Immunotherapy for human cancer : from bedside to bench and back

Kelderman, S.

Immunotherapy for human cancer

From bedside to bench and back

Sander Kelderman

Cover design: "Diverging roads" by Tomasz Ahrends
Lay-out and printing: Gildeprint, Enschede

ISBN: 978-90-9029972-3.

The research described in this thesis was performed at the Division of Immunology at the Netherlands Cancer Institute – Antoni van Leeuwenhoek Hospital (NKI-AVL).

The printing of this thesis was financially supported by the NKI-AVL.

Immunotherapy for human cancer

From bedside to bench and back

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus prof. mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 30 november 2016
klokke 13.45 uur

door

Sander Kelderman
geboren te Heemskerk
in 1986

Prof. dr. Ton N.M. Schumacher (promotor)
Prof. dr. Christian U. Blank (co-promotor)

Leden promotiecommissie:
Prof. dr. Sjoerd H. van der Burg
Prof. dr. John B.A.G. Haanen
Dr. Mirjam H.M. Heemskerk
Prof. dr. Jannie Borst (Universiteit van Amsterdam)
Prof dr. Gemma G. Kenter (Vrije Universiteit)

To all cancer patients and their families
who decide to participate in clinical trials
and donate tissue for scientific research

TABLE OF CONTENTS

Chapter 1

Scope of the thesis

"As the anecdotes coalesce into data, there's another layer, too, a sense of paradigms shifting. Immunotherapy marks an entirely different way of treating cancer—by targeting the immune system, not the tumor itself. Oncologists, a grounded-in-reality bunch, say a corner has been turned and we won't be going back."

With these words, the editors of *Science* elected cancer immunotherapy in 2013 as the scientific breakthrough of the year[1]. To say, however, that this breakthrough occurred in one year, or even several years, would not do justice to the decades of efforts that have led to our current understanding of the fundamentals of the immune system and its complex interaction with cancer.

The development of cancer immunotherapy as it stands today can roughly be divided into three distinct stages. The first stage, between 1900 and 1990, was one of discovery and compilation of knowledge, as the notion germinated that, somehow, the immune system may protect us against the development of cancer or even mediate spontaneous tumor regression in already afflicted individuals[2-4]. Seminal work in the '50s and '60s supported this idea by demonstrating immune-mediated tumor rejection in mouse models[5,6], and it was around this time that histocompatibility antigens in mice were discovered[7]. However, it was not until the late '80s when the first non-viral tumor antigen that was recognized by T cells, again in mice, was described[8]. The second stage of cancer immunotherapy development runs from 1990 until 2010, and is characterized by increasing efforts to understand tumor specific immune responses in humans and attempts to translate this knowledge into clinical interventions. In the early '90s, the first human tumor antigen recognized by T cells was identified[9]; a collection that was rapidly expanded in the following years by the SEREX approach[10]. In that same period, administration of high-dose interleukin-2 (IL-2) showed a moderate survival benefit in metastatic melanoma and renal cell carcinoma patients, and was approved in 1992 as the first form of immunotherapy for patients with cancer[11,12]. The discovery and subsequent characterization of T-cell checkpoint molecules, such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and programmed cell death protein 1 (PD-1) and its ligand PD-L1, sparked renewed enthusiasm for the potential of cancer immunotherapy[13-17]. Work from Steven Rosenberg and colleagues in the late '90s and 2000s showed that immunotherapy could also be implemented as a direct treatment approach. The adoptive cell transfer (ACT) of autologous tumor infiltrating lymphocytes (TIL) or T-cell receptor (TCR) gene-modified lymphocytes that had been cultured and expanded *ex vivo* showed profound clinical effects in some metastatic melanoma patients[18,19]. Importantly, cytotoxic T cells appeared to form an important component of this anti-tumor immune response[20], explaining much of the focus on this cell type in this thesis. However, the effects of these therapies on overall survival in the metastatic setting were only limited, or restricted to a single disease histology. That was until 2010, when cancer immunotherapy reached its

most recent stage of development, as treatment with a fully human monoclonal antibody targeting CTLA-4 was found to significantly prolong survival of patients with metastatic melanoma, leading to FDA and EMA approval in the following year[21,22]. In 2014 and 2015, reports came out that showed even more impressive clinical results when therapeutically targeting the PD-1/PD-L1 axis alone or in combination with anti-CTLA-4[23-25]. Importantly, these and other studies showed that clinical efficacy was not limited to melanoma, traditionally considered the most immunogenic of malignancies, but could likewise be observed in more common cancer types such as lung and colorectal cancer[26-28]. Similarly, ACT approaches are now reaching beyond melanoma, as TIL and TCR gene therapy have demonstrated clinical effectiveness in patients with human papilloma virus (HPV)-induced cervical cancer and synovial cell sarcoma, respectively[29,30], and CAR-modified T cells show strong clinical activity in B-cell malignancies[31,32].

Another important aspect of this third stage of cancer immunotherapy development is the availability of high-throughput next-generation sequencing (NGS) technologies[33-35], which has allowed immunologists to systematically study the composition of the tumor micro-environment, as well as the consequences of DNA damage and their interaction with the T-cell based immune system. A growing body of evidence now suggests that T-cell specific immunity is often directed against peptide antigens that result from tumor-specific mutations[36-38]. Importantly, immune responses targeting these so-called neo-antigens, that are highly patient-specific, can be influenced by therapy thereby providing an incentive to further develop personalized immunotherapies for human cancer[39-42].

In summary, cancer immunotherapy has taken up its place in oncological practice and it is likely there to stay. Although the field has come from far, much is still to learn as oncologists observe that not all patients with the same disease type respond to cancer immunotherapy, Furthermore, in some cancer types, no clinical benefit is observed at all, despite the presence of an abundant local immune infiltrate, indicating that our understanding of cancer – immune interaction is still incomplete. The overall goal of this thesis is to help address these matters in three ways. First, we have retrospectively analyzed a cohort of melanoma patients treated with ipilimumab, an anti-CTLA-4 antibody, for markers of responsiveness and review the literature to explore the limitations and curative potential of treatment strategies currently used in the clinic or under development. Second, we have developed several technology platforms that allow us to characterize and compare different types of T-cell responses that are directed against tumor-specific antigens in patient tumor material. Third, we aim to increase our understanding of the potential of cancer immunotherapy in ovarian and colorectal cancer, by analysis of the tumor-reactive compartment within these diseases.

In **Chapter 2**, we discuss the efficacy of an anti-CTLA4 blocking antibody, ipilimumab, in a cohort of patients with metastatic cutaneous melanoma treated in an expanded access

program (EAP). We find that overall survival and response rates are similar to what was observed in the initial phase III trials that led to FDA and EMA approval of ipilimumab[21,22]. Moreover, we describe the identification of a serological biomarker that correlates with survival and confirm these findings in an independent validation cohort. In **Chapter 3**, we extend our analysis of the ipilimumab EAP to a small cohort of metastatic uveal melanoma patients in which we describe that 1 out of 22 patients responded to the therapy.

Following the clinical development of anti-CTLA-4, a second immune checkpoint molecule that has received much attention recently is PD-1. Anti-PD-1 therapy has significantly extended overall survival in phase III trials for metastatic melanoma. Despite the fact that clinical responses were also observed in several other cancer types, in particular those with a high mutational burden[40,43], disease regression was only observed in a single patient with colorectal cancer[44]. A follow-up study demonstrated that anti-PD-1 therapy mainly benefitted patients with mismatch-repair deficient tumors, while being largely ineffective in mismatch-repair proficient colorectal tumors, again suggesting an important role for the expression of neo-antigens in cancer immunotherapy[27]. In **Chapter 4**, we discuss the findings of this phase II clinical trial and highlight the need to further extend anti-PD-1 therapy to other mismatch repair-deficient tumors independent of the underlying tumor type.

Although some patients clearly benefit from cancer immunotherapy, as we observed in the previous chapters, a good deal of patients does not respond or only does so temporarily. In **Chapter 5**, we review the potential mechanisms of resistance to cancer immunotherapy. Intrinsic or naturally acquired resistance is likely to play a role in patients that show no response to the treatment, whereas we can assume that therapy-induced resistance occurs in patients that initially respond but fail to do so in a durable manner. We dissect resistance to cancer immunotherapy by enumerating three discrete steps in the anti-tumor immune response: induction of the T-cell response, trafficking of T cells to the tumor, and cytolotyic activity at the intended effector site. This chapter is complementary to an earlier published review from Chen and Mellman in which they divide the cancer immunity cycle into seven distinct components[45]. At the heart of this cycle sits the antigen that is being recognized by the immune system, as its presence is first required to stimulate T cells in the lymph node and later serves as the target for tumor cell destruction. In **Chapter 6**, we review which antigens are the most suitable tumor rejection antigens and discuss why the focus on patient-specific neo-antigens is warranted in some, but should not be pursued in all cancer types.

We continue on the topic of antigen-directed cancer immunotherapy in **Chapter 7,** in which we describe the development of a highly flexible technology platform that allows us to purify antigen-specific T cells from T-cell cultures. This strategy can for instance be used to steer immune-reactivity of clinical-grade TIL infusion products towards desired target antigens and thus enhance their anti-tumor efficacy. We demonstrate that even low frequency antigen-specific T-cell populations can be effectively purified and that anti-tumor

activity is enhanced in *in vitro* assays. Using the flexibility of the platform described in the previous chapter, we were able to purify neo-antigen specific T cells from a melanoma TIL culture, and in **Chapter 8** we demonstrate superior tumor control of this enriched TIL product over a non-enriched TIL product in a preclinical setting. Furthermore, we show that tumor control is best obtained by T cells directed against mutated antigens rather than non-mutated antigens.

Data from preceding chapters indicate that the isolation and reconstruction of T-cell receptor (TCR) gene sequences with tumor-reactive potential is of great value to the field. Many of these technologies exist but these often rely on elaborate screening platforms to determine antigen-specificity or can only assess single-chain CDR3 sequences without being able to assess functionality[46]. In **Chapter 9**, we develop a novel RNA-based PCR technology that allows the unbiased identification of TCRα/β sequences at single cell resolution. Importantly, this technology bypasses the need for *in vitro* culture systems and thus allows us to assess the degree of naturally induced tumor-specific immunity in an unbiased manner for virtually every solid tumor.

We successfully applied this method to a small set of colorectal and ovarian cancer tissue (in which the presence of $CD8^+$ T cells has been linked to increased survival[47-50]) and – for a small fraction of the evaluated TCRs – could demonstrate reactivity against autologous tumor cells upon reconstruction of the full-length TCR α/β heterodimer. These data are consistent with the possibility that many of the tumor-resident T cells may not be tumor-reactive in these diseases, a topic that deserves further attention.

As the presence of $CD8^+$ T cells in ovarian cancer is linked to improved survival we hypothesized that such patients might be responsive to TIL treatment as was previously shown for melanoma. In **Chapter 10,** we demonstrate *in vitro* anti-tumor activity against autologous tumor cells in the majority of established ovarian cancer TIL cultures. These findings have led to the initiation of a phase I/II clinical trial for metastatic ovarian cancer at the Netherlands Cancer Institute.

Finally, in **Chapter 11**, the future prospects of cancer immunotherapy are discussed in light of the findings described in this thesis.

REFERENCES

1. Couzin-Frankel, J. Breakthrough of the year 2013. Cancer immunotherapy. *Science* **342**, 1432-1433 (2013).
2. Ehrlich, P. Über den jetzigen Stand der Karzinomforschung. *Ned. Tijsdchr. Geneeskd.* **5**, 273:290 (1909).
3. Cole, W.H. Spontaneous regression of cancer and the importance of finding its cause. *Natl Cancer Inst Monogr* **44**, 5-9 (1976).
4. Krikorian, J.G.*, et al.* Spontaneous regression of non-Hodgkin's lymphoma: a report of nine cases. *Cancer* **46**, 2093-2099 (1980).
5. Prehn, R.T. & Main, J.M. Immunity to methylcholanthrene-induced sarcomas. *J Natl Cancer Inst* **18**, 769-778 (1957).
6. Klein, G.*, et al.* Demonstration of resistance against methylcholanthrene-induced sarcomas in the primary autochthonous host. *Cancer Res* **20**, 1561-1572 (1960).
7. Snell, G.D. & Higgins, G.F. Alleles at the histocompatibility-2 locus in the mouse as determined by tumor transplantation. *Genetics* **36**, 306-310 (1951).
8. De Plaen, E.*, et al.* Immunogenic (tum-) variants of mouse tumor P815: cloning of the gene of tum- antigen P91A and identification of the tum- mutation. *Proc Natl Acad Sci U S A* **85**, 2274-2278 (1988).
9. van der Bruggen, P.*, et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* **254**, 1643-1647 (1991).
10. Tureci, O.*, et al.* Serological analysis of human tumor antigens: molecular definition and implications. *Mol Med Today* **3**, 342-349 (1997).
11. Dillman, R.O.*, et al.* Inpatient continuous-infusion interleukin-2 in 788 patients with cancer. The National Biotherapy Study Group experience. *Cancer* **71**, 2358-2370 (1993).
12. Fyfe, G.A.*, et al.* Long-term response data for 255 patients with metastatic renal cell carcinoma treated with high-dose recombinant interleukin-2 therapy. *J Clin Oncol* **14**, 2410-2411 (1996).
13. Walunas, T.L.*, et al.* CTLA-4 can function as a negative regulator of T cell activation. *Immunity* **1**, 405-413 (1994).
14. Krummel, M.F. & Allison, J.P. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* **182**, 459-465 (1995).
15. Ishida, Y.*, et al.* Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* **11**, 3887-3895 (1992).
16. Freeman, G.J.*, et al.* Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* **192**, 1027-1034 (2000).
17. Okazaki, T. & Honjo, T. PD-1 and PD-1 ligands: from discovery to clinical application. *Int Immunol* **19**, 813-824 (2007).
18. Dudley, M.E.*, et al.* Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* **26**, 5233-5239 (2008).
19. Johnson, L.A.*, et al.* Gene transfer of tumor-reactive TCR confers both high avidity and tumor reactivity to nonreactive peripheral blood mononuclear cells and tumor-infiltrating lymphocytes. *J Immunol* **177**, 6548-6559 (2006).
20. Dudley, M.E.*, et al.* CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clin Cancer Res* **16**, 6122-6131 (2010).

21. Hodi, F.S., *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* **363**, 711-723 (2010).
22. Robert, C., *et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* **364**, 2517-2526 (2011).
23. Robert, C., *et al.* Nivolumab in previously untreated melanoma without BRAF mutation. *N Engl J Med* **372**, 320-330 (2015).
24. Larkin, J., *et al.* Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med* **373**, 23-34 (2015).
25. Robert, C., *et al.* Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. *The Lancet* **384**, 1109-1117 (2014).
26. Brahmer, J., *et al.* Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N Engl J Med* **373**, 123-135 (2015).
27. Le, D.T., *et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* **372**, 2509-2520 (2015).
28. Herbst, R.S., *et al.* Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* **515**, 563-567 (2014).
29. Stevanovic, S., *et al.* Complete Regression of Metastatic Cervical Cancer After Treatment With Human Papillomavirus-Targeted Tumor-Infiltrating T Cells. *J Clin Oncol* **14**, 1543-1550 (2015).
30. Robbins, P.F., *et al.* Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* **29**, 917-924 (2011).
31. Porter, D.L., *et al.* Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* **365**, 725-733 (2011).
32. Maude, S.L., *et al.* Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* **371**, 1507-1517 (2014).
33. Rooney, M.S., *et al.* Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* **160**, 48-61 (2015).
34. Gentles, A.J., *et al.* The prognostic landscape of genes and infiltrating immune cells across human cancers. *Nat Med* **21**, 938-945 (2015).
35. Alexandrov, L.B., *et al.* Signatures of mutational processes in human cancer. *Nature* **500**, 415-421 (2013).
36. Linnemann, C., *et al.* High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nat Med* **21**, 81-85 (2015).
37. Lu, Y.C., *et al.* Mutated PPP1R3B is recognized by T cells used to treat a melanoma patient who experienced a durable complete tumor regression. *J Immunol* **190**, 6034-6042 (2013).
38. Wick, D.A., *et al.* Surveillance of the Tumor Mutanome by T Cells during Progression from Primary to Recurrent Ovarian Cancer. *Clin Cancer Res* **20**, 1125-1134 (2014).
39. van Rooij, N., *et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol* **31**, e439-442 (2013).
40. Rizvi, N.A., *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science* **348**, 124-128 (2015).
41. Tran, E., *et al.* Cancer Immunotherapy Based on Mutation-Specific CD4+ T Cells in a Patient with Epithelial Cancer. *Science* **344**, 641-645 (2014).
42. Snyder, A., *et al.* Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N Engl J Med* **371**, 2189-2199 (2014).

43. Champiat, S., *et al.* Exomics and immunogenics: Bridging mutational load and immune checkpoints efficacy. *Oncoimmunology* **3**, e27817 (2014).
44. Topalian, S.L*., et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* **366**, 2443-2454 (2012).
45. Chen, D.S. & Mellman, I. Oncology meets immunology: the cancer-immunity cycle. *Immunity* **39**, 1-10 (2013).
46. Calis, J.J. & Rosenberg, B.R. Characterizing immune repertoires by high throughput sequencing: strategies and applications. *Trends Immunol* **35**, 581-590 (2014).
47. Zhang, L*., et al.* Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* **348**, 203-213 (2003).
48. Galon, J*., et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **313**, 1960-1964 (2006).
49. Pages, F*., et al.* In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol* **27**, 5944-5951 (2009).
50. Fridman, W.H*., et al.* The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* **12**, 298-306 (2012).

Chapter 2

Lactate dehydrogenase as a selection criterion for ipilimumab treatment in metastatic melanoma

Sander Kelderman[1], Bianca Heemskerk[1#], Harm van Tinteren[1#], Rob R.H. van den Brom[2], Geke A.P. Hospers[2], Alfonsus J.M. van den Eertwegh[3], Ellen H.W. Kapiteijn[4], Jan Willem B. de Groot[5], Patricia Soetekouw[6], Rob L. Jansen[6], Edward Fiets[7], Andrew J.S. Furness[8], Alexandra Renn[8], Marcin Krzystanek[9], Zoltan Szallasi[9], Paul Lorigan[10], Martin E. Gore[8], Ton N.M. Schumacher[1], John B.A.G. Haanen[1], James M.G. Larkin[8], Christian U. Blank[1]

[1]Netherlands Cancer Institute NKI-AVL, Amsterdam, The Netherlands, [2]University Medical Center Groningen, Groningen, The Netherlands, [3]VU University Medical Center, Amsterdam, The Netherlands,[4] Leiden University Medical Centre, Leiden, The Netherlands, [5]Isala Clinics, Zwolle, The Netherlands, [6]Maastricht University Medical Center, Maastricht, The Netherlands, [7]Medical Center Leeuwarden, Leeuwarden, The Netherlands, [8]Royal Marsden Hospital, London, United Kingdom, [9]Technical University of Denmark, Kongens Lyngby, Denmark, [10]Christie Hospital, Manchester, United Kingdom. [#] These authors contributed equally to this work.

***Cancer Immunology Immunotherapy* 63: 449-458 (2014)**

ABSTRACT

Ipilimumab, a CTLA-4 blocking antibody, has improved overall survival in metastatic melanoma in phase III trials. However, about 80% of patients fail to respond and no predictive markers for benefit from therapy have been identified. We analysed a 'real world' population of patients treated with ipilimumab to identify markers for treatment benefit.

Patients with advanced cutaneous melanoma were treated in the Netherlands and the United Kingdom with ipilimumab at 3mg/kg. Baseline characteristics and peripheral blood parameters were assessed and patients were monitored for the occurrence of adverse events and outcomes.

A total of 166 patients were treated in the Netherlands. Best overall response and disease control rates were 17% and 35%, respectively. Median follow-up was 17.9 months, with a median progression-free survival of 2.9 months. Median overall survival was 7.5 months, and overall survival at 1 year was 37.8% and at 2 years 22.9%. In a multivariate model, baseline serum lactate dehydrogenase (LDH) was demonstrated to be the strongest predictive factor for overall survival. These findings were validated in an independent cohort of 64 patients from the UK.

In both the NL and UK cohorts, long-term benefit of ipilimumab treatment was unlikely for patients with baseline serum LDH greater than twice the upper limit of normal. In the absence of prospective data, clinicians treating melanoma may wish to consider the data presented here to guide patient selection for ipilimumab therapy.

INTRODUCTION

Ipilimumab is a fully human monoclonal IgG1 antibody that blocks the immune-checkpoint molecule cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) thereby reversing its immunosuppressive effect on T cells, although its exact mechanism of action is still under investigation[1].

Ipilimumab was approved for the treatment of advanced melanoma by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) in 2011, on the basis of increased overall survival (OS) in two phase III studies[2,3]. Treatment with ipilimumab at 3mg/kg as a second and subsequent line of therapy resulted in a median OS of 10.1 months compared to 6.4 months in the vaccine control arm together with a 1-year survival of 45.6% versus 25.3%, respectively in one of the studies[2]. Best overall response rate (BORR) was 11%.

Subgroup analysis for OS revealed that the hazard ratio for ipilimumab in comparison to the control arm was only significant in patients with normal serum lactate dehydrogenase (LDH) levels[2]. Raised serum LDH is known to be a poor prognostic factor in a number of cancers including melanoma for reasons that remain unknown. This is reflected in the 2009 AJCC staging system for melanoma, which subdivides metastatic disease into: M1a, skin, lymph node or subcutaneous metastases only; M1b, lung metastases only; and M1c, visceral metastases or metastases at any site with a raised LDH[4].

Ipilimumab is currently under evaluation in several phase II trials for other malignancies including renal cell carcinoma, non-small cell lung cancer and metastatic castration-refractory prostate carcinoma[5-8]. While potentially effective, ipilimumab can be toxic as a consequence of reduction in peripheral tolerance to self-antigens, which is reflected in the induction of immune-related Adverse Events (irAEs) such as colitis, dermatitis, hepatitis and hypophysitis[2,3].

Several clinical parameters, such as high absolute lymphocyte count (ALC) after two courses of ipilimumab, a rise of ALC over baseline and the occurrence of grade 3 or 4 irAEs have been suggested to correlate with OS and response to therapy[9-15]. A major drawback of these potential markers is that they become evident only during the course of treatment, thereby making them unsuitable for upfront patient selection. The aim of this study was, therefore, to identify baseline patient characteristics for response to ipilimumab, to assess their independent prognostic value, and compare them with the markers described above[16,17].

PATIENTS AND METHODS

Patient inclusion and treatment plan

Patients with advanced melanoma and progression on systemic therapy were treated in expanded access programs in the Netherlands (NL EAP) (NCT00495066) and in the United Kingdom after licensing. Consequently, the NL cohort included patients exclusively treated within the EAP, whereas the UK cohort comprised of both EAP and post-licensing patients. A modified World Health Organization (mWHO) performance status of 0, 1, or 2 was required. An mWHO performance status of 0 indicates that the patient has no restrictions in carrying out pre disease activities; a mWHO performance status of 1 indicates that the patient has restrictions in more strenuous activities but is completely ambulatory and is able to carry out work of a light nature; a mWHO performance status of 2 indicates that the patient is in bed for less than 50% during the day but is still ambulatory and capable of all self care. Treatment protocols were approved according to local regulations and ethical committees. Data acquisition and subsequent analyses mentioned hereafter apply to the NL cohort unless stated otherwise. Data from the UK cohort were obtained to validate findings on baseline LDH and survival probability. All patients received four cycles of 3mg/kg ipilimumab every three weeks, unless severe side effects or rapid disease progression occurred. M-stage was defined according to site of metastasis in combination with elevated LDH levels, as described previously[4].

Safety measures

Patients underwent clinical evaluation prior to each ipilimumab infusion. Immune-related Adverse Events (irAEs) were scored using the Common Terminology Criteria for Adverse Events (CTCAE) version 4.02 and patients received standard of care accordingly. A list of irAEs was pre-specified in the protocol and a serious adverse event was determined as grade 3 or 4. Haematological and serum parameters were determined at baseline, every three weeks during treatment, and three-monthly during follow-up. These included ALC, S100, erythrocyte sedimentation rate (ESR), LDH, liver, kidney and endocrine function. Upper limits of normal (ULN) were normalised for the reference range at each participating centre.

Response evaluation and follow-up

Radiologic evaluation (CT or PET/CT scanning) was performed at baseline and week 12 (i.e. three weeks after the fourth infusion) if patients had undergone all four cycles of ipilimumab. Responses were scored according to RECIST 1.1 criteria as well as immune-related Response Criteria (irRC), the latter to determine BORR[18]. Responses were confirmed at least four weeks later and during follow-up every three months thereafter or when disease progression was clinically suspected. Survival status from patients that withdrew from follow-up was obtained from primary care physicians.

Statistical analysis

Data were analysed using SPSS statistical software version 20.0 and R statistical software version 2.15.0. Univariate analysis for clinical and laboratory parameters was performed with respect to OS and progression-free survival (PFS). Survival curves of categorical variables were drawn using the Kaplan-Meier technique and compared by the log-rank test. Continuous variables in association with survival outcomes were explored by means of martingale residuals. LDH and S100 were log-transformed. Multivariate models of patient and tumour characteristics in association with PFS and OS were based on Cox-proportional hazards regression analyses. PFS was defined as time from start of ipilimumab to the onset of progression or death. Patients without progression and still alive at time of analysis were censored. OS was defined as time from start of ipilimumab to death of any cause. Patients still alive at analysis were censored. OS analyses were identical for both cohorts. The association of parameters measured at six weeks with either PFS or OS were analysed by means of a landmark method (ignoring time and events before six weeks) or as time-dependent variable in a Cox proportional hazards model. Decision trees were generated using recursive partitioning.

RESULTS

Patient characteristics

166 patients received ipilimumab between April 2010 and December 2011 within the Netherlands expanded access program (NL EAP). As of August 2012, 28% of patients were still alive. The median follow-up of the cohort was 17.9 months; clinical characteristics are listed in Table 1. Median patient age at the time of study inclusion was 55 years and 58% were male. Modified WHO performance status 0, 1, and 2 was scored in 59, 36, and 5% of patients respectively; 83% of the patients had stage M1c disease. Baseline levels of LDH were elevated between the ULN and 2x ULN in 28% of patients and >2x ULN in 16% of patients. From the UK cohort 64 patients were analysed. The median follow-up of this cohort was 19.0 months and patient characteristics were similar as shown in Table 1. In this cohort, 39% of patients had baseline levels of LDH elevated between ULN and 2x ULN, while 23% of patients had elevated LDH >2x ULN.

Table 1. Patient characteristics. WHO World Health Organization. NA not available. LDH lactate dehydrogenase. ULN upper limit of normal.

Variable	NL (N=166)	UK (N=64)
Age		
Median (range)	55 (22-88)	58 (18-84)
Sex		
female	69 (42%)	27 (42%)
male	97 (58%)	37 (58%)
WHO status		
WHO 0	98 (59%)	21 (33%)
WHO 1	60 (36%)	37 (58%)
WHO 2	8 (5%)	6 (9%)
Breslow		
<1mm	10 (6%)	5 (8%)
1-2mm	23 (14%)	14 (22%)
2-4mm	50 (30%)	14 (22%)
>4mm	24 (14%)	7 (11%)
NA	59 (36%)	24 (38%)
M stage		
M1a	8 (5%)	NA
M1b	20 (12%)	NA
M1c	138 (83%)	NA
Site metastasis		
single	31 (19%)	5 (8%)
multiple	135 (81%)	59 (92%)
Cycles of ipilimumab		
1	7 (10%)	17 (27%)
2	19 (11%)	11 (17%)
3	22 (13%)	11 (17%)
4	114 (66%)	25 (39%)
Responders	17 (11%)	7 (11%)
Non-responders	149 (89%)	57 (89%)
Immune-related adverse events (grade III/IV)		
Responders	3/17 (18%)	3/7 (43%)
Non-responders	25/149 (17%)	2/57 (4%)
Baseline LDH		
≤ 1x ULN	87 (52%)	24 (38%)
1-2x ULN	46 (28%)	25 (39%)
> 2x ULN	27 (16%)	15 (23%)
NA	6 (4%)	0 (0%)

Prior and post ipilimumab treatment modalities

All patients in the NL cohort had received prior systemic treatment of which 80% received only dacarbazine (DTIC), while 20% underwent one other systemic treatment or had more

than one prior treatment including DTIC (Supplementary Table 1). Most patients (75%) did not receive any other treatment after ipilimumab therapy. Post-ipilimumab treatment modalities comprised BRAF-inhibitors (13%) or reinduction therapy with ipilimumab (3%). One out of five patients experienced disease stabilisation upon reinduction with ipilimumab.

Toxicities

The frequencies of irAEs in the NL cohort are listed in Supplementary Table 2. We observed serious (grade 3 or 4) adverse events in 28/166 (16%) of the patients. The majority (75%) comprised dermatitis, hypophysitis and colitis, of which only the latter two led to discontinuation of treatment with ipilimumab in 8% of patients. There was one treatment-related death (1%) due to severe colitis resulting in bowel perforation. Grade 1 and 2 immune-related toxicity events mainly comprised dermatitis for which topical intervention was sufficient.

Response to therapy

The clinical responses in the NL cohort measured using RECIST or irRC (including BORR) are listed in Supplementary Table 3. Response rates at 12 weeks were 10% by RECIST and 11% by irRC, indicating no initial difference between the two response evaluation methods. However, when looking at the BORR, which takes into account the late development of anti-tumour immune effects, we observed an increase in the RR to 17%. The median time to achieve BORR was 3.7 months (range 2.5 – 18.6), which is beyond the standard time point for response evaluation at week 12 and underscores the relevance of a week-16 response re-evaluation. Four patients eventually became complete responders after initially being scored as SD or PR and one patient was scored with progressive disease (PD) at 12 weeks but eventually became a complete responder 18 months after treatment initiation. A substantial number of patients (27%) were not evaluable (NE) for response due to rapid deterioration and death (39/166) before week 12 or serious adverse events (5/166) resulting in withdrawal from ipilimumab treatment. There was no correlation between the occurrences of grade 3 and 4 irAEs and response to therapy (P = 0.62).

Survival analysis and biomarker assessment

Data on all patients was available for survival analysis. The median progression free survival (PFS) in the NL cohort was 2.9 months (95% CI: 2.8 – 3.2, Fig. 1A). Median OS of the patients in the NL cohort was 7.5 months (95% CI: 6.1 – 10.5, Fig. 1B). The 1-year survival was 37.8% (95% CI: 31.1 – 46.0) and 2-year survival 22.9% (95% CI: 16.4 – 32.1). The median OS in the UK cohort was 4.1 months (95% CI: 3.6 – 5.1, Fig. 1C). The 1-year survival was 15.6% (95% CI: 8.8 – 27.6) and 2-year survival 14.1% (95% CI: 7.67 – 25.8).

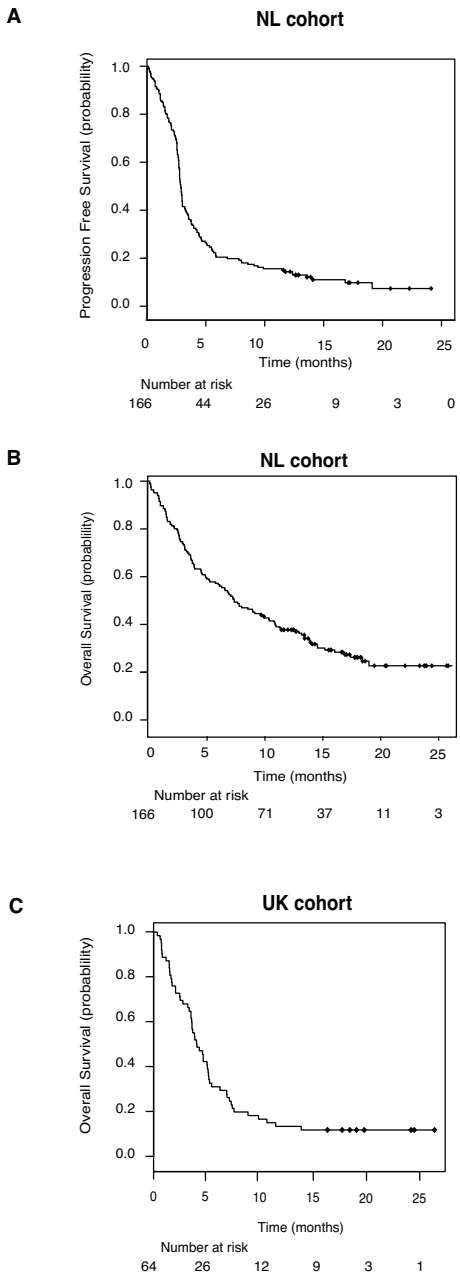


Figure 1. Kaplan-Meier curves for progression free survival and overall survival of NL and UK cohort. (a) Progression free survival curve for the NL cohort and overall survival curves for **(b)** the NL and **(c)** UK patient cohorts are shown.

Univariate analysis of the NL cohort for patient and disease characteristics such as gender, age, Breslow thickness and location of primary tumour, as well as prior treatment revealed no significant association with OS. Both mWHO performance score 0 and disease stage M1a/b, univariately correlated with a favourable outcome for OS (P = 0.001 and P = 0.04, respectively). Baseline ALC and week 6 ALC measurements showed a significant difference for survival when stratifying high versus low (P = 0.01 and P = 0.001, Fig. 2A and B, respectively). When assessing the slope in ALC as identified by Martingale residuals, we found that patients with greater than or equal to 1.35 fold higher value compared to baseline after two cycles of treatment, determined six weeks after the start of therapy, had a significantly better OS than patients with a lower increase (P = 0.02, Fig. 2C). Low baseline levels of respectively LDH, S100 and ESR, were also significantly associated with improved OS (all P <0.0001, Fig. 2D-F).

When patient characteristics (M-stage and mWHO performance status), baseline LDH, S100, ESR, and ALC were examined in a multivariate model, only ESR and LDH remained as significant independent negative prognostic factors (P < 0.01). Using this approach we identified a subset of patients with a significantly better OS. The majority of patients with low OS were identified when separating the cohort by elevated baseline levels of LDH (median OS 14.7 months for LDH normal versus 3.7 months for LDH >ULN; P < 0.001), with some further discrimination after stratification by baseline ESR (Supplementary Fig. 1). Of note, only 17/44 patients not evaluable for response were available for baseline ESR assessment while 40/44 were available for baseline LDH assessment. Within the poor prognosis group (elevated LDH above ULN) a small number of patients, including four with a partial response and one complete response (5/73), survived beyond the first year. The response rate in this subgroup was 9% compared to 23% in the ≤1x ULN group, indicating some potential predictive value of this marker. When stratifying by 2x ULN of baseline LDH values, only one patient survived beyond 12 months in the LDH-high group who had a partial response and was censored at 12.9 months follow-up. In total, only two partial responses occurred in this subgroup whereas all other responding patients occurred in the LDH-low group. Median OS was 2.9 months versus 10.0 months in the LDH greater than twice ULN versus less than twice ULN group, respectively (P < 0.001, Fig. 3).

To investigate the broader value of these findings, we analysed an independent cohort of 64 patients treated in the UK at two academic centres. Only baseline LDH values were assessed in this cohort and, unlike in the Dutch cohort, stratification on 1x ULN baseline LDH did not significantly influence OS. However, a significant difference in survival was observed when stratifying patients on their 2x ULN baseline LDH values (Fig. 3). Median OS in the LDH-high group was 3.2 months compared to 5.0 months in the LDH-low group (P = 0.004). Only within the LDH ≤2x ULN group survivors were observed beyond a year.

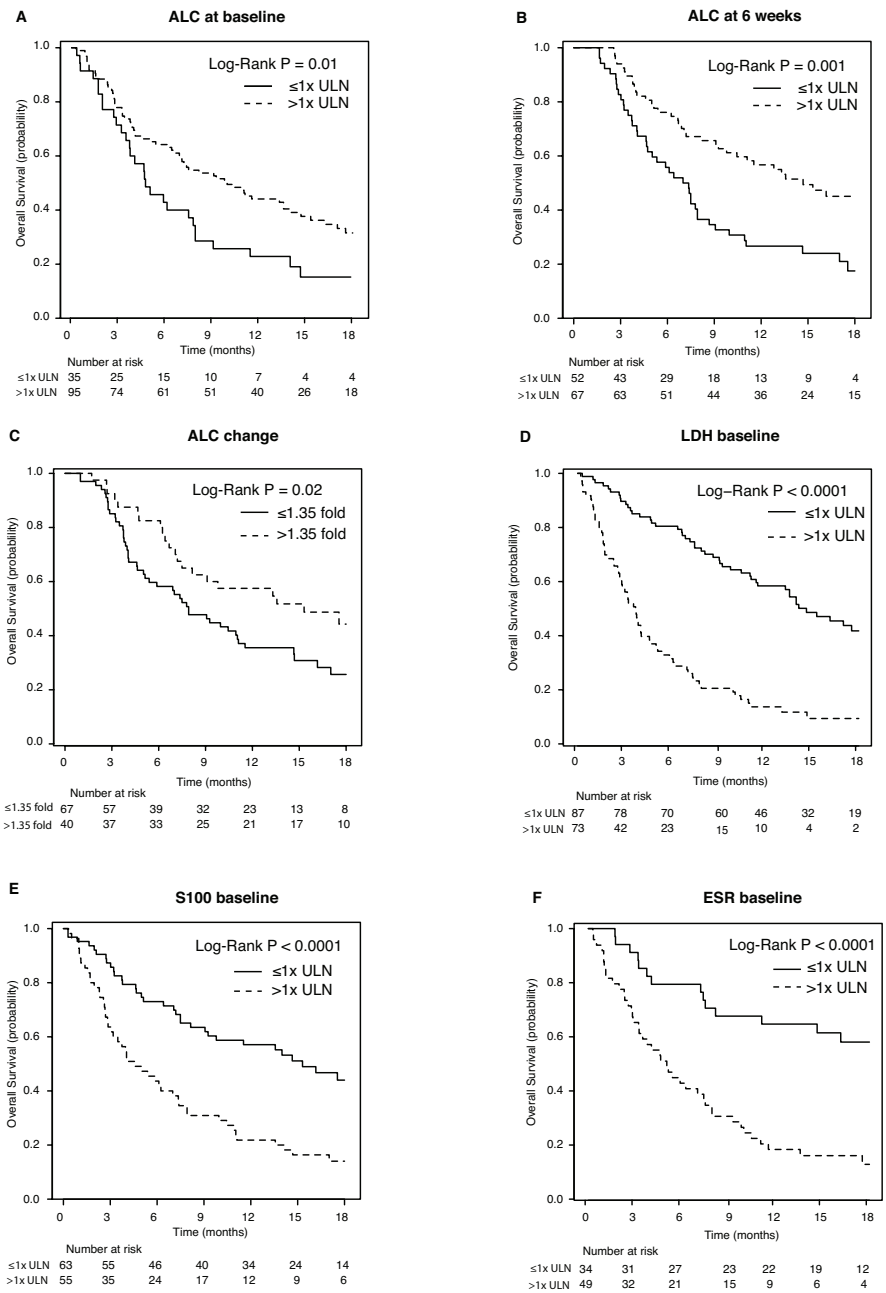


Figure 2. Kaplan-Meier curves for overall survival stratified by blood parameters. (a) Overall survival is shown when stratifying for blood values of baseline absolute lymphocyte count (ALC), **(b)** ALC at week 6, **(c)** slope of ALC after two infusions, **(d)** baseline lactate dehydrogenase (LDH), **(e)** S100 and **(f)** baseline erythrocyte sedimentation rate (ESR). ULN upper limit of normal. P < 0.05 is statistically significant.

