Modelling the role of cytotoxic T lymphocytes in tumour regression

Beck, R.J.

Chapter 1

Introduction, aim and scope of the thesis

Summary

- Immunotherapies are an emerging treatment paradigm with potential application to many cancer types.
- Currently, only a subset of patients responds to immunotherapy. Moreover, only a subset of cancers are currently treatable with immunotherapies.
- Greater insight into T cell interactions with cancer will inform and improve immunotherapeutic strategies.
- In this thesis, mathematical and computational models are applied to *in vivo* or *in vitro* datasets containing measurements of T cells.
- By quantifying T cell interactions using models, this thesis aims to improve understanding of T cell behaviour and thus contribute to the rational design of immunotherapies.

Immunotherapies for cancer

The “immune surveillance” hypothesis was developed in the 1950’s and 1960’s in response to a number of studies which showed that mice could develop immunity to chemically induced tumours[1–3], as well as an increased understanding developed from homografts that the immune system could discriminate between cells which were native and non-native to the host[4]. The immune surveillance hypothesis was first proposed by Thomas Lewis[5] and later developed by Sir Macfarlane Burnet, who stated the following in 1964[6]:

“The phenomena manifested in homograft immunity, tolerance and the like are based on the existence of a process of immunological surveillance, which eliminates cells with surface antigenic structure recognizably different from that normal to the individual. Any carcinogenic process will be successful only if this control can be overcome: (i) by inhibition of the effector process of control which is presumed to be by the direct action of immunologically competent cells; (ii) by loss of any antigens recognizable as foreign; (iii) by the development of growth potential capable of overriding any immunological control.”

The immune surveillance hypothesis seemed to provide an explanation for several observations about the incidence of cancer in humans. Cancers occurred most frequently in the very young and old - when the immune system was just developing, or was in decline. Moreover, it had been noted that tumours occurred more frequently in patients with immune-deficiency disorders or those who had been administered immunosuppressive drugs[7]. In the following years a surge of interest followed, which is well exemplified by a rather pointed quote published in *Immunological Reviews* in 1971[8]:

“The theory of immunosurveillance of neoplasia is so well established that its further discussion and demonstration risk becoming rather boring. Any-one with the temerity to question its overriding importance is likely to be the subject of discrete but possibly well-deserved ridicule.”

A body of scientists willing to risk ridicule apparently existed, because by the mid 1970’s the immune surveillance hypothesis was under attack. Other explanations for increased cancer frequency in immune-compromised humans were given: for example it was considered that cancer and immune-deficiency disorders may share a common cause, or that perhaps immunosuppressive drugs may themselves have had carcinogenic effects[9]. Studies which claimed to have demonstrated immunogenicity of tumours in mouse models were also called into question. Most evidence came from either allograft, chemically induced, or virally induced tumours in mice. It was argued that the process of allografting may have potentiated an immune response[10], or that tumours of chemical or viral origin were abnormally immunogenic and thus unrepresentative of spontaneously arising tumours[11]. The discovery of the nude mouse, which lacked a thymus and therefore was severely deficient in mature thymus cells (T cells)[12], provided evidence against the immune surveillance hypothesis: Nude mice showed no deficits in their ability to control chemically induced tumours[13] and no enhanced frequency of spontaneous tumour formation[14]. The immune surveillance hypothesis fell from favour, since the prevailing wisdom at the time was that the immune response simply discriminated between “self” and “nonself” - cancers were “self”, thus not usually recognised by the immune system.

Several developments in the late 1980’s and the 1990’s led to renewed interest in the possibility that tumours could be recognised by the immune system. First, tumour infiltrating lymphocytes - isolated from human melanoma tumours and expanded *ex vivo* - exerted cytolytic activity against fresh melanoma cells[15]. Second, certain antigens were identified on tumour cells to which T cells reacted[16,17]. Third, perforin and interferon-γ (IFN-γ), known components of the immune system, were shown to be important in defending the host against tumorigenesis[18–21]. Fourth, tumours which developed in immunocompromised hosts were significantly more immunogenic[22], showing that tumours were sculpted by an immunogenic environment.

