows an accurate and sensitive quantification of the number of T cells responding to a given TAA. Culture times usually required do not exceed 24 hours. A drawback is the impossibility of using further study “positive cells” for additional functional and phenotypic characterization [56].

More recently, additional monitoring techniques based on the detection of cytokine gene expression following short term exposure of T cells to antigens by quantitative PCR TaqMan technologies have also been developed [57, 58]. Table 3 presents an overview of the current monitoring techniques.

The main question mark in the monitoring of ASAI is represented by the nature of the cells to be monitored. Usually accessible T cells are from peripheral blood. However, since activated cells are known to extravasate, some groups advocate the monitoring of satellite lymph nodes or tumour tissues by fine needle aspirates [59].

Toxicity of ASAI

A large majority of the ASAI protocols cited above are devoid of significant toxicity and have now been safely applied in a relatively large number of patients. The most frequently detected side effects range from autoimmunity (mostly vitiligo) of varying extent to those attributable to supporting cytokines or adjuvants, ranging between skin rash and flu-like symptoms. Rarely, severe autoimmunity manifestations with transient disability

(uveitis [51], melanoma associated retinopathy and visual field impairment) and life threatening leak-syndromes related to high-dose IL-2 adjuvant therapy have been described, with a single case of immunotherapy related death reported. So far, most clinical trials have been conducted in ambulant settings, solely concomitant administration of high-dose IL-2 therapy requiring hospitalisation.

Clinical results of ASAI

Reviewing the clinical results reported so far in ASAI is a challenging task, due to the high heterogeneity of the trials and different approaches developed by immuno-oncologists. Moreover, most of the trials published so far are phase I trials, not designed to assess clinical responses but to rule out toxicity, with few phase II trials attempting to evaluate efficacy. No phase III trial has compared an ASAI protocol to a control group. Thus, no randomized controlled trial (RCT) or evidence based data exist to assess ASAI reliably. Nevertheless, most of the protocols were able to generate an antigen specific CTL response and many groups around the world reported some clinical results, ranging from a prolonged survival despite high tumour load, compared to historical control, to long lasting clinical response, the latter being the exception rather than the rule. Evidence of tumour regression of varying extent has been obtained in percentages of patients ranging between 10% and >40% of those enrolled in clinical ASAI trials. Bearing in mind that the natural history of melanoma comprises up to 1% spontaneous regression, it is hard to definitely attribute these anecdotal clinical responses to a particular protocol or to chance. On the other hand, small scale non randomized clinical trials are subject to subtle bias in patient recruitment, whose impact is difficult to

quantify. Thus, evidence of clinical effects still has to be considered anecdotal, in the absence of randomized control groups [60].

Phase III randomized controlled trials in ASAI have been delayed for many reasons. Obviously, logistical and financial problems play a major role in the implementation of a RCT. Other issues, however, should also be considered. Firstly, there is a lack of standard treatment of metastatic melanoma against which to compare the outcome of immunotherapy. Interferon- α is indeed widely used in the United States despite lack of definitive supporting evidence, but administration protocols differ largely in timing and dosages, while, considering its inherent toxicity, many oncologists (especially in Europe) chose not to use it. Biochemotherapy has gained some popularity, but again clear evidence is lacking and proposals for the use of biochemotherapy outside clinical trials or compassionate use is rare.

Finally, immunotherapy and monitoring protocols show wide variations and are difficult to standardize. The numbers of patients required to provide statistically significant results are high and impose the development of multicentre trials. In addition, the unwillingness of patients with advanced tumours to participate in randomized studies should not be underestimated.

Although we are unable to provide formal proof of their clinical effectiveness, we can now try to provide preliminary explanations of their failures.

As detailed above, not only does CTL generation remain a challenge, but also the mere "self" nature of most TAA provides additional hurdles, because of existing tolerance. Current trials mostly recruit patients in advanced stages of their disease, who are possibly immunodepressed. Large tumour

burdens are unlikely targets of CTL, because of the unfavourable relationship between numbers of specific T cells and tumour cells. Lastly, but perhaps most importantly, a number of strategies have been shown to be developed by tumour cells to escape recognition by the immune system, ranging between down-regulation of TAA or HLA molecules expression to the active killing of CTL (for a review see [62]).

Perspectives

Where should we go from here? In our opinion, the main advantage of ASAI as compared to other immunotherapy strategies resides in the possibility of establishing solid relationships between immune responses to molecularly defined antigens and clinical responsiveness. This is still a major as yet unfulfilled goal of current translational research.

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

A tentative clinical ASAI agenda urgently requires the performance of rigorously controlled phase III trials. Such trials would, by definition, imply the engagement of a number of different clinical centres, and considerable financial efforts considering that the necessary funds are usually too high for academic institutions or public research agencies. Notably, the interest of pharmaceutical companies in this area is still limited.

Most importantly, however, performing phase III trials mandates the choice of easily standardisable immunogens and immunization techniques. Furthermore, monitoring technologies should also be refined and standardized, if possible avoiding *in vitro* culture steps without jeopardizing sensitivity. Patients with a high risk of recurrence (UICC stage IIb) or surgically rendered tumour free should be specifically considered.

On the other hand, the data obtained so far underline our incomplete understanding of the rules

governing generation and maintenance of CTL immune response 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 [62] with the persistence of low numbers of memory cells, characterized by a slow renewal.

Consistent with these investigations, the few published ASAI clinical trials reporting the kinetics of immune responses eventually induced [29, 35, 40, 41] indicate that responsiveness is indeed mostly short lived and rarely sustained. CTL frequencies usually decline at the end of the immunization protocols. Admittedly, this ephemerality could also be related to inappropriate vaccine formulations and immunization procedures or to the low sensitivity of monitoring techniques.

While sharing the same tools of virus specific immune responses, tumour specific therapeutic vaccination presents particular class I restricted CTL features. Short lasting responses appear to be ideally tailored to face viral infections potentially resulting in the clearance of the aggressor or, alternatively, in the death of the host. However, in the presence of substantial tumour burdens, clearance of neoplastic cells might represent an unlikely outcome even following successful immunization. Developing clinically applicable protocols that allow the maintenance of large numbers of specific memory CTL alert represents an important challenge for tumour immunologists.

Conclusions

Immunotherapy is slowly finding its place in the antitumour weaponry. In a field typically characterized by waves of enthusiasm and disappointment, we are presently witnessing an unusual reflective phase. Beyond the era of molecularly undefined reagents, we are now able to use the science of immunology to the benefit of our patients. Although the complexities of this science could

justifiably lead to scepticism [63], the continuous refinements to the art of the induction and maintenance of cellular immune responses might provide a reasoned, low key optimism, for the next future.

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