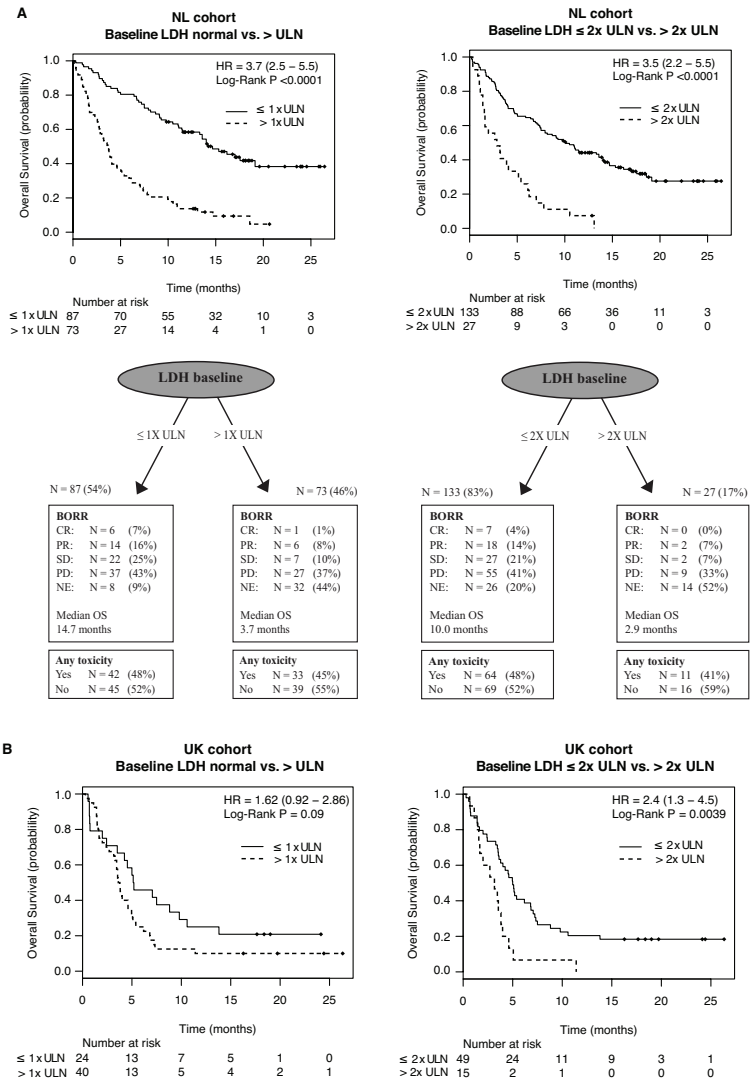


Figure 3. Patient selection based on baseline LDH for NL and UK cohort. Upper part of panel A shows the stratification of patients based on their upper limit of normal (ULN) baseline values of serum lactate dehydrogenase (LDH) in the NL cohort. For six patients baseline LDH values were not available and these were excluded from further analyses. Lower part shows patients grouped by their response to therapy and the occurrence of toxicities. A statistically significant difference in overall survival (OS) was observed when stratifying by 1x ULN baseline LDH. However, seven patients with a clinical response upon ipilimumab treatment would have been missed when implementing this cut-off. Only two patients with a clinical response remained in the LDH-high subgroup when stratifying by 2x ULN of baseline LDH. Toxicities in the high and low LDH subgroups were similar. Panel B shows the stratification by baseline LDH values of patients from the UK cohort. At a cut-off of 1x ULN of baseline LDH there is no significant influence on OS. Stratification by 2x ULN baseline LDH does significantly increase OS and no patients in the LDH-high subgroup survive beyond the first year after treatment initiation. Percentages may not add up to 100 due to rounding. HR hazard ratio. CR complete response. PR partial response. SD stable disease. PD progressive disease. NE non-evaluable.

Overall, 1- and 2-year survival rates of the combined NL and UK cohorts in the LDH-low group (≤2x ULN; N=182) were 51.2% and 34.6%, respectively, whereas they were 4.8% and 0% in the LDH-high group (>2x ULN; N=42) (Supplementary Table 4). We found that 66% of patients in the LDH-low group received all four cycles, whereas in the LDH-high group only 24% of patients completed the full treatment (data not shown).

DISCUSSION AND CONCLUSIONS

The aim of our analysis was to identify, from a 'real world' melanoma patient cohort, parameters used in routine clinical practice that may identify patients most likely to benefit from ipilimumab therapy prior to the start of treatment. We found that a high ESR and in particular a high LDH at baseline are statistically significant independent prognostic factors. The use of LDH alone at the cut-off at 2x ULN, as a negative prognosticator, was confirmed in an external cohort of patients. Patient characteristics such as M-stage and performance status did not have an independent significant effect on overall survival.

While ESR was also identified as an independent prognostic factor, only a small subgroup of twelve patients would additionally be excluded when implementing this cut-off, three of which had a partial response upon ipilimumab treatment. Therefore, we chose to focus on the more clinically relevant stratification based on baseline LDH alone. However, it cannot be excluded based on these data, that the combination of low LDH / low ESR is of superior value to low LDH alone. Also, ESR data were not available for the UK cohort to confirm these findings. A planned meta-analysis on all EAP treated patients in Europe should give more insight into the prognostic value of an inflammatory marker such as ESR combined with LDH.

The data presented here indicate that patients with elevated LDH, for the NL cohort at 1x ULN and for both cohorts at 2x ULN, are unlikely to benefit from ipilimumab therapy. High disease load and cell turnover, resulting in high serum levels of LDH, may negatively influence the potency of an immune response at the tumor site. Rapidly growing tumors are often poorly vascularized, resulting in anaerobic glycolysis as an alternative energy source, which in turn is mediated by activity of LDH that converts pyruvate into lactate[19,20]. This switch of tumor cells to a glycolytic phenotype, which can even occur under normoxic conditions, gives rise to lactate accumulation and a subsequent decrease in extracellular pH, negatively affecting the function of lymphocytes present in the tumor microenvironment[21,22]. Alternatively, elevated LDH may simply form a measure of disease state and thereby be correlated with likelihood of response.

We observed that the median OS in the >2x ULN group (hereafter referred to as LDH-high) was significantly lower than in the ≤2x ULN group (hereafter referred to as LDH-low): 2.9 months versus 10.0 months in the NL cohort and 3.2 months versus 5.0 months in the UK cohort. Interestingly, in both phase III trials leading to FDA and EMA approval of ipilimumab,

a non-elevated LDH was associated with an increased OS[2,3]. This fact, and the observation that in two other ipilimumab EAPs with smaller patient numbers, reported by Wilgenhof et al. and Delyon et al., elevated LDH showed a negative association with survival further supports our findings[13,14]. Overall survival in our cohorts was lower than what was reported in the phase III studies. This is not surprising because the inclusion criteria were less strict for the expanded access programmes than for the phase III studies. Furthermore, patients in the UK cohort benefited less from therapy than those in the NL cohort, potentially because fewer patients had a performance status of 0: 33% versus 59%, respectively.

The 1- and 2-year survival rates of 51.2% and 34.6% for the LDH-low patients of the combined NL and UK cohort indicate that the survival rates are comparable to the phase III study cohorts[2,3]. In the LDH-high group, however, we found 1- and 2-year survival rates of 4.8% and 0%, respectively, indicating no long-term benefit at all; except for one patient censored at 12.9 months. Taken together, our data show that treatment benefit from ipilimumab is almost exclusively concentrated in the LDH-low group. Patients in the high-LDH group, which is 19% of patients from both cohorts combined, that carry the BRAF V600E mutation might benefit more from targeted therapies such as BRAF- and MEK-inhibitors that are capable of inducing rapid anti-tumour responses. Recent data from an open label multicentre safety study of vemurafenib showed that patients with elevated LDH at baseline were *on therapy* for a median duration 4.1 months, which is longer than the median OS for the ipilimumab patients with elevated LDH here[23]. The efficacy of sequential treatment with BRAF inhibitors and ipilimumab should be evaluated in a randomized trial. Sequencing targeted therapies with ipilimumab might be especially helpful to patients with more aggressive disease where the delayed tumour responses generally observed with immunotherapies take too long for the patient to benefit[18,23-26].

Our analysis clearly has a number of limitations of which the most obvious is its retrospective nature and consequent possibility of bias. A strength of our dataset however is that it involves a 'real world' population as opposed to the more selected group of patients that enter phase III registration trials. Furthermore, the observation of a similarly strong predictive value of elevated LDH in two independent cohorts lends further credibility to our observations. Ideally, our data would stimulate a prospective analysis of the value of elevated LDH as a patient selection criterion. However, in view of the rapidly changing landscape in melanoma immunotherapy, with the likely registration of agents targeting programmed cell death 1 (PD1) and programmed cell death ligand 1 (PDL1)[27-29] we consider such prospective studies unlikely to happen. Without such data, we feel that is important to carry out similar analyses on other large cohorts of ipilimumab-treated melanoma patients treated in routine clinical practice.

In November 2013, the EMA approved ipilimumab as a first-line therapy in metastatic melanoma at a 3mg/kg dosing schedule[30]. This registration will extend the availability of ipilimumab to previously untreated patients throughout the European Union. After decades

of limited and mostly failing therapies, treating oncologists are now faced with the difficulty of having to decide patient eligibility at both earlier and later stages of the disease. This will consequently have an effect on the number of responding patients as well as overall survival. Related to this, the expected administration of ipilimumab to patients at earlier disease stages, due to its recent approval as a first-line treatment, warrants continued assessment of the prognostic value of the markers proposed here.

In conclusion, our results show efficacy of ipilimumab comparable to that reported in the phase III studies. Furthermore, we propose that elevated levels of baseline LDH at a 2x ULN cut-off may help physicians to select patients before treatment initiation. Serum LDH has been established for some time as a negative prognostic factor in advanced melanoma[16,17]. From our data, in the absence of a control arm, we are unable to determine whether LDH forms either a predictive or a prognostic marker for benefit from ipilimumab. However, we would assert that in routine clinical practice the difference is not relevant, and that using serum LDH to stratify patients for ipilimumab treatment should simply be considered pragmatic.

ACKNOWLEDGEMENTS

No funds were received to conduct this study. The authors want to acknowledge the members of the Dutch Working Group on Immunotherapy of Oncology (WIN-O) for their assistance in obtaining the data and their constructive discussion concerning the outcomes of this study.

CONFLICT OF INTEREST

G.A.P. Hospers, A.J.M. van den Eertwegh, E.W. Kapiteijn, J.W. de Groot, P. Lorigan, M.E. Gore, J.B.A.G. Haanen, J.M.G. Larkin and C.U. Blank have participated in advisory board meetings of Bristol-Myers Squibb for which the faculty has received compensation. P Lorigan has received support for travel and compensation for educational and speaker bureau activities. J.M.G. Larkin and M.E. Gore acknowledge National Health Service funding to the National Institute for Health Research Biomedical Research Centre at the Royal Marsden Hospital. J.B.A.G. Haanen and T.N.M. Schumacher are members of the Bristol-Meyers Squibb Immuno-Oncology network and have furthermore received a grant for translational research from Bristol-Meyers Squibb. C.U. Blank receives funding for an investigator-initiated study from Bristol-Myers Squibb. All other authors declared that they have no conflict of interest.

REFERENCES

1. Simpson, T.R.*, et al.* Fc-dependent depletion of tumor-infiltrating regulatory T cells co-defines the efficacy of anti-CTLA-4 therapy against melanoma. *J Exp Med* **210**, 1695-1710 (2013).
2. Hodi, F.S.*, et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* **363**, 711-723 (2010).
3. Robert, C.*, et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* **364**, 2517-2526 (2011).
4. Balch, C.M.*, et al.* Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol* **27**, 6199-6206 (2009).
5. Yang, J.C.*, et al.* Ipilimumab (anti-CTLA4 antibody) causes regression of metastatic renal cell cancer associated with enteritis and hypophysitis. *J Immunother* **30**, 825-830 (2007).
6. Tomasini, P.*, et al.* Ipilimumab: its potential in non-small cell lung cancer. *Ther Adv Med Oncol* **4**, 43-50 (2012).
7. Slovin, S.F.*, et al.* Ipilimumab alone or in combination with radiotherapy in metastatic castration-resistant prostate cancer: results from an open-label, multicenter phase I/II study. *Ann Oncol* **24**, 1813-1821 (2013).
8. Grosso, J.F. & Jure-Kunkel, M.N. CTLA-4 blockade in tumor models: an overview of preclinical and translational research. *Cancer Immun* **13**, 5 (2013).
9. Thompson, J.A.*, et al.* Ipilimumab in treatment-naive and previously treated patients with metastatic melanoma: retrospective analysis of efficacy and safety data from a phase II trial. *J Immunother* **35**, 73-77 (2012).
10. Ku, G.Y.*, et al.* Single-institution experience with ipilimumab in advanced melanoma patients in the compassionate use setting: lymphocyte count after 2 doses correlates with survival. *Cancer* **116**, 1767-1775 (2010).
11. Attia, P.*, et al.* Autoimmunity correlates with tumor regression in patients with metastatic melanoma treated with anti-cytotoxic T-lymphocyte antigen-4. *J Clin Oncol* **23**, 6043-6053 (2005).
12. Di Giacomo, A.M.*, et al.* Ipilimumab experience in heavily pretreated patients with melanoma in an expanded access program at the University Hospital of Siena (Italy). *Cancer Immunol Immunother* **60**, 467-477 (2011).
13. Wilgenhof, S.*, et al.* Single-center experience with ipilimumab in an expanded access program for patients with pretreated advanced melanoma. *J Immunother* **36**, 215-222 (2013).
14. Delyon, J.*, et al.* Experience in daily practice with ipilimumab for the treatment of patients with metastatic melanoma: an early increase in lymphocyte and eosinophil counts is associated with improved survival. *Ann Oncol* **24**, 1697-1703 (2013).
15. Di Giacomo, A.M.*, et al.* Long-term survival and immunological parameters in metastatic melanoma patients who responded to ipilimumab 10 mg/kg within an expanded access programme. *Cancer Immunol Immunother* **62**, 1021-1028 (2013).
16. Weide, B.*, et al.* Serum markers lactate dehydrogenase and S100B predict independently disease outcome in melanoma patients with distant metastasis. *Br J Cancer* **107**, 422-428 (2012).
17. Manola, J.*, et al.* Prognostic factors in metastatic melanoma: a pooled analysis of Eastern Cooperative Oncology Group trials. *J Clin Oncol* **18**, 3782-3793 (2000).
18. Wolchok, J.D.*, et al.* Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res* **15**, 7412-7420 (2009).
19. Fantin, V.R.*, et al.* Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. *Cancer Cell* **9**, 425-434 (2006).

20. Gatenby, R.A. & Gillies, R.J. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* **4**, 891-899 (2004).
21. Lardner, A. The effects of extracellular pH on immune function. *J Leukoc Biol* **69**, 522-530 (2001).
22. Ratner, S. Motility of IL-2-stimulated lymphocytes in neutral and acidified extracellular matrix. *Cell Immunol* **139**, 399-410 (1992).
23. Larkin, J., Vecchio M. Del, Ascierto PA, Schachter J., Garbe C., Neyns B. Open-label, multicenter safety study of vemurafenib in patients with BRAFV600E mutation-positive metastatic melanoma: interim analysis. in *2013 ASCO Annual Meeting* (Chicago, Illinois).
24. Ascierto, P.A.*, et al.* Sequencing of BRAF inhibitors and ipilimumab in patients with metastatic melanoma: a possible algorithm for clinical use. *J Transl Med* **10**, 107 (2012).
25. Chapman, P.B.*, et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**, 2507-2516 (2011).
26. Flaherty, K.T.*, et al.* Improved survival with MEK inhibition in BRAF-mutated melanoma. *N Engl J Med* **367**, 107-114 (2012).
27. Topalian, S.L.*, et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* **366**, 2443-2454 (2012).
28. Brahmer, J.R.*, et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* **366**, 2455-2465 (2012).
29. Hamid, O.*, et al.* Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med* **369**, 134-144 (2013).
30. European Commission - Community Register of Medicinal Products. http://ec.europa.eu/health/documents/community-register/html/h698.htm. Last updated Novenber 2013 (2011).

Supplementary Table 1.

	n	(%)
Prior Treatment		
DTIC	133	80%
BRAF inhibitor	5	3%
DTIC and BRAF inhibitor	3	2%
DC/DNA/peptide vaccination	3	2%
Other chemotherapy	2	1%
Other systemic therapy	1	1%
Other several lines	18	11%
After Treatment		
BRAF inhibitor	18	11%
MEK inhibitor	6	4%
Ipilimumab	2	1%
TIL	2	1%
BRAF inhibitor and ipilimumab	2	1%
BRAF inhibitor and TIL	2	1%
Ipilimumab and TIL	2	1%
DC/DNA/peptide vaccination	1	1%
Other chemotherapy	3	2%
Other systemic therapy	3	2%
None	125	75%

Supplementary Table 2.

	n	(%)
Colitis		
Grade 0	134	81%
Grade 1	10	6%
Grade 2	7	4%
Grade 3	10	6%
Grade 4	4	2%
Grade 5	1	1%
Dermatitis		
Grade 0	134	81%
Grade 1	21	13%
Grade 2	7	4%
Grade 3	4	2%
Hypophysitis		
Grade 0	161	97%
Grade 2	2	1%
Grade 3	3	2%
Hepatitis		
Grade 0	158	95%
Grade 1	2	1%
Grade 2	5	3%
Grade 3	1	1%
Uveitis		
Grade 0	164	99%
Grade 2	2	1%
Other toxicity		
Grade 0	138	83%
Grade 1	15	9%
Grade 2	7	4%
Grade 3	4	2%
Grade 4	2	1%

Supplementary Table 3.

	PD	SD	PR	CR	NE	Total
Total	79 (48%)	26 (16%)	15 (95%)	2 (1%)	44 (27%)	166
irRC						
PD	66 (40%)					66 (40%)
SD	12 (7%)	26 (16%)				38 (23%)
PR	1 (1%)		15 (95%)			16 (10%)
CR				2 (1%)		2 (1%)
NE					44 (27%)	44 (27%)
BORR						
PD	64 (39%)					64 (39%)
SD	12 (7%)	18 (11%)				30 (18%)
PR	2 (1%)	6 (4%)	13 (8%)			21 (13%)
CR	1 (1%)	2 (1%)	2 (1%)	2 (1%)		7 (4%)
NE					44 (27%)	44 (27%)

Supplementary Table 4.

Survival (%)	LDH normal	LDH >1X ULN	LDH >2X ULN
1 year NL	58.4%	13.7%	7.4%
2 year NL	38.3%	0%	0%
1 year UK	25.0%	10.0%	0%
2 year UK	20.8%	10.0%	0%
1 year NL + UK	51.2%	12.4%	4.8%
2 year NL + UK	34.6%	8.0%	0%

Chapter 3

Ipilimumab in pretreated metastatic uveal melanoma patients. Results of the Dutch working group on immunotherapy of oncology (WIN-O)

Sander Kelderman[1#], Monique K. van der Kooij[2#], Alfonsus J.M. van den Eertwegh[3], Patricia M.M.B. Soetekouw[3], Rob L.H. Jansen[4], Rob R.H. van den Brom[5], Geke A.P. Hospers[5], John B.A.G. Haanen[1], Ellen H.W. Kapiteijn[2], Christian U. Blank[1]

[1]Netherlands Cancer Institute NKI-AVL, Amsterdam, The Netherlands, [2]Leiden University Medical Center, Leiden, The Netherlands, [3]VU University Medical Center, Amsterdam, The Netherlands, [4]Maastricht University Medical Centre, Maastricht, The Netherlands, [5]University Medical Center Groningen, Groningen, The Netherlands. [#]These authors contributed equally.

***Acta Oncologica* 52: 1786-1788 (2013)**

INTRODUCTION

Uveal melanoma arises from melanocytes that reside in the iris, ciliary body or choroid of the eye. Local treatment can be divided into 'radical' enucleation and 'conservative' treatment. About 50% of patients develop metastasized disease and in up to 95% of these cases the liver is affected, due to the absence of lymphoid structures in the uvea. Once metastasized to the liver, surgical resection may be beneficial for small lesions, but less than 9% of patients fall into this category[1].

The blockade of cytotoxic T lymphocyte-associated antigen-4 (CTLA4) by ipilimumab has become standard for pretreated patients with cutaneous melanoma based on a randomized phase III study[2]. This drug significantly improved the overall survival resulting in 20-25% of the patients still being alive after more than two years.

Due to its distinct biological and clinical nature (fast progression) uveal melanoma patients are often excluded from melanoma studies. Uveal melanoma patients have been allowed to be included in ipilimumab expanded access programs, in which some clinical activity has been described[3-6].

In our study, 22 pretreated metastatic uveal melanoma patients were treated homogenously with 3mg/kg ipilimumab in the named patient program (NPP) by the Dutch immunotherapy working group (WIN-O) in The Netherlands. We describe here the toxicity and efficacy of ipilimumab at a 3mg/kg in a real world patient cohort of uveal melanoma patients.

METHODS

Patients

Patients were treated by the Dutch immunotherapy working group (WIN-O) in a NPP of ipilimumab (NCT00495066) in which uveal melanoma patients were allowed to be included. Patients had to have unresectable, metastatic uveal melanoma (with or without brain metastases) and were required to have received at least one prior treatment regimen for metastatic disease. They had to be at least 16 years of age with a WHO performance status of 0, 1, or 2. A 28-day interval since the last treatment was required before inclusion. Evaluable patients that had given their written informed consent underwent radiologic evaluation of their tumor burden at baseline and at twelve weeks after their first ipilimumab course. The treatment protocol was approved by the local medical ethical committees.

Treatment

Ipilimumab was administered at 3 mg/kg in week 1, 4, 7 and 10. Prior to every infusion, hemoglobin, leucocytes and differentiation, platelets, liver function, renal function, thyroid and adrenal function were assessed for safety reasons and monitoring of toxicity. Immune-related adverse evants (IrAEs) were scored using Common Terminology Criteria for Adverse Events (CTCAE) version 3.0.

Response and survival evaluation

At baseline and after four courses of ipilimumab at week 12, a computed tomography (CT) scan was made to evaluate the tumor response. We used the following radiological scoring systems; Immune-related response criteria (irRC) and RECIST version 1.1. The response rates were termed as partial remission (PR) and complete remission (CR). BOR was also assessed using irRC to capture delayed anti-tumor responses often observed with immunotherapy. Clinical benefit was defined as the response proportion of patients plus SD lasting longer than 24 weeks. Estimates of OS and PFS were obtained using the Kaplan-Meier method.

Data-analysis

Data were retrospectively collected from all Dutch centers organized in the Dutch immunotherapy working group (WIN-O) participating in the Dutch expanded access program and having treated uveal melanoma patients (see also co-authors affiliations). Patients' data were retrospectively collected into a predefined SPSS database by each center individually. Descriptive statistics were performed using SPSS statistical software (version 17.0 for Windows, SPSS, Chicago). The final data were graphed and analyzed using GraphPad Prism Version 5.0.

RESULTS

Twenty-two metastatic uveal melanoma patients were treated in an NPP, which was open in The Netherlands from May 2010 until August 2011. The patient characteristics of this cohort are described in Supplementary Table 1. Median follow-up was 177 days (6.3 months). Twelve patients (55%) completed the four infusions of ipilimumab. Of the remaining ten patients, nine had to discontinue treatment because of clinical deterioration due to disease progression (two of them died) and one because of severe adverse events (Fig. 1).

In Table 1 the response to treatment is described. Of the 22 patients who received at least one ipilimumab infusion, 13 patients showed progressive disease (PD) and one patient had a PR. There was no SD or CR achieved according to RECIST 1.1. Eight patients were not evaluable (NE). Following irRC there were twelve patients with PD, one with SD, one with PR and no CRs.

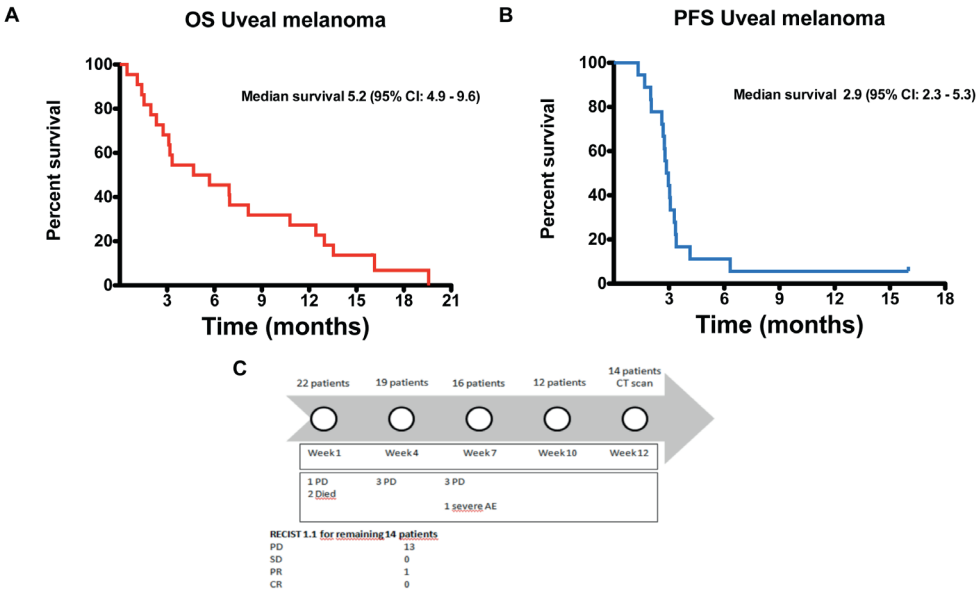


Figure 1. OS and PFS of uveal melanoma patients treated with ipilimumab 3mg/kg. All uveal melanoma patients treated in the Dutch expanded access program were evaluated retrospectively for **(a)** OS and **(b)** PFS. All 22 patients were included for PFS analysis, and the patients not evaluable at week 12 were defined to be progressive at the date of clinical deterioration. **(c)** The detailed follow up of the patients during treatment is shown.

Table 1. Response to treatment

RECIST after 12 weeks			**Clinical benefit (based on BOR)**		
Progressive disease	13	(59.1%)	PD/NE	20	(90.9%)
Stable disease	0	(0%)	SD >24w/PR/CR	2	(9.1%)
Partial response	1	(4.5%)			
Complete response	0	(0%)	**Response rate (based on BOR)**		
Not evaluable	8	(36.4%)	SD/PD/NE	21	(95.5%)
			CR/PR	1	(4.5%)
IRRC after 12 weeks					
Progressive disease	12	(54.5%)			
Stable disease	1	(4.5%)			
Partial response	1	(4.5%)			
Complete response	0	(0%)			
Not evaluable	8*	(36.4%)			
Best overall response					
Progressive disease	12	(54.5%)			
Stable disease	1	(4.5%)			
Partial response	1	(4.5%)			
Complete response	0	(0%)			
Not evaluable	8*	(36.4%)			

* Not evaluable due to fast disease progression and death within 65 days after start of treatment.

At the time of manuscript preparation one patient (4.5%) was still alive with ongoing SD (+16 months). The patient observing a PR was eligible for ipilimumab re-induction due to disease progression 7 months after ipilimumab initiation. Unfortunately, the re-induction did not result in a renewed response. The OS and PFS curves of our 22 patients are depicted in Fig. 1. The Kaplan-Meier analyses show a median PFS of 2.9 months. The median OS was 5.2 months with a 1-year survival of 27%. As shown in Supplementary Table 2 most adverse events were immune-related. Here, we only describe the grade 3 irAEs, as grade 1 or 2 was not considered clinically relevant. Grade 3 colitis was seen in two patients. One patient developed grade 3 hepatitis. All patients received corticosteroid treatment (1mg/kg predisolon) after which irAEs quickly resolved.

DISCUSSION

In our study, 22 M1c uveal melanoma patients were treated by the Dutch immunotherapy working group (WIN-O) in an ipilimumab NPP in The Netherlands. Only twelve patients (55%) completed the treatment course consisting of four infusions of ipilimumab at the dose of 3mg/kg. Within the cohort of the 22 patients, only one patient had a PR according to RECIST and another patient had SD according to irRC.

In another recently published study performed by Danielli *et al.*, nine out of thirteen patients (69%) completed the course of four infusions and two patients showed SD that remained until week 36 [4]. Median OS was 36 weeks (9 months), in contrast to 21 weeks (5.2 months) in our cohort.

Three other, so far unpublished, retrospective analyses have evaluated the efficacy of ipilimumab in uveal melanoma patients. A single center analysis of twenty uveal melanoma patients treated at the Memorial Sloan-Kettering Cancer Center observed within a group of 20 patients that received a median of four infusions of ipilimumab (20%) two PRs (one at week 12 and one at week 24) and seven SD. This resulted in a median survival of 8.6 months (95% CI, 3.5-NR), with two ongoing PRs (3+ yrs and 24+ wks)[3]. The other expanded access programs, the Italian and the US, observed a 1-year OS rate of 32% and 34%, respectively, which were comparable to the 1-year OS rate observed in our study (27%)[5,6].

Furthermore, initial phase 1 studies indicated a correlation between the presence of grade 3-4 irAEs and response[7], that was not confirmed in the phase 3 studies[2,8]. Similarly, no such correlation was found in our analysis.

In conclusion, our retrospective analysis from the Dutch expanded access program indicate limited clinical activity of ipilimumab in pretreated patients with metastatic uveal melanoma at a dose of 3mg/kg. Currently, two single-arm phase II clinical trials are testing ipilimumab in uveal melanoma patients (www.clinicaltrials.gov Identifier: NCT01355120

and NCT01034787). In addition a phase Ib/II study exploring the combination of ipilimumab with radiofrequency ablation (RFA) in uveal melanoma patients has been started recently at the Netherlands Cancer Institute (NKI-AVL), Amsterdam (www.trialregister.nl Identifier: NTR3488). Intensive patient characterization and biomarker research in these studies will hopefully be able to identify predictive factors for response and survival to targeted therapy and immunotherapy in metastatic uveal melanoma.

REFERENCES

1. Sato, T., Han, F. & Yamamoto, A. The biology and management of uveal melanoma. *Curr Oncol Rep* **10**, 431-438 (2008).
2. Hodi, F.S.*, et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* **363**, 711-723 (2010).
3. Khan, S.A.*, et al.* Ipilimumab in the treatement of uveal melanoma: The Memorial Sloan-Kettering Cancer Center experience. *Abstract #8549, ASCO Annual Meeting* (2012).
4. Danielli, R.*, et al.* Ipilimumab in pretreated patients with metastatic uveal melanoma: safety and clinical efficacy. *Cancer Immunol Immunother* **61**, 41-48 (2012).
5. Maio, M.*, et al.* Efficacy and Safety of Ipilimumab in Patients with Pretreated, Ocular Melanoma: Experience from Italian Clinics Participating in the European Expanded Access Programme. *Abstract #1133P, ESMO Annual Meeting* (2012).
6. Lawrence, D.*, et al.* Treatment of patients (pts) with stage III or IV melanoma on an ipilimumab (Ipi) expanded access program (EAP): results for 3mg/kg cohort. *Poster, SMR meeting* (2012).
7. Maker, A.V.*, et al.* Tumor regression and autoimmunity in patients treated with cytotoxic T lymphocyte-associated antigen 4 blockade and interleukin 2: a phase I/II study. *Ann Surg Oncol* **12**, 1005-1016 (2005).
8. Robert, C.*, et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* **364**, 2517-2526 (2011).

Supplementary Table 1. Patient characteristics (N=22)

Gender of the patient			**Treatment before ipi**		
Male	10	(45%)	DTIC	19	(86.4%)
Female	12	(55%)	Other chemo	1	(4.5%)
			Other several	2	(91.%)
Age					
Median	54	(37-73)	**Number of ipi courses**		
			1 course	3	(13.6%)
Mstage			2 courses	3	(13.6%)
M1a	0	(0%)	3 courses	4	(18.2%)
M1b	0	(0%)	4 courses	12	(54.5%)
M1c	0	(100%)			
			Median follow-up		
WHO status			6.3 months		
0	13	(59.1%)			
1	8	(36.4%)			
2	1	(4.5%)			

Supplementary Table 2. Immune-related adverse events (irAEs)

Colitis			**Hepatitis**		
Grade 0	20	(90.9%)	Grade 0	20	(90.9%)
Grade 1	0	(0%)	Grade 1	0	(0%)
Grade 2	0	(0%)	Grade 2	1	(4.5%)
Grade 3	2	(9.1%)	Grade 3	1	(4.5%)
Dermatitis			**Uveitis**		
Grade 0	15	(86.2%)	Grade 0	21	(95.5)
Grade 1	5	(22.7%)	Grade 1	0	(0%)
Grade 2	2	(9.1%)	Grade 2	1	(4.5%)
Grade 3	0	(0%)	Grade 3	0	(0%)
Hypophysitis			**Other**		
Grade 0	22	(100%)	Grade 0	14	(63.6%)
Grade 1	0	(0%)	Grade 1	3	(13.6%)
Grade 2	0	(0%)	Grade 2	2	(9.1%)
Grade 3	0	(0%)	Grade 3	3	(13.6%)

Chapter 4

Mismatch repair-deficient cancers are targets for anti-PD-1 therapy

Sander Kelderman[1], Ton N. Schumacher[1], Pia Kvistborg[1]

[1]The Netherlands Cancer Institute NKI-AVL, Amsterdam, The Netherlands

***Cancer Cell* 28: 11-13 (2015)**

ABSTRACT

Immune checkpoint-blocking therapies have yielded positive clinical data in a series of human malignancies. Recent work from Le and colleagues strongly supports the use of these therapies for mismatch repair-deficient tumors, independent of underlying tumor type. These data suggest the importance of sensing the consequences of DNA damage in cancer immunotherapy.

PREVIEW

Over the past few years, therapies that interfere with T cell checkpoints and, in particular, the PD-1/PD-L1 axis, have reached center stage in oncology. Clinical trials with anti-PD-1 and anti-PD-L1 antibodies have now shown objective responses in a series of human malignancies, including non-small cell lung cancer (NSCLC), bladder cancer, renal cell carcinoma, and Hodgkin's lymphoma[1]. In non-melanoma tumors, clinical development of anti- PD-1 therapy is most advanced for NSCLC, with recent approval by FDA as second-line therapy. A proposed explanation for the activity of T cell checkpoint blockade in tumors such as melanoma and NSCLC has been the boosting of T cell reactivity against ''neo-antigens'', T cell epitopes that are newly formed as a consequence of tumor-specific mutations[2]. In line with this, T cell checkpoint blockade has been shown to enhance neo-antigen-specific T cell responses in both diseases[3,4].

Contrary to the data in a number of other tumor types, clinical testing of anti-PD-1 has thus far not shown encouraging results in colorectal cancer (CRC). In two trials, a total of 33 CRC patients were treated with anti-PD-1 therapy and only 1 patient (3%) experienced an objective response, which was, however, a complete response[1]. Interestingly, the tumor of this patient displayed a hyper-mutated phenotype, a characteristic of approximately 4% of metastasized colorectal cancers.

Based on these data, Le and colleagues hypothesized that patients with mismatch repair-defects might be particularly responsive to checkpoint targeting. They now provide compelling data in favor of this hypothesis in a recent publication in the New England Journal of Medicine[5]. In a phase II study that evaluated the activity of PD-1 blockade in 41 patients, 3 cohorts of patients were included: 11 patients with mismatch repair-deficient CRC, 21 patients with mismatch repair-proficient CRC, and 9 patients with mismatch repair-deficient cancers other than CRC. DNA mismatch- repair deficiency results in large numbers of mutations, particularly in regions of repetitive DNA sequences, a phenomenon known as microsatellite instability (MSI). Mismatch-repair deficiency can arise as a consequence of inheritance of an inactive allele of one of the mismatch repair genes, with subsequent loss of the remaining wild-type copy, a genetic disorder known as Lynch syndrome. Alternatively, sporadic inactivation of mismatch-repair genes may occur, and, in this trial, patients with both types of mismatch- repair deficiency were included. Strikingly, 40% (4 out of 10) of mismatch repair-deficient CRC patients and 71% (5 of 7 patients) of mismatch repair-deficient non-CRC patients experienced an objective clinical response, whereas none of the patients with mismatch repair-proficient tumors responded (0 of 18). Furthermore, CRC patients with mismatch-repair deficiency had a significantly longer progression free survival ($p < 0.001$) as well as overall survival ($p = 0.03$). An unexpected observation in this study is that patients with Lynch syndrome appear to do less well as compared to mismatch repair-

deficient tumors not linked to Lynch syndrome (3 out of 11 versus 6 out of 6 responders). Before speculating about possible biological explanations, it will be important to further address this issue in future studies.

Consistent with a large body of literature on genomic alterations in mismatch repair-deficient and -proficient tumors, the authors demonstrated a profound difference in mutational load between the two. On average, 1,782 somatic mutations were identified in mismatch repair-deficient tumors (n = 9) (by far surpassing the mutational load in melanoma and NSCLC), whereas, on average, only 73 somatic mutations were observed in mismatch repair-proficient tumor (n = 6). A prior analysis of patients treated with anti-PD-1 has shown that patients with NSCLC with a high mutational load have a significantly higher likelihood of clinical benefit than patients with less mutated tumors[3]. A similar, albeit weaker, correlation was seen for melanoma patients treated with anti-CTLA-4 therapy[6]. Thus, the data from Le and coworkers add to the increasing body of evidence that, in at least some malignancies, high mutational load can increase the likelihood of clinical response to immune checkpoint-blocking therapies.

The most straightforward explanation for the heightened activity of anti-PD-1 therapy in mismatch repair-deficient tumors is the increased probability of a strong neo-antigen-driven T cell response (model A in Fig. 1). Nevertheless, as also pointed out by the authors, other potential mechanisms should not be excluded. First, DNA repair-deficient tumors could potentially be characterized by changes in signaling transduction pathways that lead to a more inflamed tumor micro-environment, for instance, through altered cytokine or chemokine expression (model B). Alternatively, the high level of DNA damage in mismatch repair-deficient tumors may potentially cause cellular stress that could, for instance, be sensed by innate immune cells (model C). Nevertheless, Occam's razor would tell us that, in the absence of additional evidence for such models, mismatch repair-deficient tumors may simply look more ''foreign'' because of the high number of neo-antigens they carry.

A remarkable aspect of mismatch repair-deficient CRC is the frequent loss of MHC class I expression (in up to 60% of MSI CRC tumors[7]). How can this be reconciled with the current clinical data? First, clinical activity may primarily be seen in the subset of MHC class I proficient tumors, in which case straightforward MHC class I immunohistochemistry could be used as a potential biomarker. Alternatively, it is worth considering that PD-1 therapy may also work in the absence of tumor-expressed MHC class I. First, antigen-presenting cells within the tumor microenvironment may cross-present MHC class I-restricted tumor antigens to tumor infiltrating $CD8^+$ T cells in situ, facilitating an immune response that can target the tumor microenvironment. Data from mouse models support the potential role of such a mechanism in tumor regression. In addition or alter- natively, anti-PD-1 therapy may work through tumor-specific $CD4^+$ T cells.

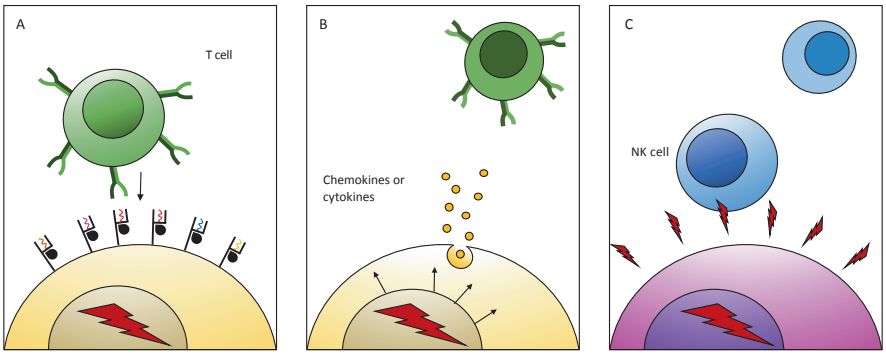


Figure 1. Potential mechanisms of action of anti-PD-1 therapy in mismatched repair-deficient tumors. (a) MMR deficiency results in a more diverse neo-antigen repertoire, increasing the chances of a tumor-specific T cell response. **(b)** MMR deficiency is associated with the activation of signaling pathways, which leads to a more inflammatory tumor micro-environment. **(c)** MMR deficiency leads to cellular stress, which, for instance, promotes T or NK cell accumulation or tumor recognition.

Indirect support for the latter model is provided by the observation that $CD4^+$ TIL in MSI tumors express higher levels of PD-1 than $CD4^+$ T cells in MSS (microsatellite stable) tumors[8] and by the observation that $CD4^+$ TIL in melanoma—a tumor type with a lower mutational load—frequently recognize mutated antigens[9]. Furthermore, work from Kreiter et al. has shown that MHC class II-restricted T cell responses can play an important role in tumor control in a pre-clinical colorectal cancer model[10].

The data from Le and colleagues strongly suggest that immune check- point blockade can form an attractive therapy for mismatch repair-deficient tumors independent of the underlying tumor type. The fraction of mismatch repair-deficient tumors for metastasized cancers is unfortunately not very high: for instance, roughly 4% of CRC, up to 11% of ovarian carcinomas, and 18% of endometrial cancers. In future studies, it will be important to under- stand if it is feasible to extend cancer immunotherapy to colorectal tumors with less profound DNA damage, for instance through combination check- point blockade. From a more mechanistic point of view, it will be of interest to understand whether clinical efficacy within the mismatch repair-deficient patient group is primarily restricted to MHC class I expressing tumors, or whether anti-PD-1 therapy can also work in the absence of tumor-expressed MHC class I.

ACKNOWLEDGMENTS

Work in the laboratory of T.N.S is supported by the Dutch Cancer Society (Queen Wilhelmina Research Award) and a Stand Up To Cancer-Cancer Research Institute Cancer Immunology Translational Cancer Research Grant. Stand Up To Cancer is a program of the Entertainment Industry Foundation Administered by the American Association for Cancer Research.