On the basis of these results, new theoretical frameworks were proposed. The “laws of lymphotics” gave an alternative perspective on the requirements for T cell responses - rather than simply responding to “self” or “nonself”, it was proposed that the immune system should respond instead to “danger”[23]. The idea was that T cells exist with the capacity to recognise a broad range of antigens, including those derived from the host. However, when a T cell meets an antigen-presenting cell there is a requirement for costimulatory signals in order for the T cell to become activated. If these signals are not supplied, tolerance is promoted instead. A refined version of the immunosurveillance hypothesis, the immunoediting hypothesis, was put forward to explain 1) why immunocompetent individuals experience cancer and 2) why most tumours are immunologically silent[24]. In the immunoediting hypothesis, nascent tumours are surveilled by the immune system and may be eliminated. However, the immune system exerts a strong selection pressure on tumours, so that any tumour which has progressed enough to be clinically detectable must have acquired features which allow it to evade an immune response. The knowledge that tumours are potentially immunogenic, but have evolved strategies to suppress

and evade an immune response, suggests that a broad range of cancers may be treatable if only the relevant immunosuppressive mechanisms can be identified and removed.

Today, research efforts focussed on immunotherapy aim to characterise the interaction between the immune system and malignancies, to identify factors which may be limiting the immune response, and to devise strategies to augment the immune response to tumours. These strategies can be contextualised and understood by considering the "cancer-immunity cycle"[25]. The cancer-immunity cycle is a modern framework proposed to describe the self-reinforcing process which occurs after recognition of a malignancy by the immune system. Tumour antigens arrive in the draining lymph nodes where they are sampled by dendritic cells and then presented to naive CD8+ T cells. When presented with cognate antigen in the presence of appropriate costimulatory signals, T cells become activated and undergo rapid clonal expansion. The resulting clones recognize the antigen which triggered the expansion, thus they can attack the tumour. After T cells infiltrate the tumour and begin to kill tumour cells, further tumour antigens are released, thereby reinforcing the immune response. Any of these steps in the cancer-immunity cycle may be defective and could represent a therapeutic target. In a recent series of publications attempting to define the clinical immuno-oncology landscape[26–28], immunotherapeutic strategies for cancer treatment were stratified into 6 categories:

1. **Cell therapies** utilise engineered T cells to attack cancer cells.
2. **T cell targeted immunomodulators** modify T cell activity by activating stimulatory receptors or inhibiting suppressive receptors expressed on T cells.
3. **Other immunomodulators** enhance immunity by modulating immune cells other than T cells (e.g. tumour associated macrophages).
4. **Cancer vaccines** *prime the immune system to respond to tumour-associated antigens[29]*
5. **Oncolytic viruses** selectively infect and kill tumour cells, resulting in subsequent engagement of the immune system at the site of the tumour[30].
6. **CD3 targeted bispecific antibodies** are designed to simultaneously engage tumour antigens and the T cell co-receptor CD3, thus redirecting the immune response towards a tumour[31].

Some of these strategies have now begun to see clinical success, and amongst the most successful have been a class of T cell targeted immunomodulators known as immune checkpoint inhibitors which function by suppressing inhibitory receptors expressed on T cells. Ipilimumab, an antibody targeting the inhibitory receptor cytotoxic-T-lymphocyte-antigen-4 (CTLA-4), was the first immune checkpoint inhibitor to be approved for treatment of advanced melanoma in 2011. A phase III study showed that median overall survival increased to 11.1 months in the study group treated with ipilimumab plus dacarbazine, compared with 9.1 months in the trial arm treated with dacarbazine plus placebo[32]. In another phase III study conducted at around the same time, ipilimumab was compared to the glycoprotein 100 vaccine and improved survival from 6.4 months to 10.1 months[33]. Since the approval of ipilimumab, other immune checkpoint inhibitors have been approved, most notably inhibitors for the programmed death receptor-1 (PD-1) and its ligand, PD-L1. The checkmate 067 trial (ClinicalTrials.gov Identifier: NCT01844505) initiated in 2013 tested ipilimumab and the PD-1 inhibitor nivolumab either as monotherapies or in combination. At the recently published five year follow up, median overall survival was 19.9

months in the ipilimumab treated group; 36.9 months in the nivolumab treated group, and had not been reached in the combination treatment group (thus was greater than 60 months)[34].

