FUNDAMENTAL PRINCIPLES OF RAISING AN ANTITUMOUR IMMUNE RESPONSE *IN VIVO*: A COMPLEX MODEL, A CASE REPORT, AND A PERSPECTIVE

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ABSTRACT

In preclinical model systems, the fundamental principles underlying a successful and durable anti-tumour immune response are well demonstrated. In clinical practice, significant successes in Phase III trials have been few over the last decades, but the field has gained tremendous interest following recent advances showing the activity of checkpoint blockade inhibitors. Still, at this time we do not fully understand why some people respond while others do not; nor do we completely understand which clinical and immunological monitoring tools we need to put in place to make immunotherapy a more controlled medical science. Reviewing recent evidence suggests that for a successful and controlled immunotherapy, we may need to juggle with several conditions at the same time; there is a need for the endogenous or exogenous addition of tumour antigens for a favourable tumour microenvironment, and for an immune system which remains actionable towards T cell (effector) activity by checkpoint blockade inhibitors.

Keywords: Immune response, vaccines, tumour antigens, tumour microenvironment, checkpoint blockade.

INTRODUCTION

Mellman et al.,[1] in their review: '*Cancer immunotherapy comes of age*', explore the advances in the understanding and regulation of anti-tumour immune responses, and explain the sequence of events that need to occur for a successful immune response to take place. Immune tolerance and immunosuppression are frequent features in cancer patients, but these can be bypassed and immunity restored as supported by recent successes,[2] which strongly suggest that active immunotherapy does indeed represent a valid therapeutic option and is a path towards a durable and long-lasting response in cancer patients. In the present review we shall compare the fundamental theoretical basis to the clinical practice of immunotherapy, the past failures in large Phase II and III clinical trials, incipient results, and fledgling success stories. We can model our endeavours to harness the immune system in the right direction by equating immunotherapy to the multi-parametric model of sustained flight with a device heavier than air.

Theoretical Basis behind the Biology of the Immune Response

Over the past decades, most of our clinical efforts were directed towards providing sufficient fuel in the form of tumour specific antigens, exogenously in a variety of flavours (tumour cell lysates, peptides, whole genes in viral carriers, DNA vaccines, etc.) or endogenously by tumour-cell lysis following standard targeted therapies or oncolytic viruses.[2] The immune-stimulatory process is enhanced by the association of a so-called 'adjuvant', which is meant to deliver a maturation signal to antigen presenting cells (APCs) such as immature dendritic cells (DCs). This 'adjuvant' might be pictured as a catapult, able to provide uplift to our model plane (a glider here) from the ground. The endogeneous approach also necessitates the recruitment of APC into the tumour area. Following effective maturation, DCs may

move towards immune organs such as tumour-draining lymph nodes, where they prompt T cells to become engaged in the battle against specific antigen-bearing cancer cells and to mature into effector T cells.

Effector T cells recirculate - passing fleetingly in circulation where they may be monitored - and re-enter the tumour bed, usually facing an array of immunosuppressive defence mechanisms such as suppressive myeloid cells and T regulatory cells (Tregs), which oppose their lytic function.[1] If the suppressive microenvironment can be blocked, and if all necessary steps of immune activation are fulfilled according to plan, T cells may infiltrate the tumour and lyse tumour cells. In our model, tolerigenic or immunosuppressive actions might be pictured as strong crosswinds or violent thunderstorms, which are known to jeopardise take offs and landings.

THE REALITY OF IMMUNE THERAPY IN CLINICAL PRACTICE

The detection of a viable tumour signifies that the immune system has failed. This may be due to failed antigen recognition. Human leukocyte antigen (HLA) dysregulation, as well as absent HLA Type 1 glycoprotein expression (preventing antigen-specific T cell recognition), have been described in tumours and cancer cell lines[3] while HLA Type 2, which is normally not expressed (for instance in normal squamous epithelium), has been shown to be expressed in >80% of carcinomas.[4,5] There exists now a wealth of data in the literature documenting active immunosuppression through the secretion of immunosuppressive mediators by the tumour and leading to induction of Tregs and subtypes of suppressive myeloid-derived cells in close association with growing tumours.[6] While natural Tregs have a role in maintaining self-tolerance and in regulating responses to infectious agents, transplantation of atypical glandular cells (AGCs), and tumour AGCs, induced Tregs are able to prevent a robust antitumour cytotoxic T lymphocyte (CTL) response.[7]