REFERENCES

1. Topalian, S.L.*, et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* **366**, 2443-2454 (2012).
2. Schumacher, T.N. & Schreiber, R.D. Neoantigens in cancer immunotherapy. *Science* **348**, 69-74 (2015).
3. Rizvi, N.A., *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science* **348**, 124-128 (2015).
4. van Rooij, N.*, et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol* **31**, e439-442 (2013).
5. Le, D.T.*, et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* **372**, 2509-2520 (2015).
6. Snyder, A.*, et al.* Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N Engl J Med* **371**, 2189-2199 (2014).
7. Dierssen, J.W.*, et al.* HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. *BMC Cancer* **7**, 33 (2007).
8. Llosa, N.J.*, et al.* The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov* **5**, 43-51 (2015).
9. Linnemann, C.*, et al.* High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nat Med* **21**, 81-85 (2015).
10. Kreiter, S.*, et al.* Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* **520**, 692-696 (2015).

Chapter 5

Acquired and intrinsic resistance in cancer immunotherapy

Sander Kelderman[1], Ton N.M. Schumacher[1], John B.A.G. Haanen[1]

[1]The Netherlands Cancer Institute NKI-AVL, Amsterdam, The Netherlands

***Molecular Oncology* 8: 1132-1139 (2014)**

ABSTRACT

A number of immunotherapies, in particular immune checkpoint targeting antibodies and adoptive T-cell therapies, are starting to transform the treatment of advanced cancers. The likelihood to respond to these immunotherapies differs strongly across tumor types, with response rates for checkpoint targeting being the highest in advanced melanoma, renal cell cancer and non-small cell lung cancer. However, also non-responsiveness is observed, indicating the presence of intrinsic resistance or naturally acquired resistance. In addition, a subgroup of patients that do initially respond to immunotherapy will later recur, thereby also pointing towards a role of therapy-induced acquired resistance.

Here, we review our current understanding of both intrinsic and acquired resistance mechanisms in cancer immunotherapy, and discuss potential strategies to overcome them.

INTRODUCTION

For many tumor types, including melanoma, renal cell cancer, colon cancer, ovarian cancer, and some subtypes of breast cancer, the presence of lymphocytic infiltrates within the tumor is highly correlated with improved outcome[1-8]. These infiltrates mostly consist of $CD4^+$ and $CD8^+$ T cells, and especially for melanoma it has been well established that part of these T cells recognize tumor-associated antigens[9,10]. The fact that these cells can have direct tumoricidal potential is well illustrated by the clinical effects of adoptive transfer of *ex vivo* expanded tumor-infiltrating lymphocytes (TIL) in metastatic melanoma patients. In several small clinical trials, response rates varying from 40% to 70% have been observed in highly selected metastatic melanoma patients[11,12]. In a more recent intent-to-treat analysis in a TIL trial for melanoma, a response rate of 30% has been reported[13]. Within these studies, the absolute numbers of $CD8^+$ T cells infused is strongly correlated with response to treatment, suggesting an important role for MHC class I restricted, cytotoxic T-lymphocyte (CTL) mediated tumor killing[13,14]. Direct evidence in support of such a role has been obtained through the administration of TIL products enriched for $CD8^+$ T cells, which showed a response rate comparable to that seen with unselected TIL products[15]. In addition to tumor-reactive $CD8^+$ T cells, it is clear that TIL products can also contain $CD4^+$ T-cell populations that are tumor-reactive, and there is evidence for an anti-tumoral effect of such tumor-reactive $CD4^+$ populations in melanoma and cholangiocarcinoma[16,17].

A second, much more widely used, group of immunotherapeutic strategies that target the same cellular compartment focuses on the administration of antibodies that bind to immune checkpoint molecules, thereby (re)activating an endogenous tumor-specific T-cell immune response. Administration of ipilimumab, an antibody that binds the inhibitory receptor cytotoxic T-lymphocyte antigen 4 (CTLA4) on T cells, has shown a four month increase in median overall survival in phase III trials, leading to FDA and EMA approval[18,19]. An analysis of a large cohort of melanoma patients treated following this registration shows a long-term survival in 20-25% of treated metastatic melanoma patients[20], a number that compares favorably to the 8-10% seen previously in patients treated with chemotherapy. More recently, objective response rates up to 50% have been reported in phase I/II trials testing antibodies that target another checkpoint molecule, programmed cell death protein 1 (PD-1) or its ligand (PD-L1). Importantly, clinical responses upon PD-1 – PD-L1 targeting have been observed in malignancies other than melanoma, such as renal cell carcinoma (RCC) and non-small cell lung cancer (NSCLC)[21-23].

These encouraging clinical results have rightfully put immunotherapy at the forefront of oncological practice. Nevertheless, it is important to note that a substantial number of patients still derive no or only limited benefit for reasons largely unknown, sometimes at the cost of severe toxicities. The disparity in response rates observed between different

immunotherapeutic treatment modalities, but also across tumor types strongly suggests a role for immune resistance. Further evidence for such resistance comes from patients treated with immunotherapy who experience an initial decrease in overall tumor burden but eventually succumb to disease recurrence. In the following sections we describe the relevance of different classes of immunotherapy resistance in oncology and contrast this with therapy resistance seen with targeted therapies. Furthermore, we describe the strategies that may be taken to obtain a better understanding of immunotherapy resistance, and how this knowledge can be used clinically.

Requirements for an optimal anti-tumor T-cell response

To understand at which levels resistance to T cell-based cancer immunotherapy may occur, it is important to first describe the key elements that are required for a successful T-cell response that leads to cancer regression. To do so, we subdivide this process into three discrete steps (Fig. 1).

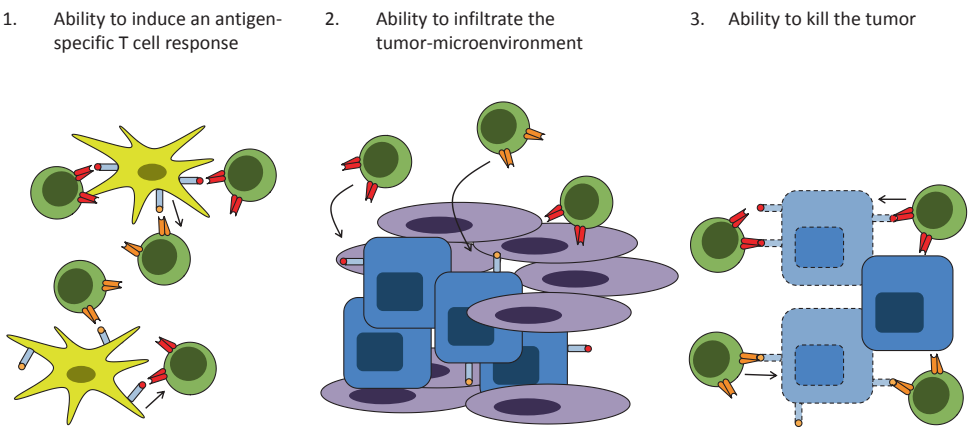


Figure 1. Key elements for an effective anti-tumor T-cell response. The development of an effective anti-tumor T-cell response follows three distinct steps: 1.) Priming and activation of naïve antigen-specific T cells; 2.) Migration and infiltration of activated T cells through the vasculature and tumor-surrounding stroma; 3.) Recognition of cognate peptide in the context of MHC and release of cytoloytic granules to mediate tumor cell killing.

First, T cells need to be properly activated by professional antigen-presenting cells (APCs) in peripheral lymphoid organs. For this to occur, two things are required: A). Dendritic cells (DCs) need to display tumor antigens (derived from apoptotic or necrotic tumor cells) in the context of MHC class I or II for which an antigen-specific T-cell repertoire is present. B). These DCs need to have received maturation signals that instruct the development of an effector T-cell response, rather than T-cell anergy or the expansion of regulatory T (Treg) cells.

Second, following priming in peripheral lymphoid organs, the activated T cells need to home to the tumor, extravasate through the endothelium and infiltrate via the surrounding stromal tissue into the tumor before they can bind to their target. This both requires certain phenotypic characteristics, such as expression of chemokine receptors, on the T cells and the expression of cell adhesion molecules/ chemokines by the vascular endothelium for cells to pass the endothelial barrier and invade the tumor[24]. T cells that have been inefficiently activated, because of lack of costimulatory molecule expression on APCs, or as a result of ineffective priming, can become anergic. By the same token, also when T-cell priming is efficient, but the tumor lacks the inflammatory signals to attract these cells, the tumor-specific immune response will be of little value.

Third, the T-cell receptors on the infiltrating T cells need to contact peptide MHC complexes on the tumor cell surface, in the case of $CD8^+$ cells, to release lytic granules in the immune synapse thereby mediating tumor destruction. Furthermore, the environment that the T cells encounter needs to permit such cytolytic activity. Negative feedback loops that regulate T-cell activity at effector sites are abundant and are essential to prevent run-away immune responses, but can also inhibit T-cell mediated tumor regression.

Having described the requirements for an optimal anti-tumor immune response, we can now make a subdivision of cancer immune resistance into three distinct classes (Fig. 2A) that are further detailed below.

Intrinsic resistance

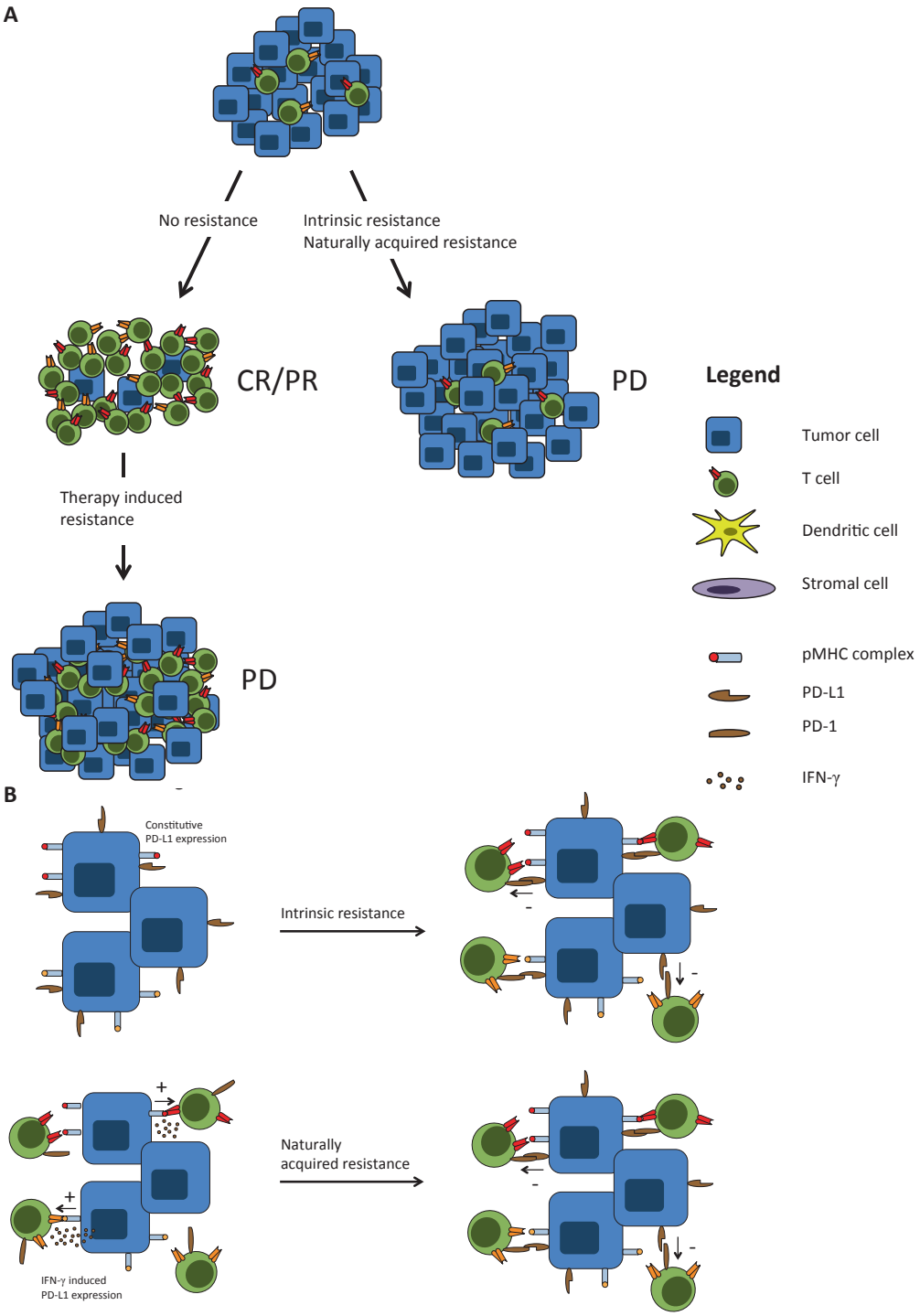
First, there are non-responding patients that lack anti-tumor immune activity and that also fail to elicit a T-cell response that has substantial tumoricidal potential upon immunotherapy, indicating *intrinsic resistance*. This form of resistance can be the result of a failing antitumor immune response either locally or systemically. A first class of systemic immune failure is observed in patients that are unable to elicit a potent immune response to a large variety of truly foreign antigens, such as viruses. This is for example observed in severely immune-compromised HIV patients or transplantation patients who carry an increased risk of virally induced neoplasias[25]. Also elderly people may lose the capacity to mount a sufficiently strong systemic immune response upon foreign antigen exposure, possibly caused by a decrease in the diversity of the total T-cell pool[26-28]. This effect is demonstrated in elderly patients who suffer from the reactivation of silent viruses, such as VZV that causes shingles, or infection with Merkel cell polyomavirus that can cause Merkel cell carcinoma[29]. Although speculative, it is conceivable that such patient subgroups are less capable of mounting an anti-tumor immune response that is efficient enough to eradicate cancer cells. A second class of systemic intrinsic resistance is formed by tumors that express few antigens that can be seen as foreign by the immune system. Tumor antigens can be subdivided in distinct antigen classes that together form the antigenic landscape of a particular tumor. Specifically,

many of the antigens that are (over-) expressed by tumors are also expressed on healthy tissues. For at least some of these 'self antigens', the avidity of the available T-cell repertoire will be low because of T-cell tolerance[30]. As a second class of antigens, human tumors can express epitopes that are truly foreign to the immune system, either derived from viral proteins or from mutant epitopes formed as a consequence of mutations. Recent evidence suggests that recognition of such neo-antigens may be of particular importance for tumor control[31-35]. Consequently, non-viral tumors with a low mutational load, such as pediatric and many of the liquid tumors, may be more likely to evade immune detection than tumors with a high mutational load, such as melanoma or smoking-related non-small cell lung cancer (NSCLC)[36,37].

Local intrinsic immune resistance may manifest itself in several ways. First, in some patients tumors may completely lack lymphocytic infiltrates. Assuming that in at least some of these patients, a systemic tumor-specific T-cell response was induced (data are presently lacking on this), this would signify the presence of a non-inflammatory tumor micro-environment that hampers infiltration of immune cells that would otherwise be able to recognize the tumor[38,39].

Assuming that tumor-specific T cells are properly activated and capable of homing to the tumor, the tumor microenvironment can pose the last barrier for T cells to exert their effector functions thereby giving rise to intrinsic resistance. It has been described that expression of PD-L1, which is the main ligand for the T-cell inhibitory molecule PD-1, can be induced upon loss of the tumor-suppressor gene PTEN and activation of the PI3K pathway in glioblastoma cell lines[40] (Fig. 2B).

Figure 2. Categories of immune resistance. (a) Several categories of immune resistance can be distinguished in either treatment-naïve or treatment experienced cancer patients. In the first group, tumor-infiltrating immune cells are either absent or scanty indicating indicative of *intrinsic resistance* or *naturally acquired resistance*. These patients are unlikely to respond to immune modulatory treatments. In the second group of patients, the degree of immune infiltration is sufficient to establish tumor regression upon immunotherapy initiation. However, due to several potentially overlapping mechanisms the tumor becomes resistant to this immune pressure and *therapy-induced resistance* ensues. **(b)** Mechanisms of intrinsic and naturally acquired resistance are exemplified by PD-L1 expression and subsequent effector function inhibition of antigen-specific T cells. Upper panel shows tumor cells that constitutively express PD-L1 as a result of genetic alterations related to the oncogenic process. Lower panel shows induced expression of PD-L1 mediated by IFN-γ producing T cells. ➔


A
No resistance
Intrinsic resistance
Naturally acquired resistance
CR/PR
PD
Therapy induced
resistance
PD
Legend
Tumor cell
T cell
Dendritic cell
Stromal cell
pMHC complex
PD-L1
PD-1
IFN-γ
B
Constitutive
PD-L1 expression
Intrinsic resistance
Naturally
acquired resistance
IFN-γ induced
PD-L1 expression

Additionally, the secretion of inhibitory molecules such as TGF-β, IL-10 and IDO can have a direct negative effect on T-cell function in the microenvironment[41-44], but also indirectly via the recruitment of tolerogenic immature DCs, myeloid derived-suppressor cells (MDSCs) or (inducible) regulatory $CD4^+$ T cells[45-48]. It is important to point out though that the presence of a T-cell infiltrate within a progressing tumor does not necessarily imply local inhibition of T-cell function as the mechanism of intrinsic resistance. Specifically, for most cancer types where T-cell infiltration is apparent we do not presently know to what extent this T-cell infiltrate consists of tumor-specific T cells or of bystander cells, and only in the former case, local inhibition needs to be considered as a barrier to immune control.

Intrinsic resistance is not unique to immunotherapy but can also be observed in patients treated with targeted therapies. A well-described example of this is the different sensitivities of tumors that carry the BRAF V600E mutations to drugs such as vemurafenib that bind the mutant BRAF protein. Specifically, whereas the majority of melanoma patients with a BRAF V600E mutation show a rapid (albeit often transient, see below) tumor regression upon treatment with BRAF inhibitors, patients with BRAF V600E colorectal cancer are unresponsive to these drugs. Recent work demonstrates that this intrinsic resistance is due to EGFR expression in the BRAF mutant colorectal tumors, and that sensitivity can be imposed by concomitant EGFR inhibition[49,50]. While obtained in an entirely different therapeutic field, these data illustrate that intrinsic resistance can occur as a coincidental side effect of the oncogenic process, and can be overcome upon a better understanding of this process.

Naturally acquired resistance

Naturally acquired resistance is special in that it is unique to immunotherapy. This form of resistance is defined as a reduced sensitivity that is not induced by cancer immunotherapy but that develops as a consequence of naturally occurring immune pressure. In this group of patients, there will generally be signs of an ongoing immune response in peripheral blood or tumor tissue, but they will fail to derive benefit from immune modulatory treatment.

In the case of naturally acquired resistance, there is presently little evidence for altered T-cell activation or homing. Rather, this form of resistance may mostly manifest itself as mechanisms that interfere with T-cell activity within the tumor micro-environment. Multiple inhibitory feedback mechanisms can play a role here, including the expression of a variety of (potentially overlapping) checkpoint molecules that dampen the immune response, such as LAG-3, TIM-3 and BTLA[51]. As an example, when tumor-infiltrating effector T cells start to produce IFN- γ upon binding of cognate antigen, this will induce PD-L1 expression on the tumor cell surface, which serves to limit further T-cell effector function by engaging the immune checkpoint molecule PD-1[39] (Fig. 2B).

In addition, a naturally occurring immune response may select for tumor cell subpopulations with loss of MHC class I expression, or other defects in the antigen processing machinery, thereby cloaking the tumor cell from the immune system[52-54]. A similar immune evasive effect may be achieved through selection of tumor subclones present within heterogeneous tumors lacking one or multiple antigens that are subject to strong Darwinian selection, a process called immune-editing[55-57]. Strong evidence for immune editing has been obtained in mouse model systems. However, other murine studies suggest that antigen loss may be less of an issue in cases in which the release of IFN-γ and TNF-α by CTLs leads to the destruction of tumor stroma [58]. Human data on this topic are at present lacking but may conceivably be obtained with the recently developed abilities to describe T-cell responses against (mutant) antigens within individual patients.

Therapy-induced resistance

A third class of resistance is observed when patients that initially respond to immunotherapy relapse, which we define as *therapy-induced resistance*. This type of resistance is well known in patients treated with classical cytotoxic agents or with targeted agents, such as BRAF inhibitors[59]. Natural resistance upon treatment with such targeted therapies, where virtually all patients eventually relapse, can be due to selection of resistant tumor clones already present at low numbers at the start of treatment, or of newly mutated resistant clones. This stands in stark contrast with immunotherapy-treated patients where durable complete responses are often already observed after a single-modality treatment. Although immunotherapy-induced clinical responses can last up to years, a subgroup of patients experiences only temporary disease regression[20,60,61]. The general mechanisms of therapy-induced resistance will be very similar to those mentioned previously in the setting of naturally acquired resistance: When a properly activated T-cell pool with homing capacity is present, an equilibrium between effector T cells and the tumor is reached locally, which at some point in time tips the balance in favor of renewed tumor growth.

Strategies to study resistance mechanisms

To increase our understanding of immunotherapy resistance, we suggest to analyze this process on the basis of the three different nodes that are involved in an effective anti-tumor immune response.

First, a diverse T-cell pool is required that can respond to a wide variety of tumor-associated antigens. The currently used immunotherapeutic strategies that exploit the activity of the endogenous T-cell compartment appear predominantly effective in tumors with median to high mutational loads, consistent with a role of neo-antigen recognition in tumor control. While the occurrence of neo-antigen reactive T cells appears to be a common trait in human melanoma[31,32], more direct evidence for their role in tumor control

is still lacking. Longitudinal immune monitoring of neo-antigen specific T cells in a setting of cancer immunotherapy, using polychromatic flow cytometry or mass cytometry[62] should be of value here. In a similar manner, assessment of immune competence (i.e. the ability to elicit a polyfunctional T-cell response) on a per patient basis could help guide eligibility for immunotherapeutic intervention.

Second, to study the homing capacity of endogenously activated T cells in the context of immune escape, we need to address to what extent the tumor microenvironment is capable of triggering T-cell infiltration. To study this, pre-therapy tumor biopsies can be taken from patients included in immunotherapy trials and predictive gene-expression signatures established that correlate with ongoing or subsequent T-cell infiltration or with clinical benefit[63].

The third and final step that needs to be analyzed is the ability of T cells to release their effector functions at the site where it is needed. Several feedback mechanisms are at play here. Importantly though a hierarchy has not yet been determined, and such a hierarchy 1). Is likely to differ between tumor types; 2). Is within tumor types likely to differ depending on the specific genetic alterations; 3). May for a given tumor conceivably even vary depending on the site of metastasis. Recent work has emphasized the role of PD-L1 expression as an important regulator of local T-cell effector function. Using PD-L1 expression as a biomarker grouped responding patients in an anti-PD-1 phase I clinical study, although absence of expression did not exclude a response to therapy[64]. These data underscore the value of biomarker discovery not only for the early phases of the endogenous immune response (e.g. local inflammation) but also for the later effector phase. Notably though, patients with colorectal cancer only infrequently show responses to PD-1 blockade, even though these tumors have high mutational loads and T-cell infiltrates within these tumors has been shown to form a prognostic factor superior to the standard TNM classification[65,66]. These data are consistent with the hypothesis that within these tumors, another inhibitory pathway could be dominant.

Strategies to overcome resistance

The efficacy of many immunotherapeutic strategies is dependent on the strength of the endogenous T-cell response, including the level of tolerance towards the antigens recognized. Therefore, patients with an impaired capacity to mount immune responses, or who carry tumors that express few strong T-cell antigens may gain most clinical benefit from strategies that create the missing tumor-reactive T-cell pool. This may be achieved by the adoptive transfer of T cells genetically modified to express an exogenous CAR or TCR capable of effective target killing[67,68 69-71]. In patients with a weak T-cell response against tumor antigens, low-frequency tumor-specific T-cell populations may be enriched from PBMNC or, perhaps preferable, from TIL, in order to steer reactivity towards predefined

tumor-associated epitopes and thereby augment the anti-tumor response. Alternatively, expression of co-stimulatory and co-inhibitory markers in fresh tumor digest has been shown to define the tumor-reactive T-cell subset in melanoma lesions, offering a potential means to create TIL products that are enriched for tumor reactivity without the need for prior knowledge on antigen-specificity[72,73].

In patients that have the capacity to mount a systemic T-cell response but where tumors do not permit the infiltration of immune cells or prevent the initiation of a local endogenous immune response, are unlikely to benefit from treatment regimens that rely on such local immune responses. Such patients might benefit more from pre-conditioning regimens that promote an immune supportive tumor microenvironment by providing 'danger signals' and the establishment of an inflammatory signature[74]. This may conceivably be achieved through the induction of immunogenic cell death either by chemotherapy, radiotherapy or even local injection of Toll-like receptor (TLR) agonists[75]. For cancer types with a relatively low mutational load, such as ovarian, breast or pancreatic cancer, the use of DNA damaging agents could lead to an increase in the mutation frequency and as such broaden (albeit in a non-clonal manner) the epitope landscape[76,77].

Patients that do exhibit an endogenous anti-tumor immune response in all the relative compartments discussed before are eligible for at least several immunotherapeutic interventions such as ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1). As with many classical cytotoxic agents, combination therapy could be of importance here in preventing escape from immune pressure. In this context, the nodes in the tumor-immune interaction that are targeted should be as little overlapping as possible and preferably complementary in nature. The combination of ipilimumab (thought to be involved in the early priming phase of T-cell activation) and nivolumab (thought to be involved in the later effector phase of T-cell activation) has already shown higher response rates than either treatment modality alone[78]. Presently, little is known about optimal timing or sequencing of available therapies but there is increasing evidence that patients failing one type of immunotherapy can respond to another, indicating independently operating resistance mechanisms than can be targeted accordingly. As an example, patients that have not benefitted from ipilimumab treatment still can have a meaningful objective response to anti-PD-1 treatment[22] and vice versa[64]. Patients failing ipilimumab treatment can likewise develop a durable complete remission upon TIL therapy[13].

CONCLUSION

In cancer immunotherapy, future rational treatment decision-making should be based on the specific node that is affected in the tumor-immune system interaction: 1.) Are antigen-specific T cells efficiently activated in the treatment-naïve host? 2.) Is there infiltration of those T cells into the tumor? and 3.) Are the tumor-infiltrating T cells able to exert their function at that site? To achieve this, efforts should be focused on the discovery and implementation of simple biomarkers at each stage in the immune response that can predict whether a patient is likely to respond to a specific type of immunotherapy or not. Considering the variation in response rates of immunotherapy within one tumor entity and between tumor types, immunotherapy finds itself at the point where a patient-specific approach is required in order to achieve durable tumor control in a larger group of patients.

ACKNOWLEDGEMENTS

This work was supported by Dutch Cancer Society grant KWF 2012-5463 to T.N.M.S., and by a SU2C-CRI Cancer Immunology Translational Cancer Research Grant to T.N.M.S.

REFERENCES

1. Clemente, C.G.*, et al.* Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer* **77**, 1303-1310 (1996).
2. Erdag, G.*, et al.* Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res* **72**, 1070-1080 (2012).
3. Galon, J.*, et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **313**, 1960-1964 (2006).
4. Zhang, L.*, et al.* Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* **348**, 203-213 (2003).
5. Hwang, W.T.*, et al.* Prognostic significance of tumor-infiltrating T cells in ovarian cancer: a meta-analysis. *Gynecol Oncol* **124**, 192-198 (2012).
6. Nakano, O.*, et al.* Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res* **61**, 5132-5136 (2001).
7. Alexe, G.*, et al.* High expression of lymphocyte-associated genes in node-negative HER2+ breast cancers correlates with lower recurrence rates. *Cancer Res* **67**, 10669-10676 (2007).
8. Mahmoud, S.M.*, et al.* An evaluation of the clinical significance of FOXP3+ infiltrating cells in human breast cancer. *Breast Cancer Res Treat* **127**, 99-108 (2011).
9. Coulie, P.G.*, et al.* A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med* **180**, 35-42 (1994).
10. Kawakami, Y.*, et al.* Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc Natl Acad Sci U S A* **91**, 6458-6462 (1994).
11. Dudley, M.E.*, et al.* Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* **26**, 5233-5239 (2008).
12. Dudley, M.E.*, et al.* CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clin Cancer Res* **16**, 6122-6131 (2010).
13. Besser, M.J.*, et al.* Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. *Clin Cancer Res* **19**, 4792-4800 (2013).
14. Radvanyi, L.G.*, et al.* Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* **18**, 6758-6770 (2012).
15. Dudley, M.E.*, et al.* Randomized selection design trial evaluating CD8+-enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. *J Clin Oncol* **31**, 2152-2159 (2013).
16. Tran, E.*, et al.* Cancer Immunotherapy Based on Mutation-Specific CD4+ T Cells in a Patient with Epithelial Cancer. *Science* **344**, 641-645 (2014).
17. Hunder, N.N.*, et al.* Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. *N Engl J Med* **358**, 2698-2703 (2008).
18. Hodi, F.S.*, et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* **363**, 711-723 (2010).
19. Robert, C.*, et al.* Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* **364**, 2517-2526 (2011).

20. Prieto, P.A., *et al.* CTLA-4 blockade with ipilimumab: long-term follow-up of 177 patients with metastatic melanoma. *Clin Cancer Res* **18**, 2039-2047 (2012).
21. Topalian, S.L., *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* **366**, 2443-2454 (2012).
22. Hamid, O., *et al.* Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med* **369**, 134-144 (2013).
23. Brahmer, J.R., *et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* **366**, 2455-2465 (2012).
24. Harlin, H., *et al.* Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. *Cancer Res* **69**, 3077-3085 (2009).
25. Butel, J.S. Viral carcinogenesis: revelation of molecular mechanisms and etiology of human disease. *Carcinogenesis* **21**, 405-426 (2000).
26. Khan, N., *et al.* Herpesvirus-specific CD8 T cell immunity in old age: cytomegalovirus impairs the response to a coresident EBV infection. *J Immunol* **173**, 7481-7489 (2004).
27. Akbar, A.N. & Fletcher, J.M. Memory T cell homeostasis and senescence during aging. *Curr Opin Immunol* **17**, 480-485 (2005).
28. Messaoudi, I., *et al.* Age-related CD8 T cell clonal expansions constrict CD8 T cell repertoire and have the potential to impair immune defense. *J Exp Med* **200**, 1347-1358 (2004).
29. Feng, H., *et al.* Clonal integration of a polyomavirus in human Merkel cell carcinoma. *Science* **319**, 1096-1100 (2008).
30. Kvistborg, P., *et al.* Human cancer regression antigens. *Curr Opin Immunol* **25**, 284-290 (2013).
31. van Rooij, N., *et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol* **31**, e439-442 (2013).
32. Robbins, P.F., *et al.* Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med* **19**, 747-752 (2013).
33. Heemskerk, B., *et al.* The cancer antigenome. *EMBO J* **32**, 194-203 (2013).
34. Champiat, S., *et al.* Exomics and immunogenics: Bridging mutational load and immune checkpoints efficacy. *Oncoimmunology* **3**, e27817 (2014).
35. Brown, S.D., *et al.* Neo-antigens predicted by tumor genome meta-analysis correlate with increased patient survival. *Genome Res* **24**, 743-750 (2014).
36. Alexandrov, L.B., *et al.* Signatures of mutational processes in human cancer. *Nature* **500**, 415-421 (2013).
37. Vogelstein, B., *et al.* Cancer genome landscapes. *Science* **339**, 1546-1558 (2013).
38. Gajewski, T.F., *et al.* Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. *Cancer J* **16**, 399-403 (2010).
39. Taube, J.M., *et al.* Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* **4**, 127ra137 (2012).
40. Parsa, A.T., *et al.* Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. *Nat Med* **13**, 84-88 (2007).
41. Geissmann, F., *et al.* TGF-beta 1 prevents the noncognate maturation of human dendritic Langerhans cells. *J Immunol* **162**, 4567-4575 (1999).
42. Pickup, M., *et al.* The roles of TGFbeta in the tumour microenvironment. *Nat Rev Cancer* **13**, 788-799 (2013).
43. Steinbrink, K., *et al.* Interleukin-10-treated human dendritic cells induce a melanoma-antigen-specific anergy in CD8(+) T cells resulting in a failure to lyse tumor cells. *Blood* **93**, 1634-1642 (1999).

44. Braun, D., *et al.* A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation. *Blood* **106**, 2375-2381 (2005).
45. Lutz, M.B. & Schuler, G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* **23**, 445-449 (2002).
46. Vukmanovic-Stejic, M., *et al.* Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. *J Clin Invest* **116**, 2423-2433 (2006).
47. Gabrilovich, D.I., *et al.* Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol* **12**, 253-268 (2012).
48. Strauss, L., *et al.* A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. *Clin Cancer Res* **13**, 4345-4354 (2007).
49. Prahallad, A., *et al.* Unresponsiveness of colon cancer to BRAF(V600E) inhibition through feedback activation of EGFR. *Nature* **483**, 100-103 (2012).
50. Sun, C., *et al.* Reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. *Nature* **508**, 118-122 (2014).
51. Pardoll, D.M. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* **12**, 252-264 (2012).
52. Khong, H.T., *et al.* Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. *J Immunother* **27**, 184-190 (2004).
53. del Campo, A.B., *et al.* Immune escape of cancer cells with beta2-microglobulin loss over the course of metastatic melanoma. *Int J Cancer* **134**, 102-113 (2014).
54. Restifo, N.P., *et al.* Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. *J Natl Cancer Inst* **88**, 100-108 (1996).
55. Matsushita, H., *et al.* Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. *Nature* **482**, 400-404 (2012).
56. Dunn, G.P., *et al.* Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **3**, 991-998 (2002).
57. Khong, H.T. & Restifo, N.P. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* **3**, 999-1005 (2002).
58. Zhang, B., *et al.* IFN-gamma- and TNF-dependent bystander eradication of antigen-loss variants in established mouse cancers. *J Clin Invest* **118**, 1398-1404 (2008).
59. Chapman, P.B., *et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**, 2507-2516 (2011).
60. Rosenberg, S.A., *et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* **17**, 4550-4557 (2011).
61. Di Giacomo, A.M., *et al.* Long-term survival and immunological parameters in metastatic melanoma patients who responded to ipilimumab 10 mg/kg within an expanded access programme. *Cancer Immunol Immunother* **62**, 1021-1028 (2013).
62. Bendall, S.C., *et al.* Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* **332**, 687-696 (2011).
63. Ji, R.R., *et al.* An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunol Immunother* **61**, 1019-1031 (2012).
64. Weber, J.S., *et al.* Safety, efficacy, and biomarkers of nivolumab with vaccine in ipilimumab-refractory or -naive melanoma. *J Clin Oncol* **31**, 4311-4318 (2013).
65. Pages, F., *et al.* In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol* **27**, 5944-5951 (2009).

66. Galon, J.*, et al.* Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol* **232**, 199-209 (2014).
67. Morgan, R.A.*, et al.* Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* **314**, 126-129 (2006).
68. Robbins, P.F.*, et al.* Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* **29**, 917-924 (2011).
69. Grupp, S.A.*, et al.* Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. *N Engl J Med* **368**, 1509-1518 (2013).
70. Kalos, M.*, et al.* T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* **3**, 95ra73 (2011).
71. Porter, D.L.*, et al.* Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. *N Engl J Med* **365**, 725-733 (2011).
72. Gros, A.*, et al.* PD-1 identifies the patient-specific CD8+ tumor-reactive repertoire infiltrating human tumors. *J Clin Invest* **124**, 2246-2259 (2014).
73. Ye, Q.*, et al.* CD137 accurately identifies and enriches for naturally occurring tumor-reactive T cells in tumor. *Clin Cancer Res* **20**, 44-55 (2014).
74. Gajewski, T.F. Cancer immunotherapy. *Mol Oncol* **6**, 242-250 (2012).
75. Kroemer, G.*, et al.* Immunogenic cell death in cancer therapy. *Annu Rev Immunol* **31**, 51-72 (2013).
76. Segal, N.H.*, et al.* Epitope landscape in breast and colorectal cancer. *Cancer Res* **68**, 889-892 (2008).
77. Zhang, G.N.*, et al.* TP53 K351N mutation-associated platinum resistance after neoadjuvant chemotherapy in patients with advanced ovarian cancer. *Gynecol Oncol* **132**, 752-757 (2014).
78. Wolchok, J.D.*, et al.* Nivolumab plus ipilimumab in advanced melanoma. *N Engl J Med* **369**, 122-133 (2013).

Chapter 6

Tumor antigens in human cancer control

Sander Kelderman[1], Pia Kvistborg[1]

[1]The Netherlands Cancer Institute NKI-AVL, Amsterdam, The Netherlands

***Biochimica et Biophysica Acta* 1865: 83-89 (2016)**

ABSTRACT

The body of evidence that is supporting the role of T cells in human tumor control is substantial and it is now beyond doubt that T cells can be crucial in the clinical response to cancer immunotherapies such as adoptive T cell therapy and checkpoint blockade. This has been proven in particular for melanoma and non-small cell lung cancer. Strikingly, while clinical experience with these therapies is extensive, what these T cells detect on the tumors remains largely unknown. An extensive effort has been put into the characterization of tumor antigens and based on the recent successes of immunotherapies Cancer/Germline, mutated and viral antigens appear rather promising targets for tumor control. Furthermore, it is becoming evident that the most potent antigen in tumor control is highly dependent on the type of malignancy and may also vary even within malignancies.

WHY T CELLS ARE RELEVANT

It is now beyond doubt that the endogenous T cell based immune system can recognize cancer cells and in some situations control the disease. Pre-clinical data from numerous mouse models have demonstrated the importance of T cells in tumor control by depleting either the adaptive immune response or T cells alone resulting in abrogation of tumor rejection to various degrees. The importance of T cells in the human setting is supported by the correlation of tumor infiltrating T cells with good prognosis in a substantial number of different cancers[1-4]. In particular, it is well described that the location and activation status of T cells in colorectal cancer is of high prognostic value. Galon and colleagues have shown that quantifying the infiltration of antigen experienced $CD8^+$ T cells in the tumor invasive margin and the center of the tumor as a prognostic tool is equally strong (or potentially even better) as the currently used staging system[5,6]. Furthermore, work from Ribas and colleagues has shown that tumor infiltration and location of $CD8^+$ positive cells in human melanoma can function as a predictive biomarker for clinical outcome to anti-PD-1 therapy[7]. Nevertheless, the mere infiltration of T cells into cancer and the correlation with prognosis or clinical outcome is not providing direct evidence for T cells being an active contributor to the control of human cancer.

As a direct proof for the tumoricidal potential of patient autologous T cells, Rosenberg and colleagues have shown that infusion of autologous *ex vivo* expanded tumor infiltrating lymphocytes (TIL) can induce objective clinical responses in melanoma patients[8,9]. Similar response rates are achieved when infusing TIL products enriched for $CD8^+$ T cells providing direct evidence for the tumor killing capacity of these cells[10-12]. Within the last few years, the evidence for the activity of T cells in tumor control has extended beyond melanoma to a number of other human malignancies. This has in particular been demonstrated in clinical trials showing responses to anti-PD-1 therapy in a number of cancers including non-small cell lung cancer (NSCLC), bladder cancer, renal cell carcinoma and Hodgkin's lymphoma[13-16]. Furthermore, it was shown in a recent case report that TIL therapy in a patient with metastatic cholangiocarcinoma was able to mediate tumor regression[17].

The main point that can be inferred from these clinical trial data is that a proportion of tumor cells must express antigens that allow endogenous T cells to specifically recognize and kill them. Having established that T cells can play a pivotal role in human cancer control, the next step is to assess which antigens that can be recognized by T cells leading to tumor regression.

WHICH ANTIGENS ARE DETECTED

T cells can recognize antigens that are presented on the surface of tumor cells in the context of HLA class I and II molecules and thereby mediate tumor cell destruction. These stretches of antigen bound to HLA molecules, epitopes, are a result of protein degradation in the cytosol. The fragmented antigens are transported into the ER lumen where they can be loaded onto an HLA molecule that will be translocated to the surface of the cell. Therefore, the epitopes bound to the HLA molecule forms a representation of cellular content. The first appreciation of such antigens recognized by autologous T cells came from van der Bruggen and colleagues in 1991 with the identification of MAGE-A1[18]. Since this first discovery a huge number of T cell epitopes have been characterized from proteins with an aberrant expression in tumor cells (Fig. 1).

The characterized T cell epitopes were identified through either the use of patient-derived T cell populations or by the 'reverse immunology' approach. Using the approach of dissecting what tumor reactive T cell clones from cancer patients recognize directly provides evidence for the immunogenicity of the identified epitopes. The reverse immunology strategy does not provide a similar validation and it is therefor central to demonstrate that T cell epitopes identified with such a strategy can be recognized by T cells from cancer patients directly *ex vivo*, and very importantly that these T cells recognize the autologous tumor.

Tumor antigens can be divided into two main classes. The first being the tumor associated antigens (TAA), which includes proteins that are shared between tumor and healthy tissue and to which tolerance is incomplete. These antigens can be further divided into categories based on their expression pattern in healthy tissues. One category is the over-expressed antigens[19,20]. These are proteins expressed by various healthy tissues and tumor cells and differ at the expression level of the proteins. A second category is the cell lineage-specific group of proteins including the melanocyte differentiation antigens that are expressed by the vast majority of melanomas[21,22]. A third category is the Cancer/Germline (C/G) antigens. These proteins are encoded by genes mainly expressed in germline cells and can be re-expressed in tumor due to dysregulation of demethylation in tumor cells[23].