Despite the extremely encouraging results demonstrated by the marked improvement in median survival time in the checkmate 067 study, not all patients responded to the therapy. Patient response was graded according to the RECIST criteria[35] which has 4 categories: Complete Response (CR), where no evidence of the disease remains; Partial Response (PR), where there is a measurable reduction in disease burden and no new lesions; Progressive Disease (PD), where there is a greater than 50% increase in the size of any existing lesion or there are new lesions; Stable Disease (SD), where none of the three other criteria have been met. The best responses achieved for the ipilimumab plus nivolumab combination in the checkmate 067 study were[34]: CR - 22%; PR - 36%; SD - 12%; PD - 24%, thus a significant number of patients did not respond to treatment. Indeed, a significant outstanding question in the field of immunotherapy is whether biomarkers can be found which predict which patients are most likely to benefit from treatment. Another question is whether other combinations exist that might yield enhanced clinical benefit, for example other immune checkpoints such as TIM-3 and LAG-3 which are both under investigation in combination with PD-1 inhibitors[36]. Further, although most successful so far in melanoma, immunotherapies are currently also employed in many other other types of cancer e.g. bladder cancer[37] and there is work to be done to expand the scope of immunotherapies further to other types of tumour. Improvements in our understanding of the interactions of T cells with tumours will be vital for the rational design of immunotherapies.

The role of Computational Models

Theories provide an objective framework for interpreting experimental data[38]. An important feature of theories is that by logically following their consequences, predictions can be made. The iterative process of developing and revising theories, and then testing their consequences, is the basis for advancement of scientific knowledge. Therefore, theories are an indispensable component of the scientific method. Mathematical and computational models can be regarded as a class of theory, whose predictions are quantitative, specific, and precise. As such, mathematical modelling of the interaction between cancer and the immune system has an important role in guiding experimentation and generating new hypotheses. Mathematical modelling can incorporate processes believed to explain the dynamics of the system, and test whether these are indeed sufficient to explain what is actually observed. If the developed model can explain all the dynamics, the model is a cheap and convenient tool to study and predict the expected effect of different perturbations to the system. If the model cannot explain the system dynamics, new processes can be introduced into the model. In either case, the model should generate new and specific predictions which can be experimentally verified, in turn leading to new knowledge.

Computational Models of Cytotoxic T Cells

Computational models have been developed to address several of the obstacles facing the development of successful immunotherapies[39]. Examples are models which have identified patient specific parameters such as antigenicity[40] or tumour size[41] which might be predictive of response to treatment. Other models have been developed to identify optimal dosage and

scheduling for immunotherapies[42], or to identify promising combination strategies for immunotherapeutic treatments[43]. Different model formalisms are typically employed depending on the studied phenomena of interest. For example, for the modelling of homogenous cell populations ordinary differential equation (ODE) models are typically applied[40,43,44]. When modelling populations of cells which are spatially heterogeneous but homogeneous otherwise, partial differential equation (PDE) models are employed[45,46]. When heterogeneous populations of cells are under consideration, agent based models (ABM) are applicable[47,48]. Different types of ABM are typically employed depending on the granularity required to describe the phenomena of interest. Cells are often represented as entities on two-dimensional or three-dimensional lattices, with a set of rules specified to determine permissible interactions between individual entities. When only the spatial location of a cell is of interest each cell may be adequately described by assigning it only a single lattice site[49,50]. In contrast, when a more realistic description of the interaction between individual cells is desired[51,52], formalisms such as the cellular Potts model[53] may be employed in which many lattice sites are assigned to represent a single cell.

In this thesis, we develop ODE models and ABMs of Cytotoxic T Lymphocytes (CTLs), otherwise known as "killer T cells" or $CD8^+$ T cells. CTLs are key players in the immune response, since their specificity combined with their ability to form a long lasting memory holds promise for long lasting and highly targeted interventions. In broad terms, there are only two ways by which a tumour may conceivably be controlled. Tumour cells may either be killed, or their proliferation may be suppressed. This leads to a very simple calculus for describing the evolution of a tumour over time, when considering only the dynamics of the tumour:

$$\frac{dT}{dt} = (g - d)T, \qquad \text{Eq. 1}$$

where T is the number of tumour cells, g is the proliferation rate of the tumour cells, and d is the death rate of tumour cells. Noting that g and d are not necessarily constant and may depend on other cell types (see Eq's 2-3 below), the model is extremely general and can be adapted to a wide range of realistic scenarios, and assumes only that "tumour cells" can be clearly defined and separated from normal (non malignant) cells. The model can exhibit 4 different types of behaviour corresponding to biologically relevant scenarios and analogous to the RECIST criteria for evaluating tumour response to therapy[35]. If $g > d$, then tumour cells proliferate faster than they die, so the tumour is progressing analogously to the PD RECIST evaluation. When $g = d$, the proliferation rate of tumour cells is exactly matched by their death rate, analogously to the SD RECIST evaluation. In the case where $g < d$, tumour cells die at a rate greater than they proliferate, so the tumour is in a regressing state, analogously to the PR RECIST evaluation. Should the tumour remain in the regressing state for a sufficient duration, then $T \to 0$ and the tumour will be eliminated, corresponding to the CR RECIST evaluation.

