Modelling the role of cytotoxic T lymphocytes in tumour regression

Beck, R.J.

Chapter 6

Discussion

Summarizing discussion

In this thesis we have studied the effector functions of cytotoxic T lymphocytes in various *in vitro* and in vivo experiments, with emphasis on the role of CTLs as mediators of immunotherapies for cancer. To study CTL effector functions, we analysed experimental data in order to derive statistics and parameters informative about CTL function. Moreover, using these parameters, we developed mechanistic models and studied them in order to obtain insights about the behaviour of CTLs. In the following sections, we revisit the questions originally posed in the introduction to this thesis.

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

In chapter 2, we used stochastic models and bayesian inference to study the expected kinetics of CTL killing when observed under the microscope. In particular, we sought to define the measurements required in order to accurately determine the killing rate of cells based on imaging data. Early models treated CTL cytotoxicity as a Poisson process[1], but others have suggested that multiple hits from CTLs may be required in order to effectively lyse target cells (i.e., the multiple-hitting hypothesis)[2–4]. We found that the multiple hitting hypothesis was compatible with recent unexplained observations of non-Poisson killing dynamics for CTLs *in vitro*[5], and established a bayesian inference procedure which could be applied to test the multiple-hitting hypothesis. We also determined that the ability to track the contact history of CTLs with individual target cells was a requirement for accurate assessment of multiple hitting based on imaging data.

We had data available from three different cell lines with which we could address the question of CTL killing rate. In an *in vitro* assay in which CTLs killed Epstein-Barr virus transformed B cells presenting the pp65 peptide[5] (chapter 2), CTLs killed with an average rate of k=8 (kills CTL^{-1} day^{-1}). In an *in vivo* setup employing EL4 lymphoma[6] (chapter 3), CTLs killed with an average rate of k=4 (kills CTL^{-1} day^{-1}). In *in vivo* experiments using the B16F10 melanoma cell line[7] (chapter 4), CTLs killed with an average rate of k=0.75 (kills CTL^{-1} day^{-1}). These estimates for CTL killing rates towards tumour cells are comparable to other reports in the literature. In another murine experiment we have analysed[8] (not included in this thesis), CTLs were estimated to kill B cell lymphoma cells in the bone marrow at a rate of k=4.8 (kills CTL^{-1} day^{-1}). Another study using the B16F10 melanoma cell line reported different rates of killing depending on the site of injection[9], with k=1.24 (kills CTL^{-1} day^{-1}) when melanoma cells were injected into the liver, but k=3.18 (kills CTL^{-1} day^{-1}) when cells were injected into the spleen. Overall, in this thesis we have studied the killing rate of CTLs in two tumour cell lines *in vivo*, as well as one cell line in *an vitro* setting. Moreover, we have laid out a framework for analysing the killing kinetics of CTLs in imaging data in future.

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

An important focus of this thesis was to assess the sufficiency of CTL killing to account for tumour control. In chapter 3, we investigated *in vivo* experiments employing the EL4 lymphoma cell line which underwent rapid regression following adoptive transfer of CTLs[6]. For these EL4 tumours, conflicting reports existed in the literature regarding the mechanisms used by adoptively transferred CTLs to eliminate the tumours. Imaging of mixed tumours, containing both patches of antigen-positive and antigen-negative tumour, showed selective elimination of antigen-positive regions after CTL transfer[6]. This result from mixed tumours suggested an important role for contact dependent, antigen specific recognition and destruction of tumour cells by the transferred CTLs. However, another study using the same EL4 tumour cell line showed that perforin and FAS-L doubly deficient T cells were not significantly compromised in their ability to control the same tumours[10]. Since either perforin or FAS-L are expected to be required for contact dependent killing, effective tumour control in the absence of both mechanisms seemed incompatible with the imaging study using mixed tumours.

Estimates for the killing rate of CTLs were available from two photon imaging of the regressing EL4 tumours, with a value for the killing rate of k=4 (kills CTL^{-1} day^{-1}). Thus, we asked whether the observed CTL killing rate was sufficient to explain the rapid tumour regression which was also reported. To address this question we developed both an ordinary differential equation model and a spatially explicit agent based model, to describe CTL killing inside the tumours. We found that the observed rates of CTL killing were well below those required to explain the rapid tumour regression evident in the data. This was true even when we simulated multiple-hitting CTLs, for which the associated variable killing rate over time[11] (chapter 2) could have implied that the reported killing rate (k=4 kills CTL^{-1} day^{-1}) was an underestimate due to imaging being performed at an early time point. However, the discrepancy between the measured killing rate and the killing rate we predicted necessary to result in tumour regression was too large to be accounted for by multiple-hitting.