The second class of antigens is formed by the tumor specific antigens (TSAs) also referred to as neo-antigens. These antigens include mutated antigens that arise as a consequence of tumor specific DNA damage as well as antigens resulting from the expression of oncogenic viral proteins.

This review will mainly focus on C/G and neo-antigens and discuss the promising evidence for their clinical relevance in the context of cancer immunotherapy.

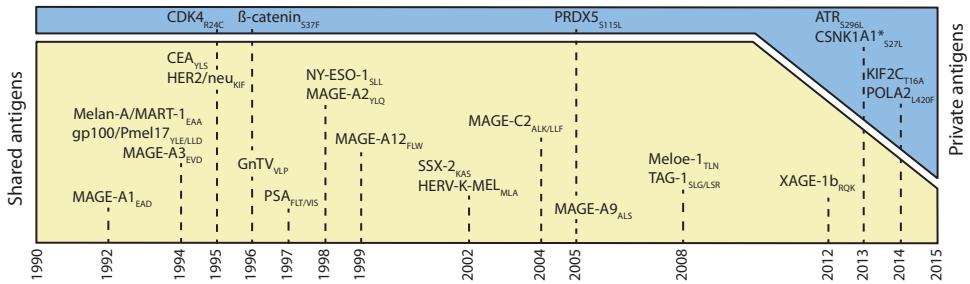


Figure 1. Time frame of antigen discovery. Over the course of 25 years, a multitude of tumor antigens have been discovered using various technologies such as cDNA library cloning, SEREX, reverse-immunology and, more recently, whole tumor-exome sequencing. The colored parts contain a non-exhaustive list of tumor antigens for which T-cell responses have been detected, in yellow for shared antigens and in blue for private antigens, with a more dominant focus on the latter antigen class in recent years. Data was retrieved from the Cancer Immunity Peptide Database[74]. T cell responses detected against the neo-antigens ATR, CSNK1A1 and, KIF2C and POLA2 were retrieved from [40, 41] and [42], respectively. Asterisk indicates that multiple neo-antigen specific T-cell responses were detected but only one here is shown.

Cancer/Germline antigens

Proteins are stratified to the group of C/G antigens based on their expression in both germ line cells and cancers. The C/G proteins are widely expressed during fetal development but become silenced for a substantial part by methylation of the genes in adult tissue, except for in germ cells. These genes are often re-expressed in cancers likely caused by changes in genomic methylation[23]. The first C/G antigen described was MAGE1 (now known as MAGEA1, Fig. 1). This antigen was identified using patient-derived tumor-reactive T cell clones isolated from a melanoma patient with an unusual favorable clinical course[18]. In the following decades >200 antigens belonging to this family have been identified. This discovery was in particular fast-tracked with the development of the SEREX technology (serological analysis of cDNA expression libraries).

The expression of C/G antigens varies greatly between different malignancies. As an example, NY-ESO-1 is expressed in NSCLC, ovarian carcinoma, breast carcinoma and melanoma with varying frequency[24]. Furthermore, within each tumor lesion the expression of a certain C/G antigen can be highly heterogeneous. Hence, the expression of C/G antigens appears to be somewhat tumor 'private' even though it is a shared antigen.

The reason for the heterogeneous expression pattern of C/G antigens has not yet been established but might reflect an ongoing Darwinian pressure together with redundant functionality. For some C/G antigens it has been demonstrated that the expression can be beneficial for tumor cells. As an example, proteins of the MAGE family have been found to modulate cell survival by suppressing the function of p53[25] and linked to acquired drug resistance in relapsing multiple myeloma patients[26,27]. However, for the vast majority of C/G

antigens there is no clear function known and thus their expression may be a stochastic event caused by tumor related changes in chromosomal stability. Regardless of their function that may or may not be linked to survival benefit, these antigens still form a useful group of targets.

The restricted expression profile of C/G antigens in healthy tissue and their aberrant expression in various tumors has made this group of antigens theoretically highly attractive targets for anti-cancer immune interventions. This is reflected by the high number of early clinical trials that have used members of this protein family as targets. The vast majority of these trials was based on vaccination strategies and did not result in notable toxicities nor did they result in sufficient clinical efficacy to be further developed. Despite the conceptually attractive characteristics of these antigens there are three crucial points to consider before developing therapies that will steer potent immune responses towards them.

First, more recent studies mapping the expression pattern of these proteins revealed that a good number are expressed at varying levels on healthy tissues accessible to the immune system. Examples are MAGE-A9 expressed on brain tissue and MAGE-A11 on lung tissue[28]. Based on these data a proportion of antigens that have previously been regarded as tumor-restricted targets might potentially cause severe toxicities when targeted with potent immune interventions. As a matter of fact, such toxicities have been encountered in clinical trials making use of T cell receptor (TCR) gene-engineered T cells for adoptive cell therapy. Severe safety issues were observed in a clinical trial in which melanoma patients were treated with a MAGE-A3 specific TCR[29]. This TCR cross-reacted with an epitope derived from MAGE-A12, which is expressed at low levels in the gray matter of the brain. This resulted in severe brain toxicity in 1/3 of the treated patients. More encouragingly, an NY-ESO-1 specific TCR was used in a clinical trial without toxicity issues in melanoma patients and synovial cell sarcoma patients achieving high objective response rates[30]. Taken together, these data illustrate that members of this antigen family can be potent tumor regression antigens. However, it is important not to view this antigen group as uniform concerning expression pattern but rather thoroughly investigate the expression of each protein in healthy tissue before targeting them. A second issue to consider is that, as previously mentioned, the expression of these antigens within the same tumor can be rather heterogeneous[31]. Patients included in the NY-ESO-1 TCR trial were selected for high homogeneous NY-ESO-1 expression and this may well be an important factor that determined the clinical success of this trial, as it is not known if sustained tumor control can be achieved when targeting heterogeneously expressed antigens. Finally, there is evidence that at least a part of C/G antigens can be expressed by thymic epithelial cells indicating that also for those C/G antigens that are truly restricted to immune privileged healthy tissue and tumor cells there may exist some level of T cell tolerance.

Neo-antigens

All tumor specific antigens that are truly foreign to the immune system belong to the class of neo-antigens. These are antigens that either arise as a consequence of tumor specific DNA damage or are derived from viral proteins expressed in virus-induced cancers. Because of their foreign nature central tolerance towards these antigens is expected to be absent, which is in contrast with the self-antigens discussed earlier. Conceptually, this class of antigens is highly attractive as they are truly foreign to the immune system, because they are strictly restricted to tumor tissue, and no central tolerance should exist[32].

Cancer is, in essence, a genetic disease characterized by an accumulation of mutations over time. In general, human cancers are caused by mutations in proto-oncogenes either as a single event or in combination with loss of tumor suppressor genes. In most malignancies, as described in the hallmarks of cancer, hits in multiple genes are required for the malignant process to fully develop[33]. The genes involved in the malignant transformation comprise only a small fraction of the exome with additional mutations occurring at random locations. This chance driven process means that an accumulation of mutations in passengers or essential passenger genes will occur during tumor formation (reviewed in[34]). Therefore, the vast majority of mutations in each tumor will be in non-essential targets and tumor specific. Non-synonymous or miss-sense mutations result in an amino acid change in protein sequences. When translated, such mutated protein sequences can produce a mutated antigen that can be recognized by the immune system. However, the fact that a miss-sense mutation can result in an epitope that can successfully bind to one of the patient specific HLA alleles and thereby be presented to the T cell based immune system does not necessarily make it an immunogenic antigen. Whether the endogenous T cell pool is reactive against these antigens is again a chance-driven process due to the random nature of TCR alpha and beta chain rearrangements that will not automatically result in a cognate T cell receptor for every expressed neo-antigen.

This class of antigens has long been regarded highly attractive candidates for tumor rejection antigens and a landmark study Wölfel *et al.* was the first to describe a T cell clone in a melanoma patient that recognized a mutated antigen within the CDK4 gene (Fig. 1)[35]. At that point in time though, technical limitations prevented a systematic approach to query large datasets for the presence of patient-specific putative neo-antigens. In recent years, our understanding of the mutational landscapes of human tumors, both at the genomic and protein-encoding level, has immensely increased with the development of next-generation sequencing technologies that have by now become a mainstream application in tumor biology. These advances, together with the development of computational algorithms to predict peptide binding chances for large numbers of both human and mouse HLA alleles, have allowed immunologists to study the interaction of the consequences of DNA damages with the T cell based immune system[36].

Seminal work from Schreiber and colleagues nicely demonstrates how next-generation sequencing of the tumor exome can lead to identification of immunogenic neo-antigens in a mouse sarcoma model. These identified neo-antigens were found to be involved in tumor control in immune competent mice either alone or after checkpoint blockade[37,38]. As a side note, these two studies also show that there is a hierarchy within neo-antigens recognized by T cells within a tumor. Some epitopes have the capacity to be a tumor rejection antigen alone, and these are more prone to be lost in e.g. clonal selection, whereas others need help in form of e.g. checkpoint blocking or vaccine strategies to confer tumor control. Other studies have shown that a multi-epitope vaccination strategy utilizing neo-epitopes derived from the B16F10 melanoma cell line can elicit protective immune responses in a therapeutic setting of a mouse model[39]. Following these initial preclinical findings, we and others have demonstrated that both $CD4^+$ and $CD8^+$ T cells interact with the consequences of DNA damage in particular in melanoma but also NSCLC, ovarian cancer and gastro-intestinal cancers[17,40-45]. Furthermore, it has been shown that immunotherapy can induce and enhance such T cell responses[40,43]. However, the fact that T cells can recognize these neo-antigens does not necessarily imply a role in human tumor control. Even though direct evidence of neo-antigens as tumor rejection antigens is lacking from the human setting it has been shown that patients with NSCLC with high mutational load have significantly higher likelihood of clinical benefit from anti-PD-1 therapy[43]. A similar correlation, nevertheless not as strong, was seen for melanoma patients treated with anti-CTLA-4 therapy[46]. In line with these findings, PD-1 blockade was shown to be clinically effective in metastatic colorectal cancer patients with mismatch-repair deficiency. This group had a more than 20-fold higher mutational load on average compared to patients that were mismatch-repair proficient and in whom no objective clinical responses were observed[47]. These findings, together with the evidence of T cell responses towards neo-antigens, strongly suggest that neo-antigens are an important component in the clinical activity of immunotherapy in some human malignancies.

A second group of neo-antigens is the viral antigens. These are as foreign to the immune system as mutated antigens, and perhaps even more so, since there is no expected overlap between viral epitopes and non-mutated self-epitopes. Viral epitopes are created when a part of the viral genome becomes integrated in the host genome upon infection. This is often the case in HPV-related cancers such as head and neck cancer and cancers of the male and female urogenital region. To date, several immunotherapeutic strategies are in development to combat these tumors including DNA or synthetic long peptide vaccines that encode immunogenic epitopes of the most oncogenic virus types or the adoptive transfer of autologous TIL[48-50]. One example of such an effective strategy is the administration of a synthetic long-peptide vaccine targeting the HPV-16 oncogene E6 and E7 in women with vulvar intraepithelial neoplasia. Clinical responses were observed in 60% of patients and

a correlation was found with the induction of HPV-16 specific immunity. Furthermore, for tumors with a known viral origin it is possible to develop prophylactic interventions as was done for HPV-based cancers for which a vaccine is now incorporated into the children vaccine program in multiple countries.

Human endogenous retroviruses

We have now described two families of antigens, the C/G and the neo-antigens, which have been linked to clinical benefit either directly (NY-ESO-1) or indirectly (neo-antigens). In addition to the better-characterized groups of antigens there may well be additional types that could be potent tumor rejection antigens for some malignancies.

One such type of antigens could be the human endogenous retroviruses (HERVs). Extensive studies have shown that all vertebrate genomes analyzed thus far contain exogenous elements closely related to retroviruses[51]. These sequences are referred to as endogenous retroviruses after they infected and integrated into host germ line cells and are passed on to offspring in a Mendelian fashion. The presence of ERV elements in humans was first discovered in the early 1980s and there is evidence for expression of HERVs or HERV elements in many different types of human malignancies[52,53]. This expression may either suggest a functional role for elements of HERVs or is simply reflecting deregulation by hypomethylation in tumor cells in general (reviewed in[54]).

A very attractive trait of HEVRs is their viral origin as retroviral infections can be sensed by the innate immune system and it can be speculated that HERV elements expressed in cancers may even serve as adjuvant or 'danger signal' inducing or enhancing anti-tumor immunity.

In addition to potentially providing 'danger signals' epitopes derived from expressed HERV proteins can also function as targets for T cell recognition of the tumor. It is not known to what level these proteins are expressed in the thymus (if they are expressed at all), however, it is known that part of the HERV elements can be expressed to various levels in healthy tissue indicating that tolerance can exist. In a recent study, it was shown that the expression of HERVs correlated with a cytolytic signature in a number of human malignancies including kidney cancer and ovarian cancer[55], although these data do not elucidate if the T cells are specific for HERV derived epitopes. However, other studies have provided evidence for the presence of a T cell repertoire specifically recognizing HERVs[56-58]. The first study to describe such a T cell response came from Coulie and colleagues in 2002 showing $CD8^+$ T cells isolated from a melanoma patient were specific for an epitope from HERV-K-mel restricted to HLA-A2, and these T cell clones could recognize the autologous tumor as well[58]. Furthermore, we have shown the presence of T cell populations specific for HERV-K-mel within TILs[56]. At this point in time only a few T cell epitopes derived from HERVs have been described and considerably more work is warranted to elucidate how common

the expression of T cell epitopes from HERVs are in human malignancies and if they can play a role in tumor control.

Importantly for these antigens, the uncertainties regarding tumor restriction warrant for caution when considering them as potential targets for immunotherapies much like the situation for the C/G antigens as a whole.

Bacterial components

As a more speculative side note; several studies support a role of gut bacteria in playing a part in the development of colorectal cancer[59,60]. Taken together with the evidence that the integrity of the mucosal barrier has been compromised it may be speculated that bacterial antigens can be part of the cancer antigenome of these cancers. As these antigens are truly foreign to the immune system that would make them as conceptually attractive as the previously described neo-antigens and perhaps even more so as they can also serve as danger signal to the immune system, thus combining two attractive components. Nevertheless, this is at a rather speculative state and it needs to be established if T cell responses towards these bacterial antigens indeed exist and can cause tumor regression while at the same time not cause severe toxicity.

WHICH ANTIGENS TO TARGET

When considering which antigens are the most successful targets to obtain tumor control, it is noteworthy that the most successful immunotherapies to date are those that do not target specific antigens but rather systemically activate the endogenous T cell pool. These therapies include the checkpoint targeting therapies and the reinvigoration of tumor infiltrating T cells *ex vivo* in TIL therapy[11,61]. In contrast, the strategies specifically targeting molecular defined tumor antigens have for the vast majority lacked clinical success thus far. This was already eluded to in a review by Neller et al. from 2008 in which response rates were compared between cancer vaccines based on a set of molecular defined antigens or bulk tumor cells as antigen source[62]. This study revealed that the response rate for melanoma patients treated with whole tumor cell as antigen source was approximately 2-fold higher compared to the molecular defined antigens, albeit still rather low. Taken together, these observations warrant re-visiting which antigens are selected as targets.

As outlined in the above sections, there are clinical data supporting a potential role in tumor control for antigens belonging to the two main groups described (neo-antigens and C/G antigens). Even though these two groups of antigens have not been compared for their quality as tumor rejection antigens in a systematic way, there are data that provide hints as to which is the better target. A recent analysis of thousands of TCGA samples from 18

different human malignancies showed a correlation between mutational load and intra-tumoral cytolytic activity in multiple cancer types including lung cancer, colorectal cancer and cervical cancer[55]. Interestingly, this correlation was not found when performing the same analysis for the expression of C/G genes. Nevertheless, a correlation between cytolytic activity and a few individual C/G antigens were observed in some types of malignancies e.g. melanoma, lung, head and neck, stomach, and ovarian cancer. Similar findings were made in a large set of colorectal cancer specimens in a study using T cell effector markers as a read-out for T cell function[63]. Furthermore, as described in the section of neo-antigens, mutational load has been found to correlate with clinical outcome to checkpoint blocking therapies in multiple malignancies. In addition to these data, we have shown in one melanoma case that neo-antigen specific TCRs can be superior compared to C/G specific TCRs in tumor control when tested in a patient derived xenograft model (Kelderman *et al.,* unpublished). Collectively, these data suggest a strong role for neo-antigens in disease control in a number of malignancies with a certain level of mutational load. Based on the outlined evidence and the previously discussed characteristics of neo-antigens, these are highly attractive tumor antigens to target. Nevertheless, there are malignancies with a low mutational load that are less likely to yield an immunogenic neo-antigen repertoire but that still contain a high level of immune infiltrates. This at least indicates the presence of an ongoing immune dialogue, although it is most likely not based on the recognition of neo-antigens. For these malignancies other classes of antigens including the C/G antigens and the HERVs may play a more important role in tumor control. Furthermore, even within the malignancies generally characterized by a high mutational load, there is a large spread in the number of mutations (Fig. 2)[64,65], suggesting that modulating T-cell responses against mutated antigens might not be an effective strategy in all cases. Regardless of which antigen is selected to target, the most important criteria include tumor restriction for safety and lack of pre-existing tolerance. In addition, it is beneficial if the target is crucial for tumor cell survival to diminish the risk of clonal escape variants, however, by targeting multiple antigens simultaneously this may be of less importance.

Some 20 years ago, the field set out to design vaccines that could be used to treat many patients with the same malignancy. Currently, we know that the optimal targets for immunotherapy can differ greatly between patients due to the diversity in mutational load as well as the simultaneous expression of several antigen classes within the same group of malignancies. Encouragingly, with the current advances in deep-sequencing technologies and high-throughput processing of bioinformatics data, it is conceivable that a more personalized approach is soon within reach. One possible scenario that we envision (Fig. 3) is the mapping of each patient's individual antigenome using tumor-specific RNA sequence data in combination with analyses on the tumor-immune interaction in the tumor-microenvironment (TME). The expressed antigens will be ranked based on the desired

characteristics e.g. level of expression and their potential to bind to the patient's specific HLA alleles with high affinity. As a side note, it has been shown by Sahin and colleagues that the strength of predicted binding is an important factor for immunogenicity[66]. In case of the C/G antigens, it will be important to understand the expression pattern of each protein and likewise for the HERVs. Regarding the mutated antigens we still need to acquire an understanding of which mutated antigens are most immunogenic. At this point in time, we know that only a minor percentage of mutated epitopes are recognized by T cells in melanoma, and we do not know how to specifically select those, or if other mutated epitopes can function as better tumor rejection antigens despite the lack of preexisting immune recognition. To address this, it is extremely important to verify that therapy-induced neo-antigen specific T cell responses indeed can recognize autologous tumor. Based on such knowledge, the field can gain insight in how to select mutated epitopes for e.g. vaccines. An additional ranking can be made by using information from the TME e.g. whether there is an active or rather suppressed immune dialogue and to which degree the level of T cell infiltration e.g. by measuring clonality of TIL is predictive for a pre-existing anti-tumor immune response. Finally, a treatment plan can be devised that incorporates potential pre-conditioning regimens to boost the endogenous immune response or epitope vaccinations in combination with checkpoint blockade to induce new T cell responses.

Another consideration to take into account in this final step is whether it is preferable to target antigens for which a T cell response already exists. The benefit when targeting antigens by boosting pre-existing immunity is that the antigen is indeed immunogenic, i.e. it is presented by the tumor to a sufficient level for T cell detection and a T cell repertoire exists that can recognize the antigen. The advantage of targeting antigens not already targeted by the immune system is mainly a lowered risk for clonal selection to have taken place against those tumor cell clones expressing the antigen.

In summary, since the discovery of the first tumor antigen, now more than two decades ago, the field has made immense progress in identifying, characterizing and classifying vast numbers of antigens. Together with high-throughput deep-sequencing approaches that are becoming readily available personalized therapies are a reality. Venturing into patient-specific strategies utilizing these new therapeutic tools in a systematic approach will be crucial to understand how to specifically select the true tumor rejection antigens.

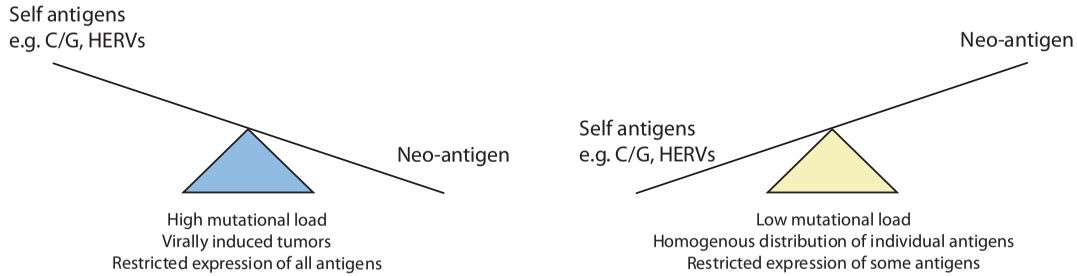


Figure 2. C/G versus neo-antigens. Several considerations should be taken into account when selecting the most optimal target antigen in a given tumor type or for each individual tumor within that tumor type.

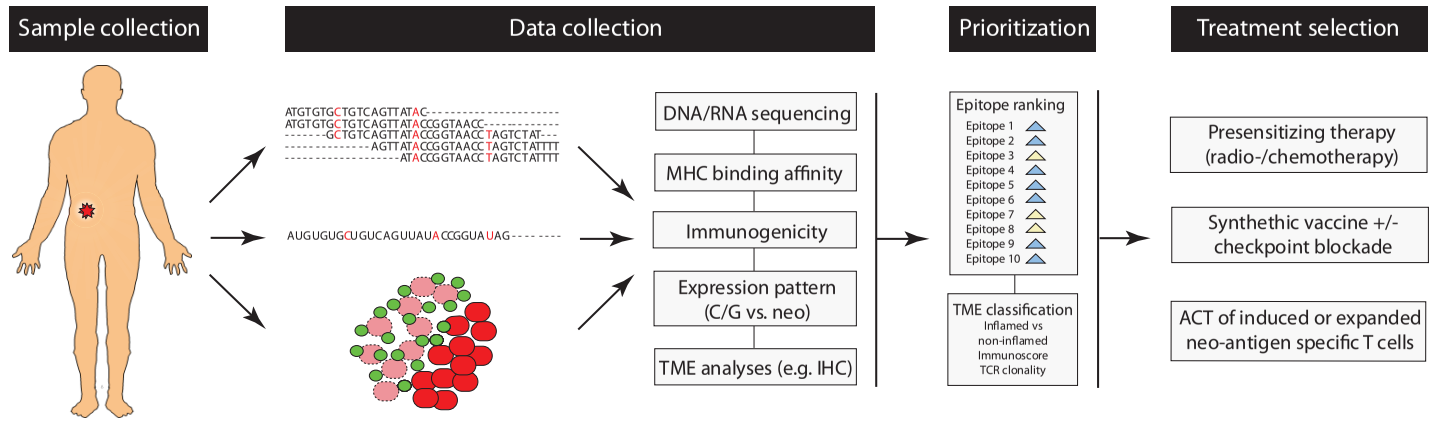


Figure 3. Towards highly personalized cancer immunotherapy. Several phases can be discriminated towards identifying the most optimal strategy for future patient-specific cancer immunotherapy. Tumor material is collected and sequenced for DNA (whole-exome) and RNA, as well as collecting information e.g. by means of immunohistochemistry (IHC) on the local tumor microenvironment (TME). Algorithms developed to weigh each of these factors with respect to expected immunogenicity etc. would yield an epitope ranking list (blue indicates neo-antigens, yellow indicates C/G antigens), which in combination with TME analyses provides an immunotherapeutic strategy specifically tailored for each individual patient.

Antigen-independent factors that determine outcome to cancer immunotherapy

Provided the relevant antigens are abundantly presented, many factors can subsequently short circuit the so-called cancer-immunity cycle leading to failed or incomplete anti-tumor immunity. This process can be divided in seven distinct steps as proposed by Chen and Mellman and these will be briefly discussed here[67]. First, the nature of antigen release in the tumor microenvironment must be immunogenic rather than tolerogenic. More specifically, pro-inflammatory cytokines such as TNF-α, IFN-α and IL1 will stimulate, whereas IL-10, IL-4 and IL-13 will hamper DC maturation. Second, should immunogenic cell death occur, this will then result in the maturation of DCs and their subsequent migration to peripheral lymph nodes where they present antigens in the context of MHC class I and II molecules. Third, the nature of costimulatory signals provided in this priming and activation phase will determine the fate of naïve T cells. For example, CD28:B7.1 and CD27:CD70 ligation will stimulate whereas CTLA4:B7.1 interaction will inhibit T cell activation. Fourth and fifth, activated T cells depend on complex chemokine gradients to migrate to the effector site and extravasate through the endothelial barrier into the tumor. Sixth, effector cells require engagement of the TCR with their cognate peptide in order to kill cancer cells. This crucial step might be hampered by loss of MHC class I expression due to mutations in beta-2-microglobulin, which is likely to play a major role in tumor immune-escape[68-70]. This might be particularly expected in those tumors with a hypermutated phenotype[71,72]. Additionally, in several cancer types, including colorectal and kidney clear cell cancer, it was demonstrated that the number of predicted neo-epitopes was much lower than what was expected based on the silent mutation rate[55]. Taken together with a strong body of evidence in experimental mouse models (reviewed in[73]), this is highly suggestive of cancer immunoediting in which the ongoing immune response can shape the antigenome through selection of non-immunogenic epitopes or tumor cells that fail to express MHC class I molecules altogether. Finally, expression of inhibitory ligands such as PD-L1 on the tumor cell surface can prevent the release of T cell granule content thereby escaping immune-mediated destruction in the last phase of the cancer-immunity cycle.

ACKNOWLEDGEMENTS

No funds were received to conduct this study. The authors would like to thank Ton Schumacher and members of his lab for useful discussions.

REFERENCES

1. Fridman, W.H., Pages, F., Sautes-Fridman, C. & Galon, J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* **12**, 298-306 (2012).
2. Zhang, L.*, et al.* Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* **348**, 203-213 (2003).
3. Galon, J.*, et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **313**, 1960-1964 (2006).
4. Erdag, G.*, et al.* Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res* **72**, 1070-1080 (2012).
5. Galon, J.*, et al.* Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol* **232**, 199-209 (2014).
6. Pages, F.*, et al.* In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol* **27**, 5944-5951 (2009).
7. Tumeh, P.C.*, et al.* PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* **515**, 568-571 (2014).
8. Dudley, M.E.*, et al.* CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clin Cancer Res* **16**, 6122-6131 (2010).
9. Dudley, M.E.*, et al.* Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* **26**, 5233-5239 (2008).
10. Dudley, M.E.*, et al.* Randomized selection design trial evaluating CD8+-enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. *J Clin Oncol* **31**, 2152-2159 (2013).
11. Rosenberg, S.A.*, et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* **17**, 4550-4557 (2011).
12. Besser, M.J.*, et al.* Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. *Clin Cancer Res* **19**, 4792-4800 (2013).
13. Brahmer, J.*, et al.* Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N Engl J Med* **373**, 123-135 (2015).
14. Ansell, S.M.*, et al.* PD-1 Blockade with Nivolumab in Relapsed or Refractory Hodgkin's Lymphoma. *N Engl J Med* **372**, 311-319 (2014).
15. McDermott, D.F.*, et al.* Survival, Durable Response, and Long-Term Safety in Patients With Previously Treated Advanced Renal Cell Carcinoma Receiving Nivolumab. *J Clin Oncol* **33**, 2013-2020 (2015).
16. Brahmer, J.R.*, et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N Engl J Med* **366**, 2455-2465 (2012).
17. Tran, E.*, et al.* Cancer Immunotherapy Based on Mutation-Specific CD4+ T Cells in a Patient with Epithelial Cancer. *Science* **344**, 641-645 (2014).
18. van der Bruggen, P.*, et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* **254**, 1643-1647 (1991).
19. Fisk, B., Blevins, T.L., Wharton, J.T. & Ioannides, C.G. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *J Exp Med* **181**, 2109-2117 (1995).
20. Kessler, J.H.*, et al.* Efficient identification of novel HLA-A(*)0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis. *J Exp Med* **193**, 73-88 (2001).

21. Coulie, P.G.*, et al.* A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J Exp Med* **180**, 35-42 (1994).
22. Kawakami, Y.*, et al.* Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc Natl Acad Sci U S A* **91**, 6458-6462 (1994).
23. Chomez, P.*, et al.* An overview of the MAGE gene family with the identification of all human members of the family. *Cancer Res* **61**, 5544-5551 (2001).
24. Jungbluth, A.A.*, et al.* Immunohistochemical analysis of NY-ESO-1 antigen expression in normal and malignant human tissues. *Int J Cancer* **92**, 856-860 (2001).
25. Yang, B.*, et al.* MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. *Cancer Res* **67**, 9954-9962 (2007).
26. Monte, M.*, et al.* MAGE-A tumor antigens target p53 transactivation function through histone deacetylase recruitment and confer resistance to chemotherapeutic agents. *Proc Natl Acad Sci U S A* **103**, 11160-11165 (2006).
27. Nardiello, T.*, et al.* MAGE-A inhibits apoptosis in proliferating myeloma cells through repression of Bax and maintenance of survivin. *Clin Cancer Res* **17**, 4309-4319 (2011).
28. Hofmann, O.*, et al.* Genome-wide analysis of cancer/testis gene expression. *Proc Natl Acad Sci U S A* **105**, 20422-20427 (2008).
29. Morgan, R.A.*, et al.* Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother* **36**, 133-151 (2013).
30. Robbins, P.F.*, et al.* A pilot trial using lymphocytes genetically engineered with an NY-ESO-1-reactive T-cell receptor: long-term follow-up and correlates with response. *Clin Cancer Res* **21**, 1019-1027 (2015).
31. Barrow, C.*, et al.* Tumor antigen expression in melanoma varies according to antigen and stage. *Clin Cancer Res* **12**, 764-771 (2006).
32. Gilboa, E. The makings of a tumor rejection antigen. *Immunity* **11**, 263-270 (1999).
33. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
34. Heemskerk, B., Kvistborg, P. & Schumacher, T.N. The cancer antigenome. *EMBO J* **32**, 194-203 (2013).
35. Wolfel, T.*, et al.* A p16INK4a-lnsensitive CDK4 Mutant Targeted by Cytolytic T Lymphocytes in a Human Melanoma *Science* **269**, 1281-1284 (1995).
36. Segal, N.H.*, et al.* Epitope landscape in breast and colorectal cancer. *Cancer Res* **68**, 889-892 (2008).
37. Matsushita, H.*, et al.* Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. *Nature* **482**, 400-404 (2012).
38. Gubin, M.M.*, et al.* Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. *Nature* **515**, 577-581 (2014).
39. Castle, J.C.*, et al.* Exploiting the mutanome for tumor vaccination. *Cancer Res* **72**, 1081-1091 (2012).
40. van Rooij, N.*, et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol* **31**, e439-442 (2013).
41. Robbins, P.F.*, et al.* Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med* **19**, 747-752 (2013).
42. Lu, Y.C.*, et al.* Mutated PPP1R3B is recognized by T cells used to treat a melanoma patient who experienced a durable complete tumor regression. *J Immunol* **190**, 6034-6042 (2013).
43. Rizvi, N.A., *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science* **348**, 124-128 (2015).

44. Linnemann, C*., et al.* High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nat Med* **21**, 81-85 (2015).
45. Wick, D.A*., et al.* Surveillance of the Tumor Mutanome by T Cells during Progression from Primary to Recurrent Ovarian Cancer. *Clin Cancer Res* **20**, 1125-1134 (2014).
46. Snyder, A*., et al.* Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N Engl J Med* **371**, 2189-2199 (2014).
47. Le, D.T*., et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* **372**, 2509-2520 (2015).
48. Kenter, G.G*., et al.* Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* **361**, 1838-1847 (2009).
49. Oosterhuis, K*., et al.* Preclinical development of highly effective and safe DNA vaccines directed against HPV 16 E6 and E7. *Int J Cancer* **129**, 397-406 (2011).
50. Stevanovic, S*., et al.* Complete Regression of Metastatic Cervical Cancer After Treatment With Human Papillomavirus-Targeted Tumor-Infiltrating T Cells. *J Clin Oncol* **14**, 1543-1550 (2015).
51. Belshaw, R*., et al.* Long-term reinfection of the human genome by endogenous retroviruses. *Proc Natl Acad Sci U S A* **101**, 4894-4899 (2004).
52. Buscher, K*., et al.* Expression of human endogenous retrovirus K in melanomas and melanoma cell lines. *Cancer Res* **65**, 4172-4180 (2005).
53. Gibb, E.A*., et al.* Activation of an endogenous retrovirus-associated long non-coding RNA in human adenocarcinoma. *Genome Med* **7**, 22 (2015).
54. Kassiotis, G. Endogenous retroviruses and the development of cancer. *J Immunol* **192**, 1343-1349 (2014).
55. Rooney, M.S., Shukla, S.A., Wu, C.J., Getz, G. & Hacohen, N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* **160**, 48-61 (2015).
56. Kvistborg, P*., et al.* TIL therapy broadens the tumor-reactive CD8(+) T cell compartment in melanoma patients. *Oncoimmunology* **1**, 409-418 (2012).
57. Takahashi, Y*., et al.* Regression of human kidney cancer following allogeneic stem cell transplantation is associated with recognition of an HERV-E antigen by T cells. *J Clin Invest* **118**, 1099-1109 (2008).
58. Schiavetti, F., Thonnard, J., Colau, D., Boon, T. & Coulie, P.G. A human endogenous retroviral sequence encoding an antigen recognized on melanoma by cytolytic T lymphocytes. *Cancer Res* **62**, 5510-5516 (2002).
59. Louis, P., Hold, G.L. & Flint, H.J. The gut microbiota, bacterial metabolites and colorectal cancer. *Nat Rev Microbiol* **12**, 661-672 (2014).
60. Zitvogel, L*., et al.* Cancer and the gut microbiota: an unexpected link. *Sci Transl Med* **7**, 271ps271 (2015).
61. Larkin, J*., et al.* Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med* **373**, 23-34 (2015).
62. Neller, M.A., Lopez, J.A. & Schmidt, C.W. Antigens for cancer immunotherapy. *Semin Immunol* **20**, 286-295 (2008).
63. Angelova, M*., et al.* Characterization of the immunophenotypes and antigenomes of colorectal cancers reveals distinct tumor escape mechanisms and novel targets for immunotherapy. *Genome Biol* **16**, 64 (2015).
64. Alexandrov, L.B*., et al.* Signatures of mutational processes in human cancer. *Nature* **500**, 415-421 (2013).
65. Vogelstein, B*., et al.* Cancer genome landscapes. *Science* **339**, 1546-1558 (2013).
66. Kreiter, S*., et al.* Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* **520**, 692-696 (2015).

67. Chen, D.S. & Mellman, I. Oncology meets immunology: the cancer-immunity cycle. *Immunity* **39**, 1-10 (2013).

68. Khong, H.T., Wang, Q.J. & Rosenberg, S.A. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient: tumor escape by antigen loss and loss of MHC expression. *J Immunother* **27**, 184-190 (2004).

69. del Campo, A.B*., et al.* Immune escape of cancer cells with beta2-microglobulin loss over the course of metastatic melanoma. *Int J Cancer* **134**, 102-113 (2014).

70. Restifo, N.P*., et al.* Loss of functional beta 2-microglobulin in metastatic melanomas from five patients receiving immunotherapy. *J Natl Cancer Inst* **88**, 100-108 (1996).

71. Dierssen, J.W*., et al.* HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. *BMC Cancer* **7**, 33 (2007).

72. Kloor, M*., et al.* Immunoselective pressure and human leukocyte antigen class I antigen machinery defects in microsatellite unstable colorectal cancers. *Cancer Res* **65**, 6418-6424 (2005).

73. Dunn, G.P., Koebel, C.M. & Schreiber, R.D. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* **6**, 836-848 (2006).

74. van der Bruggen, P., Stroobant, V., Vigneron, N. & Van den Eynde, B. Peptide database: T cell-defined tumor antigens. *Cancer Immun* URL: http://www.cancerimmunity.org/peptide/(2013).

Chapter 7

Antigen-specific TIL therapy for melanoma:

A flexible platform for personalized cancer immunotherapy

Sander Kelderman[1], Bianca Heemskerk[1], Lorenzo Fanchi[1], Mireille Toebes[1], Pia Kvistborg[1], Marit M. van Buuren[1], Nienke van Rooij[1], Samira Michels[1], Lothar Germeroth[2], John B.A.G. Haanen[1], Ton N.M. Schumacher[1]

[1]The Netherlands Cancer Institute NKI-AVL, Amsterdam, The Netherlands,
[2]Stage Cell Therapeutics GmbH, Göttingen, Germany

European Journal of Immunology **46: 1351-60 (2016)**

ABSTRACT

Tumor infiltrating lymphocyte (TIL) therapy has shown objective clinical response rates of 50% in stage IV melanoma patients in a number of clinical trials. Nevertheless, the majority of patients progress either directly upon therapy or after an initial period of tumor control. Recent data have shown that most TIL products that are used for therapy contain only low frequencies of T cells reactive against known melanoma-associated epitopes. Because of this, the development of technology to create T-cell products that are enriched for reactivity against defined melanoma-associated antigens would seem valuable, both to evaluate the tumoricidal potential of T cells directed against different antigen classes and to potentially increase response rates. Here, we developed and validated a conditional MHC streptamer-based platform for the creation of TIL products with defined antigen reactivities. We have used this platform to successfully enrich both high frequency (≥1%) and low frequency (<1%) tumor-specific $CD8^+$ T-cell populations, and thereby create T-cell products with enhanced tumor recognition potential. Collectively, these data demonstrate that selection of antigen-specific T-cell populations can be used to create defined T-cell products for clinical use. This strategy thus forms a highly flexible platform for the development of antigen-specific cell products for personalized cancer immunotherapy.

INTRODUCTION

In recent years, many oncological disciplines have moved towards cancer therapies that take tumor-specific characteristics into account. Within melanoma, BRAF mutation status is used to guide treatment with BRAF inhibitors[1], in lung cancer, ALK and EGFR mutation status is amongst others used to guide treatment choice[2], and a randomized phase II trial in breast cancer patients suggests the value of targeted therapy that is matched to the molecular profile of the tumor[3].

In cancer immunotherapy, the administration of *ex vivo* expanded autologous T cells can be considered the most patient-specific approach that has been developed to date. This treatment modality either comprises the adoptive transfer of tumor infiltrating lymphocytes (TIL) that have been expanded from a resected lesion, or the infusion of genetically modified PBMCs that express a newly introduced TCR (reviewed in Rosenberg & Restifo, 2015[4]). To date, the clinical activity of TIL therapy has been particularly promising, with an approximately 50% response rate observed in trials at different clinical centers. The clinical activity of TIL therapy correlates with the number of $CD8^+$ T cells that are infused and the infusion of $CD8^+$ enriched TIL has also been shown to lead to clinical responses[5-9]. As such, it is likely that the clinical activity of TIL therapy is in large part mediated by $CD8^+$ T cells. Strikingly though, the antigen reactivities of the $CD8^+$ T cells present within the infusion product is not controlled. Furthermore, as different antigen-specific T-cell pools are likely to vary significantly in their tumoricidal potential (see below), the generation of TIL products of a more defined composition would appear attractive.

Conceptually, two major classes of tumor antigens can be distinguished. First, human melanomas commonly express a series of non-mutated self-antigens, such as the cancer/germline (C/G) antigens and the melanocyte differentiation (MD) antigens, which are both expressed in a restricted set of healthy tissues[10]. Second, because of the significant amount of UV-induced DNA damage in melanomas[11], these tumors can be expected to also frequently express neo-antigens that arise as a consequence of tumor-specific mutations (reviewed in[12]). Indeed, recent work that has utilized exome-based analysis to uncover the patient-specific neo-antigen directed T-cell repertoire has shown that T-cell responses against mutated antigens are common in melanoma[13-15]. The clinical benefits of targeting these two different antigen classes are presently unclear. Specifically, while it is likely that the affinity of T-cell responses against some of the shared antigens is affected by central T-cell tolerance, it is unclear whether this applies to all shared antigens[10].

In order to directly reveal the relative value of T-cell responses against neo-antigens and specific self-antigens, it would be valuable to generate TIL products with a defined composition. Furthermore, in most TIL products, the frequency of $CD8^+$ T cells that target (shared) antigens is generally below 1%[16]. For this reason, it may be hypothesized that the

efficacy of TIL therapy could be further improved by enhancing the reactivity towards (a set of) defined tumor antigens.

To enable the development of antigen-specific T-cell products, we here created a flexible platform that allows the selection of T cells directed against defined tumor-specific antigens from bulk TIL cultures. Importantly, the high degree of flexibility of this technique should facilitate clinical implementation of antigen-specific TIL therapy for both shared antigens and patient-specific neo-antigens.