In order to introduce CTLs into this calculus, our general strategy is to consider the growth and death rates of the tumour as functions depending on the presence of CTLs inside the tumour, which are denoted E (effectors) throughout this thesis:

$$g = f_g(E, \ldots), \quad \text{Eq. 2}$$

$$d = f_d(E, \ldots), \quad \text{Eq. 3}$$

thus f_g and f_d are functions representing the tumour growth and death rates (respectively), modified by the presence of CTLs. Our methodology is to study experimental data in which measurements of E and T are available or can be estimated. We will then attempt to determine forms for the functions f_g and f_d which are capable of matching the measurements made from the experimental data, subject to any other constraints which can be placed on the model. The resulting models should contain the minimum possible set of elements required to describe a given set of observations. Thus we will be able to test whether known interactions are minimally sufficient to quantitatively describe tumour progression in the presence of CTLs. We will also be able to assess, among a group of interactions, which play the greatest role in control of a tumour. Finally, if known interactions do not appear to be consistent with observed dynamics, our models will provide insights into the type of interactions which might explain the data, which will lead to new hypotheses and directions for experimental work.

Research questions

Although the mathematical framework we have just established is simple, within it there lies scope for considerable complexity due to the plethora of pathways through which CTLs may be able to modify the proliferation or death rate of tumour cells. Within this scope, a number of specific research questions can be identified which will be addressed in this thesis. Below, the background of these questions is discussed, after which a thesis outline is provided.

How can the rate at which CTLs kill target cells be quantified and what is the rate at which CTLs kill tumour cells?

The canonical function of CTLs is their ability to recognise and kill antigen presenting targets. CTLs are able to do this in a number of ways: secretion of the cytotoxic perforin and granzyme molecules towards the target cell membrane[54,55], induction of death via Fas-ligand[20,56,57], or release of soluble factors such as tumour necrosis factor which may facilitate target cell death[58]. Although the ability to directly kill antigen presenting cells is perhaps the most well recognised function of CTLs, quantifying this behaviour may be difficult. For example, it has been reported that CTLs can require multiple hits to kill target cells[59,60] (the 'multiple-hitting hypothesis'), which can influence the dynamics of the killing process[51,61] and thus may hamper accurate determination of the underlying killing rate of the CTLs. In this thesis we devote substantial effort to characterising the killing rate of CTLs.

How important is the contribution of CTL mediated killing towards control of tumours?

In addition to the “direct” means of killing tumour cells discussed in the previous paragraph, the arrival of CTLs at the tumour may lead to further downstream events which increase the rate at which tumour cells die - for example by recruitment of innate effectors into the tumour which go on to kill tumour cells[62]. Thus in this thesis we aim to quantify the importance of direct killing of tumour cells by CTLs, and ask whether such killing is sufficient to account for the reduction in tumour growth following adoptive transfer of CTLs.

How important are the antiproliferative effects that CTLs exert upon tumour cells?

In addition to increasing the rate at which tumour cells die, there are also reported means by which CTLs may alter the growth rate of tumour cells. Activated CTLs secrete interferon-γ, which has an antiproliferative effect on some tumour cells[63–65]. Additionally, the presence of CTLs inside the tumour has been linked to destruction of tumour vasculature which should exert an antiproliferative effect on tumour cells by depriving them of nutrients required for proliferation[66]. Thus, in this thesis we aim to identify and quantify the importance of antiproliferative effects exerted by CTLs upon tumour CTLs towards tumour regression.

What is the effect of CTL stimulation on their *in vivo* functionality?

Since there is significant clinical interest in modulating the functions of CTLs to improve their anti-tumoural potential, we also ask how CTL functions could be modulated *in vivo*. In this thesis, we address that question in two ways. First, we study how CTL functions are modified after administration of a stimulating compound. For this we analyse a series of experiments wherein rates relevant for various aspects of the CTL:tumour interaction (i.e. CTL and tumour cell apoptosis/mitosis rates) are recorded in the presence or absence of agonist antibody anti-CD137. Initial clinical trials of such antibodies as a potential immune stimulatory therapy led to liver damage due to an inflammatory response in that organ, yet modified approaches that aim to target CD137 agonists specifically to the tumour are ongoing, e.g., by using bispecific constructs [67,68].

What is the contribution of immune checkpoint molecules towards CTL exhaustion?