Tregs appear to be exquisitely sensitive to chemotherapy,[8] but they tend to reappear over time in the presence of a persistent tumour. Similarly, subsets of 'M2 Type' APCs, in the tumour microenvironment (TME), were shown to be relevant to poor outcome. Depletion of tumour-associated macrophages (TAM) in an animal model, restored tumour-infiltrating CTL responses and suppressed tumour growth,[9-11] while depletion of tissue resident macrophages had no effect. It has been suggested that the presence of colony stimulating factor-1 (CSF-1), a macrophage-specific growth factor which is abundant in many tumours, will lead to TAM and may divert effective maturation from DCs, which are potent APCs, thereby perturbing efficient immune stimulation.[12,13] However, in some instances there are signs of an effective immune response occurring at the tumour site. Thus, the presence of specialised CD4+ T cells in the TME, when found to localise to the germinal centres of peri-tumoural tertiary lymphoid structures in extensively infiltrated neoplastic lesions, predicted improved outcome in breast carcinoma patients.[14,15]

Similarly, during neoadjuvant chemotherapy in patients with breast cancer, the increase of a CD25-CD127- CD4+ T cell population in circulation correlated with tumour regression.[16] In a recent paper assessing the microenvironment of cervical cancer patients, the quantification of different subsets of myeloid cells revealed that a strong intraepithelial infiltration of CD14+ cells, and more specifically, the population of CD14+ CD33- CD163- matured 'M1' (activating phenotype) macrophages, were associated with a large influx of intraepithelial T lymphocytes (p=0.008), and with improved disease-specific survival (p=0.007). This factor retained an independent prognostic value for improved survival in a multi-parametric analysis (p=0.033).[17] Recently, 84-gene signature on genes involved in immune function was shown as being able to predict outcomes in patients with melanoma.[17] In practical terms, the immunological monitoring of patients is most often based on sampling of lymphocytes from circulation, rather than from the tumour or from tumour draining lymph nodes, while a more accurate assessment of tumour infiltrating lymphocytes would be based on a biopsy at the crucial tumour site.

PAST CLINICAL DEVELOPMENTS IN CANCER IMMUNOTHERAPY

There are many different types of immunotherapies, and a great variety of reagents to choose from. What did we accomplish in a century of immunotherapy and what should be adapted to future clinical trials? A recent review article listed 41 Phase II or Phase III trials, some with quite significant numbers of patients, all asking questions

concerning clinical efficacy and a prolonged disease-free period, or overall survival.[2] In many cases, trials were abandoned at interim analysis because there was no significant survival benefit in the intention to treat population, compared with an active comparator or best supportive care.

When subgroups of patients were analysed, signs of activity could be seen in so-called 'favourable' patient groups, with either a favourable prognostic score, as in a prostate cancer group with a Halabi score predicted survival of >18 months,[18,19] or for patients who had combined treatments with hormone therapy, chemotherapy, or radiation. Importantly, after many early failures, some recent very clear successes in clinical trial responses[20,21] could be achieved by using so called 'checkpoint modulators' directed against programmed death-1 (PD-1) T cell co-receptor and its ligands. PD-1 is expressed on antigen-experienced T cells in the periphery, and serves in the normal host to limit the activity of T cells at the time of an inflammatory response, thereby protecting normal tissues from collateral destruction. By blocking its effect in cancer patients, the immunosuppressive effect in the TME may be lifted.

A Model Perspective

While controlled flight has been a preoccupation of mankind over many centuries, it was only at the beginning of the last century (1903) that we grasped the multiple parameters that need to be fulfilled to not only lift an object heavier than air into the air but, more importantly, to control all actions of safe flying. To put immunotherapy in historical perspective, at the time the Wright brothers were building their plane, the New York surgeon William Coley used live bacteria as antigens to immunise against tumours (1893). What are the essential ingredients for controlled flight and can we transpose this model to immunotherapy? To fly an airplane, we need fuel for propulsion, we need wings, and we need balance; we need those three main ingredients all together at the same time. Each one, taken on its own, will not allow an airplane to fly (Figure 1). Ifwe try to transpose that idea to immunotherapy, we may submit that antigens and adjuvants represent the fuel, whilst lift/wings to remain airborne may be equated with an 'adjuvant', and with correct DC maturation.