RESULTS

Enrichment of high and low frequency antigen specific T-cell populations from TIL by UV-exchange MHC streptamers

As described previously, MHC streptamers that are generated through the use of MHC molecules tagged with an epitope that displays a moderate affinity for streptavidin can bind antigen-specific T-cell populations in a reversible manner[17,18], and can be used to create virus-specific T-cell products for therapy. In order to provide this platform with the required flexibility to create antigen-enriched T-cell products for a variety of T-cell antigens, we generated HLA-A*02:01 streptamers loaded with a UV-sensitive ligand[19,20]. Validation experiments demonstrated that the resulting 'conditional MHC streptamers' could be loaded with peptides of interest in one-hour UV exchange reactions (data not shown).

To determine whether the resulting MHC streptamers could successfully be used to generate antigen-specific T-cell populations from bulk TIL cultures, we enriched a total of five T-cell specificities in eleven independent experiments from donors NKIRTIL006, NKIRTIL012 and NKIRTIL025. The majority of enrichments were done for T cells reactive with the MART-1_{ELA} and $gp100_{KTW}$ epitopes (Fig. 1A). In addition, enrichments were performed for T-cell populations reactive with the MAGE-$C2_{LLF}$, MAGE-$C2_{VIW}$, MAGE-$C2_{ALK}$, MAGE-$A10_{GLY}$ and Meloe-1_{TLN} epitopes. Single antigen-specific T-cell populations with a frequency of 1% or higher in the bulk TIL could be enriched with a 29-fold median increase in frequency, to an average purity of 73%. Furthermore, enrichment of T-cell populations that were present at a very low frequency (<1%) within the TIL population was effective in two out of three cases, resulting in a purity of >50% (Fig. 1B). Simultaneous enrichment of multiple high and low frequency antigen-specific T-cell populations was also feasible, and increased the mean purity of the T-cell product from 3.8% pre-enrichment to 74.7% post-enrichment (Fig. 1C). Median recovery of the target T-cell populations for high and low frequency antigen-specific T-cell populations after enrichment was 23% and 33%, respectively (Fig. 1D).

To evaluate whether subsequent expansion of the enriched TIL product substantially influenced its composition, a total of eight enriched cell cultures containing 25 enriched antigen-specific T-cell populations were expanded according to the rapid expansion protocol (REP) that is used to create TIL products for therapy. Following expansion, there was on average a modest drop in the frequency of enriched antigen-specific T-cell populations (mean 42% pre-REP versus 36.2% post-REP, Fig. 1E). This was however unlikely to be explained by negative effects of the enrichment procedure, as the frequency of these antigen-specific cells in control non-enriched bulk TIL cultures likewise decreased upon expansion (mean 1.6% pre-REP versus 0.9% post-REP, Supplementary Fig. 1A). Notably, the magnitude of T-cell expansion was not affected by T-cell enrichment, with a 1087-fold mean expansion for antigen-enriched TIL, as compared to a 937-fold mean expansion for non-enriched bulk TIL (Supplementary Fig. 1B).

Phenotypic analysis of antigen-enriched TIL cultures

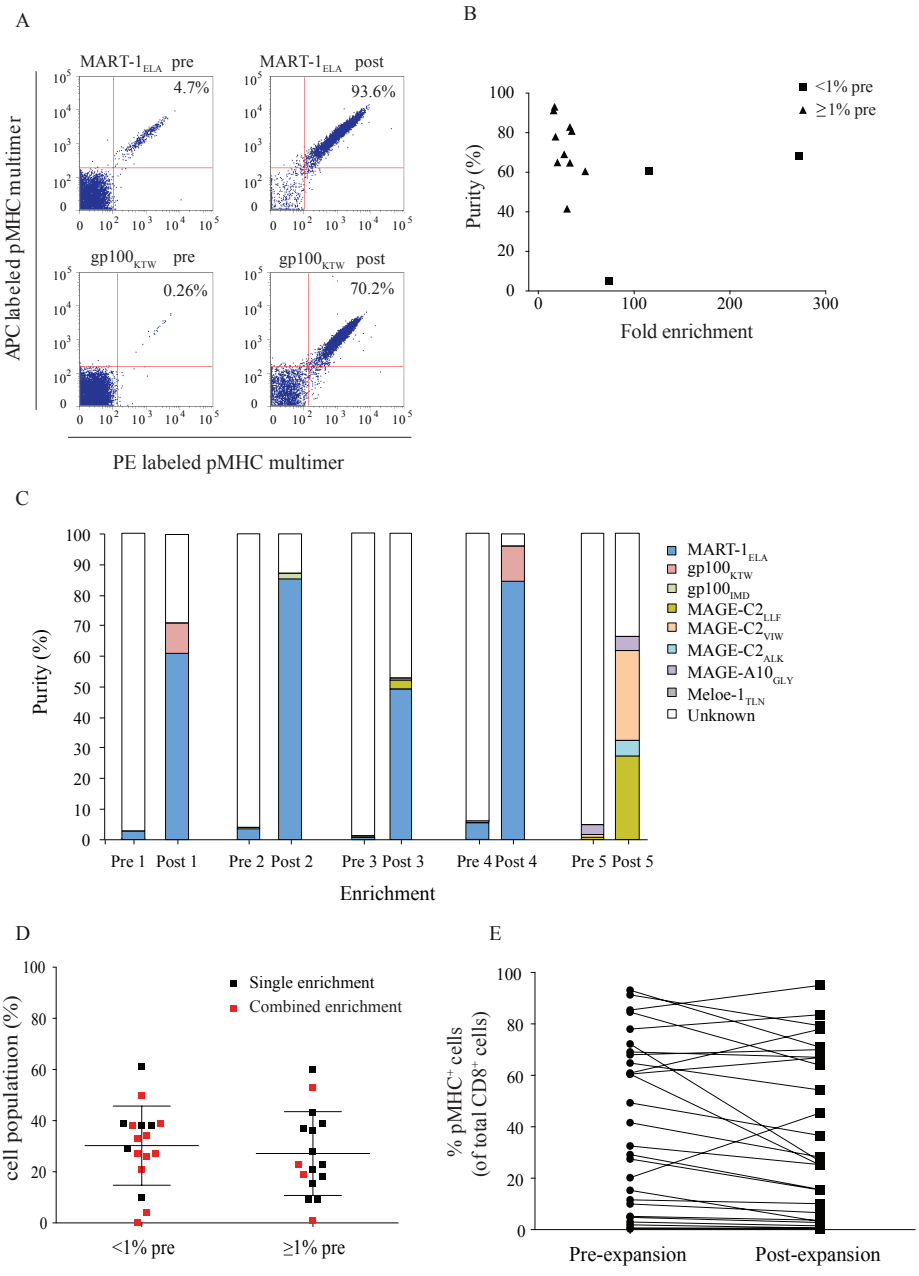
Next, we measured the effect of antigen-specific T-cell selection on the phenotypic characteristics of the T cells that were obtained. For this, we performed phenotypic analyses of both enriched and non-enriched antigen-specific TILs from patient NKIRTIL025. A total of five antigen-specific T-cell populations (three independent enrichment experiments) were analyzed for the expression of CCR7, CD27, CD127, CD95, CD45RA, CD45RO and CD62L. No significant difference in expression of CCR7, CD95, CD127, CD45RA, CD45RO and CD62L, was observed within the antigen-specific T-cell populations after enrichment and subsequent expansion (Fig. 2A). Expression of CD27 on antigen-specific cells was increased following enrichment and subsequent expansion compared to non-enriched antigen-specific cells ($P < 0.05$, Fig. 2A and 2B), resulting in a cell product with a more favorable phenotype.

Functional reactivity of antigen-enriched TIL cultures

Having demonstrated the feasibility of antigen-specific T-cell selection, we aimed to determine whether enriched TIL cultures were more reactive than non-enriched bulk TIL against autologous and partially HLA-matched tumor lines. To this purpose, we generated three TIL cultures from donor NKIRTIL012 that were enriched for MART-1- and gp100-specific T cells, either separately or in combination. We then compared the functional activity of the enriched cell products with the non-enriched cell product by measurement of T-cell degranulation upon coculture with four melanoma lines (Fig. 2C). As a first test, we compared reactivity of the enriched and non-enriched TIL products against the autologous tumor line. In this setting, the effect of enrichment of the intended antigen-specific T-cell populations is seen, but also the effect of the concomitant depletion of other $CD8^+$ T-cell populations that are reactive with the autologous tumor. Analysis of $CD8^+$ T-cell reactivity of standard and antigen-enriched TIL against autologous tumor demonstrated that 23% of

non-enriched CD8$^+$ T cells were reactive against the autologous tumor cell line. Notably, reactivity of TIL cultures that had been enriched for T cells specific for the gp100 antigen, MART-1 antigen, or both antigens combined was higher (32%, 46% and 46%, respectively). Second, we assessed recognition of bulk TIL and enriched TIL against the HLA-A*02:01 matched cell line mel526 to directly test the effect of T-cell enrichment on reactivity towards shared HLA-A*02:01-restricted epitopes. This analysis demonstrated that enrichment of T cells specific for the gp100$_{KTW}$ antigen, MART-1$_{ELA}$ antigen, or both antigens combined resulted in an approximately 10-fold increased reactivity against these antigens.

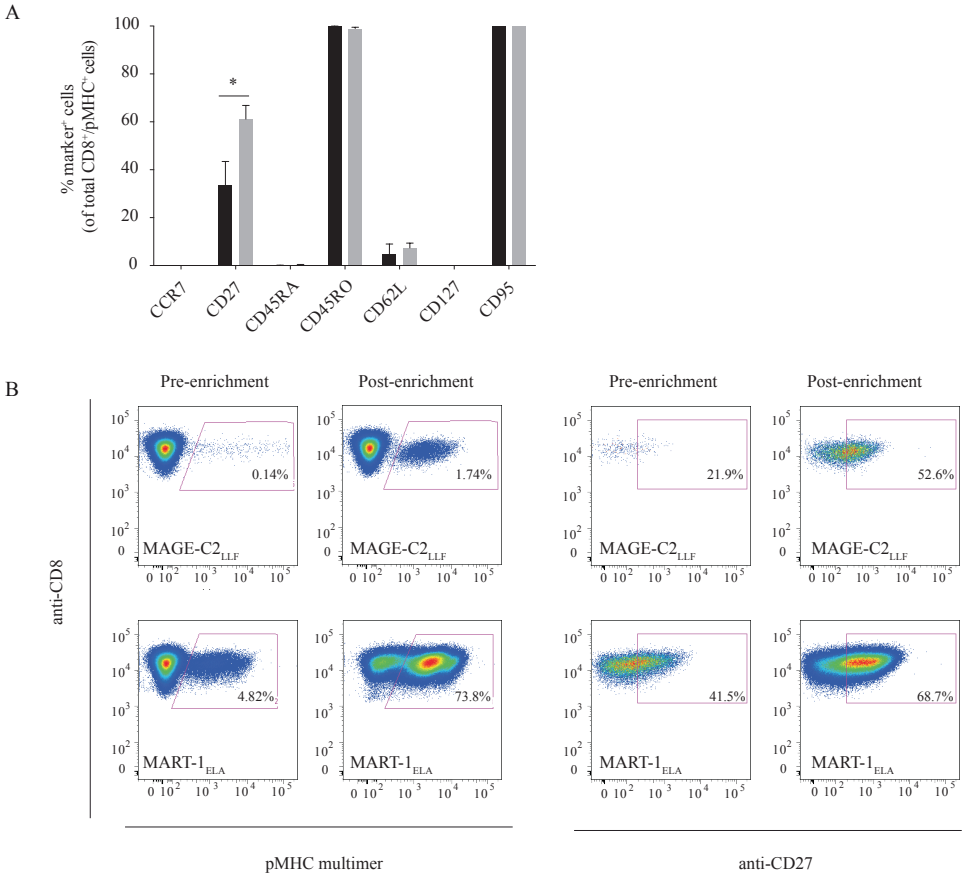
Figure 1. Enrichment and expansion of high and low frequency antigen-specific T-cell populations from bulk melanoma TIL cultures. (a) Examples of two representative enrichments (total N = 11) of a high frequency MART-1$_{ELA}$ specific T-cell population (upper panel) and a low frequency gp100$_{KTW}$ specific T-cell population (lower panel) are depicted. Enrichment was performed using antigen-specific streptamers loaded with the peptide of interest and a subsequent MACS column pull-down. Antigen-specific T-cell populations are visualized by flow cytometry using pMHC complexes generated with a UV-induced ligand exchange and fluorochrome conjugation. Pre indicates pre-enrichment, post indicates post-enrichment. Numbers reflect the percentage of pMHC multimer$^+$ CD8$^+$ cells of total CD8$^+$ cells. Gating strategy is shown in Supplementary Fig. 2B. **(b)** Overview of enrichments of 13 antigen-specific T-cell populations in a singular fashion is depicted. Squares indicate pre-existing frequencies of less than 1%, whereas triangles indicate pre-existing frequencies of 1% or higher. Purity is determined as the frequency of pMHC multimer$^+$ CD8$^+$ cells of total CD8$^+$ cells; fold enrichment is calculated by dividing the post-enrichment frequency over the pre-enrichment frequency. **(c)** Results of five independent enrichments are depicted, in which several high and low frequency antigen-specific T-cell populations were purified simultaneously. A cumulative bar shows the percentage purity of each antigen-specific T-cell population. Colored bars represent TIL that recognize the defined shared antigens that collectively formed the targeted T-cell population; the white bar represents the remaining TIL that were not targeted. **(d)** Recovery of antigen-specific T cells upon enrichment is shown in squares for high (≥1%, N=16) and low (<1%, N=17) frequency antigen-specific T-cell populations. Recovery was calculated as the fraction of antigen-specific T cells that was obtained after enrichment from the total antigen-specific T cells present in the bulk TIL culture prior to enrichment. Black squares indicate T-cell populations that were enriched in a singular manner; red squares indicate T-cell populations that were enriched simultaneous with one or up to four other antigen-specific T-cell populations. Horizontal line indicates mean recovery of antigen-specific cells from all enrichment procedures ± standard deviation. **(e)** Percentage of the targeted antigen-specific T-cell populations directly after enrichment and following a subsequent 14-day expansion (N=25, 8 independent experiments). Note that in most cases the percentage of antigen-specific T cells is not substantially affected by T-cell expansion. Average expansion was 1,087-fold. ➔


A
MART-1ELA pre
4.7%
MART-1ELA post
93.6%
gp100KTW pre
0.26%
gp100KTW post
70.2%
APC labeled pMHC multimer
PE labeled pMHC multimer
B
<1% pre
≥1% pre
Purity (%)
Fold enrichment
C
Purity (%)
Pre 1 Post 1 Pre 2 Post 2 Pre 3 Post 3 Pre 4 Post 4 Pre 5 Post 5
Enrichment
MART-1ELA
gp100KTW
gp100IMD
MAGE-C2LLF
MAGE-C2VIW
MAGE-C2ALK
MAGE-A10GLY
Meloe-1TLN
Unknown
D
Recovery of target cell population (%)
Single enrichment
Combined enrichment
<1% pre
≥1% pre
E
% pMHC+ cells (of total CD8+ cells)
Pre-expansion
Post-expansion

Clinical-scale enrichment of antigen-enriched TIL products

Having established that enrichment of antigen-specific T-cell populations present within TIL is feasible and can be used to steer the antigen reactivity of TIL products, we aimed to determine the feasibility of clinical-scale antigen-specific T-cell enrichment in a closed system. To this purpose, three antigen-specific T-cell populations, specific for MART-1_{ELA}, MAGE-$C2_{LLF}$ and $gp100_{KTW}$, were enriched from a pre-REP TIL culture of $1x10^8$ cells from patient NKIRTIL025 using the CliniMACS system. The total purity of the obtained cell product was 94% directly after enrichment, and 87% after a subsequent 924-fold expansion (Fig. 3A). Overall recovery of the target T-cell populations was 22% (Fig. 3B). Reactivity against the autologous tumor cell line was increased from 7% in the non-enriched TIL product, to 48% in the enriched TIL product (Fig. 3C). Furthermore, reactivity against the HLA-A*02:01 matched cell line mel526 increased from 4% to 79% after antigen-specific T-cell enrichment, corresponding with the frequency of antigen-reactive T cells in the final cell product.

Figure 2. Functional analyses of antigen-enriched TIL cultures. (a) Phenotypic analysis of antigen-specific cells after enrichment and expansion, comparing non-enriched and enriched TIL cultures. Five antigen-specific T-cell populations of donor NKIRTIL025 enriched in three independent experiments were assessed by flow cytometry using pMHC-multimers in combination with a panel of phenotypic markers indicated in the graph. Black bars indicate expression of phenotypic markers on antigen-specific T cells in non-enriched control cultures; grey bars indicate expression of markers on antigen-specific T cells in enriched cultures. Expression of CD27 was significantly higher, indicated by asterisk, on antigen-specific cells following enrichment and subsequent expansion, as compared to CD27 expression on the same antigen-specific T-cell population in the non-enriched TIL culture ($P<0.005$, paired Student's t test). **(b)** Two representative examples showing expression of CD27 in expanded T-cell products that were enriched for the indicated antigen-specific T-cell populations. Top panels show results for a low frequency MAGE-$C2_{LLF}$ specific T-cell population, bottom panels show results for a high frequency MART-1_{ELA} specific T-cell population. Left panels show efficacy of enrichment, right panels show CD27 expression on the antigen-specific T-cell population. Percentages indicate positive population within the gates. **(c)** Expression of CD107a of a non-enriched TIL culture (black bar) and three independently enriched TIL cultures (red bar indicates $gp100_{KTW}$ specific T cell enrichment; blue bar indicates MART-1_{ELA} specific T cell enrichment; green bar indicates combined $gp100_{KTW}$/MART-1_{ELA} specific T cell enrichment) from donor NKIRTIL012 upon a 5-hour co-culture with autologous tumor (NKIRmel012), an HLA-A mismatched tumor cell line (NKIRmel084), and a tumor cell line (mel526) matched for the HLA*A02:01 allele that presents the $gp100_{KTW}$/MART-1_{ELA} antigens. Non-stimulated T cells are shown as a control. Gating strategy for CD107a expression is shown in Supplementary Fig. 2A. The percentage of CD107a positive cells was significantly higher in all three enriched TIL cultures, as compared to the non-enriched TIL culture (indicated by asterisks). Mean values of technical triplicates plus standard deviation are depicted. Results are representative of two independent experiments. Samples were compared using a two-tailed paired Student's t test. A p-value < 0.05 was considered statistically significant. ➔


A
% marker+ cells
(of total CD8+/pMHC+ cells)
100
80
60
40
20
0
*
CCR7
CD27
CD45RA
CD45RO
CD62L
CD127
CD95
B
Pre-enrichment
Post-enrichment
Pre-enrichment
Post-enrichment
anti-CD8
0.14%
1.74%
21.9%
52.6%
MAGE-C2LLF
4.82%
73.8%
41.5%
68.7%
MART-1ELA
pMHC multimer
anti-CD27
C
% CD107a+ cells
(of total CD8+ cells)
60
40
20
0
*
Non-enriched
gp100KTW enriched
MART-1ELA enriched
Double enriched
NKIRmel012
A1+/A2+
NKIRmel084
A1-/A2-
Mel 526
A1-/A2+
No target

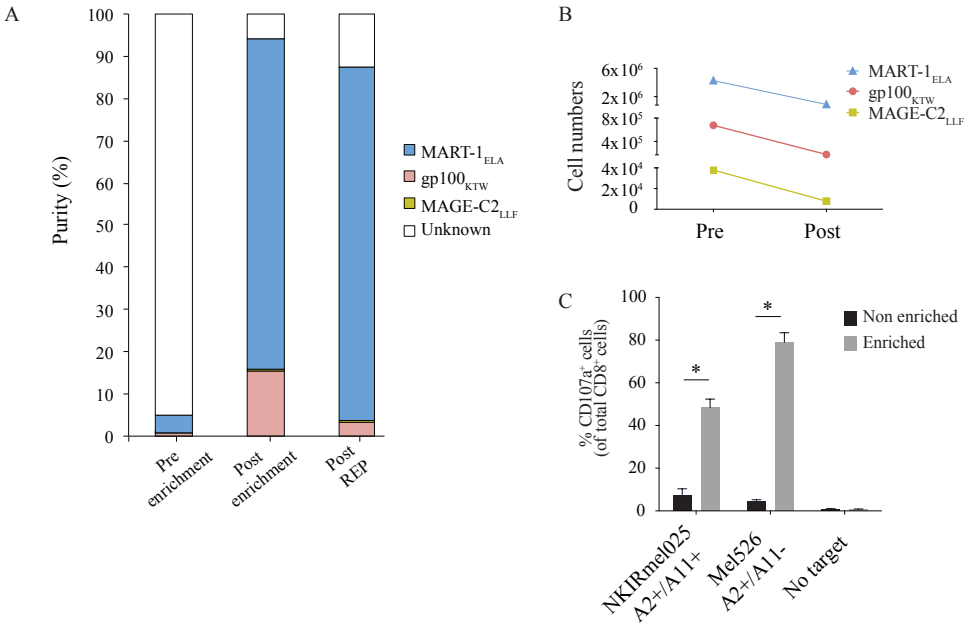


Figure 3. Generation of clinical grade enriched TIL products. (a) Simultaneous enrichment of three antigen-specific T-cell populations from a bulk TIL culture from patient NKIRTIL025 using the CliniMACS. Samples were processed in a similar fashion as described above, but enriched in a closed system instead of on MACS columns to assess feasibility of GMP-grade enriched TIL production. Composition of the cell product pre-enrichment, post-enrichment and post-expansion (REP) is shown. Antigen-specific T-cell populations are depicted as colored stacked bars; white bar depicts non-targeted T-cell populations of unknown specificity. Purity was determined by pMHC multimer staining. Data are representative of two independent CliniMACS runs. **(b)** Cell numbers pre- en post-enrichment of MART-1_{ELA}, $gp100_{KTW}$ and MAGE-$C2_{LLF}$ antigen specific T-cell populations are shown. Average recovery was 22%, calculated as the ratio of antigen-specific T cells before and after the enrichment procedure. **(c)** Expression of CD107a in non-enriched and enriched TIL cultures from donor NKIRTIL025 upon coculture with autologous tumor (NKIRmel025) and a tumor cell line (mel526) matched for the HLA*A02:01 allele that presents the $gp100_{KTW}$/MART-1_{ELA} antigens. Non-stimulated T cells are shown as a control. Black bar depicts mean percentages with standard deviations of non-enriched TIL cultures, grey bar depicts enriched TIL cultures. The percentage CD107a positive cells was significantly higher for the enriched TIL product ($P<0.001$, indicated by asterisks). Mean values of triplicates plus standard deviation are depicted. Samples were compared using a two-tailed paired Student's t test. A p-value < 0.05 was considered statistically significant.

Enrichment of neo-antigen reactive T-cell populations

The effectiveness of cancer immunotherapy is positively correlated with mutational burden, likely due to T-cell recognition of the neo-antigens that arise as a consequence of tumor-specific mutations[12]. As neo-antigens are entirely tumor-specific, and as the affinity of neo-antigen specific T cells is not capped by thymic tolerance, the steering of immune reactivity towards this particular class of antigens may be particularly attractive. To assess the feasibility of streptamer-based enrichment of T cells directed against patient-specific neo-antigens, we

aimed to enrich neo-antigen reactive T-cell populations from a bulk melanoma TIL culture. For patient NKIRTIL027, we performed exome and RNA sequencing of the autologous tumor cell line which was overlaid with exome sequence data of healthy reference TIL to identify tumor-specific mutations. In this way, we identified 909 non-synonymous mutations, of which 582 were present within expressed genes. Using our epitope prediction pipeline [13], we predicted 318 putative neo epitopes for the HLA-A*01:01 allele for which streptamer reagents were available. Subsequently, a bulk TIL culture was screened for the presence of neo-antigen reactive T cells, resulting in the identification of two T-cell responses, directed against the mutated gene products of $ENTPD4_{(P85L)}$ (0.23% of $CD8^+$ T cells), and $TTC37_{(A692V)}$ (0.45% of $CD8^+$ T cells) (Fig. 4A). Utilizing HLA-A*01:01 streptamers, we could enrich $ENTPD4_{(P85L)}$ and $TTC37_{(A692V)}$ specific T-cell responses by 55- and 45-fold, respectively, to a combined frequency of 29.4% (Fig. 4B). During the subsequent rapid expansion culture, $TTC37_{(A692V)}$ specific T cells expanded further, whereas the frequency of $ENTPD4_{(P85L)}$ specific T cells slightly dropped, resulting in a combined purity of neo-antigen specific T cells of 60%. In line with this enrichment, we demonstrated a 29-fold increase in recognition of autologous tumor cells (Fig. 4B). These data demonstrate that the production of enriched antigen-specific TIL cultures is not limited to T cells reactive against shared antigens but can likewise be implemented to target parts of the patient autologous mutanome.

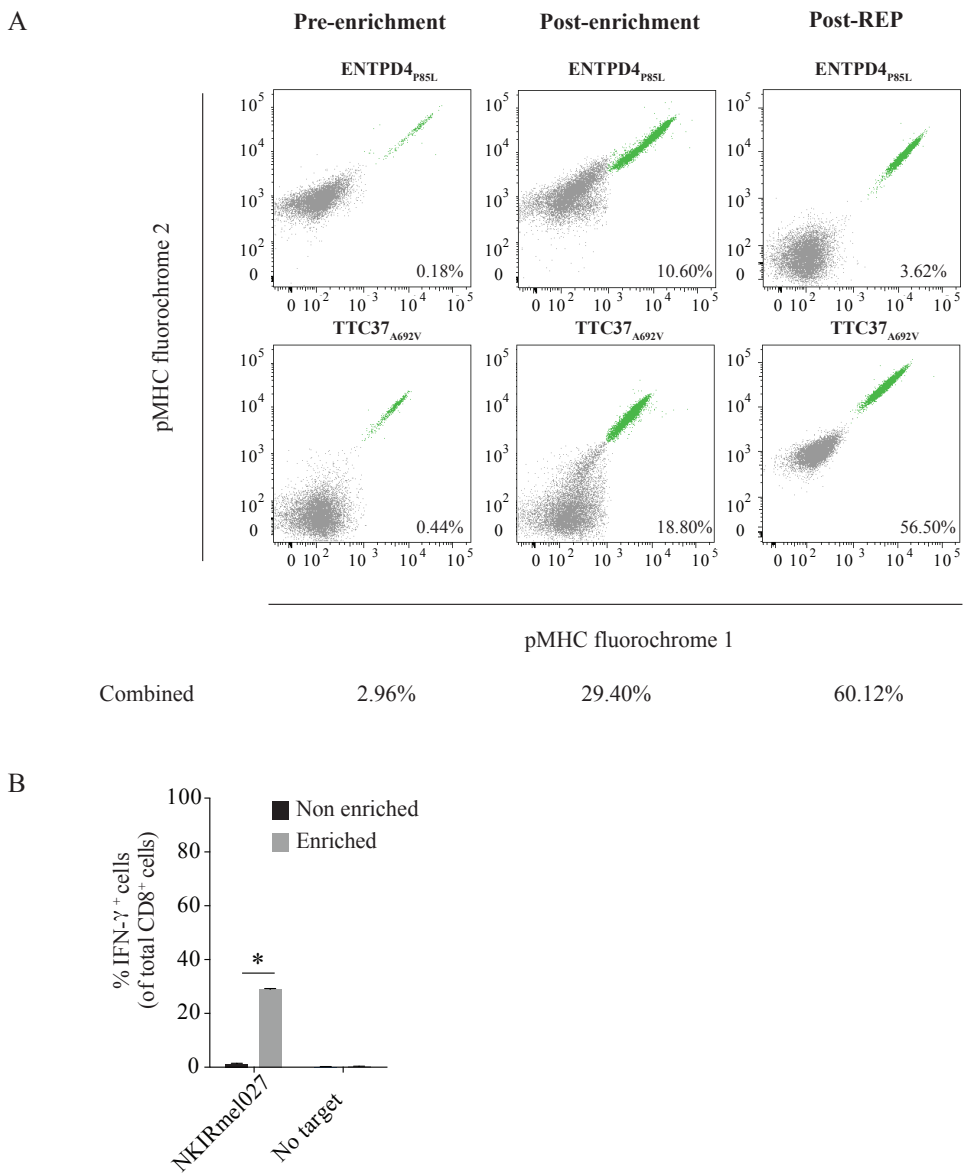


Figure 4. Enrichment of neo-antigen specific T-cell population. (a) Exome sequencing and neo-epitope predictions were performed for NKIRTIL027. A subsequent neo-antigen specific T cell screen utilizing pMHC multimer combinatorial encoding technology revealed two distinct HLA-A*01:01 restricted T-cell responses with a combined frequency of 2.96% of total $CD8^+$ T cells. Reactivity was directed towards the mutated gene products of *ENTPD4* with a proline to leucine substitution at position 85 (P85L) and *TTC37* with an alanine to valine substitution at position 692 (A692V). The total purity of neo-antigen reactive T cells was 29.4% directly after enrichment and 60.1% following a 14-day rapid expansion culture. Grey population in lower left corner depicts pMHC negative T cells; green population in upper right corner depicts double pMHC positive T cells of total $CD8^+$ T cells (indicated in percentages).

Negative populations are unevenly distributed across FACS plots because different combinations of pMHC multimers were used in enrichment and expansion experiments. Combined purity is shown below flow cytometry plots. **(b)** Following REP, both the enriched TIL culture (grey bar) as well as a control non-enriched TIL culture (black bar) was tested for reactivity against the autologous tumor cell line NKIRmel027. Following a 5-hour incubation, cells were fixed, permeabilized, and stained for intracellular IFN-γ expression. Data are depicted as mean percentages of technical triplicates plus standard deviations for cytokine positive cells in total $CD8^+$ T cells. Unstimulated TIL were used as controls. Enriched TIL showed significantly higher recognition of autologous tumor cells compared to non-enriched TIL ($P<0.05$, Student's t test). Representative data from two experiments are shown.

DISCUSSION

In this study, we demonstrate the feasibility of enriching antigen-specific T-cell populations from bulk melanoma TIL cultures. Both high frequency and low frequency T-cell populations can successfully be enriched, resulting in clinical-grade cell products with a high degree of purity and enhanced anti-tumor activity. Because of its flexible nature, this approach is feasible for the isolation of T cells reactive against non-mutated self-antigens and patient-specific mutated antigens, making it feasible to directly compare the relative value of T-cell responses against both antigen classes (Kelderman et al., ms submitted).

The clinical value of T-cell products enriched for defined antigen-specific T-cell populations has already been demonstrated in patients suffering from recurrent CMV infections after allogeneic stem-cell transplantations[21-23]. Furthermore, studies from Lang and colleagues have demonstrated the enrichment of T-cell populations reactive to melanoma antigens by an MHC-based platform[24,25]. The current work builds on these efforts by developing an 'open' system for MHC-based T-cell purification, in which purification reagents can be charged with any epitope of interest, including patient-specific antigens.

Phenotypic analysis of antigen-specific T cells from enriched and non-enriched TIL cultures revealed that overall phenotypic characteristics were unaltered after selection and expansion. Following enrichment, there was a moderate increase in the fraction of CD27-positive antigen-specific cells. Prolonged TCR-MHC interaction has been shown to correlate with a decrease rather than an increase in CD27 expression[26], which makes the possibility of a persistent interaction between remaining multimer reagents and antigen-specific cells an unlikely explanation, also since multimer reagents are dissociated directly after enrichment. Potentially, the short-duration TCR-MHC interaction leads to the preferential outgrowth of the $CD27^+$ subset, which could be beneficial for clinical purposes as the number of $CD8^+CD27^+$ TILs infused in melanoma patients has been shown to correlate with clinical response[27].

Following enrichment and expansion, we observed that enriched TIL cultures are more reactive towards autologous and a partially HLA-matched melanoma cell line than non-enriched TIL cultures. It is important to realize though that the effects of antigen enrichment

are two-fold. First, total tumor reactivity of the TIL product can increase, which is especially beneficial in cases of low-level reactivity against autologous tumor. Second, the reactivity of the obtained TIL products will be focused towards those epitopes for which selection is performed, at the expense of any others. As an example, enrichment of TIL products that already have a very high reactivity is unlikely to increase total reactivity substantially. In such cases, enrichment will only be attractive when it focuses the resulting cell product on T-cell reactivities with above average tumoricidal potential. At present, our understanding of the relative tumoricidal potential of different tumor antigen specific T-cell populations is still limited. However, the platform described here makes it feasible to generate TIL products steered towards a wide variety of antigen classes, and thereby test the potential of different antigen classes in preclinical models. In this regard, $CD8^+$ T-cell reactivity against patient-specific mutated antigens, which appears common in human melanoma[13-15] is of particular interest. Clinical data suggest the potential relevance of such neo-antigens in cancer immunotherapy. However, the evidence is thus far only correlative[28,29]. Utilizing the UV-exchange streptamer technology described here, we have directly compared the ability of a neo-antigen directed TIL product and 'standard' unselected TIL product to control autologous tumor in a preclinical model of human melanoma (S. Kelderman et al. ms submitted). These experiments revealed superior tumor control by neo-antigen directed TIL for this patient, thereby providing a strong rationale to target neo-antigens in a clinical setting. Other work has demonstrated that TIL may also be enriched based on the expression of one or several co-stimulatory and co-inhibitory receptors such as CD137 and PD-1[30,31]. While this approach has the advantage of maintaining the diversity of the tumor antigen-specific T-cell response[32], it does not allow one to specifically focus reactivity towards T-cell responses that are considered most valuable. As such, side by side (pre-)clinical evaluation of both approaches appears most attractive.

The approach developed here relies on already established clinical protocols and can be performed under Good Manufacturing Practice conditions, and clinical implementation should therefore be relatively straightforward. Taking into account the rapid advances in tumor exome sequencing and immunomonitoring, the production of antigen-specific TIL by MHC-based enrichment does appear feasible when dedicated equipment and appropriate infrastructure are in place[33], in particular when targeted therapies such as BRAF-inhibitors with a median progression-free survival of 5.6 months are utilized as a bridging treatment[34], while antigen-specific TIL are being produced.

MATERIALS AND METHODS

Patient material and cell cultures. TIL cultures NKIRTIL006, NKIRTIL012, NKIRTIL025, NKIRTIL027, NKIRTIL084 and corresponding melanoma lines were established from patient material obtained in-house following informed consent and in accordance with local guidelines. Cell line mel526 was a kind gift of Dr. S. Rosenberg (Surgery Branch, NIH). Tumor lines were cultured in RPMI1640 supplemented with 10% FCS and penicillin (100 IU/ml) and streptomycin (100 µg/ml). T cells were cultured in complete medium (CM; RPMI-1640 supplemented with human AB serum (10%), IL-2 (6000 IU/ml, Novartis), penicillin (100 IU/ml, Roche), streptomycin (100 µg/ml, Roche) and glutamax (200mM, Life Technologies). Expansion of TIL cultures was performed according to the standardized Rapid Expansion Protocol (REP) that is also used for clinical applications[9]. Cells were cryopreserved in FCS with 10% DMSO and stored in liquid nitrogen.

Deep sequencing and epitope predictions. Screens to identify neo-antigen reactive T-cell populations were performed as described previously[13]. In short, genomic DNA was extracted from NKIRmel027 using a DNeasy kit (Qiagen) and processed for deep-sequencing on an Illumina HiSeq DNA Analyzer (75bp paired-end reads). Resulting reads were aligned to a human reference genome. Subsequently, epitope predictions were performed for the patient's corresponding HLA alleles (A*01:01, A*26:01, B*08:01 and B*44:02) using NetChop c-term 3.0 and NetMHCpan2.8[35-37].

MHC-multimer reagents and T-cell staining. Peptides were synthesized in-house and stored in 100% DMSO at -20°C. MHC multimer-reagents were generated in-house as described previously [38]. In brief, HLA-A*02:01 or HLA-*01:01 monomers containing a UV-conditional ligand (100µg/ml) were exposed to UV-irradiation (360nm, Camag) for 1 hr in the presence of a rescue peptide (200µM). The resultant peptide-MHC (pMHC) complex was conjugated to streptavidin-conjugated fluorochromes (Life Technologies). After 30 min of incubation, NaN_3 (0.02% wt/vol) was added, and an excess of D-biotin (26.4 mM, Sigma) was added to block residual binding sites. T cells were stained with titrated amounts of fluorochrome conjugated pMHC complexes and 2 µl anti-CD8 FITC (BD Biosciences, clone SK1) in a total volume of 135 µl. Cells were incubated at 37 °C for 15 minutes. Cells were washed two times with FACS buffer and stained with DAPI (5 µl) in standard enrichment experiments or LIVE⁄DEAD® Fixable Violet Dead Cell Stain Kit (0.5 µl) in neo-antigen specific T-cell screens. Prior to flow cytometric analysis, cells were washed twice in 200 µl FACS buffer, 1500 RPM for 3 minutes.

Streptamer-based T-cell enrichment. Streptagged HLA-A*02:01 and HLA-A*01:01 reagents were loaded with peptides of interest by UV-induced exchange reactions, as described above. Subsequently, the resultant MHC monomers were conjugated to Strep-Tactin-loaded nanobead-particles (IBA) according to the manufacturer's protocol. Cryopreserved TIL were thawed 1 hr prior to antigen-specific T-cell enrichment, incubated for 20 mins with benzonase (25 IU/ml), and washed with IS buffer (IBA). MHC/bead complexes (1µg MHC / $1x10^7$ cells) were loaded on a pre-washed LS MACS column (Miltenyi) to remove unbound MHC molecules. The conjugated MHC/beads mixture was eluted with IS buffer from the column and mixed with the cells in suspension. The resultant mixture was incubated at 4°C for 45 min on a roller. Cells were washed twice with IS buffer to remove unbound MHC/bead complexes and subsequently loaded on a pre-washed LS column with pre-filter. The column was washed twice with IS buffer and cells were subsequently eluted and washed twice with IS buffer containing D-biotin (100mM, IBA) to dissociate MHC multimer reagents. Recovery was defined as the number of the targeted antigen-specific cells obtained after purification, relative to the number of the targeted antigen-specific cells within the starting material. Clinical-scale enrichments were performed on a CliniMACS (Miltenyi), using a Tubing Set 150 and the selection program "Enrichment 1.1." Eluted cells were expanded using the Rapid Expansion Protocol (REP) that is used for TIL production for adoptive cell therapy, in 50/50 medium (50% CM / 50% AIM-V, Life Technologies), in the presence of irradiated autologous feeder cells (200 fold excess), IL-2 (3000IU/ml), and anti-CD3 (30ng/ml OKT3, Janssen-Cilag).

T-cell functionality assays. Enriched and non-enriched TIL cultures were stained for expression of a panel of phenotypic markers, using 1 µl anti-CD8 V500 (BD Biosciences, clone RPA-T8), 2 µl anti-CD27 APC (BD Biosciences, clone M-T271) or 2 µl anti-CD27 BV-421 (BD Biosciences, clone M-T271), 2 µl anti-CD127 PE (BD Biosciences clone hIL-7R-M21), 5 µl anti CD95 FITC (BD Biosciences clone DX2) or 2 µl anti-CD62L FITC (BD Biosciences, clone DREG-56), 0.25 µl anti-CD45RA PE-Cy5.5 (Life Technologies, clone MEM-56), 0.5 µl anti-CD45RO PE-CF594 (BD Biosciences, clone UCHL1), 1 µl anti-CCR7 PE-Cy7 (BD Biosciences, clone 3D12), 5 µl DAPI or 0.5 µl of LIVE/DEAD Fixable IR Dead Cell Stain Kit (Invitrogen, L10119) and 1 µl PE-pMHC or 3 µl Q605-pMHC and 2 µl Q655-pMHC. Cells were incubated for 20 min at 4°C in a total staining volume of 50 µl.

T-cell reactivity assays were set-up by co-culturing $2x10^5$ T cells and the indicated tumor cells in a 1:1 ratio, in 96-well plates for 5 hrs at 37°C in the presence of anti-CD107a PE antibody (BD Biosciences, clone H4A3). After one hour, Monensin and Brefeldin A (BD Biosciences) were added. Following incubation, cells were washed and stained with 1 µl anti-CD3 FITC (BD Biosciences, clone SK7) and 1 µl anti-CD8 PerCP Cy5.5 (BD Biosciences, clone SK1), and subsequently fixed and permeabilized according to the manufacturer's protocol (BD Biosciences Fix/Perm kit). After fixation, intracellular cytokine stains were performed

with 0.5 µl anti-IFN-γ APC (BD Biosciences, clone B27) in permeabilization buffer. Cells were washed twice prior to data acquisition.