In chapters 4-5, we studied two datasets derived from the B16F10 melanoma model after adoptive CTL transfer. The different datasets were generated by two different experimental groups, with each group having adopted a slightly different experimental setup. In one series of experiments[7] (used in chapter 4) tumours were injected and then observed by means of long (1-4 hours) three-dimensional two-photon microscopy imaging through dorsal skinfold windows implanted into the mice. CTLs recognised ovalbumin (OVA) expressed by the B16F10 melanoma cells. Data from this series of experiments had the advantage that direct measurements of the killing rate of tumour cells by CTLs could be made (k=0.75 kills CTL^{-1} day^{-1}), along with measurements of the mitosis rate of tumour cells. However, the presence of the imaging windows limited the size of tumours that could be studied and it was unclear whether the presence of the windows influenced the growth of the tumours or the response of the CTLs.

Complementing these data were data from other experiments[12] (used in chapter 5) using the B16F10 melanoma cell line, except with pmel-1 transgenic T cells recognising the gp100 peptide expressed on the tumour cells. In this series of experiments, *ex vivo* cryosections rather than an

imaging window was used, permitting study of larger tumours but having the disadvantage that no direct measurements of CTL killing rate or tumour mitosis rate were available. Nevertheless, using model fitting we were able to infer values for these parameters from the available data. In these data, immunofluorescence imaging of the cryosections was used to quantify the number of tumour infiltrating CTLs. Moreover, in combination with the Fucci cell cycle reporter, the *ex vivo* sections allowed discrimination between tumour cells in the G_1 phase of the cell cycle and tumour cells in other (S-G_2-M) phases, which made it possible to estimate the rate of tumour cell mitosis. Since we could infer the tumour mitosis rate from the ratio of tumour cells in G_1:S-G_2-M phases, we were able to make an estimate of the CTL killing rate: we did this by asking how much additional CTL killing, in addition to the reduced mitosis, would be needed in order to explain the volumetric progression of the tumours. By comparing the estimate of tumour cell mitosis to the volumetric progression of the tumours, we inferred a value for the killing rate of the CTLs, i.e., k=1 kill CTL^{-1} day^{-1}. Interestingly, in spite of the different antigen (gp100) recognised by CTLs in these experiments, this killing rate estimate was consistent with the experiments where CTLs recognised the OVA antigen (chapter 4), suggesting that the exact antigen recognised by CTLs does not have a major impact on the rate at which they can kill target cells.

We developed ODE models to describe the data generated by each set of B16F10 experiments. Despite the differences in experimental settings, our analysis and modelling of each set of data gave consistent conclusions: in each case the cytotoxic function of CTLs had only a small effect on tumour progression. Thus our results from studying the B16F10 melanoma cell line (chapters 4-5) were consistent with those obtained in our study of the EL4 lymphoma cell line (chapter 3), i.e. CTL killing was insufficient to account for the majority of the reduction in tumour size or progression concomitant with the adoptive transfer of CTLs.

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

Since in each of the *in vivo* experiments studied (chapters 3-5) we found that CTL mediated killing at the reported or inferred rates was insufficient to explain the extent of the tumour regression reported, we asked in each case to what extent an antiproliferative effect of the transferred CTLs could explain the data. In chapter 3, we used our spatial agent based model, but applied a constraint that our model should also be able to describe the results from mixed EL4 tumours where selective destruction of antigen expressing cells was observed. We found that including a mechanism whereby CTLs secrete a soluble cytokine with antiproliferative effects on tumour cells would allow us to simultaneously describe all the available data. Our model suggested that the antiproliferative effect of the cytokine was quantitatively more important, due to the ability of CTLs to control the proliferation of many tumour cells, given that the antiproliferative effect is mediated by a soluble molecule which can diffuse away from CTLs. This would explain how double knockout of perforin and FAS-L had little impact on tumour control, as shown experimentally in an earlier study[10]. Nevertheless, our model also explained the selective destruction of antigen expressing cells in mixed tumours, which occurred for two reasons. Firstly, the antiproliferative effect would affect the OVA-expressing cells more strongly than non-OVA-expressing bystander cells, due to the selective localisation of CTLs leading to high concentrations of cytokine in areas inhabited by antigen expressing tumour cells. Second, although the reported CTL killing rates were quite low, they eventually led to appreciable reduction in the number of tumour cells, provided the tumour

cells could not proliferate. Together these two effects impose a strong selection pressure on the tumour cells, explaining the selective elimination of antigen expressing cells.