In our second approach to understanding potential for modulating CTL function, we studied the development of the “exhausted” phenotype among adoptively transferred CTLs. CTL exhaustion is characterised by a progressive loss of effector function alongside upregulation of inhibitory receptors among chronically stimulated populations of CTLs[69–71]. CTL exhaustion is currently of particular relevance due to the large number of immune checkpoint inhibitors currently being explored as immunotherapeutic strategies which aim to inhibit suppressive receptors expressed on CTLs and thereby reinvigorate exhausted CTLs. In this thesis we examine how CTL effector functions in vivo are diminished as the expression of several well known immune molecules increases, in order to characterise the contribution of these different immune checkpoints towards CTL exhaustion.

Thesis outline

In the first section of this thesis (chapter 2), we study the killing behaviour of individual CTLs using stochastic models. Moreover, we develop statistical procedures which could be used to test for the multiple hitting hypothesis in future. In the second section of the thesis (chapters 3-5), we apply models to various *in vivo* datasets where CTLs were observed after adoptive transfer into tumours. By integrating data from different modalities to estimate values for key parameters which should determine tumour progression (e.g. the killing rate of CTLs and the proliferation rate of the tumour), we investigate the relationship between the estimated parameters and the progression of the tumours. This thesis concludes with a discussion of our findings, limitations of the work, and future research directions (chapter 6).

In chapter 2, we develop stochastic models of individual CTLs in order to better characterise the expected killing kinetics of multiple hitting CTLs. With the aid of these models, we re-examined a previously published *in vitro* dataset where CTLs were confined with antigen presenting targets and their killing kinetics were monitored over a period of 12 hours. In that dataset, the killing kinetics of the CTLs could not be explained by existing models. Therefore, a subpopulation of "high rate killer" CTLs had been invoked to explain the kinetics, despite the fact that no other evidence could be provided for such a hypothesis. Applying our models to this data, we show that the multiple hitting hypothesis was sufficient to account for the unexplained CTL kinetics, without any requirement to invoke a subpopulation of "high rate killer" CTLs. Moreover, we developed statistical procedures to be used for identification of multiple hitting CTLs in imaging data, and suggested experimental strategies for determining the presence of multiple hitting in future experiments.

In chapter 3, we study progression of a murine thymoma after adoptive transfer of CTLs. We parameterised spatial and nonspatial models with estimates of tumour proliferation rate, CTL killing rate, and estimates of the density of CTLs inside the tumours. In doing so, we showed that the reported killing rate of the CTLs was insufficient to account for the tumour regression that occurred in the experimental data. After also investigating whether uncertainties in the killing estimate due to multiple hitting could account for the apparent insufficiency in killing, we found that the discrepancy between the estimated versus observed rates of killing were too large to permit this explanation. Using a spatially explicit agent based model, we showed how an antiproliferative effect exerted by CTLs on the tumour could account for the discrepancy.

In chapter 4, we develop ordinary differential equation models applied to an experimental murine model of B16F10 melanoma. In these experiments, CTLs were adoptively transferred to melanoma bearing mice in the presence or absence of a stimulating antibody targeting the CD137 receptor. Our analysis revealed an extremely low killing rate of CTLs, and our models demonstrated that such a low killing rate combined with relatively low infiltration of CTLs should not have any important impact on tumour progression whatsoever. We also investigated the mechanisms underpinning the reduced rate of tumour progression in mice treated with CTLs alongside the stimulatory CD137 antibody. We found that CD137 antibody stimulation did not enhance the killing of transferred CTLs, but rather found that an improved antiproliferative effect or enhanced recruitment of CTLs to the site of the tumour was most compatible with the data.

In chapter 5, we again apply ordinary differential equation models applied to another experimental murine model of B16F10 melanoma following adoptive transfer of CTLs. In this series of experiments, a Fucci sensor was used allowing tracking of melanoma cells through the cell cycle. Additionally, transcript data was available to quantify the production of IFN-γ inside the tumour. These data allowed us to refine the models developed in chapters 3-4 to include an explicit description of the cell cycle and the effect of IFN-γ thereupon. The results obtained with this second B16F10 dataset agreed with those in chapter 4, i.e. an extremely low killing rate of CTLs meant that the IFN-γ mediated antiproliferative effect of CTLs had the most substantial effect on tumour progression. Moreover, we found evidence of the development of an exhausted state amongst the tumour infiltrating CTLs, and using transcriptomics data we characterised the immune checkpoint molecules which best defined the development of the exhausted state amongst tumour infiltrating CTLs. This thesis concludes with a discussion of our findings, limitations of the work, and future research directions (chapter 6).

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