Flow-cytometry. Data acquisition was performed on an LSRFortessa or LSR-II flow cytometer (Becton Dickinson) with FacsDiva software. The following instrument settings on the LSRFortessa were used: Blue laser (488nm): FITC, 505LP, 530/30. Violet laser (405nm): DAPI, 450/50; V500, 505LP, 525/50. Red laser (640nm): APC, 670/14. Yellow-green laser (561nm): PE, 586/15; PE-CF594, 600lP, 610/20; PE-Cy5.5, 685LP, 710/50; PE-Cy7, 750LP, 870/60. The following gating strategy for T cells in standard enrichment experiments was applied: selection of live single-cell lymphocytes (FSC-W/H low, SSC-W/H low, FSC/SSC-A), followed by selection of $CD8^+$ cells (Supplementary Fig. 2A). Data was analyzed using FlowJo software (Treestar Inc.). Instrument settings on the LSR-II flow-cytometer in neo-antigen screens have been described previously[39].

Statistical analyses. Data are expressed as means plus standard deviations. A paired Student's t-test was performed to compare pre and post variables, and a p-value of <0.05 was considered statistically significant.

Accession codes. DNA and RNA sequencing data for melanoma specimen NKIRTL027 have been deposited in the European Genome-Phenome Archive with accession codes EGAD00001000243 and EGAD00001000325.

ACKNOWLEDGEMENTS

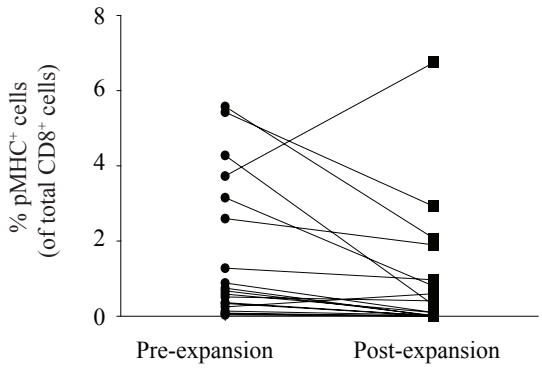
The authors would like to thank Anna-Sophia Wiekmeijer for preliminary work on this project. SK designed and performed experiments, analyzed and interpreted data and wrote the paper. BH designed and interpreted experiments, collected patient material and was involved in the early development of this project. LF, DP and MT provided technical assistance. PK and MB performed and analyzed T-cell antigen screens. NR and SM collected patient material. LG provided MHC streptamers. JH interpreted data and was involved in clinical translational efforts. TS designed experiments, interpreted data, co-wrote the paper and supervised the project.

This work was supported by Dutch Cancer Society grant KWF 2012-5463 to T.N.M.S., and by a Stand Up To Cancer–Cancer Research Institute Immunology Translational Cancer Research Grant to T.N.M.S. SU2C is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research.

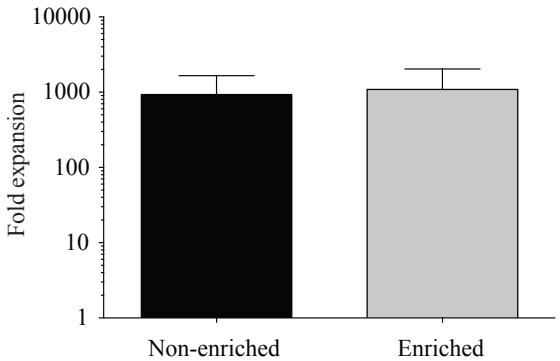
CONFLICT OF INTEREST

LG is shareholder and co-founder of Stage Cell therapeutics, which produces the streptamers used in this project. All other authors declare no competing financial interest.

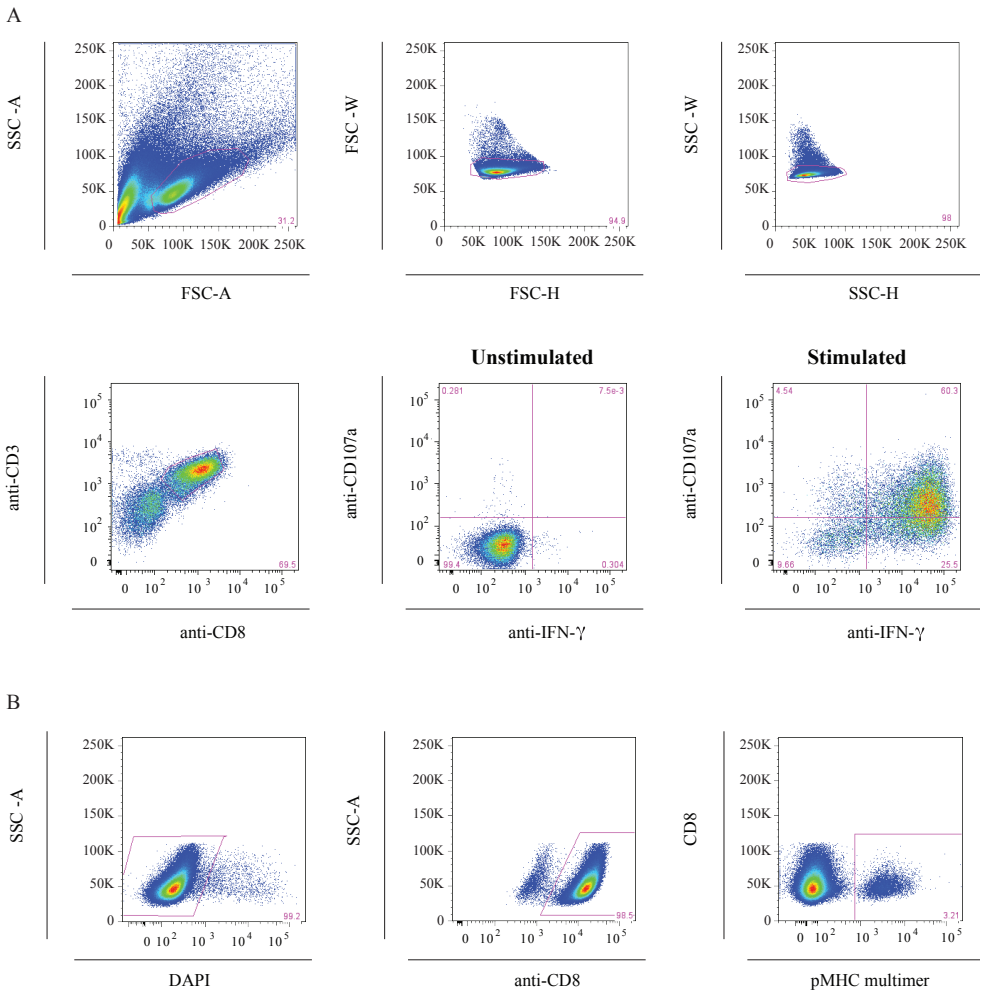
A



B



Supplementary Figure 1. Expansion of TIL cultures. (a) Effect of T-cell expansion on the frequency of defined antigen-specific T-cell populations in non-enriched TIL cultures (1,087-fold mean expansion for the antigen-enriched TIL versus 937-fold mean expansion for non-enriched bulk TIL). **(b)** Fold expansion of 16 paired non-enriched and enriched TIL cultures. Bars represent mean values plus standard deviation. Samples were compared using a two-tailed paired Student's t test. No significant difference in fold expansion between non-enriched and enriched TIL cultures was observed (p = 0.26, Student's t test).



Supplementary Figure 2. Gating strategy. General gating strategy applied in all flow cytometry experiments, as described in Methods section. **(a)** First, a lymphocyte gate was set, followed by a duplicate exclusion. For reactivity assays, gates were set to identify $CD3^+$ and $CD8^+$ T cells, followed by CD107a and IFN-γ gates set on unstimulated control cells. **(b)** In pMHC multimer screens, as well as T-cell enrichment experiments, gates were set on single live $CD8^+$ T cells, positive for either one (shown here) or two (not shown) fluorochrome-conjugated pMHC complexes.

REFERENCES

1. Chapman, P.B.*, et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**, 2507-2516 (2011).
2. Shames, D.S. & Wistuba, II. The evolving genomic classification of lung cancer. *J Pathol* **232**, 121-133 (2014).
3. Le Tourneau, C.*, et al.* Randomised proof-of-concept phase II trial comparing targeted therapy based on tumour molecular profiling vs conventional therapy in patients with refractory cancer: results of the feasibility part of the SHIVA trial. *Br J Cancer* **111**, 17-24 (2014).
4. Rosenberg, S.A. & Restifo, N.P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* **348**, 62-68 (2015).
5. Radvanyi, L.G.*, et al.* Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* **18**, 6758-6770 (2012).
6. Besser, M.J.*, et al.* Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. *Clin Cancer Res* **19**, 4792-4800 (2013).
7. Dudley, M.E.*, et al.* Randomized selection design trial evaluating CD8+-enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. *J Clin Oncol* **31**, 2152-2159 (2013).
8. Besser, M.J.*, et al.* Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. *Clin Cancer Res* **16**, 2646-2655 (2010).
9. Dudley, M.E.*, et al.* CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clin Cancer Res* **16**, 6122-6131 (2010).
10. Kvistborg, P., van Buuren, M.M. & Schumacher, T.N. Human cancer regression antigens. *Curr Opin Immunol* **25**, 284-290 (2013).
11. Alexandrov, L.B.*, et al.* Signatures of mutational processes in human cancer. *Nature* **500**, 415-421 (2013).
12. Schumacher, T.N. & Schreiber, R.D. Neoantigens in cancer immunotherapy. *Science* **348**, 69-74 (2015).
13. van Rooij, N.*, et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol* **31**, e439-442 (2013).
14. Robbins, P.F.*, et al.* Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med* **19**, 747-752 (2013).
15. Lu, Y.C.*, et al.* Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. *Clin Cancer Res* **20**, 3401-3410 (2014).
16. Kvistborg, P.*, et al.* TIL therapy broadens the tumor-reactive CD8(+) T cell compartment in melanoma patients. *Oncoimmunology* **1**, 409-418 (2012).
17. Knabel, M.*, et al.* Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med* **8**, 631-637 (2002).
18. Neudorfer, J.*, et al.* Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *J Immunol Methods* **320**, 119-131 (2007).
19. Toebes, M.*, et al.* Design and use of conditional MHC class I ligands. *Nat Med* **12**, 246-251 (2006).
20. Rodenko, B.*, et al.* Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. *Nat Protoc* **1**, 1120-1132 (2006).

21. Schmitt, A., *et al.* Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. *Transfusion* **51**, 591-599 (2011).
22. Cobbold, M., *et al.* Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *J Exp Med* **202**, 379-386 (2005).
23. Odendahl, M., *et al.* Clinical-scale isolation of 'minimally manipulated' cytomegalovirus-specific donor lymphocytes for the treatment of refractory cytomegalovirus disease. *Cytotherapy* **16**, 1245-1256 (2014).
24. Bouquie, R., *et al.* A fast and efficient HLA multimer-based sorting procedure that induces little apoptosis to isolate clinical grade human tumor specific T lymphocytes. *Cancer Immunol Immunother* **58**, 553-566 (2009).
25. Labarriere, N., *et al.* A full GMP process to select and amplify epitope-specific T lymphocytes for adoptive immunotherapy of metastatic melanoma. *Clin Dev Immunol* **2013**, 932318 (2013).
26. Hintzen, R.Q., *et al.* Regulation of CD27 expression on subsets of mature T-lymphocytes. *J Immunol* **151**, 2426-2435 (1993).
27. Rosenberg, S.A., *et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* **17**, 4550-4557 (2011).
28. Rizvi, N.A., *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science* **348**, 124-128 (2015).
29. Snyder, A., *et al.* Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N Engl J Med* **371**, 2189-2199 (2014).
30. Gros, A., *et al.* PD-1 identifies the patient-specific CD8+ tumor-reactive repertoire infiltrating human tumors. *J Clin Invest* **124**, 2246-2259 (2014).
31. Ye, Q., *et al.* CD137 accurately identifies and enriches for naturally occurring tumor-reactive T cells in tumor. *Clin Cancer Res* **20**, 44-55 (2014).
32. Wolfl, M., Kuball, J., Eyrich, M., Schlegel, P.G. & Greenberg, P.D. Use of CD137 to study the full repertoire of CD8+ T cells without the need to know epitope specificities. *Cytometry A* **73**, 1043-1049 (2008).
33. Cohen, C.J., *et al.* Isolation of neoantigen-specific T cells from tumor and peripheral lymphocytes. *J Clin Invest* **125**, 3981-3991 (2015).
34. Larkin, J., *et al.* Vemurafenib in patients with BRAF(V600) mutated metastatic melanoma: an open-label, multicentre, safety study. *Lancet Oncol* **15**, 436-444 (2014).
35. Lundegaard, C., *et al.* NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11. *Nucleic Acids Res* **36**, W509-512 (2008).
36. Nielsen, M., *et al.* Improved prediction of MHC class I and class II epitopes using a novel Gibbs sampling approach. *Bioinformatics* **20**, 1388-1397 (2004).
37. Calis, J.J., Reinink, P., Keller, C., Kloetzel, P.M. & Kesmir, C. Role of peptide processing predictions in T cell epitope identification: contribution of different prediction programs. *Immunogenetics* **67**, 85-93 (2015).
38. Toebes, M., Rodenko, B., Ovaa, H. & Schumacher, T.N. Generation of peptide MHC class I monomers and multimers through ligand exchange. *Curr Protoc Immunol* **Chapter 18**, Unit 18 16 (2009).
39. Kvistborg, P., *et al.* Anti-CTLA-4 therapy broadens the melanoma-reactive CD8+ T cell response. *Sci Transl Med* **6**, 254ra128 (2014).

Chapter 8

Neo-antigen enriched TIL therapy mediates superior tumor control in a xenograft model of human melanoma

Sander Kelderman[1#], Laura Bies[1#], Marit M. van Buuren[1], Nienke van Rooij[1], Daisy Philips[1], Mireille Toebes[1], Laura J.A. van Dijk[1], Samira Michels[1], Lorenzo Fanchi[1], Bianca Weißbrich[4], Kristel Kemper[1], Daniel S. Peeper[1], Sam Behjati[2], Michael R. Stratton[2], Lothar Germeroth[3], Bianca Heemskerk[1], Dirk H. Busch[4], Chantale Bernatchez[5], Laszlo G. Radvanyi[5], Christian U. Blank[1], Patrick Hwu[5], John B.A.G. Haanen[1], Pia Kvistborg[1], and Ton N.M. Schumacher[1]

[1]The Netherlands Cancer Institute NKI-AVL, Amsterdam, The Netherlands, [2]Cancer Genome Project, Wellcome Trust Sanger Institute, Hinxton, United Kingdom, [3]Stage Cell Therapeutics GmbH, Göttingen, Germany, [4]Technische Universität München, Munich, Germany, [5]MD Anderson Cancer Center, Houston, USA. [#] These authors contributed equally to this work

Manuscript submitted

INTRODUCTION

In cancers with a high mutational load, such as melanoma, lung and colorectal cancer[1,2], large numbers of mutated peptides (hereafter referred to as neo-antigens) are potentially presented by MHC class I and II molecules on the surface of tumor cells[3-5]. Furthermore, recent reports have demonstrated that neo-antigen-specific T-cell reactivity is frequently observed in melanoma[5-9]. However, the contribution of neo-antigen specific T-cell reactivity to tumor control is only partially understood. In this report, we explore the potential clinical relevance of neo-antigens that are recognized by $CD8^+$ T cells. We first demonstrate how treatment with a clinically effective TIL product can lead to the marked dominance of a neo-antigen specific T-cell response in a patient with melanoma. We then provide direct evidence in a patient-derived xenograft model of human melanoma that T-cell responses against neo-antigens can mediate superior tumor regression as compared to bulk TIL. Finally, we show that within this patient, the anti-tumor potential of neo-antigen specific T-cell receptors is greater than that of T-cell receptors directed against the Cancer/Germline antigen family. Collectively, these data provide support for the selective targeting of neo-antigens in cancer immunotherapy.

RESULTS

For this study, we obtained tumor tissue from a 46-year old female patient (MDACCTIL2379) suffering from metastatic melanoma who underwent treatment with $100x10^9$ autologous TIL according to a previously described clinical protocol[10]. Radiologic evaluation six weeks post TIL infusion showed a rapid clinical response, with a more than 50% decrease of baseline tumor burden. Seven months post-therapy, the patient was in complete remission, and continues to be so at two years of follow-up (Supplementary Fig. 1A). Based on the fact that T-cell responses against neo-antigens are not hampered by central T-cell tolerance, an important role of such T-cell reactivities in anti-tumor immune responses has previously been postulated[6,11-13]. Furthermore, through the development of cancer exome sequencing approaches and high-throughput immunomonitoring technology, it is now possible to evaluate T-cell reactivity against neo-antigens within individual patients. By this method, T-cell responses against neo-antigens have been observed in T-cell products successfully used for therapy, including one case in which infusion of a $CD4^+$ T-cell population with high reactivity towards a neo-antigen was shown to lead to a reduction in tumor burden[14]. To address the potential involvement of neo-antigen directed T-cell reactivity in the tumor response of patient MDACCTIL2379, we performed whole-exome sequencing on a primary melanoma cell line obtained from this patient to identify somatic tumor mutations (Fig. 1A).

A total of 960 non-synonymous mutations were found, (953 substitution and 7 insertions/deletions, FDR 0.07). As expected, substitutions were predominantly C>T/G>A transitions enriched at dipyrimidine sequences indicative of a UV-induced mutational signature[1] (Supplementary Fig. 1B, left). Next, mutations present within expressed genes were identified using RNAseq data, and 39 amino-acid stretches encompassing these mutations were used for the prediction of potential neo-antigens (Fig. 1A). Using a low cut-off to avoid false negatives, this resulted in 1,008 potential CD8 T-cell epitopes for HLA-A*01:01, HLA-A*23:01 and HLA-B*55:01. Subsequently, the autologous TIL culture of this patient was analyzed for the presence of neo-antigen reactive T-cell populations against any of these peptides, by combinatorial coding with a library of peptide-MHC (pMHC) complexes generated through UV-induced peptide exchange[15-17]. This screen revealed the presence of two neo-antigen reactive T-cell populations within the TIL infusion product: one low-level response (0.17% of $CD8^+$ T cells), specific for a mutation in the RASSF1 (RAS association domain family member 1) gene product, and one very dominant response (23% of $CD8^+$ T cells within the infusion product), specific for a mutation in the *DHX33* (DEAH box polypeptide 33) gene product (Fig. 1B).

To reveal whether T-cell reactivity against these neo-antigens was substantially influenced by TIL therapy, pre- and post-therapy PBMC samples were analyzed by staining with MHC multimers loaded with the $RASSF1_{R244C}$ and $DHX33_{R186W}$ neo-epitopes. Within pre-therapy PBMC, total T-cell responses against the two neo-antigens were barely detectable (0.011% of $CD8^+$ T cells). However, at day 7 post-infusion, approximately 50% of peripheral blood T cells were directed against the single neo-antigen within *DHX33*, and this T-cell response expanded further in the subsequent weeks to approximately 62%, a more than 6,800-fold increase relative to pre-therapy levels (Fig. 1C and Supplementary Fig. 1C).

Prior data have shown the common occurrence of neo-antigen specific $CD8^+$ T-cell responses in TIL products[8,9,18], and the above data demonstrate that T-cell responses against a mutated antigen can subsequently dominate the T-cell compartment at the time of clinical response. Nevertheless, this type of clinical data can only provide correlative evidence for the importance of neo-antigen reactive T cells. To evaluate the relevance of neo-antigen specific T-cell reactivity in a more direct manner, we aimed to develop a system in which the activity of different T-cell populations against autologous tumor could be analyzed in parallel. For this purpose, we studied tumor tissue and TIL obtained from a 61-year old female patient with metastatic melanoma (NKIRTIL006) who had undergone a palliative metastasectomy in 2005 and never received any form of immunotherapeutic intervention. From the tumor digest, TIL and tumor cell line cultures were established in parallel and tumor tissue was used for both whole exome sequencing and to generate a PDX model in NOD-SCID-IL2R gamma-chain deficient (NSG) mice. Within the tumor of this patient, a total of 350 non-synonymous mutations were identified (Supplementary Fig. 1B, right) and, using

our epitope prediction pipeline, 252 potential neo-antigens were predicted. Subsequent screening of autologous TIL with an MHC multimer library containing these epitopes led to the identification of three neo-antigen reactive T-cell populations within this patient: (1) a T-cell response (1.604% of $CD8^+$ T cells) directed against an R24L mutation in the *CDK4* (cyclin-dependent kinase 4) gene product; (2) a T-cell response (0.407% of $CD8^+$ T cells) directed against a mutation in the *GCN1L1* (general control of amino-acid synthesis 1-like 1) gene product; (3) a low-magnitude (0.003%) T-cell response directed against a mutation in the *DNAH17* (dynein axonemal heavy chain 17) gene product (Fig. 1D).

In order to compare the relative activity of neo-antigen specific T cells versus that of standard TIL, we developed a T-cell enrichment procedure that uses HLA-A*0201 UV-exchange streptamers to purify T-cell populations of interest and utilized this to enrich for the $GCN1L1_{L2330P}$ and $CDK4_{R24L}$ -specific HLA-A*02:01 restricted T-cell responses[19]. Both the enriched cell culture (92.4% total $GCN1L1_{L2330P}$ and $CDK4_{R24L}$ reactivity) and the standard bulk culture (2.78% total $GCN1L1_{L2330P}$ and $CDK4_{R24L}$ reactivity) were expanded according to a rapid expansion protocol (REP)[10] that is used to create TIL products for treatment (Supplementary Fig. 1D). High functional activity of the enriched T-cell product towards the two mutant epitopes was established by analysis of intracellular cytokine secretion upon coculture of T cells with HLA-A*02:01 restricted target cells loaded with either the mutant epitope or the wild type counterpart (Fig. 1E). Notably, a subsequent coculture of the standard TIL and the enriched TIL with the autologous tumor cell line revealed a more modest increase in reactivity and tumor cell killing (Fig. 1F and Supplementary Fig. 1E).

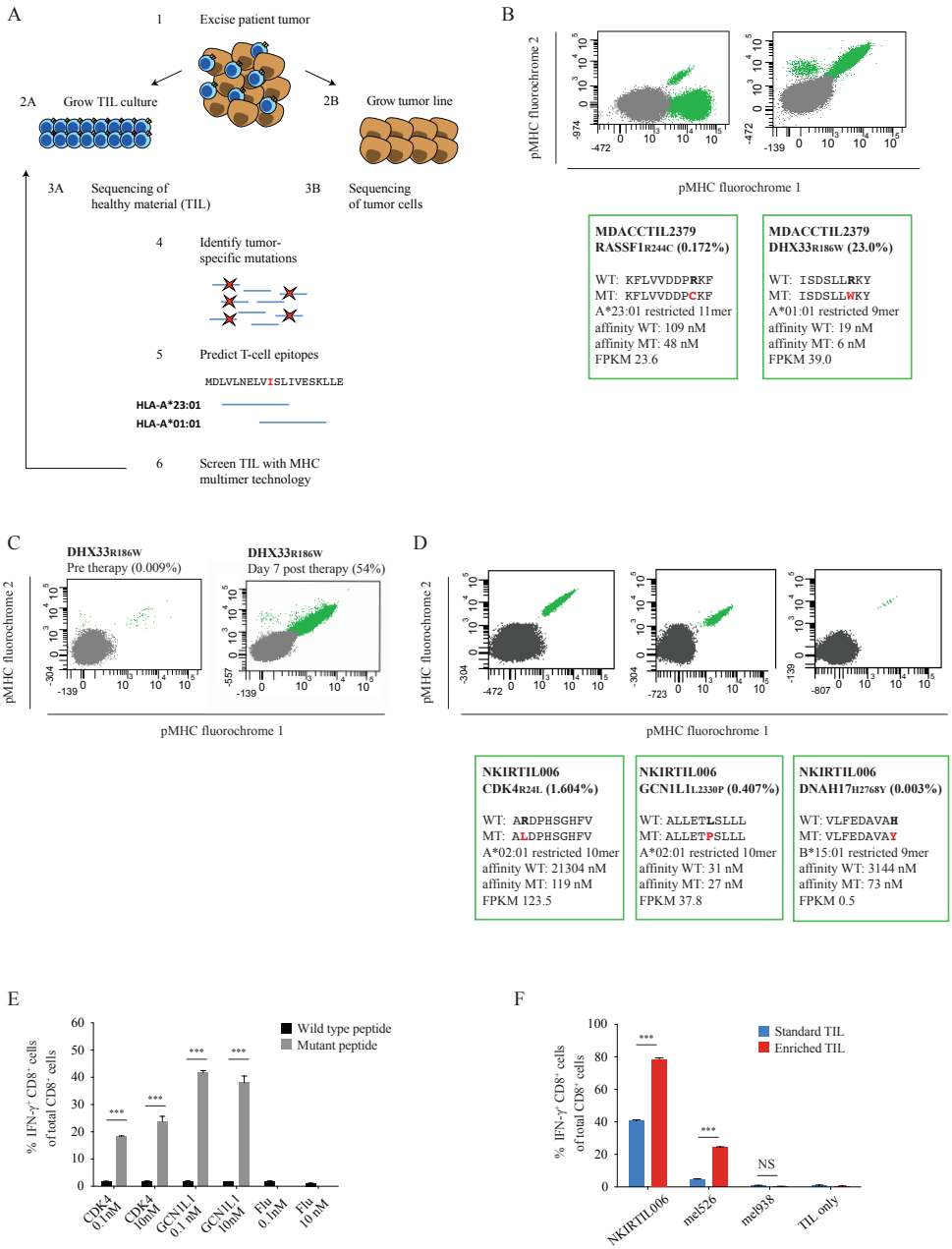
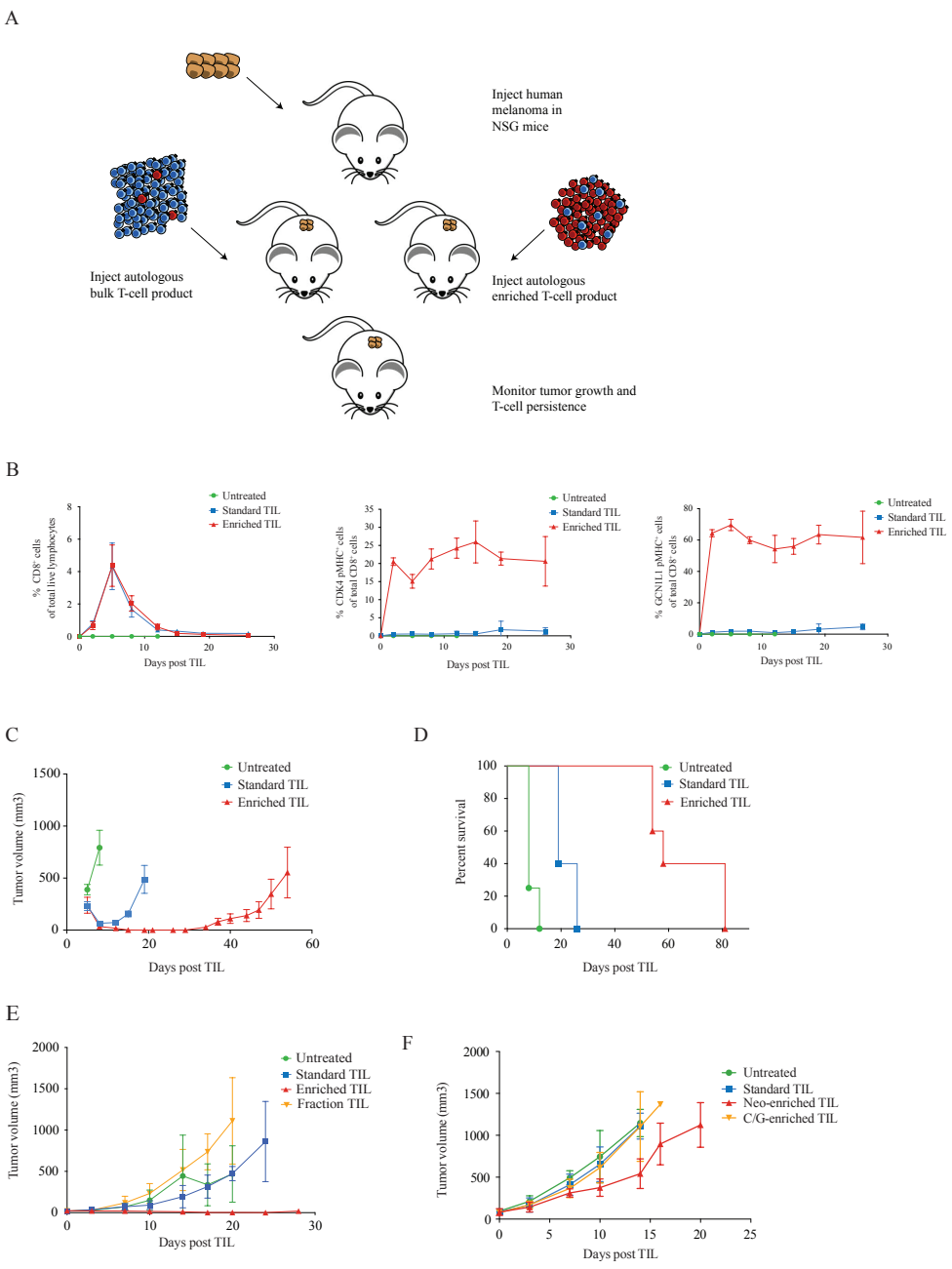


Figure 1. Neo-antigen reactive T-cell populations in human melanoma TIL cultures. (a) Overview of methods used to identify neo-antigen reactive CD8⁺ T cells in bulk melanoma tumor-infiltrating lymphocytes (TIL) cultures. Brown cells: tumor cells; blue cells: TIL; red stars: tumor-specific mutations. **(b)** Flow cytometric analysis of neo-antigen reactive T-cell populations in TIL of MDACCTIL2379. Top: neo-antigen reactive T-cell populations specific for mutant RASSF1 (left) and DHX33 (right). Percentages of pMHC double-positive cells within total CD8⁺ cells are indicated. Bottom: amino-acid sequences,

MHC-restriction and MHC-binding affinities for wild type (WT) and mutant (MT) peptides, as well as RNA expression levels in Fragments Per Kilobase of exon per Million fragments mapped (FPKM). **(c)** Flow cytometric analysis of mutant DHX33-specific T cells present in peripheral blood pre-therapy (left) and seven days post-therapy (right). Percentages of pMHC double-positive cells within total $CD8^+$ cells are indicated. **(d)** Flow cytometric analysis of neo-antigen reactive T-cell populations in TIL of NKIRTIL006. Top: neo-antigen reactive T-cell populations specific for mutant CDK4 (left), mutant GCN1L1 (middle), and mutant DNAH17 (right). Percentages of pMHC double-positive cells within total $CD8^+$ cells are indicated. Bottom: epitope characteristics as in Fig. 1B. **(e)** Functional activity of enriched TIL, analyzed by intracellular IFN-γ cytokine staining upon coculture with wild type and mutant peptide-loaded T2 cells. Data are presented as percentages of IFN-γ producing $CD8^+$ T cells. Bars represent means of triplicates plus standard deviation. **(f)** Functional activity of standard TIL and enriched TIL, analyzed by intracellular IFN-γ cytokine staining upon coculture with autologous (NKIRTIL006) melanoma, and two allogeneic HLA-A*02:01 matched (mel526, which is known to express the previously described $CDK4_{R24C}$ mutation) and mismatched (mel938) melanoma cell lines. Data are presented as percentages of IFN-γ producing $CD8^+$ T cells. Bars represent means of triplicates plus standard deviation. Data are representative of two independent experiments. Samples were compared using an unpaired two-sided Student's t-test (*** $P < 0.001$; NS, not significant).

To evaluate the potency of neo-antigen specific T cells in an in vivo setting, NSG mice were injected with tumor cells of patient NKIRTIL006, and either the autologous standard TIL product or the autologous enriched TIL product was administered once when tumors were palpable (Fig. 2A). Engraftment of $CD8^+$ T cells in peripheral blood of mice treated with standard TIL and enriched TIL was comparable, with a peak in T-cell frequencies at day 5 and a subsequent rapid decline (Fig. 2B). Importantly, the composition of the engrafted T-cell compartment was significantly different, with dominance of $GCN1L1_{L2330P}$ and $CDK4_{R24L}$ -specific T cells only seen in mice treated with enriched TIL (2% versus 85% mean combined $GCN1L1_{L2330P}$ and $CDK4_{R24L}$ reactivity at day 5, Fig. 2B). Strikingly, while a slight decrease in tumor growth was initially seen in both treatment groups, prolonged tumor control and survival was only observed in mice treated with enriched TIL (Fig. 2C and 2D). This superior activity of neo-antigen selective TIL is directly due to the higher number of these cells, rather than the removal of potentially inhibitory T-cell subsets, as shown by analysis of tumor growth of mice treated with the fraction of neo-antigen reactive cells present within the bulk TIL product (Fig. 2E). Interestingly, while in vivo retreatment had no effect on tumor outgrowth in two out of three mice previously treated with enriched TIL (Supplementary Fig. 2A), all recurring tumors still expressed MHC class I (Supplementary Fig. 2B) and were recognized efficiently in vitro (Supplementary Fig. 2C), suggesting that escape of antigen-loss variants did not occur.

Figure 2. Neo-antigen reactive TIL mediate superior tumor control over standard 'bulk' TIL in a mouse model of human melanoma. (a) In vivo set-up to compare tumor control upon treatment with standard TIL and TIL enriched for neo-antigen reactivity. Brown cells: tumor cells; blue cells: bulk TIL; red cells: neo-antigen specific TIL. **(b)** Flow cytometric analysis of tail vein-derived blood taken from mice treated with standard (n = 5, blue square) or enriched TIL (n = 5, red triangle), or from untreated control mice (n = 4, green circle) at the indicated time points post-TIL infusion (16 x 10^6 total cells per mouse administered i.v.). All mice received high-dose IL-2 (7.2 x 10^5 IU) twice daily for three days after TIL infusion. Left: percentage of human $CD8^+$ T cells of total live lymphocytes; middle: percentage of mutant $CDK4_{R24L}$ -specific T cells within the total $CD8^+$ T-cell population; right: percentage of mutant $GCN1L1_{L2330P}$ -specific T cells within the total $CD8^+$ T-cell population. Data are presented as mean ± s.d. There was no statistically significant difference between engraftment of total $CD8^+$ T cells from standard TIL and enriched TIL. Frequencies of neo-antigen specific cells were significantly higher in recipients of enriched TIL as compared to recipients of standard TIL at all time points ($P < 0.001$). **(c)** Tumor growth in mice treated with standard TIL or enriched TIL, or in untreated control mice. Data are presented as mean ± s.d. **(d)** Survival analysis of mice treated with standard TIL or enriched TIL, or of untreated control mice. Survival distribution was analyzed by log-rank test (standard vs. enriched $P < 0.005$). **(e)** Tumor growth in non-treated mice (n = 4, green circles), and in mice treated with standard TIL (n = 4, 10 x 10^6 cells per mouse, blue squares), neo-antigen enriched TIL (n = 5, 10 x 10^6 cells per mouse, red triangles), or an equivalent number of neo-antigen reactive TIL as present in the bulk TIL (n = 5, 2.72 x 10^5 cells per mouse, yellow triangles). In this setup, the standard TIL group and fraction TIL group received equal numbers (1.95 x 10^5) of neo-antigen reactive T cells. All mice received high-dose IL-2 (7.2 x 10^5 IU) twice daily for three days after TIL infusion. Data are presented as mean ± s.d. There was a statistically significant difference in tumor control from day 17 onward for neo-antigen enriched TIL vs. standard TIL ($P < 0.005$) and neo-antigen enriched TIL vs. fraction TIL ($P < 0.005$). At day 17, mice treated with standard TIL had significantly lower tumor burden compared to mice treated with fraction TIL ($P = 0.02$). Samples were compared using an unpaired two-sided Student's t-test. **(f)** Tumor growth in untreated control mice (n = 7, green circles), or in mice treated with standard TIL (n = 6, blue squares), neo-antigen enriched TIL (n = 5, red triangles), or C/G-antigen enriched TIL (n = 6, yellow triangles). All treated mice received 20 x 10^6 total cells i.v. Contribution of the aimed for antigen-specific T-cell population, as assessed by MHC multimer staining, was 37.5% for both recipients of C/G-antigen and neo-antigen enriched TIL. All mice received high-dose IL-2 (7.2 x 10^5 IU) twice daily for three days after infusion. Previously expanded TIL cultures were used, which were re-expanded (REP2) after enrichment to generate the TIL infusion products. Data are presented as mean ± s.d. There was a statistically significant difference in tumor control at day 14 for neo-antigen enriched TIL vs. standard TIL ($P = 0.02$) and neo-antigen enriched TIL vs. C/G-antigen enriched TIL ($P = 0.02$), but not for standard TIL vs. C/G-antigen enriched TIL ($P = 0.98$). Samples were compared using an unpaired two-sided Student's t-test. ➔


A
Inject human melanoma in NSG mice
Inject autologous bulk T-cell product
Inject autologous enriched T-cell product
Monitor tumor growth and T-cell persistence
B
Untreated
Standard TIL
Enriched TIL
% CD8+ cells of total live lymphocytes
% CDK4 pMHC+ cells of total CD8+ cells
% GCN1L1 pMHC+ cells of total CD8+ cells
Days post TIL
C
Tumor volume (mm3)
Days post TIL
D
Percent survival
Days post TIL
E
Fraction TIL
F
Neo-enriched TIL
C/G-enriched TIL

Next, we aimed to address whether TIL products enriched for reactivity against other classes of potentially relevant tumor rejection antigens could achieve a similar anti-tumor effect as TIL enriched for neo-antigen reactivity. Analysis of NKIRTIL006 by staining with a panel of HLA-A*02:01 multimers containing shared melanoma antigens had revealed the presence of T-cell reactivity against the Cancer/Germline (C/G) antigens MAGE-A10$_{GLY}$, MAGE-C2$_{LLF}$, MAGE-C2$_{VIW}$ and MACE-C2$_{ALK}$ (3.16%, 0.09%, 0.59% and 0.89% of CD8^{+} T cells, respectively)[20]. To analyze the potential value of these C/G-antigen reactive T-cell responses, we independently enriched TIL cultures for either neo-antigen (CDK4$_{R24L}$ and GCN1L1$_{L2330P}$) reactivity or C/G-antigen (MAGE-A10 and MAGE-C2) reactivity (Supplementary Fig. 3A). For both groups, the TIL cultures were again expanded after enrichment (necessary to obtain sufficient numbers of MAGE-A10/ MAGE-C2 enriched T cells from the low number of starting cells). Subsequently, frequencies of MHC multimer positive cells were adjusted to the same level for both groups (resulting in a combined frequency of either neo-antigen or C/G-antigen reactive T cells of 37.5%) and the C/G and neo-antigen specific T cell-enriched TIL products were used for treatment of NSG mice carrying established autologous melanoma. Following infusion, a similar engraftment of TIL was observed across all treatment groups (Supplementary Fig. 3B). While overall anti-tumor activity was lower than in experiments using TIL expanded only a single time, also in this setting a significant difference in tumor outgrowth between mice treated with standard TIL and neo-antigen enriched TIL was observed ($P < 0.05$). In contrast, infusion of C/G-antigen enriched TIL was not superior over treatment with standard TIL ($P = 0.98$, Fig. 2F). Furthermore, we observed a significant difference in tumor outgrowth between mice treated with neo-antigen enriched TIL as compared to mice treated with C/G-antigen enriched TIL ($P = 0.02$, Fig. 2F), indicative of a qualitative difference between epitope classes targeted by TIL therapy in this patient. However, the relative fitness of the enriched cell populations could be a major confounder for their tumoricidal potential. To enable a full qualitative comparison between TCR specificities, we sequenced the genes of each respective TCR alpha/beta chain, cloned them into a retroviral vector for expression on peripheral blood mononuclear cells (PBMCs) (Supplementary Fig. 4A) and confirmed their reactivity against the autologous tumor cell line (Supplementary Fig. 4B). We then treated tumor-bearing NSG mice with either a mixture of the two neo-antigen reactive TCR transduced PBMCs or a mixture of the four C/G antigen reactive TCR transduced PBCMs or with non transduced PBMCs as a control (Supplementary Fig. 4C). Upon infusion, all mice treated with neo-antigen reactive TCRs experienced complete tumor control (Fig. 3A), without late recurrence as was initially observed in the TIL treatment setting. Mice treated with C/G antigen reactive TCRs experienced some initial tumor control but eventually all mice were sacrificed because of tumor recurrence (Fig. 3B). To dissect differences with respect to tumor clearance within the group of neo-antigen reactive TCRs, we treated two additional groups with either CDK4 or GCN1L1 reactive

PBMCs. To our surprise, only mice treated with the CDK4 TCR transduced PBCMs completely rejected the tumor (Fig. 3C). This may be a TCR intrinsic feature, as a subsequent k_{off}-rate assay[21] showed a significant difference in binding affinity of the CDK4 TCR in comparison with the GCN1L1 TCR and two of the four MAGE TCRs that we were able to test (Fig. 3D). These data indicate that within the group of neo-antigen reactive T cells there still may be heterogeneity in, although this is certainly not limited to, the quality of the T-cell response. Validation of TCR binding affinity may thus provide important information on tumor-control efficacy of reconstructed autologous TCRs for adoptive cell therapy approaches.