A limitation of our study in EL4 tumours (chapter 3) was lack of any direct data concerning the proliferation rate of tumour cells. This limitation was addressed in chapters 4-5, since in both datasets estimates of tumour cell mitosis were available. By examining hypothetical deviations in the estimated rates of tumour cell mitosis after adoptive transfer of CTLs, we were able to make inferences about the impact of CTL transfer on the proliferation rate of the tumour cells. After incorporating these data into our ODE models, we saw in each case the mitosis rate of tumour cells was substantially diminished concomitant with the presence of the CTLs. Indeed, we found that the reduced mitosis rates after CTL transfer were quantitatively sufficient to account for the majority of reduced volume progression. Overall, we again found that the cytotoxic function of CTLs had a negligible effect on tumour progression when quantitatively compared with the antiproliferative effect. Thus in all the *in vivo* data we studied, we reached the conclusion that an antiproliferative effect associated with transferred CTLs is more important than any direct killing of tumour cells by CTLs.

Since we identified a potential antiproliferative effect of CTLs as having a large effect on tumour progression, it is important to understand the mechanisms through which such an effect occurs. Based on reports that the CTL secreted cytokine interferon-γ (IFN-γ) can cause cell cycle arrest[12–14], and also because the antiproliferative effect of IFN-γ was explicitly explored and confirmed in one of the studies we used to develop our models[12] (chapter 5), it seems that the cytokine IFN-γ is an important mediator of the antiproliferative effect in the B16F10 cell line. Although IFN-γ was shown to have no effect on the proliferation of EL4 cells *in vitro*[12], when we simulated a similar antiproliferative effect from IFN-γ in our spatial ABM (chapter 3) we were able to describe the time course of the EL4 lymphoma tumour size. This result suggests that there may be other means, in addition to IFN-γ mediated cell cycle arrest, by which transferred CTLs can reduce the proliferation of tumour cells. There are plausible mechanisms by which this could occur - for example IFN-γ promotes expression of the chemokines CXCL9, CXCL10, and CXCL11, which all have angiostatic effects[15]. As another example, nitric oxide is secreted by stromal cells after exposure to IFN-γ[16] and can reduce proliferation of EL4 cells *in vitro*. Due to the importance of antiproliferative effects of CTLs suggested and highlighted by our models, an important focus of future work should be to clarify and quantify these effects.

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

In addition to quantifying the relative importance of killing and antiproliferative effects of CTLs towards tumour regression in the B16F10 tumours, additional data available from the studies used in chapters 4-5 allowed us to further investigate other aspects of CTL function. In the series of experiments where CTLs were observed through dorsal imaging windows using two photon microscopy (chapter 4), an additional experimental condition was studied in which CTLs were adoptively transferred in the presence of an agonist antibody targeting the costimulatory CD137 receptor[7]. CD137 is expressed on both innate and adaptive immune cells and stimulation of $CD8^+$ T cells via the CD137 receptor may improve their proliferation and resistance to apoptosis[7,17,18]. In the data we studied[7], several differences had already been observed in the CD137 stimulated

condition compared to the unstimulated condition: increased tumour cell apoptosis and reduced tumour cell mitosis after CD137 stimulation, alterations in the apoptosis and mitosis kinetics of the CTLs, and improved tumour control in the CD137 stimulated case. Moreover, CD137 stimulation led to increases in expression of molecules related to enhanced CTL cytolytic function, such as the transcription factors T-bet and eomesodermin[7]. It was therefore suggested that CD137 improved the *in vivo* killing capacity of the transferred CTLs. However, there had been no attempt to integrate these differences into a coherent model and thus it was not clear which of the differences in dynamics at the cellular level accounted for enhanced tumour control. Thus, we utilised our ODE model describing the behaviour of the CTLs inside the tumour, and fit our model separately to each condition in order to explore differences in the fitted model parameters and resulting dynamics between the CD137-stimulated and non-CD137-stimulated CTLs. With our modelling approach we found that enhanced antiproliferative effects of the CTLs at the site of the tumour were likely to account for the majority of the improvement in tumour control after stimulation with the CD137 targeted agonist antibody. Thus our overall conclusion was that stimulation of T cells via the CD137 axis did not have an appreciable impact on their cytolytic function *in vivo*. Rather, the most important impact of stimulation upon CTL function was an improvement in their ability to prevent tumour cells from proliferating.