Powerful crosswinds can be likened to an unfavourable TME, favouring DC maturation toward tolerance, rather than toward activation, while violent thunderstorms would be the immunosuppressive microenvironment mediated by Tregs and myeloid derived suppressor cells. Finally, the last, but crucial aspect of flying has to do with balance and centre of gravity, which also affect the stability of the aircraft. When the centre of gravity is 'out of range', the aircraft may pitch uncontrollably down or up. This tendency may exceed the control capacity of the pilot and cause a loss of control. To ensure the aircraft is safe to fly, the centre of gravity must fall within specified limits established by the aircraft manufacturer, and may be compared in our model to immune checkpoints whose normal function is to prevent excessive and uncontrolled immune responses. An extreme loss of control through a checkpoint modulation is already reported in the literature in 2006, when six healthy volunteers (within 90 minutes of receiving a single intravenous dose of an anti-CD28 antibody), all had a systemic inflammatory response, characterised by a rapid induction of proinflammatory cytokines and accompanied by headache, myalgias, nausea, diarrhoea, erythema, vasodilatation, and hypotension.[22]

Within hours, they became critically ill with pulmonary infiltrates and lung injury, renal failure, and disseminated intravascular coagulation. Severe and unexpected depletion of lymphocytes and monocytes from circulation occurred within 24 hours. All six patients were transferred to an intensive care unit, where they received intensive cardiopulmonary support. Despite evidence of the multiple cytokine-release syndrome, all six patients survived. Immune checkpoint modulation may thus be compared to releasing a brake on immune control. While interfering with CD28 was very poorly tolerated, similar actions on other checkpoints such as cytotoxic T lymphocyte antigen 4 (CTLA4) or PD-1 have been shown to be an advantage in the treatment of cancer patients.

Modulations – adaptations to the individual immune response settings

- Possible actions to avoid turbulence are to fly on a calm day. By analogy, only treat patients with a good Halabi score, or take DCs out of the compromised TME and grow them *in vitro*, and re-inject matured (finally differentiated) DCs that are loaded with the desirable antigen. A number of clinical trials using DCs have shown some activity in the clinic, leading to

the registration of Provenge.[23] A variant from this scheme is to expand T lymphocytes *in vitro* from the patient T lymphocytes which have already shown some ability to kill the patient's own tumour or natural killer T cells directed against a tumour antigen. Steve Rosenberg's laboratory has been a leader in that field and has done remarkable work for many years.[24]

- A good pilot may also make use of flaps and elevator trim to give some leverage in controlling crosswinds. Flaps may be equated to assistance by standard therapies in the control of Tregs and myeloid suppressors. It is documented that myelosuppressive chemotherapy[25] may improve the disturbances in the microenvironment, rendering it less tolerigenic. *In vivo* measures, other than myelosuppressive chemotherapy, to specifically block the differentiation of inflammatory macrophages may be considered in the future via specific blockade of the CSF1 receptor. CSF1 is a chemotactic cytokine for the recruitment of monocytes to an area of intense tissue turnover, and for their differentiation into macrophages. It has a major role in wound repair. We have previously shown that it modifies DC differentiation prior to the terminal differentiation step by tumour necrosis factor, while fully mature DCs will not be modified by CSF1.[13]
- Finally, we can use the notion of checkpoint modification as analogous to the balance of the centre of gravity of the immune response. Similarly, just as we carefully monitor the stability of an aircraft, we can stimulate activating receptors on T cells or block inhibitory receptors such as block interaction of PD1 with PD-L1, or expand T cell numbers with anti CTLA4. Establishing an immunological 'grade' for tumour stratification and therapeutic decision before treatment with vaccines +/- checkpoint inhibitors could be equivalent to the pilot doing preflight checks, particularly for weather conditions, prior to take-off.