This report provides both indirect and direct support for the (pre-) clinical relevance of neo-antigen reactive T-cell populations in two melanoma patients. It is, however, important to point out that neo-antigen specific $CD8^+$ T-cell reactivity will not be a dominant factor in all patients that respond to immunotherapy. For instance, in tumors with a more modest mutational load, T-cell reactivity against non-mutant antigens could be a more critical factor, for the simple reason that the available repertoire of mutant antigens is limited. The observation that the clinical activity of recently developed immunotherapeutics is particularly pronounced in tumors with high mutational loads suggests that at least for these tumors an increased focus on patient-specific mutated antigens is warranted[22-25]. Clinical studies performed by Rosenberg and colleagues have already demonstrated that neo-antigen reactive T-cell populations enriched from clinical TIL products can exert profound clinical effects and that such a procedure is feasible at a clinical-grade level. Unfortunately, data from such trials will only provide correlative evidence for the involvement of T cells targeting neo-antigens, whereas the combination of our in vivo model system and T-cell receptor affinity assessment makes it possible to compare the quality of distinct T-cell responses targeting putative tumor-rejection antigens in a direct manner. As mentioned earlier, the differences we observed in tumor-clearance efficacy are likely not limited to TCR affinity alone but can also be related to whether a driver or a passenger mutation is being targeted or to differences in antigen expression levels. Furthermore, analyses on additional patients are required to gain insights in the tumoricidal fraction of the total neo-antigen reactive T-cell pool. Ultimately, mouse model systems might still be required as a learning strategy for the effects of hierarchical targeting of neo-antigens[26] before we will be able to discern which form the more superior targets in obtaining tumor control in each individual patient.

In summary, ongoing developments in next-generation sequencing platforms and in silico prediction algorithms have made it feasible to identify patient-specific mutant epitopes within individual patients, and should make it feasible to enhance T-cell responses against such epitopes in a therapeutically relevant time frame in future studies.

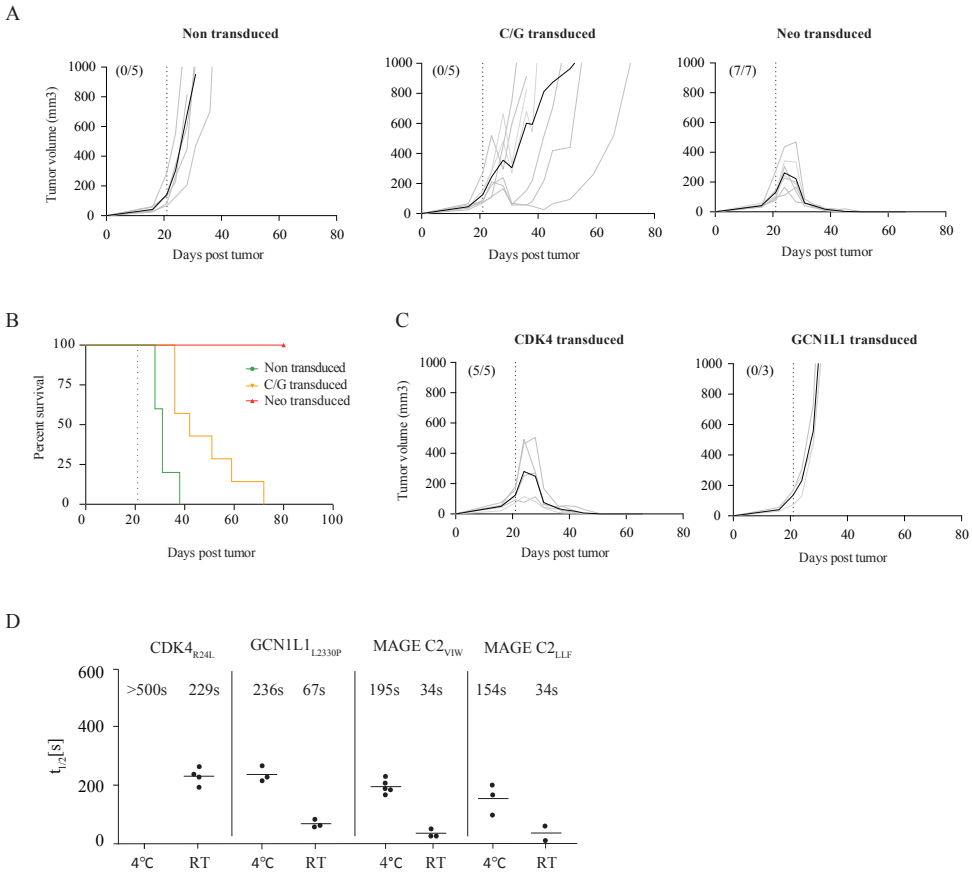


Figure 3. (a) Tumor growth in mice treated with control non transduced T cells (left), C/G TCR transduced T cells (middle) or neo TCR transduced T cells (right). Graphs show tumor growth of each individual mouse (grey line) and the average of the group (black line). Dotted line indicates time-point of T-cell infusion. Number of complete responders per group is indicated between brackets. Mice received 1e6 total $CD8^+TCR^+$ T cells. (b) Survival analysis per group is shown. (c) Tumor growth in mice treated with either $CD8^+CDK4^+$ T cells or $CD8^+GCN1L1^+$ T cells. **(d)** k_{off}-rate assay was performed at 4 °C or room temperature (RT) for 4 out of 6 TCRs. TCR staining with streptamers was not detectable for MAGE-$C2_{ALK}$ and MAGE-$A10_{GLY}$ TCRs.

METHODS

Patient material and cell cultures. Material was obtained from patients (NKITRIL006 and MDACCTIL2379) with progressive stage IV melanoma after informed consent had been signed and with approval of the local medical ethical authorities. MDACCTIL2369 was treated in a phase II clinical trial study of TIL therapy (NCT00338377) at the MD Anderson Cancer Center under study protocol 2004-006910. Response evaluation was performed

according to standard RECIST criteria as well as irRC[27]. TIL and tumor cell line cultures from both institutes were established from resection material according to identical protocols. Expansion of TIL cultures was performed according to a Rapid Expansion Protocol[10]. Cells were cryopreserved in 10 % DMSO and stored in liquid N2.

Exome sequencing and detection of somatic mutations. Genomic DNA was extracted from cell pellets using the QIAGEN DNeasy purification kit. Libraries of genomic DNA were prepared using the Illumina Paired-End Sample Prep kit following the manufacturer's guidelines. Resulting gDNA libraries were enriched for exonic sequences as described previously using the Agilent Sure Select Human All Exon 50Mb target enrichment system[28]. Sequencing was performed on an Illumina HiSeq DNA Analyzer (75bp paired-end reads), according to the manufacturer's instructions. Reads were aligned to the human reference genome (NCBI Build 37) using the Burrows-Wheeler Aligner algorithm[29]. Unmapped reads, read mapping outside the targeted region and PCR-derived duplicates were excluded from further analyses.

To call substitutions and indels, the CAVEMAN and PINDEL algorithms were used, respectively, as implemented by the Cancer Genome Project[30]. The precision of substitution calling was determined by re-sequencing DNA from one sample (NKIRTIL006) at the NKI Genomics Core Facility, using the same sequencing platform but calling substitutions using Somatic SNIPER31. Indels were validated by manual inspection.

RNA sequencing and gene expression. RNA was isolated using the QIAGEN RNeasy purification kit. Poly-A selected RNA libraries were then prepared, using the TruSeq RNA library protocol (Illumina, San Diego, CA, USA), and the resulting libraries were sequenced on an Illumina HiSeq2000 using 75bp paired-end reads. Reads were aligned using Tophat (version 1.3.3)[32]. Expression values were calculated as FPKM using Cufflinks (version 1.0.2)[33].

Epitope Predictions. Amino acid stretches of 39 aa with the mutated amino acid at position 20 were used to perform predictions of proteasomal cleavage (NetChop[34],) and MHC class I binding (NetMHC3.2 and NetMHCpan2.4[35,36]). For those mutations located within 20 amino acids from the N- or C-terminus of the protein, shorter fragments were used. The following peptides were selected; 1) those epitopes that contain the mutated amino acid; and 2) derived from genes with FPKM values > 0 (as there is little known about the importance of RNA expression levels, this low threshold was chosen); and 3) with a predicted C terminal cleavage probability of > 0.5; and 4) with predicted binding affinities of: < 8,500nM for the HLA-A*01:01 allele, <3,100 nM for the HLA-A*23:01 allele, <13,000 nM for the HLA-B*55:01 allele, < 1,000nM for the HLA-A*02:01 allele, and <600 nM for the HLA-B*15:01 allele. The latter cut-offs were chosen such that per 100 mutations, a total of 35 peptides/allele (a

sum of 9-, 10- and 11-mers) are included in the screen for each allele, a number based on predictions using a model set of mutations.

Generation of pMHC multimers. Panels of pMHC multimers were generated by coupling each pMHC complex to a defined combination of two out of eight different fluorescent streptavidin (SA) conjugates (Invitrogen, Carlsbad, CA, USA). For each 10 µl of pMHC monomer (100 µg ml^{-1}), the following amount of SA-conjugates were utilized: 1.5 µl SA-QD605, 1.0 µl SA-QD625, 1.5 µl SA-QD655, 1.5 µl SA-QD705, 1.0 µl SA-QD800, 0.9 µl SA-PE (1 mg ml^{-1}) and 0.6 µl SA-APC (1 mg ml^{-1}). Mixtures were incubated on ice for 30 min. NaN3 (0.02% wt/vol) and an excess of D-biotin (26.4 mM, Sigma-Aldrich, St. Louis, MO, USA) were added, the latter to block residual binding sites.

T-cell staining with pMHC multimers. Thawed cells were incubated with 25 U/ml benzonase (Novagen, EMD Millipore, Billerica, MA, USA) for 1 hr at 37 °C. For T-cell staining, the following amounts of fluorescently labeled pMHC complexes were pooled together for combinatorial coding: 1 µl for each PE-labeled pMHC complex, 2 µl for each APC-labeled pMHC complex, 3 µl for each QD605-labeled pMHC complex, 2 µl for each QD625-labeled pMHC complex, 2 µl for each QD655-labeled pMHC complex, 4 µl for each QD705-labeled pMHC complex, 4 µl for each QD800-labeled pMHC complex, and 3 µl for each PE-Cy7-labeled pMHC complex. Cells were incubated in a total volume of 135 µl at 37 °C for 15 min. 2 µl anti-CD8-FITC (clone SK1, BD Bioscience, San Diego, CA, USA), 1 µl anti-CD4-AF700 (clone S3.5, Invitrogen, Carlsbad, CA, USA) and 0.5 µl LIVE⁄DEAD® Fixable Violet Dead Cell Stain Kit (Invitrogen, Carlsbad, CA, USA) was subsequently added, and cells were incubated on ice for 30 min. Prior to flow cytometric analysis, cells were washed twice in 200 µl FACS buffer, 1500 RPM, 3 minutes.

Flow cytometry. Data acquisition of T-cell screens was performed on an LSR-II flow cytometer (BD Bioscience, San Diego, CA, USA) with FacsDiva software, utilizing the following eleven color instrument setting: UV laser (355 nm): QD605, 595LP, 605/12; QD705, 685LP, 710/50; QD800, 750LP, 780/60. Violet laser (405 nm): QD625, 610LP, 625/20; QD655, 635LP, 655/8. Blue laser (488 nm): FITC, 505LP, 525/50. Yellow-green laser (561 nm): PE, 585/15; PE-Cy7, 750LP, 780/60. Red laser (640 nm): APC, 670/14; AF700, 685LP, 710/50; IR-Dye, 750LP, 780/60. To identify antigen-specific T cells, the following gating strategy was used. 1) Selection of live (IR-dye negative) single cell lymphocytes (FSC-W/H low, SSC-W/H low, FSC/SSC-A). 2) Selection of anti-CD8-$FITC^+$ and 'dump' (anti-CD4) negative cells. 3) Selection of $CD8^+$ T cells that were positive in two and only two MHC multimer channels.

T-cell enrichment. Enrichments were performed using MHC-exchange molecules equipped with a strep-tag III19. Complexes were exposed to 366 nm UV light (CAMAG, The Netherlands) at 4 °C in the presence of a rescue peptide. MHC streptamers were generated by conjugating the resulting exchange reaction to magnetic microbeads, according to the manufacturer's protocol (IBA, Göttingen, Germany). T cells were labeled using 1 µg of MHC streptamer per $1x10^7$ cells. Antigen-specific cells were positively selected using LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany). After enrichment, cells were incubated with 10 mM D-biotin (IBA, Göttingen, Germany) to dissociate MHC reagents.

TCR gene expression on PBMCs. TCR genes from single-cell sorted T-cell clones were determined by high-throughput deep-sequencing using a previously published method and subsequently cloned into a retroviral vector construct37. Transduction efficiency was determined by pMHC multimer and murine constant domain staining (anti-mouse-beta-chain-PE, clone H57-597, BD Pharmingen, San Diego, CA, USA).

K_{off}-rate assay. TCR binding affinity was assessed in a k_{off}-rate assay as previously described[21]. In short, transduced T cells were stained with pMHC Alexa488 and *Strep*-Tactin APC beads (Superflow, 50 % suspension, IBA). The decay of fluorescence signal was measured after the addition of D-biotin using real-time fluorescence microscopy, followed by the decay of fluorescence signal reflecting the dissociation of monomeric MHC molecules. k_{off}-rate and half-life time ($t_{½}$) values were calculated using MetaMorph Offline image analysis software (Molecular Devices).

Functional analyses. To determine antigen reactivity (analyses performed in triplicate), indicated effector cells were cultured at a 1:1 ratio with target cells for 5 hr at 37 °C and 5% CO2 (BD Pharmingen, San Diego, CA, USA). Target cell lines were either autologous (NKIRTIL006), HLA-A*02:01 matched (mel526), HLA-A*02:01 mismatched (mel938) or peptide-loaded T2 cells. Peptide loading was performed for 1 hr at 37 °C and 5% CO2 and cells were washed twice after incubation. After a 5 hr coculture, cells were stained with anti-CD8 PerCP-Cy5.5 (clone SK-1, BD Pharmingen, San Diego, CA, USA) and anti-CD3 FITC (clone SK7, BD Pharmingen, San Diego, CA, USA), fixed with Cytofix/Cytoperm according to the manufacturer's guidelines (BD Bioscience, San Diego, CA, USA), and stained for intracellular IFN-γ expression using anti-IFN-γ APC (clone B27, BD Pharmingen, San Diego, CA, USA). Samples were measured on a Fortessa flow cytometer (BD Bioscience, San Diego, CA, USA) with FacsDiva software and analyzed using FlowJo software version (version 9.4, Treestar Inc., San Carlos, CA, USA).

Chromium release assays were performed by labeling target cells for 1 hr at 37 °C with 100 mCi (3.7 MBq) ^{51}Cr (Amersham, Ghent, Belgium). Labeled target cells were washed three times with RPMI 1640 medium containing 5% FCS and were then incubated with effector

cells at the indicated ratios for 4 hr at 37 °C and 5% CO2 in 200 µL medium. Maximal and spontaneous release was determined by the addition of 1 % Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) or addition of medium alone, respectively. ^{51}Cr release was determined by transfer of 50 µl of supernatant to a Lumaplate (Perkin-Elmer, Waltham, MA, USA) and measurement in an automatic counter (Topcount, Perkin-Elmer). The percentage of specific release was calculated as: ((cpm experimental release – cpm spontaneous)/(cpm maximal – cpm spontaneous)) x 100.

In vivo model. NSG mice (The Jackson Laboratory, Bar Harbor, ME, USA) were housed in the Experimental Animal Department of The Netherlands Cancer Institute. All mouse experiments were performed in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee (DEC) of The Netherlands Cancer Institute.

Female mice of at least 8 weeks old were challenged by subcutaneous injection of 1 x 10^6 tumor cells embedded in matrigel (BD Bioscience, San Diego, CA, USA) in the flank. When tumors were palpable, mice were ranked by tumor size and randomized to ensure equal average tumor size per group with at least four mice per experimental group (no power calculations were performed). Subsequently, the indicated numbers of autologous T cells were injected intravenously, immediately followed by high-dose (7.2 x 10^5 IU) IL-2 (Proleukin, Novartis) intraperitoneally twice daily for three consecutive days. Control mice received no T cells or non transduced PBMCs. Tumors were measured twice per week by a researcher blinded to the experimental condition, using a digital caliper. Animals were sacrificed when tumors exceeded 15 mm in any dimension or when the average of two dimensions was higher than 12 mm. Blood samples were taken from the tail vein twice weekly. Recurring tumors were enzymatically digested and stained with anti-HLA-ABC-PE (clone G46-2.6, BD Pharmingen, San Diego, CA, USA). Immune monitoring was performed on a Fortessa flow-cytometer (BD Bioscience, San Diego, CA, USA).

Statistical analysis. Samples were compared using an unpaired, two-sided Student's t-test, unless specified otherwise. P-values <0.05 were considered statistically significant.

Data deposition. DNA and RNA sequencing data are deposited: EGAD00001000243 (https://www.ebi.ac.uk/ega/studies/EGAS00001000216) and EGAD00001000325 (https://www.ebi.ac.uk/ega/studies/EGAS00001000251). Data on MDACCTIL2379 can be found under PD13414a, PD13414b and PR13414a. Data on NKIRTIL006 can be found under PD9029a, PD9029b and PR9029a.

ACKNOWLEDGEMENTS

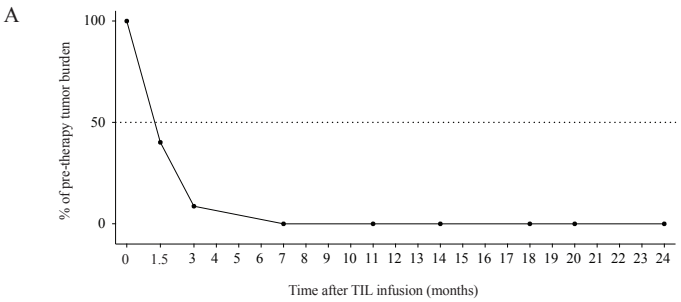
The authors would like to thank D. El Atmioui and H. Hilkmann of the Netherlands Cancer Institute Peptide Synthesis facility for technical support. We thank A. Velds and R. Kerkhoven for support on sequence analyses, H. van Tinteren for support with statistical analyses, C. Kesmir for advice on bio-informatics analyses, and R. Mezzadra for technical assistance. We would like to thank C. Linnemann and G. Bendle for critical reading of the manuscript and members of the Schumacher and Haanen laboratories for useful discussions. S.B. receives a Wellcome Trust Research Training Fellowship for Clinicians. This work was partly supported by the NIH, NCI grant R01 CA111999 and accompanying ARRA supplement to P.H. and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation award titled “Interactive program to identify predictive biomarkers in TIL therapy” to L.G.R. and C.B. This work was supported by Dutch Cancer Society grant KWF 2012-5463 to T.N.M.S., and by a Stand Up To Cancer–Cancer Research Institute Immunology Translational Cancer Research Grant to T.N.M.S. SU2C is a program of the Entertainment Industry Foundation administered by the American Association for Cancer Research.

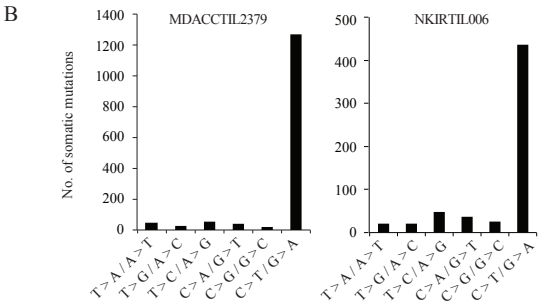
AUTHOR CONTRIBUTIONS

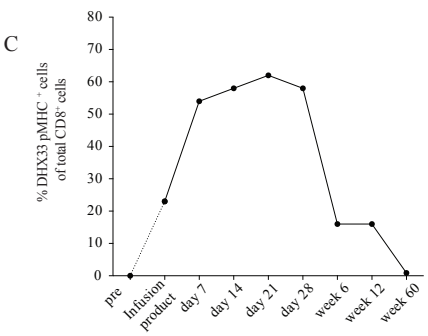
S.K. and L.B. designed, performed, analyzed and interpreted experiments, and wrote the paper. M.v.B. performed bio-informatics analyses and epitope predictions. M.v.B., N.v.R. and D.P. performed and analyzed T-cell neo-antigen screens. M.T. and L.J.A.D. synthesized MHC reagents. S.M. and L.F. provided technical assistance. K.K., L.B. and D.S.P. developed patient-derived xenograft systems for human melanoma. S.B. and M.R.S. designed, performed and interpreted sequencing experiments and provided bio-informatics support. L.G. designed MHC streptamers, B.W. and D.H.B. performed and interpreted k_{off}-rate experiments and B.H. contributed to the streptamer enrichment technology. C.B., L.G.R., C.U.B., P.H. and J.B.A.G.H. were involved in clinical trials, patient material collection and interpreted patient data. P.K. designed and performed T-cell neo-antigen screens, analyzed and interpreted data, and provided manuscript corrections. T.N.M.S. supervised the project, designed and interpreted all experiments, and wrote the paper.

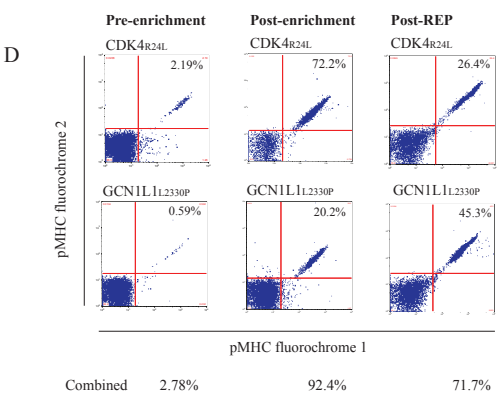
Competing financial interests. L.G. is shareholder and co-founder of Stage Cell therapeutics, which produces the MHC streptamers used in this project. All other authors declare no competing financial interest.

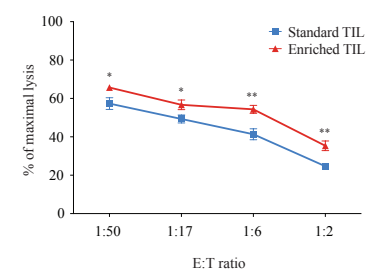
Supplementary Figure 1. Enrichment and functional analysis of neo-antigen reactive T cells in bulk melanoma TIL culture. **(a)** Change in tumor burden of patient MDACCTIL2379 relative to that at start of TIL therapy (1 x 10^{11} cells plus high-dose IL-2) is depicted. Measurements were performed on a target lesion in the right long using computed-tomography (CT) scanning images and reported according to immune-related response criteria (irRC) and RECIST. **(b)** Mutational profiles of patient MDACCTIL2379 (left) and NKIRTIL006 (right). Total number of somatic mutations is indicated. **(c)** Engraftment of mutant DHX33-specific T cells in peripheral blood of patient MDACCTIL2379 upon TIL therapy, as measured by MHC multimer combinatorial coding. **(d)** MHC multimer-based enrichment of neo-antigen reactive TIL from bulk melanoma TIL culture of patient NKIRTIL006. Left: pre-enrichment; Middle: post-enrichment; Right: post-REP. Data depict the percentage of pMHC double-positive cells within the total $CD8^+$ T-cell population. Combined purity of TIL product (in percentages) is depicted below. **(e)** Chromium-release assay to measure cytolytic activity against autologous tumor cells of either standard TIL or neo-antigen enriched TIL at the indicated effector to target (E:T) ratios. Data are presented as means of triplicates ± s.d. Samples were compared using an unpaired two-sided Student's t-test (*P<0.05, **P<0.01). ➔


A
100
50
0
% of pre-therapy tumor burden
0 1.5 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Time after TIL infusion (months)


B
MDACCTIL2379
NKIRTIL006
No. of somatic mutations
1400
1200
1000
800
600
400
200
0
500
400
300
200
100
0
T>A/A>T
T>G/A>C
T>C/A>G
C>A/G>T
C>G/G>C
C>T/G>A

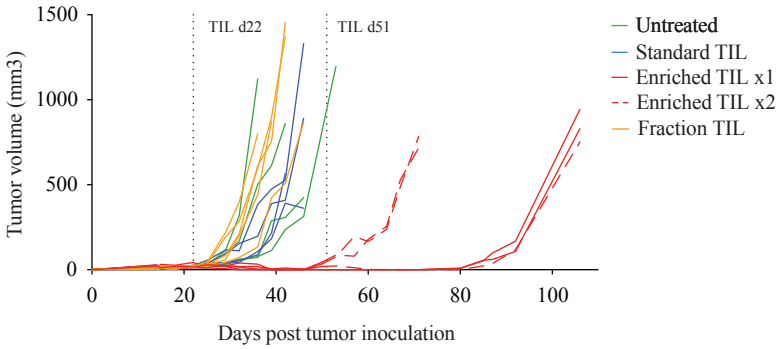

C
% DHX33 pMHC+ cells of total CD8+ cells
80
70
60
50
40
30
20
10
0
pre
Infusion product
day 7
day 14
day 21
day 28
week 6
week 12
week 60


D
Pre-enrichment
Post-enrichment
Post-REP
CDK4R24L
2.19%
72.2%
26.4%
GCN1L1L2330P
0.59%
20.2%
45.3%
pMHC fluorochrome 2
pMHC fluorochrome 1
Combined 2.78% 92.4% 71.7%

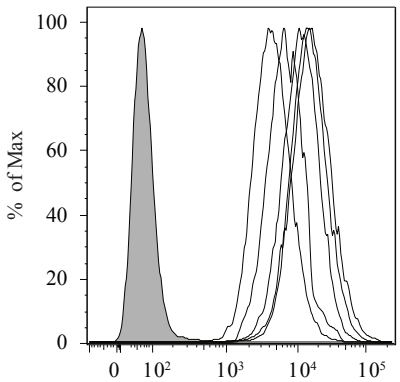

E
100
80
60
40
20
0
% of maximal lysis
Standard TIL
Enriched TIL
*
*
**
**
1:50
1:17
1:6
1:2
E:T ratio

Supplementary Figure 2. Disease recurrence in the absence of immune editing. (a) Tumor growth in individual untreated control mice (n = 4, green), or in mice treated (first dotted vertical line) with standard TIL (n = 4, blue), neo-antigen enriched TIL (n = 5, red), or fraction TIL (n = 5, yellow). Three recipients of neo-antigen enriched TIL group were retreated (TIL x2, second dotted vertical line) with neo-antigen enriched TIL (10×10^6 total cells per mouse) and high-dose IL-2 (7.2×10^5 IU) twice daily for three days at the time of tumor recurrence (day 51). The remaining two mice received no additional infusion of TIL but did receive high-dose IL-2 (7.2×10^5 IU) twice daily for three days at the time of tumor recurrence (day 85). **(b)** Recurring tumors were analyzed for expression of MHC class I by flow cytometry. Overlays of histograms are shown, grey filled area are unstained control cells. MFI denotes median fluorescence intensity. TIL x1 denotes mice that were treated with a single TIL infusion and had complete tumor regression until day 80; TIL x2 (R) denotes the mouse that, after initial tumor control received a second TIL infusion at day 51 and experienced complete regression until day 80; TIL x2 (NR) denotes mice that, after initial tumor control, received a second TIL infusion at day 51 to which they did not respond. **(c)** Analysis of (neo-) antigen presentation by recurring tumors, as measured by intracellular IFN-γ cytokine staining of standard TIL and enriched TIL upon coculture with tumors from control mice, or mice treated with standard TIL or neo-antigen enriched TIL. Data are presented as percentages of IFN-γ producing $CD8^+$ T cells, black lines indicate mean values. ➔

A

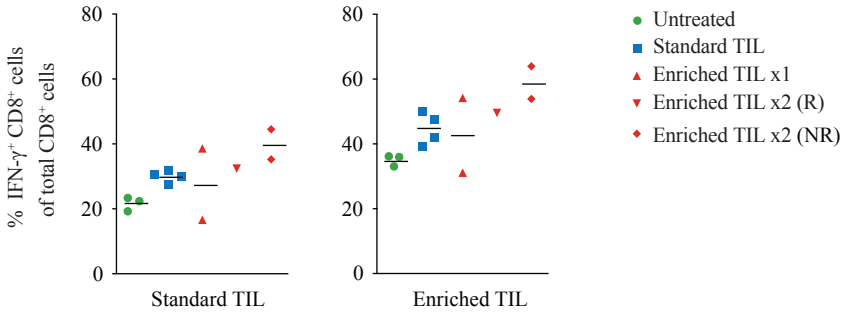


B

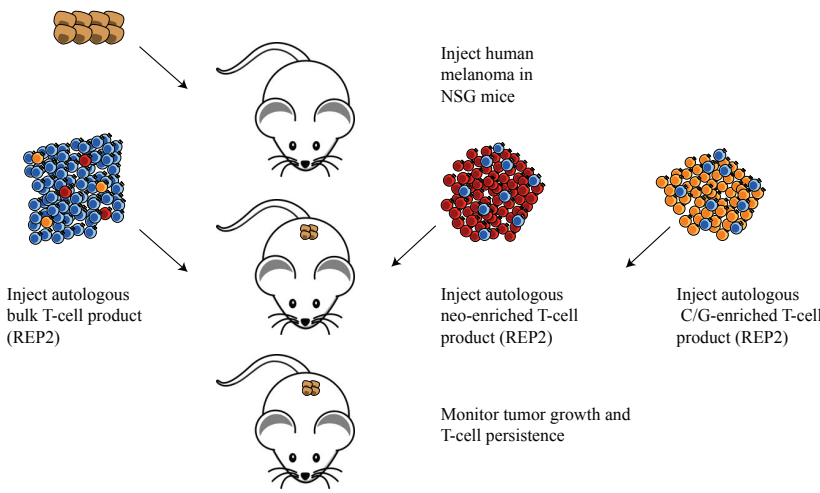


Mouse	MFI MHC class I
Enriched TIL X1	4372
Enriched TIL X1	6892
Enriched TIL x2 (R)	11687
Enriched TIL x2 (NR)	14873
Enriched TIL x2 (NR)	16738

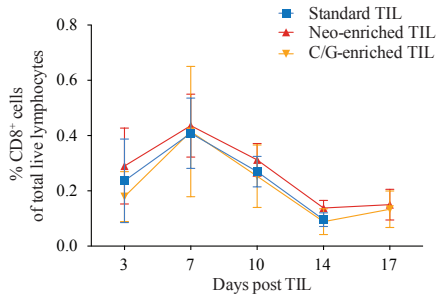
C



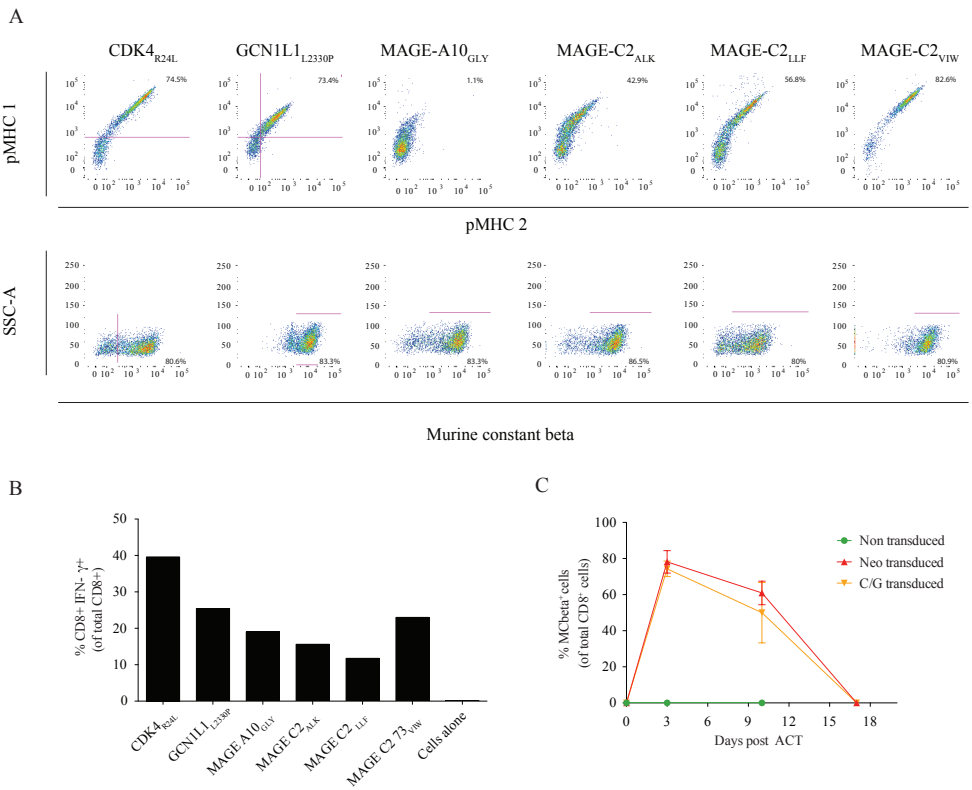
A



B



Supplementary Figure 3. Comparison of neo-antigen enriched T-cell product with C/G antigen enriched T-cell product. (a) In vivo set-up to compare tumor control upon treatment with standard TIL, neo-antigen enriched TIL and C/G-antigen enriched TIL. Brown cells: tumor cells; blue cells: bulk TIL; red cells: neo-antigen specific TIL; yellow cells: C/G-antigen specific TIL. For this experiment, previously expanded TIL cultures were enriched and re-expanded (REP2) to generate the TIL infusion products. **(b)** Flow cytometric analysis of tail vein-derived blood taken from untreated control mice (n = 7, green circles), or mice treated with standard TIL (n = 6, blue squares), neo-antigen enriched TIL (n = 5, red triangles), or C/G-antigen enriched TIL (n = 6, yellow triangles). All treated mice received 20 x 10^6 total cells i.v. plus high-dose IL-2 (7.2 x 10^5 IU) twice daily for three days. Numbers depict the percentage human $CD8^+$ T cells of total live lymphocytes. There was no statistically significant difference between engraftment of total $CD8^+$ T cells between all treatment groups. Data are presented as mean ± s.d. Samples were compared using an unpaired two-sided Student's t-test.



Supplementary Figure 4. Comparative analysis of isolated TCRs. (a) Transduction efficiency of each indicated TCR is shown by pMHC multimer staining (upper panel) or Murine constant domain staining (lower panel). **(b)** Reactivity of transduced T-cell cultures was determined by measuring intracellular IFN- γ cytokine staining upon coculture with the autologous tumor cell line. **(c)** Flow cytometric analysis of tail vein-derived blood from mice treated with non transduced (green circles), neo TCR transduced (red triangles) or C/G TCR transduced T cells (yellow triangles).

REFERENCES

1. Alexandrov, L.B.*, et al.* Signatures of mutational processes in human cancer. *Nature* **500**, 415-421 (2013).
2. Vogelstein, B.*, et al.* Cancer genome landscapes. *Science* **339**, 1546-1558 (2013).
3. Segal, N.H.*, et al.* Epitope landscape in breast and colorectal cancer. *Cancer Res* **68**, 889-892 (2008).
4. Govindan, R.*, et al.* Genomic landscape of non-small cell lung cancer in smokers and never-smokers. *Cell* **150**, 1121-1134 (2012).
5. Linnemann, C.*, et al.* High-throughput epitope discovery reveals frequent recognition of neo-antigens by $CD4^+$ T cells in human melanoma. *Nat Med* **21**, 81-85 (2015).
6. Lennerz, V.*, et al.* The response of autologous T cells to a human melanoma is dominated by mutated neoantigens. *Proc Natl Acad Sci U S A* **102**, 16013-16018 (2005).
7. van Rooij, N.*, et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol* **31**, e439-442 (2013).
8. Robbins, P.F.*, et al.* Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nat Med* **19**, 747-752 (2013).
9. Lu, Y.C.*, et al.* Mutated PPP1R3B is recognized by T cells used to treat a melanoma patient who experienced a durable complete tumor regression. *J Immunol* **190**, 6034-6042 (2013).
10. Radvanyi, L.G.*, et al.* Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* **18**, 6758-6770 (2012).
11. Wolfel, T.*, et al.* A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* **269**, 1281-1284 (1995).
12. Coulie, P.G.*, et al.* A mutated intron sequence codes for an antigenic peptide recognized by cytolytic T lymphocytes on a human melanoma. *Proc Natl Acad Sci U S A* **92**, 7976-7980 (1995).
13. Robbins, P.F.*, et al.* A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *J Exp Med* **183**, 1185-1192 (1996).
14. Tran, E.*, et al.* Cancer Immunotherapy Based on Mutation-Specific $CD4^+$ T Cells in a Patient with Epithelial Cancer. *Science* **344**, 641-645 (2014).
15. Rodenko, B.*, et al.* Generation of peptide-MHC class I complexes through UV-mediated ligand exchange. *Nat Protoc* **1**, 1120-1132 (2006).
16. Toebes, M.*, et al.* Design and use of conditional MHC class I ligands. *Nat Med* **12**, 246-251 (2006).
17. Hadrup, S.R.*, et al.* Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers. *Nat Methods* **6**, 520-526 (2009).
18. Lu, Y.C.*, et al.* Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. *Clin Cancer Res* **20**, 3401-3410 (2014).
19. Neudorfer, J.*, et al.* Reversible HLA multimers (Streptamers) for the isolation of human cytotoxic T lymphocytes functionally active against tumor- and virus-derived antigens. *J Immunol Methods* **320**, 119-131 (2007).
20. Kvistborg, P.*, et al.* TIL therapy broadens the tumor-reactive $CD8(^+)$ T cell compartment in melanoma patients. *Oncoimmunology* **1**, 409-418 (2012).
21. Nauerth, M.*, et al.* TCR-ligand koff rate correlates with the protective capacity of antigen-specific $CD8^+$ T cells for adoptive transfer. *Sci Transl Med* **5**, 192ra187 (2013).
22. Champiat, S., *et al.* Exomics and immunogenics: Bridging mutational load and immune checkpoints efficacy. *Oncoimmunology* **3**, e27817 (2014).

23. Le, D.T*., et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* **372**, 2509-2520 (2015).
24. Rizvi, N.A., *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science* **348**, 124-128 (2015).
25. Snyder, A*., et al.* Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N Engl J Med* **371**, 2189-2199 (2014).
26. Kreiter, S*., et al.* Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature* **520**, 692-696 (2015).
27. Wolchok, J.D*., et al.* Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res* **15**, 7412-7420 (2009).
28. Varela, I*., et al.* Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature* **469**, 539-542 (2011).
29. Li, H. & Durbin, R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* **26**, 589-595 (2010).
30. Behjati, S*., et al.* Recurrent PTPRB and PLCG1 mutations in angiosarcoma. *Nat Genet* **46**, 376-379 (2014).
31. Larson, D.E*., et al.* SomaticSniper: identification of somatic point mutations in whole genome sequencing data. *Bioinformatics* **28**, 311-317 (2012).
32. Trapnell, C., *et al.* TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105-1111 (2009).
33. Trapnell, C*., et al.* Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* **28**, 511-515 (2010).
34. Nielsen, M., Lundegaard, C., Lund, O. & Kesmir, C. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. *Immunogenetics* **57**, 33-41 (2005).
35. Lundegaard, C*., et al.* NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8-11. *Nucleic Acids Res* **36**, W509-512 (2008).
36. Nielsen, M*., et al.* Improved prediction of MHC class I and class II epitopes using a novel Gibbs sampling approach. *Bioinformatics* **20**, 1388-1397 (2004).
37. Linnemann, C*., et al.* High-throughput identification of antigen-specific TCRs by TCR gene capture. *Nat Med* **19**, 1534-1541 (2013).

Chapter 9

Reconstructing tumor-reactivity of single cytotoxic T lymphocytes in human cancer

Sander Kelderman[1], Wouter A. Scheper[1], Carsten Linnemann[1], Gavin Bendle[1], Marije A.J. de Rooij[1], Christian Hirt[1], Ferenc A. Scheeren[1], Jorg J. Calis[1], Roelof J.C. Kluin[2], Marja Nieuwland[2], Ron M. Kerkhoven[2], Henry J.M.M.A. Zijlmans[3], Willemien J. van Driel[3], Gemma G. Kenter[3], John B.A.G. Haanen[1], Ton N.M. Schumacher[1]

[1]Division of Immunology, [2]Central Genomics Facility, [3]Department of Gynecology, The Netherlands Cancer Institute NKI-AVL, Amsterdam, the Netherlands

Manuscript in preparation

ABSTRACT

The presence of cytotoxic T lymphocytes is strongly correlated with a favorable prognosis in a number of cancer types. However, whether such infiltrates primarily consist of tumor-specific T cells or of bystander T cells has not been established. Furthermore, as intratumoral T cells are frequently characterized by an exhausted phenotype, it is challenging to address this question by classical assay systems that rely on *in vitro* expansion or testing of the tumor-resident T-cell pool.