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

In chapter 5, we exploited the availability of gene expression data at multiple time points for the B16F10 melanoma tumours to derive further insights about the behaviours of the tumour infiltrating CTLs[12]. Interestingly, according to the expression data, IFN-γ signalling decreased whilst CTLs were still present in the tumour, indicating a loss of function for the CTLs. By examining the gene expression data for candidate explanations for these dynamics, we found upregulation of several immune checkpoint molecules which suggested the development of an exhausted CTL phenotype[19–21] as a potential explanation for the loss of effector function. Thus we included CTL exhaustion in our model, in which the effector functions of the CTLs were reduced as the levels of the immune checkpoint transcripts increased. Our model showed that such a mechanism of CTL exhaustion was indeed able to account for the observed CTL and tumour dynamics. We also used our model to compare between different checkpoint molecules to identify which might be the most important determinants of the exhausted CTL state in the setting with B16F10 tumours. Interestingly, we found that the PD-1/PD-L1 axis alone was not compatible with the exhausted state, since according to the transcript data these molecules were only transiently expressed and therefore not able to explain the progressive deterioration in CTL production of IFN-γ and the progressive reduction of CTLs inside the tumour. Instead, we found that LAG3 and TIM3 were most consistent with being determinants of CTL exhaustion in B16F10 tumours. Taken together these results suggest that immune checkpoint molecules do contribute towards CTL exhaustion, however expression of PD-1 and its ligand PD-L1 do not appear to be sufficient to explain the exhausted state. Other molecules such as TIM3 and LAG3 likely also play a role and are candidates for further study.

Perspectives

In the following sections, some limitations of the work presented in this thesis are discussed. Suggestions are made for how some of those limitations might be addressed in future work, and possible avenues for extending the work in this thesis are presented.

Importance of killing in other types of tumour

Since the ability of CTLs to recognise and kill antigen presenting target cells is their most well known function, it was perhaps surprising to discover that our models predicted a negligible contribution of CTL mediated killing of tumour cells in two different *in vivo* tumour cell lines studied in this thesis (EL4 lymphoma, B16F10 melanoma). The relative importance of CTL mediated killing versus an antiproliferative effect of CTLs upon tumour cells is likely to depend on the characteristics of the tumour in question. For example, in slow-growing tumours one might expect the killing of CTLs to have a large relative contribution, simply because in such tumours there is little mitosis to suppress. This observation highlights one limitation of our studies, which is that they were based on extremely rapidly growing experimental tumour models, with growth rates of 0.4-0.9 day^{-1} corresponding to tumour cells undergoing mitosis every 1-2 days. These are not necessarily reflective of the tumour growth rates found in human cancer patients. A recent study comparing growth rates in five cohorts of human cancer patients with different types of cancer[22] found much lower growth rates, with doubling times ranging from 70-3050 days. Therefore, although our findings relating to the importance of an antiproliferative effect associated with CTL infiltration will certainly help to interpret and contextualise findings based from studies using preclinical mouse models, it is unclear whether they will also hold true in human tumours with much slower growth.

Due to the difficulties inherent in obtaining data from human cancer patients, it is important to find other ways to address the question on the contribution of killing and antiproliferative effects to tumour control, for example by using murine tumours (although it remains important to find ways to link data from animal studies to human patients - see section below on extension to human studies). A starting point would be to characterise the importance of an antiproliferative effect in murine tumours with a broader spectrum of growth rates. In one experiment we have analysed (not included in this thesis) where B cell lymphomas grew in the bone marrow of mice, the estimated doubling time of the tumour was 14 days[8]; there we found that tumour eradication was entirely consistent with the estimated killing rate of the CTLs, although we could not exclude the possibility that cell cycle arrest may have also played a role in the control of those tumours. Future work should therefore continue to analyse additional experimental tumours with a broad spectrum of growth rates in order to clarify whether the relative importance of an antiproliferative effect compared to CTL killing is indeed dependent on tumour growth rate.