Case History

While we are still some distance from curing patients with immunotherapy in a regular and controlled fashion, the way aspirin cures most headaches, there are occasional success stories which are worth exploring in depth. Ten metastatic breast cancer patients, who had previously progressed on chemotherapy, were treated in 1999 at Institut Curie, Paris, France, with the Transgene TG4010 vaccine product (containing the MUC1 gene + interleukin-2 in a Vaccinia Virus vector). Injections had been administered every 3 weeks. Two of ten patients achieved a partial response, which lasted 11 months for patient '204'. Patient '207', after surgical resection of residual disease remains in complete remission in 2014.[2] Injections were administered every 3 weeks. 15 years ago, we knew that patients with a 'healthy' immune response had on average >1,000 total peripheral lymphocyte counts, and that these have a tendency to drop during metastatic tumour progression, that immunosuppression (in relation to AIDS) was correlated with a low CD4 count in circulation, and that T effector cells were CD8+. We routinely evaluated CD4+ and CD8+ total counts in circulation, as well as serum CA153 marker and CSF1 levels.

Figure 1: Three-dimensional dynamics of anti-tumour immune response.

While treatment tolerance was excellent, patient 207 complained of increasing fatigue at 6-8 months, which led to a diagnosis of hypothyroidism. Retrospectively we tested all the stored serum samples (Table 1) showing that the increase in circulating CD4+ T cell counts paralleled the appearance of thyroid auto antibodies, a failing thyroid function, but most importantly, of tumour regression. Interestingly, CSF1 serum levels, an inflammatory macrophage differentiation, and survival factor were consistently low throughout.[26] The patient has been in complete remission for >14 years. It is striking that the unique single patient who developed signs of autoimmune thyroiditis in parallel with vaccination, which peaked with the maximal tumour shrinkage, achieved a durable remission and, very likely, a cure. Her MUC1 specific immune function could unfortunately not be tested due to a technical problem[26] but additional genomic testing of her tumour is presently ongoing. In the present case, the specificity of these CD4+ T cells is unknown, but in a recent publication, adoptive transfer of CD4+ T helper 1 cells, recognising a specific mutated epitope on cancer cells, was shown to be able to mediate a regression of metastatic epithelial lesions.[27]

Table 1: Quantitative changes in circulating PBMC of CD4/CD8 phenotype and rising autoantibodies during an effective vaccine based tumour regression.

Variations of CD4 levels of anti-thyroid and anti-nuclear antibodies, of thyroid function tests and breast cancer tumour marker CA153									
		Real-time assessment			Retrospective assessment				
					Antibodies				
Injection number	Date	**CD4 Fresh blood**	CD4/CD8 **Fresh blood**	CA153 serum	**Anti-TPO serum**	Anti-nuclear serum	Anti-DNA serum	T4 serum	TSH serum
		counts/ mm^3	ratio	U/ml	U/ml	inverse ratio	U/ml	ng/ml	ug/ml
BL	20th Jan 99	680		26	179	0	0		
1	28th Jan 99			23		0	0	10.7	1.18
2	18th Feb 99	908		18		0	0	10.3	1.94
3	11th Mar 99	1,160	4.7	18		0	0		
4	1st Apr 99	1,081	5.2	17		0	0		
5	17th May 99	1,172	5.6	16		0	0	12	2.92
6	28th June 99	1,305		18		80	14	15.2	2.23
7	9th Aug 99	1,224	4.7	17		160	15		
8	**20th Sept 99**	**1,444**	**5.5**	**18**	**11,529**	**320**	**13**	**5.8**	**51.29**
9	2nd Nov 99	1,345	3.5	18	11,052			11.9	9.14
10	13th Dec 99	966	4.7	18	6,667	260	260	12	0.97

PBMC: peripheral blood mononuclear cells; Anti-TPO: anti-thyroid peroxidase; T4: total thyroxine; TSH: thyroid stimulating hormone.

CONCLUSIONS AND PERSPECTIVES

While many isolated observations of tumour responses to immunotherapy do exist, and while some statistically significant successes in Phase III trials have been reported, we do not, at present, fully understand why some people respond and others do not; nor do we understand which clinical monitoring tools we could put in place to make immunotherapy a more controlled science. If the model of aviation holds true, then we would need to fulfil several conditions at the same time, such as the presence of tumour antigens, a favourable microenvironment, and an immune system geared towards T cell effector (lytic) activity. Modern techniques, allowing assessment of the likelihood of immune responsiveness by an immune activity signature, will be helpful for the selection of patients in a first instance.[17] To bring on board immunotherapy for all, we will need to not only add fuel, but also think about the microenvironment and checkpoint blockade. Whether it will take us another century to control immunotherapy - to expand and fold wings at leisure - remains to be seen.

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