Improved quantification of CTL killing

Another avenue for future work centres on the quantification of tumour cell death in the presence of CTLs. In chapter 2 we addressed how a requirement for multiple hits for CTLs to kill tumour cells might confound estimates of the CTL killing rate. This is relevant because some evidence

suggests that melanoma cells require multiple hits for annihilation by CTLs[3]. Moreover, as has been pointed out previously[11] and in chapter 2 of this thesis, multiple-hitting should lead to a variable observed rate at which CTLs kill target cells, which depends on the number of hits targets have already received. Our investigation of the requirements for quantifying the killing rate of CTLs based on microscopy data in the presence of multiple-hitting highlighted the importance of studying the interaction history of CTLs with tumour cells. By studying the risk of tumour cell death as a function of time spent in contact with CTLs, one can very directly assess whether target cells' risk of dying increases after having spent a significant time in contact with the CTL, or whether targets instead face a constant risk of death whilst in contact with a CTL. Although in chapter 2 we focussed on characterising multiple hitting, the principle of studying target cell risk of death in the presence of CTLs has more general applications. For example, it has been suggested that tumour infiltrating CTLs recruit innate effector immune cells and that these play a significant role in killing tumour cells[10]. In addition, cytokines secreted by CTLs may also increase the risk of death experienced by targets not directly contacted by CTLs[10,23,24]. These questions could be addressed directly by looking at the risk of tumour cell death in the absence of CTL transfer, and comparing it to the risk of death experienced by uncontacted tumour cells in tumours following adoptive CTL transfer. If innate effectors or CTL-secreted cytokines are indeed relevant, one should expect an increased risk of death even amongst uncontacted tumour cells following adoptive CTL transfer, with risk of death likely varying as a function of distance to the CTLs, since uncontacted tumour cells ought to be at enhanced risk of being killed by the innate effectors or cytokines. Future work applying similar analysis as presented in chapter 2 of this thesis to a variety of imaging datasets will thus be useful to better understand how the presence of CTLs influences target cell's risk of death in various contexts.

Modulation of CTL effector functions

The models and approach developed in this thesis establish a quantitative baseline for several important aspects of CTL effector function in tumours. We established dynamical equations and estimated the rates of several important functions, such as CTL killing of tumour cells, changes to the mitosis rate of tumour cells following adoptive CTL transfer, and also the dynamics of the CTL populations. Although our modelling approach is clearly a simplification, in which we reduce the complex dynamics occurring inside tumours to just a few equations, this is already a substantial refinement on existing frequently used methods of quantifying CTL performance, like measuring the volume progression of tumours after CTL transfer. As such, our modelling approach is highly useful for studying the effect of different modulators of CTL function. We studied costimulation of CTLs via the CD137 receptor, however there are a number of other modulators which similarly stimulate the priming and activation of CTLs. For example, OX40 (CD134), CD27, and CD28 are similar to CD137 in that they are all members of the tumour necrosis factor family of receptors, they all provide costimulatory signals to T cells, and they are all linked to enhanced T cell functions[25–27]. Thus these molecules are also candidates for future study using similar methods as those outlined in this thesis.

Extension to human studies

In a previous section (Importance of killing in other tumour models) we discussed the representability of our results based on murine models and in particular whether they might be relevant to human cancer patients. The ultimate aim of our research is to provide insights that might inform strategies for treating human cancer patients, therefore it is important to explicitly seek ways to link research in murine models to the human patient. Recent studies have examined biopsies from patients undergoing immunotherapy, to identify mechanisms underlying success or failure of treatment[28], or identify biomarkers predicting which patients are likely to respond to a particular immunotherapy [29,30]. Examples of biomarkers which have been repeatedly associated with response to immunotherapies are the tumour mutational burden[31,32], cytotoxic gene signature[33,34], or density of infiltrating immune cells[35,36]. However, the predictive value of such biomarkers and their applicability to different types of cancer is debated.

Mathematical and computational models such as those developed in this thesis are appropriate tools for linking observations in murine models to human data. A prerequisite for making this comparison is the development of mathematical and computational models which can be defined in terms of measurements which can feasibly be made in human patients. For many of the variables used in our models this should be feasible. For example, all our models rely on quantification of the frequency of tumour infiltrating CTLs, which can be obtained from tumour biopsies using immunofluorescence techniques. Given our findings that tumour infiltrating CTLs may have a potent antiproliferative effect on tumour cells (chapters 3-5), it would also be of interest to determine whether such antiproliferative effects occur and are important in cancer patients receiving immunotherapy. Single cell sequencing of cell populations from human tumours is a possible means of testing this finding[37], since cell cycle markers might reveal differences in the proportion of tumour cells in various cell cycle stages before and after immunotherapy (in case longitudinal samples from the same patients were available), or between immunotherapy treated and untreated patients (in case longitudinal samples from the same patients were not available). Future work should thus aim to validate our findings about the relevance of antiproliferative effects of CTLs, by searching for evidence of decreased tumour cell mitosis and arrested cell cycle in data from immunotherapy patients.

1. Perelson, A. S. & Macken+, A. Kinetics of Cell-Mediated Cytotoxicity: Stochastic and Deterministic Multistage Models. *Math. Biosci.* **70**, 161–194 (1984).

2. Gadhamsetty, S., Marée, A. F. M., Beltman, J. B. & de Boer, R. J. A general functional response of cytotoxic T lymphocyte-mediated killing of target cells. *Biophys. J.* **106**, 1780–1791 (2014).

3. Caramalho, I., Faroudi, M., Padovan, E., Müller, S. & Valitutti, S. Visualizing CTL/melanoma cell interactions: multiple hits must be delivered for tumour cell annihilation. *J. Cell. Mol. Med.*

13, 3834–3846 (2009).

4. Halle, S. *et al.* In Vivo Killing Capacity of Cytotoxic T Cells Is Limited and Involves Dynamic Interactions and T Cell Cooperativity. *Immunity* **44**, 233–245 (2016).

5. Vasconcelos, Z. *et al.* Individual Human Cytotoxic T Lymphocytes Exhibit Intraclonal Heterogeneity during Sustained Killing. *Cell Rep.* **11**, 1474–1485 (2015).

6. Breart, B., Lemaître, F., Celli, S. & Bousso, P. Two-photon imaging of intratumoral CD8+ T cell cytotoxic activity during adoptive T cell therapy in mice. *J. Clin. Invest.* **118**, 1390–1397 (2008).

7. Weigelin, B. *et al.* Focusing and sustaining the antitumor CTL effector killer response by agonist anti-CD137 mAb. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 7551–7556 (2015).

8. Cazaux, M. *et al.* Single-cell imaging of CAR T cell activity in vivo reveals extensive functional and anatomical heterogeneity. *J. Exp. Med.* jem.20182375 (2019).

9. Liu, L., Dai, B., Li, R., Liu, Z. & Zhang, Z. Intravital molecular imaging reveals the restrained capacity of CTLs in the killing of tumor cells in the liver. *Theranostics* **11**, 194–208 (2021).

10. Hollenbaugh, J. A., Reome, J., Dobrzanski, M. & Dutton, R. W. The rate of the CD8-dependent initial reduction in tumor volume is not limited by contact-dependent perforin, Fas ligand, or TNF-mediated cytolysis. *J. Immunol.* **173**, 1738–1743 (2004).

11. Gadhamsetty, S., Marée, A. F. M., Beltman, J. B. & de Boer, R. J. A Sigmoid Functional Response Emerges When Cytotoxic T Lymphocytes Start Killing Fresh Target Cells. *Biophys. J.* **112**, 1221–1235 (2017).

12. Matsushita, H. *et al.* Cytotoxic T lymphocytes block tumor growth both by lytic activity and

IFNγ-dependent cell-cycle arrest. *Cancer Immunol Res* **3**, 26–36 (2015).

13. Harvat, B. L., Seth, P. & Jetten, A. M. The role of p27Kip1 in gamma interferon-mediated growth arrest of mammary epithelial cells and related defects in mammary carcinoma cells. *Oncogene* **14**, 2111–2122 (1997).

14. Chin, Y. E. *et al.* Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. *Science* **272**, 719–722 (1996).

15. Keeley, E. C., Mehrad, B. & Strieter, R. M. Chemokines as mediators of tumor angiogenesis and neovascularization. *Exp. Cell Res.* **317**, 685–690 (2011).

16. Hollenbaugh, J. A. & Dutton, R. W. IFN-γ Regulates Donor CD8 T Cell Expansion, Migration, and Leads to Apoptosis of Cells of a Solid Tumor. *The Journal of Immunology* **177**, 3004–3011 (2006).

17. Yonezawa, A., Dutt, S., Chester, C., Kim, J. & Kohrt, H. E. Boosting Cancer Immunotherapy with Anti-CD137 Antibody Therapy. *Clin. Cancer Res.* **21**, 3113–3120 (2015).

18. Makkouk, A., Chester, C. & Kohrt, H. E. Rationale for anti-CD137 cancer immunotherapy. *Eur. J. Cancer* **54**, 112–119 (2016).

19. Wherry, E. J. T cell exhaustion. *Nat. Immunol.* **12**, 492–499 (2011).

20. Wherry, E. J. & Kurachi, M. Molecular and cellular insights into T cell exhaustion. *Nat. Rev. Immunol.* **15**, 486–499 (2015).

21. Blank, C. U. *et al.* Defining ‘T cell exhaustion’. *Nat. Rev. Immunol.* **19**, 665–674 (2019).

22. Talkington, A. & Durrett, R. Estimating Tumor Growth Rates In Vivo. *Bull. Math. Biol.* **77**, 1934–1954 (2015).

23. Selleck, W. A. *et al.* IFN-gamma sensitization of prostate cancer cells to Fas-mediated death: a gene therapy approach. *Mol. Ther.* **7**, 185–192 (2003).

24. Nagoshi, M., Sadanaga, N., Joo, H. G., Goedegebuure, P. S. & Eberlein, T. J. Tumor-specific cytokine release by donor T cells induces an effective host anti-tumor response through recruitment of host naive antigen presenting cells. *Int. J. Cancer* **80**, 308–314 (1999).

25. Croft, M., So, T., Duan, W. & Soroosh, P. The significance of OX40 and OX40L to T-cell biology and immune disease. *Immunol. Rev.* **229**, 173–191 (2009).

26. Hendriks, J. *et al.* CD27 is required for generation and long-term maintenance of T cell immunity. *Nat. Immunol.* **1**, 433–440 (2000).

27. Esensten, J. H., Helou, Y. A., Chopra, G., Weiss, A. & Bluestone, J. A. CD28 Costimulation: From Mechanism to Therapy. *Immunity* **44**, 973–988 (2016).

28. Grasso, C. S. *et al.* Conserved Interferon-γ Signaling Drives Clinical Response to Immune Checkpoint Blockade Therapy in Melanoma. *Cancer Cell* (2020) doi:10.1016/j.ccell.2020.08.005.

29. Havel, J. J., Chowell, D. & Chan, T. A. The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. *Nat. Rev. Cancer* (2019).

30. Braun, D. A. *et al.* Interplay of somatic alterations and immune infiltration modulates response to PD-1 blockade in advanced clear cell renal cell carcinoma. *Nat. Med.* **26**, 909–918 (2020).

31. Hellmann, M. D. *et al.* Tumor Mutational Burden and Efficacy of Nivolumab Monotherapy and in Combination with Ipilimumab in Small-Cell Lung Cancer. *Cancer Cell* **33**, 853–861.e4 (2018).

32. Goodman, A. M. *et al.* Tumor Mutational Burden as an Independent Predictor of Response to Immunotherapy in Diverse Cancers. *Mol. Cancer Ther.* **16**, 2598–2608 (2017).

33. Jiang, P. *et al.* Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nat. Med.* **24**, 1550–1558 (2018).

34. Hwang, S. *et al.* Immune gene signatures for predicting durable clinical benefit of anti-PD-1 immunotherapy in patients with non-small cell lung cancer. *Sci. Rep.* **10**, 643 (2020).

35. Mlecnik, B. *et al.* Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite Instability. *Immunity* **44**, 698–711 (2016).

36. Pagès, F. *et al.* International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* **391**, 2128–2139 (2018).

37. Hsiao, C. J. *et al.* Characterizing and inferring quantitative cell cycle phase in single-cell RNA-seq data analysis. *Genome Res.* **30**, 611–621 (2020).