To allow an unbiased functional assessment of the intrinsic tumor recognition capacity of cytotoxic T lymphocytes (CTLs) present in human solid tumors, we have developed and exploited a high-throughput screening platform that combines next-generation sequencing of T cell receptors (TCRs) from single intratumoral CTLs with *in silico* reconstruction of full-length TCRα/β heterodimers. Using this approach, we reconstructed a collection of TCRs isolated from colorectal and ovarian cancer samples. Functional analysis of a collection of isolated TCRs by TCR gene transfer experiments uncovered the presence of tumor-reactive CTLs, but also substantial tumor infiltration by T cells without detectable tumor recognition potential. Thus, the strategy presented here provides a powerful tool to query tumor-resident T-cell populations for their intrinsic tumor-reactive potential in an unbiased manner, and suggests that bystander infiltration may be a prominent feature of human tumors.

INTRODUCTION

Over the past five years, immunotherapy has become a new pillar in the treatment of human cancer[1,2]. Initially, clinical benefit of cancer immunotherapy was predominantly observed in metastatic melanoma, but subsequent clinical studies, evaluating the potential of blockade of the PD-1 – PD-L1 axis, have demonstrated activity in other common solid and non-solid tumor types[3-6]. To date, response rates in colorectal cancer (CRC) and ovarian cancer (OVC) have however been low, in spite of the previously reported strong association between patient survival and the presence of intratumoral cytotoxic T lymphocytes (CTLs)[7,8]. In CRC, anti-PD-1 therapy has shown effectiveness in a molecular subset that is characterized by deficiencies in the DNA mismatch repair machinery[9], and it is hypothesized that the high levels of neo-antigens resulting from this deficiency enhance the visibility of these tumors to the immune system[10]. In contrast, patients with OVC and mismatch repair-proficient CRC derive hardly any clinical benefit from the currently available immunotherapeutic interventions. One potential reason for this poor clinical efficacy may be a scarcity of true tumor-specific T cells among the infiltrating lymphocytes,. As an alternative possibility, the tumor-resident T-cell pool may be intrinsically tumor-reactive but rendered inactive by signals from the tumor micro-environment.

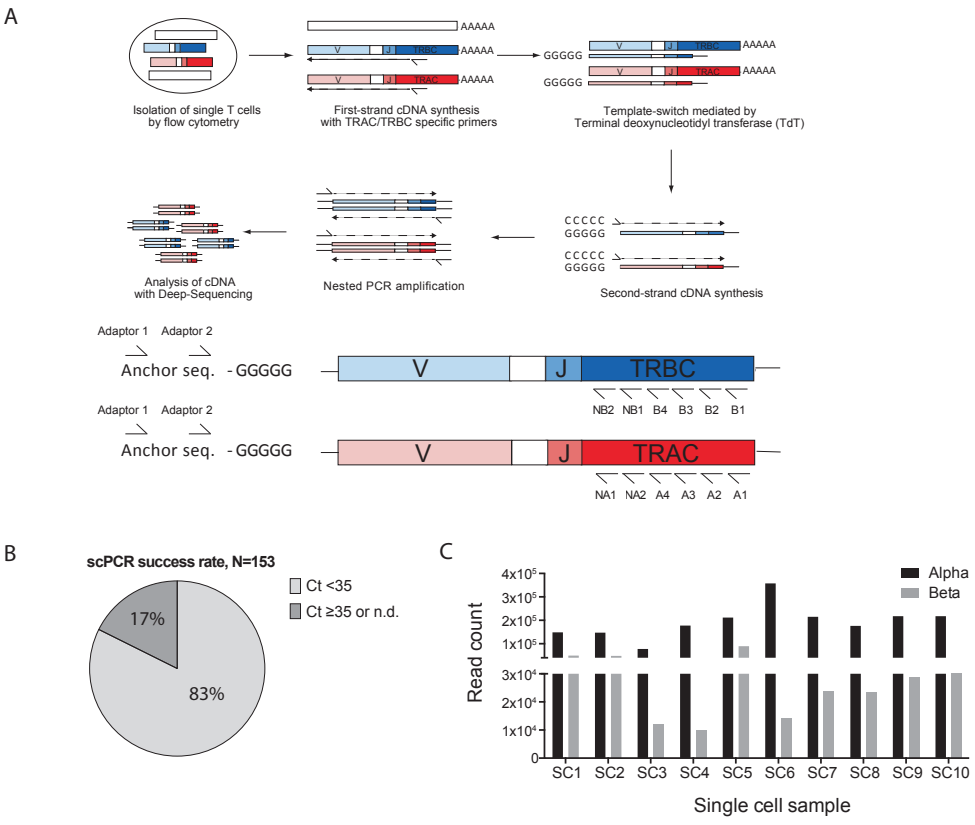
In-depth sequence analysis of the tumor-resident T-cell receptor (TCR) repertoire in combination with functional validation of these TCRs would therefore be highly relevant in these malignancies, as such an effort could be used to assess the intrinsic tumor-reactive nature of bulk tumor infiltrating CTLs. Currently, a number of technologies exist that could address this matter, but these generally depend on the use of large collections of V-gene specific primers or require extensively cultured cells of clonal origin, which unavoidably bias the resulting data[11]. Additionally, the full-length sequence of endogenously paired chains at the single cell level is required to reconstruct functional TCRα/β heterodimers, thereby hampering a bulk approach. Here, we develop a novel PCR-based next-generation sequencing (NGS) platform that allows the identification of TCR genes at single-cell resolution in an unbiased manner. Using this technology, we were able to successfully identify 106 TCR pairs (of which 66 were unique) from 189 single intratumoral T cells from patients with primary OVC and CRC, of which 35 were tested functionally. Interestingly, although reactivity against autologous tumor material was observed with TCRs isolated from both patients, the majority of TCRs in the two samples analyzed were not tumor-reactive, suggesting that the endogenous immune response in these tumors is largely composed of T cells that have no capacity to contribute to tumor control.

RESULTS

High throughput single-cell TCR sequencing technology

The functional reconstruction of endogenous TCRα/β heterodimers from a mixed CTL population is complicated by the requirement to correctly pair the two unique chains of single cells. To address this, we adapted a previously established single-cell PCR (scPCR) methodology[12] to incorporate targeted amplification of TCRα/β chains from single cell-sorted TIL (Fig. 1A). In this set-up, single alpha and beta chain sequences are identified per sample, thus automatically constituting the endogenous receptor of the original T cell. To determine whether we could correctly identify TCRα/β sequences with this approach, we employed flow cytometry based single-cell sorting of CMV pp65-specific and minor histocompatibility antigen HA2 specific T-cell clones expressing a known CDR3 sequence[13]. Real-time Taqman PCR assays on reverse-transcribed and PCR-amplified samples demonstrated successful cDNA generation and PCR amplification in 83% (126/153) of single cell samples (pooled data from 18 independent experiments) (Fig. 1B). Subsequently, for ten CMV single-cell sorted samples we tested whether cDNA yield was sufficient to reliably detect TCR sequences using next-generation sequencing (NGS). Average read counts for the TCR alpha and beta chains were $1.9x10^5$ and $3.3x10^4$, respectively, sufficient to identify both CDR3 sequences in each individual sample (Fig. 1C). Finally, we assessed whether we could correctly call endogenous TCR pairs from four previously sequenced melanoma derived T-cell clones that were mixed in a 1:1:1:1 ratio and subsequently single-cell sorted[14]. Pairing of the endogenous alpha and beta chain was correct in 88% of cases (79/90 sorted single cells) (Fig. 1D), with an overall efficiency of TCR sequence detection from single lymphocytes of 73%.

Figure 1. Establishment and validation of single-cell derived T-cell receptor deep-sequencing technology. (a) Single $CD8^+$ T cells are sorted from thawed single cell tumor digest in PCR lysis buffer (top left) containing four TCRα/β constant domain specific primers. After lysis, first-strand cDNA synthesis is performed (top middle) followed by a polyguanylation step of the reverse transcribed strand (top right). Next, a template switch and second strand cDNA synthesis is performed using a poly-(C) primer containing anchor sequences to be used in subsequent PCR steps (centre right). Finally, two rounds of nested PCRs are performed to further amplify the obtained PCR product that can be processed for NGS (centre middle and left). Schematic overview of the used primer sets is shown (bottom). **(b)** CMV or HA2 specific single T cells were used to assess the feasibility of single-cell cDNA generation using real-time PCR as a read-out. A threshold cycle value (Ct) of 35 was used as a cut-off for successful cDNA generation. Data are taken from 18 independent experiments representing a total of 153 single cells. **(c)** Ten CMV sorted single cells were processed according to the scPCR protocol and processed for NGS. Read count indicates the number of reads per sample for the CMV-specific CDR3 sequences of the alpha and beta chains. **(d)** A mixture of four tumor-antigen specific T-cell clones was sorted as single cells (N=90) and processed by scPCR for NGS. Upon analysis, 88% of the samples contained the correct alpha/beta TCR combination (left), input ratio of the 4 clones was recapitulated within this set (right). ➔


A
AAAAA
GGGGG
Isolation of single T cells
by flow cytometry
First-strand cDNA synthesis
with TRAC/TRBC specific primers
Template-switch mediated by
Terminal deoxynucleotidyl transferase (TdT)
CCCCC
GGGGG
Second-strand cDNA synthesis
Nested PCR amplification
Analysis of cDNA
with Deep-Sequencing
Adaptor 1
Adaptor 2
Anchor seq.
GGGGG
V
J
TRBC
NB2 NB1 B4 B3 B2 B1
TRAC
NA1 NA2 A4 A3 A2 A1
B
scPCR success rate, N=153
Ct <35
Ct ≥35 or n.d.
17%
83%
C
Alpha
Beta
Read count
4x10^5
3x10^5
2x10^5
1x10^5
3x10^4
2x10^4
1x10^4
0
SC1 SC2 SC3 SC4 SC5 SC6 SC7 SC8 SC9 SC10
Single cell sample
D
TAA-specific T cells, N=90
(of total single cells)
11%
1%
88%
Correctly paired
Mispaired
Chain lacking
TCR frequency
(within correctly paired)
MAGE-A10GLY
MAGE-A2YLQ
MAGE-C2KVL
SSX-2KAS

Reconstruction of TCRs from primary CRC

Having established this technology, our next aim was to assess whether we could identify TCR sequences of tumor infiltrating $CD8^+$ T cells. For this purpose, we collected material from a patient with primary CRC who had not received any prior treatment. We sorted $CD8^+$ single cells from frozen, uncultured tumor digest, performed scPCR and processed 94 of the resulting PCR products for Illumina NGS. Subsequently, sequence data were analyzed by the MiTCR algorithm to detect CDR3 regions[15] and filtered for high read counts. Resulting TCRα/β pairs were cross compared to a database containing all CDR3 amino-acid sequences identified in prior sequence runs allowing for the identification of potential contaminations. Finally, we analyzed non-recurring sequences with the TCRprimer algorithm (developed in-house) to determine a full-length consensus sequence. By this approach, we were able to call TCR pairs of 68% of total single cells from patient sample CRC11 (Fig. 2A). Interestingly, 75% of the TCR sequences identified within TIL of this patient were encountered more than once, including two dominant TCR pairs present in 39% and 11% of all called samples (Fig. 2B). To assess tumor-specificity of isolated TCRs, we randomly selected 20 TCR sequences (with the inclusion of the 2 recurring TCR pairs) and expressed these in donor PBLs (Fig. 2C). Thus far, we have assessed functionality of 16 transduced T-cell cultures and observed reactivity against autologous 3D-cultured tumor cells in five (31%) (Fig. 2D and 2E). Interestingly, one of the two dominant clones contained a TCR pair that was reactive with autologous tumor, indicating that the initiation of an endogenous immune response in this CRC patient led to clonal outgrowth of tumor-specific T cells.

Reconstructing TCRs from primary OVC

Next, we employed the same approach to an OVC sample obtained from a patient undergoing primary abdominal surgery in the absence of prior therapy. Analysis of deep sequencing data resulted in 44% of single cell samples being called (Fig. 3A). Upon crosscheck with our database we discovered that 7% of identified OVC21 samples consisted of contaminating CMV pp65 and HA2 sequences, and these were excluded from further analysis. In the set of called samples, only a single recurring TCR sequence was identified (combined 5% of total called sequences) indicating a highly heterogeneous intratumoral T-cell population in this particular patient (Fig. 3B). To assess tumor-specificity of the identified TCR pairs, we randomly selected 20 TCR pairs (with the inclusion of the duplicate sequence) and expressed these in donor PBLs (Fig. 3C). For one TCR (OVC21-9), no substantial TCR expression was observed. Upon functional validation of the remaining TCRs, one out of 19 TCRs showed production of IFN-γ above background upon exposure to uncultured autologous tumor digest (Fig. 3D and 3E). Thus, in this highly heterogeneous OVC-derived endogenous T-cell pool only low-level anti-tumor reactivity is observed.

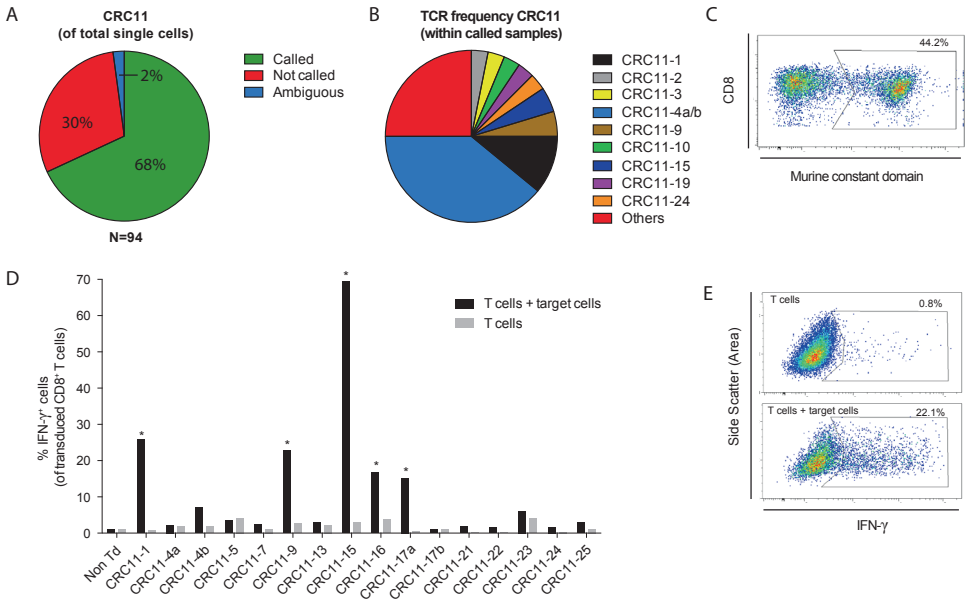


Figure 2. Reconstruction and functional testing of TCR sequences from a colorectal cancer patient. **(a)** From patient CRC 11, 180 single cells were processed by scPCR of which 94 were sequenced. Pie chart denotes analysis of all sequenced samples, resulting in 68% of samples being called. Remaining samples were not called due to low read counts (30%) or inconsistent pairing of TCRα/β chains (2%). No contaminating sequences were found. **(b)** Within the group of called samples, two dominant clones (CRC11-1 and CRC11-4a/b) were identified, constituting half of all TCR pairs, as well as a number of less frequent clones (25% of total called samples). An equal number of TCR pairs occurred only once (in red). **(c)** A selection of reconstructed TCR sequences was cloned into the pMP71 retroviral vector and expressed on healthy donor PBLs. A representative example of twenty independent transduction experiments is shown. **(d)** A total of 16 TCR constructs was tested for functional reactivity against autologous 3D cultured tumor cells in an overnight stimulation assay (black bars). Unstimulated transduced T-cell cultures and one non-transduced T-cell culture served as background controls (grey bars). Five T-cell cultures recognized autologous tumor cells. Reactivity has been corrected for transduction efficiency. **(e)** Flow cytometric analysis of CRC11-1 transduced T cells upon coculture with autologous tumor cells. Upper panel shows unstimulated T cells, lower panel shows T cells stimulated with target cells. Plots are gated on $CD3^{+}CD8^{+}$ single live lymphocytes.

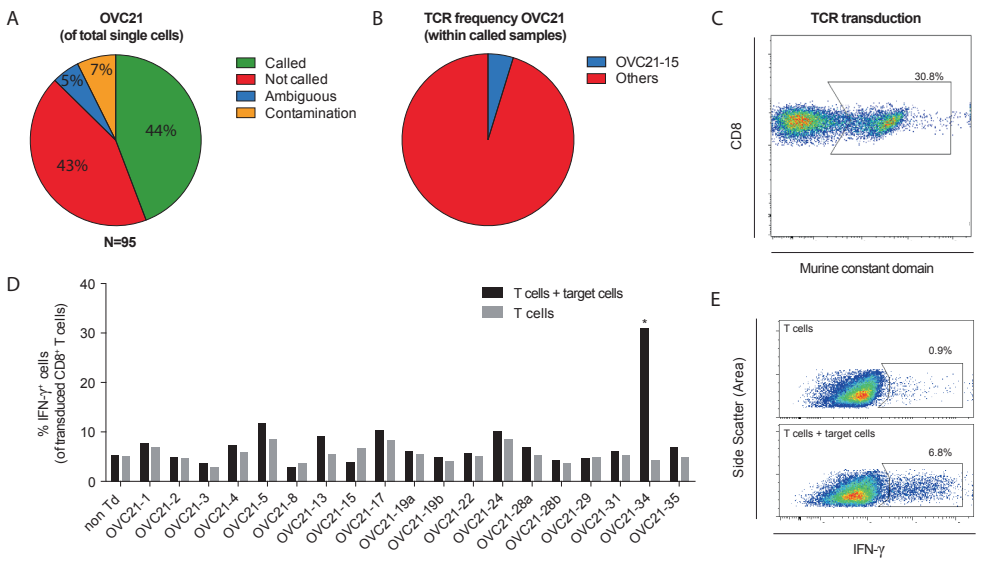


Figure 3. Reconstruction and functional testing of TCR sequences from an ovarian cancer patient. **(a)** From patient OVC21, 180 single cells were processed by scPCR of which 95 were sequenced. The pie chart denotes analysis of all sequenced samples, resulting in 44% of samples being called. Remaining samples were not called due to low read counts (43%), inconsistent pairing of TCRα/β chains (5%), or presence of contaminating sequences (7%). **(b)** Of the called samples, one TCR pair (OVC21-15, blue) occurred twice, whereas all others (red) occurred only once. **(c)** A selection of reconstructed TCR sequences was cloned into the pMP71 retroviral vector and expressed on healthy donor PBLs. A representative example of twenty independent transductions is shown. **(d)** A total of 19 TCR constructs was tested for functional reactivity against autologous uncultured tumor cells in an overnight stimulation assay (black bars). Unstimulated transduced T-cell cultures and one non-transduced T-cell culture served as background controls (grey bars). Sample OVC21-34 recognized autologous tumor cells. Reactivity has been corrected for transduction efficiency. **(e)** Flow cytometric analysis of OVC21-34 transduced T cells upon coculture with autologous tumor cells. Upper panel shows unstimulated T cells, lower panel shows stimulated T cells. Plots are gated on $CD3^+CD8^+$ single live lymphocytes.

DISCUSSION

The presence of tumor infiltrating CTLs in colorectal and ovarian cancer strongly correlates with patient survival. Despite this association, immunotherapy has thus far not shown a substantial rate of objective clinical responses in these cancer types[16-19]. The degree to which these T cells are truly tumor-reactive has remained an unanswered question in the field, although attempts have been made to address it. A recent study investigated the role of adaptive immunity in CRC by stimulating purified intratumoral T cells from 26 patients with DCs loaded with either lysate of autologous tumor digest or normal colonic mucosa and found tumor-reactivity in 44% of patients[20]. Subsequently, a panel of MHC pentamers for six shared HLA-A2 restricted antigens was used to stain cultured $CD3^+CD8^+$ cells, which revealed

0.1% to 2.5% of T cells being antigen-specific and tumor-reactive. Although this analysis likely underestimates the frequency of total tumor-reactive T cells, as non-shared antigens were not included, and as analysis was restricted to a single HLA-A2 allele, antigen-specific T cells were observed in the majority of CRC patients. Another study aimed to address this matter by assessing tumor-reactivity of CRC derived T-cell cultures, finding reactivity in three out of five patients for which autologous tumor material was available[21]. However, due to the extensive culturing of both T-cell clones and autologous tumor cell lines, tumor reactivity can be lost over time as was demonstrated in one of the three patients. In OVC, tumor-reactivity has been particularly observed in the $CD137^{+}$ fraction of intratumoral CTLs[22].

Even though these studies indicate that tumor-specific T-cell reactivity can be present in these tumors, the methodology used cannot be used to accurately determine the tumor reactivity of the intratumoral TCR pool, both because of the potential bias in T-cell outgrowth, and because of potential exhaustion of intrinsically tumor-reactive T cells.

Here, we provide the first unbiased analysis of the tumor-reactive fraction of single cytotoxic T cells in primary human ovarian and colorectal cancer. In the first patient, we observed the presence of two highly abundant T-cell populations (39% and 11%), which is indicative of clonal expansion. Indeed, one of these TCRs was reactive against autologous tumor cells. Investigation of microsatellite status revealed that CRC11 was of the stable (MSS) subtype, indicating that patients with mismatch repair proficient CRC can still be able to elicit a numerically strong endogenous tumor-specific T-cell response. Whether such patients are sensitive to immune modulation by for example immune checkpoint blockade, as is the case for MSI positive CRC, should be investigated further. Samples from a second CRC patient are currently being processed for functional testing (data not shown). In the OVC sample, a total of 19 TCRs were assessed for reactivity. We found one TCR (5%) to be reactive against uncultured autologous tumor cells. A second OVC sample is currently being processed for similar analyses (data not shown). Whether the low degree of intrinsic reactivity in OVC is a general feature should be determined on larger patient cohorts. Together, the majority of tumor-infiltrating T cells in the two samples analyzed thus far is comprised of TCRs that are not reactive to tumor-specific antigens present within the tumor cell population. If this finding is extended in larger sample collection, this observation should have profound implications for the efficacy of immunotherapeutic approaches that aim to reinvigorate the tumor-resident endogenous T-cell pool.

Despite the high sensitivity of our assay, several factors should be taken into account that could influence our analyses. First, our calling efficiency in these two samples was 68% and 44% for CRC11 and OVC21 respectively, which is lower than the 83% efficiency observed in our HA2/CMV experiments. This is likely a representation of cellular fitness in combination with RNA quality and transcript abundance in tumor-resident versus cultured T cells. In addition, the differentiated state of antigen-specific T cells may potentially lead to an underestimation of the total tumor-reactive fraction[23,24]. Second, of the 40 reconstructed

TCRs, 5 (12.5%) repeatedly failed to be expressed on PBLs and were thus excluded from functional assessment. It is conceivable that errors occasionally occur along the multi-step process of TCR reconstruction, thereby leading to potential false negatives. In order to assess the false negative rate of our technology, we aim to reconstruct TCRs from T cells that are known upfront to be reactive against autologous tumor cells. The number of TCRs that show tumor reactivity when analyzed by the strategy developed here will provide an indication on the fraction of tumor-reactivity that is successfully recovered by our experimental process.

Notwithstanding these limitations, the value of this technology with respect to our understanding of the T-cell based immune system in response to the two tumor types that we describe here is two-fold. First, it allows us to determine the fraction of tumor-reactive T cells that is present within a bulk TIL population in any solid tumor. Analyses on larger patient cohorts could provide insights whether reactivation of the endogenous T-cell pool, using for example immune-checkpoint inhibitors, will primarily be useful in those patients that have a high rate of intrinsic anti-tumor reactivity. Second, TCR sequence data in combination with matching exome data from the autologous tumor will make it possible to identify the cognate antigens that are recognized, a question of clinical relevance since it is as yet unknown which antigen class – i.e. shared antigens versus mutated neo-antigens – is preferentially recognized on these tumors. For instance, in colorectal tumors that are microsatellite instable (MSI), immune-reactivity may preferentially be targeted against mutated antigens[25], which might not be the case or apply to a lesser degree in MSS CRC. Similarly, in ovarian cancer the mutational load is on average much lower than in MSI CRC, and targeting of Cancer/Germline (C/G) or overexpressed antigens such as NY-ESO-1 or HER2/neu may there play a more significant role[26].

In conclusion, as cancer immunotherapy is continuously improving life expectancy of cancer patients, a better understanding is required of the intrinsic tumor recognition potential of endogenous tumor-resident T cells in patient groups that do not benefit from current cancer immunotherapies. Although the presence of intratumoral T cells in OVC and CRC correlates with improved prognosis, our data suggest that bystander infiltration may be a prominent feature, which warrants a more personalized approach towards the administration of cancer immunotherapy.

METHODS

Patient material. Tumor material was collected from patients treated at the Antoni van Leeuwenhoek Hospital. All patients gave informed consent in accordance with local ethical committee guidelines. Included patients did not receive any prior treatment. Collected samples were enzymatically digested and stored as single-cell suspensions in liquid nitrogen.

Primary cell cultures. Organoid cultures were established from primary CRC tumor digest[27] and subsequently injected into immunodeficient NSG mice or stored for further use. Mice were sacrificed upon outgrowth of patient-derived xenografts (PDX) and tumors were harvested. PDX tumors were passaged until sufficient numbers of target cells were obtained. Following each passage, tumor cells were analyzed for expression of MHC class I (HLA-ABC, clone G46-2.6, BD Biosciences) and epithelial cell adhesion molecule (EpCAM, clone 9C4. BD Biosciences) by flow cytometry or immunohistochemistry.

Single-cell FACS sorting. Tumor digests from CRC and OVC patients were stained with antibodies for a panel of phenotypic markers prior to single-cell sorting. These included anti-CD3 AF700 (clone UCHT1, Invitrogen), anti-CD8 FITC (clone SK1, BD Biosciences), anti-PD-1 PE (clone J105, eBioscience), anti-TIM-3 PE-Cy7 (clone F38-2E2, eBioscience), anti-LAG-3 APC (R&D Systems), anti-CD137-BV421 (clone 4B4-1, Biolegend), anti-CD103 BV711 (clone Ber-ACT8, BD Biosciences), anti-CD45RO (clone UCHL1, BD Biosciences). IR-dye (LIVE/DEAD® FixableNear-IR Dead Cell Stain, Invitrogen) was used to exclude dead cells. Gates were set on single live $CD3^{+}CD8^{+}$ lymphocytes and single cells were sorted into 96-well PCR plates using a MoFlo Astrios sorter set at 1.0 sorting stringency.

PCR methods. The PCR protocol used in this project was modified from Tang *et al.*[12]. PCR plates containing lysis buffer were prepared under RNAse free conditions. Samples were lysed immediately after sorting, followed by RT-PCR using four pairs of TCRα/β constant-domain specific primers (Supplementary Table 1). Subsequently, free primers were degraded and double-stranded DNA was obtained by addition of a poly-G tail to the first strand and template switch to synthesize the second strand. Finally, two rounds of nested PCR amplification were performed using additional constant domain primers and adaptor primers annealing to an anchor sequence introduced in the poly-G domain. Libraries were made using the Kappa Illumina kit and subsequently sequenced on an Illumina MiSeq.

Reconstruction of TCR chains. Sequence data were analysed using the MiTCR script that extracts CDR3 regions and identifies TCR V, D and J segments[14]. Called CDR3 sequences were subsequently ranked by read count and discarded if below 100 reads. Remaining sequences were considered true (called) when they comprised more than 75% of total reads for that chain in a given sample and when this TCR sequence was not observed in combination with any other chain in other samples. When there were multiple in-frame α-chains in a single sample the combined frequency of the reads had to exceed 75%, in which case multiple TCRα/β pairs per sample were generated and tested (e.g. CRC11-4a and CRC11-4b). Full-length TCR chains were reconstructed using an in-house developed script, TCRprimer, which reads upstream from the CDR3 region into the variable domain and generates a consensus

sequence by cross-linking the input sequence data to a database containing all human TCR variable domains. Output was manually verified for each sample using IMGT/V-Quest[28].

Generation of retroviral vectors and TCR expression in PBLs. The resulting consensus sequences were codon-optimized, synthesized and subcloned into the retroviral vector pMP71 (Life Technologies). In this vector, human TCRα and β constant domains are replaced by their murine counterparts to reduce mispairing with endogenous TCR chains, and to allow expression analysis by flow cytometry with anti-murine TCRβ constant domain antibody. Viral packaging cells (FLYRD18) were transfected with 10μg of plasmid DNA using Extremegene transfection reagent. Virus-containing supernatant was spinoculated onto anti-CD3/CD28 bead-activated T cells from healthy blood donors (Sanquin, The Netherlands). Transduction efficiency was measured four days later by flow cytometry using an antibody directed against the murine TCRβ constant domain (clone H57-597, BD Biosciences). T-cell cultures with at least 20% transduction efficiency were expanded in the presence of IL-2 (100 U/ml, Proleukin®, Novartis) and IL-15 (5ng/ml, Peprotech) for 14 days prior to functional validation.

Validation of TCR functionality. Tumor-reactivity of TCR transduced T-cell cultures was determined by intracellular cytokine staining. TCR-transduced T cells were cocultured with patient autologous tumor cells in a multi-well format. For CRC11, a 3D-cultured target cell line was established[27], whereas for OVC21 primary uncultured tumor cells were used as target cells. After 1 hr of stimulation, brefeldin A and monensin were added to allow intracellular cytokine accumulation. Following overnight incubation, cells were fixed, permeabilized, and stained with anti-IFN-γ-APC (clone B27, BD Biosciences). Samples were analysed on an LSR Fortessa or LSRII (BD Biosciences). Reactivity was corrected for transduction efficiency.

ACKNOWLEDGEMENTS

The authors would like to thank A. Pfauth, F. Van Diepen, P. Kvistborg and D. Philips for flow cytometric support; K.K. Dijkstra and A.M. Kuijpers for support with the 3D organoid culture system; R. Gomez, R. Mezzadra, L. Bies and L. Fanchi for technical assistance, and members from the Schumacher and Haanen laboratories for useful discussions.

Supplementary Table 1. Primer sequences utilized to amplify single-cell derived TCR-specific mRNA.

Primer name	Nucleotide sequence
TRAC-A1	TTGAGAATCAAAATCGGTGAAT
TRAC-A2	CAGAATCCTTACTTTGTGACACATT
TRAC-A3	CTAGCACAGTTTTGTCTGTGATATACA
TRAC-A4	ACTGTTGCTCTTGAAGTCCATAGAC
TRAC-N1	GACAGACTTGTCACTGGATTTAGAG
TRAC-N2	CTGGTACACGGCAGGGTC
TRBC-B1	ACCAGTGTGGCCTTTTGG
TRBC-B2	CTCAGCTCCACGTGGTCG
TRBC-B3	TGCACCTCCTTCCCATTC
TRBC-B4	TGCTCCTTGAGGGGCTGC
TRBC-N1	GAGATCTCTGCTTCTGATGGC
TRBC-N2	GACCTCGGGTGGGAACA
Poly(C)	ACAGCAGGTCAGTCAAGCAGTAGCAGCAGTTCGATAAGCGGCCGCCATGGACCCCCCCCCCCC
Adaptor 1	ACAGCAGGTCAGTCAAGCAGTA
Adaptor 2	AGCAGTAGCAGCAGTTCGATAA

TRAC: T-cell receptor alpha constant domain, TRBC: T-cell receptor beta constant domain, N denotes nested PCR primers.

REFERENCES

1. Sharma, P. & Allison, J.P. The future of immune checkpoint therapy. *Science* **348**, 56-61 (2015).
2. Rosenberg, S.A. & Restifo, N.P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* **348**, 62-68 (2015).
3. Brahmer, J.*, et al.* Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N Engl J Med* **373**, 123-135 (2015).
4. Stevanovic, S.*, et al.* Complete Regression of Metastatic Cervical Cancer After Treatment With Human Papillomavirus-Targeted Tumor-Infiltrating T Cells. *J Clin Oncol* **14**, 1543-1550 (2015).
5. Ansell, S.M.*, et al.* PD-1 Blockade with Nivolumab in Relapsed or Refractory Hodgkin's Lymphoma. *N Engl J Med* **372**, 311-319 (2014).
6. Rapoport, A.P.*, et al.* NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med* **21**, 914-921 (2015).
7. Zhang, L.*, et al.* Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* **348**, 203-213 (2003).
8. Galon, J.*, et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **313**, 1960-1964 (2006).
9. Le, D.T.*, et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* **372**, 2509-2520 (2015).
10. Schumacher, T.N. & Schreiber, R.D. Neoantigens in cancer immunotherapy. *Science* **348**, 69-74 (2015).
11. Calis, J.J. & Rosenberg, B.R. Characterizing immune repertoires by high throughput sequencing: strategies and applications. *Trends Immunol* **35**, 581-590 (2014).
12. Tang, F.*, et al.* RNA-Seq analysis to capture the transcriptome landscape of a single cell. *Nat Protoc* **5**, 516-535 (2010).
13. Heemskerk, M.H.*, et al.* Efficiency of T-cell receptor expression in dual-specific T cells is controlled by the intrinsic qualities of the TCR chains within the TCR-CD3 complex. *Blood* **109**, 235-243 (2007).
14. Linnemann, C.*, et al.* High-throughput identification of antigen-specific TCRs by TCR gene capture. *Nat Med* **19**, 1534-1541 (2013).
15. Bolotin, D.A.*, et al.* MiTCR: software for T-cell receptor sequencing data analysis. *Nat Methods* **10**, 813-814 (2013).
16. Kershaw, M.H.*, et al.* A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* **12**, 6106-6115 (2006).
17. Kandalaft, L.E.*, et al.* Autologous lysate-pulsed dendritic cell vaccination followed by adoptive transfer of vaccine-primed ex vivo co-stimulated T cells in recurrent ovarian cancer. *Oncoimmunology* **2**, e22664 (2013).
18. Topalian, S.L.*, et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* **366**, 2443-2454 (2012).
19. Hamanishi, J.*, et al.* Safety and Antitumor Activity of Anti-PD-1 Antibody, Nivolumab, in Patients With Platinum-Resistant Ovarian Cancer. *J Clin Oncol* **33**, 4015-4022 (2015).
20. Reissfelder, C.*, et al.* Tumor-specific cytotoxic T lymphocyte activity determines colorectal cancer patient prognosis. *J Clin Invest* **125**, 739-751 (2015).
21. Turcotte, S.*, et al.* Tumor-reactive CD8+ T cells in metastatic gastrointestinal cancer refractory to chemotherapy. *Clin Cancer Res* **20**, 331-343 (2013).
22. Ye, Q.*, et al.* CD137 accurately identifies and enriches for naturally occurring tumor-reactive T cells in tumor. *Clin Cancer Res* **20**, 44-55 (2014).

23. Stegle, O.*, et al.* Computational and analytical challenges in single-cell transcriptomics. *Nat Rev Genet* **16**, 133-145 (2015).
24. Buettner, F.*, et al.* Computational analysis of cell-to-cell heterogeneity in single-cell RNA-sequencing data reveals hidden subpopulations of cells. *Nat Biotechnol* **33**, 155-160 (2015).
25. Vogelstein, B.*, et al.* Cancer genome landscapes. *Science* **339**, 1546-1558 (2013).
26. Rooney, M.S.*, et al.* Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* **160**, 48-61 (2015).
27. Sato, T.*, et al.* Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* **141**, 1762-1772 (2011).
28. Brochet, X.*, et al.* IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic Acids Res* **36**, W503-508 (2008).

Chapter 10

Preclinical assessment of the potential of tumor-infiltrating lymphocyte (TIL) therapy in epithelial ovarian cancer

Sander Kelderman[1], Maaike van Zon[2], Noor Bakker[2], Joost H. van den Berg[2], Willemien J. van Driel[3], Henry J.M.M.A. Zijlmans[3], Gemma G. Kenter[3], Gabe S. Sonke[4], John B.A.G. Haanen[1,4], Ton N.M. Schumacher[1]

[1]Division of Immunology, [2]Amsterdam Biotherapeutics Unit (AmBTU),
[3]Department of Gynecology, [4]Department of Medical Oncology,
The Netherlands Cancer Institute NKI-AVL, Amsterdam, The Netherlands

Manuscript in preparation

ABSTRACT

The positive prognostic value of intratumoral T cells in primary epithelial ovarian cancer (EOC) suggests that these patients may benefit from cancer immunotherapy. However, to date, these therapies have demonstrated little clinical effectiveness. One approach that should be revisited in view of our increased understanding of T-cell differentiation is the adoptive transfer of ex vivo expanded autologous tumor infiltrating lymphocytes (TIL). To investigate the potential of such a strategy, we utilized material from six primary ovarian tumors to establish preclinical TIL cultures according to standardized protocols used for TIL therapy of melanoma. We show that expansion of ovarian cancer TIL is comparable to that seen for melanoma TIL. Furthermore, we observed both MHC-I and MHC-II restricted T-cell reactivity *in vitro* in response to autologous tumor cells. Based on these data, we propose to conduct a phase I/II trial for patients with EOC in the metastatic setting.

INTRODUCTION

Epithelial ovarian cancer (EOC) has a poor prognosis upon disease recurrence and development of resistance to platinum and taxane-based chemotherapy. The median overall survival in patients with stage III-IV disease ranges from 29 to 38 months depending on the recurrence and platinum-free interval[1], with a 5-year relative overall survival rate between 17% and 36%[2]. Attempts to improve overall survival by means of chemotherapy has for decades met with only limited clinical success[3]. Therefore, alternative approaches such as immunotherapy are currently being explored. An incentive to study EOC in the context of cancer immunotherapy is derived from a landmark study conducted by Zhang *et al.* in which the presence of intratumoral T cells positively correlated with overall survival[4]. These findings have been confirmed in subsequent independent studies as well as a meta-analysis[5-7]. Recently, T-cell checkpoint blockade with anti-PD-1/PD-L1 antibodies was shown to be able to induce clinical responses in 10-20% of heavily pretreated women with EOC, indicating that mobilization of the endogenous T-cell pool by immunotherapy has potential in this disease[8-10].

Another form of immunotherapy that may be utilized for the treatment of EOC is TIL therapy - a strategy in which patients receive large numbers of autologous *ex vivo* cultured tumor-infiltrating lymphocytes (TIL). Already in the early '90s, clinical responses were observed in several EOC patients treated with TIL therapy, although no follow-up trial data have been published[11,12]. Importantly, patients in these trials underwent treatment with extensively cultured T cells in the absence of prior lymphodepleting chemotherapy. In recent years, the body of knowledge regarding TIL therapy has increased considerably and so has our understanding of the requirements for a clinically effective autologous T-cell product. In particular, clinical trial data in melanoma patients treated with TIL therapy have shown reproducible response rates of 50% with long-term cures in 10-20%[13]. The increase in effectiveness of this form of T-cell therapy that was obtained in the past decade is thought to be largely dependent on two factors: 1) a relatively short-term *in vitro* culture period to generate less differentiated T cells; 2) systemic non-myeloablative lymphodepletion to enhance engraftment of the transferred T cells[14,15]. Taking into account that the EOC studies conducted in the '90s lacked both of these conditions, a renewed look at TIL therapy for EOC patients appears justified. To enable the development of such a study, we obtained material from six patients with primary EOC and established T-cell cultures according to currently used GMP protocols and evaluated reactivity against autologous tumor cells.

RESULTS

Expansion of EOC TIL cultures

Tumor tissue was collected from six patients with newly diagnosed EOC for which they underwent primary debulking surgery. The growth rate of these six EOC TIL cultures was comparable to that observed for eight control malignant melanoma (MM) preclinical TIL cultures, with a median fold expansion of 984 and 1311 (P = 0.30), respectively (Fig. 1A). Characterization of all TIL cultures revealed a 95-100% pure $CD3^+$ T-cell product with preferential outgrowth of $CD4^+$ T cells over $CD8^+$ T cells in EOC, 56% and 39% respectively, whereas in MM this was 29% and 63%, respectively (non-significant difference, Fig. 1B).

Reactivity of EOC TIL cultures against autologous tumor

Next, we assessed whether these EOC TIL cultures were able to recognize autologous tumor cells in standardized coculture assays. In the first set of three patients that we analyzed we observed that 12.7%, 0.64% and13.4% of $CD4^+$ T cells expressed IFN- γ in response to autologous tumor cells (Fig. 1C). In the same cultures, reactivity in the $CD8^+$ T-cell compartment was 4.6%, 22.2% and 18.3%, respectively (Fig. 1D). Overall, we observed reactivity in 5/6 EOC TIL cultures with a majority of cultures showing tumor reactivity within the $CD4^+$ T-cell compartment, whereas reactivity in the $CD8^+$ T-cell compartment was observed in half of the cultures (Fig. 1E). Flow cytometric analysis of the target cells showed expression of MHC class II (HLA-DR, DP and DQ) molecules (data not shown). Eight cultures established under a clinical protocol for melanoma TIL production revealed a similar degree of reactivity in the $CD8^+$ T-cell compartment as the EOC TIL (Fig. 1F).

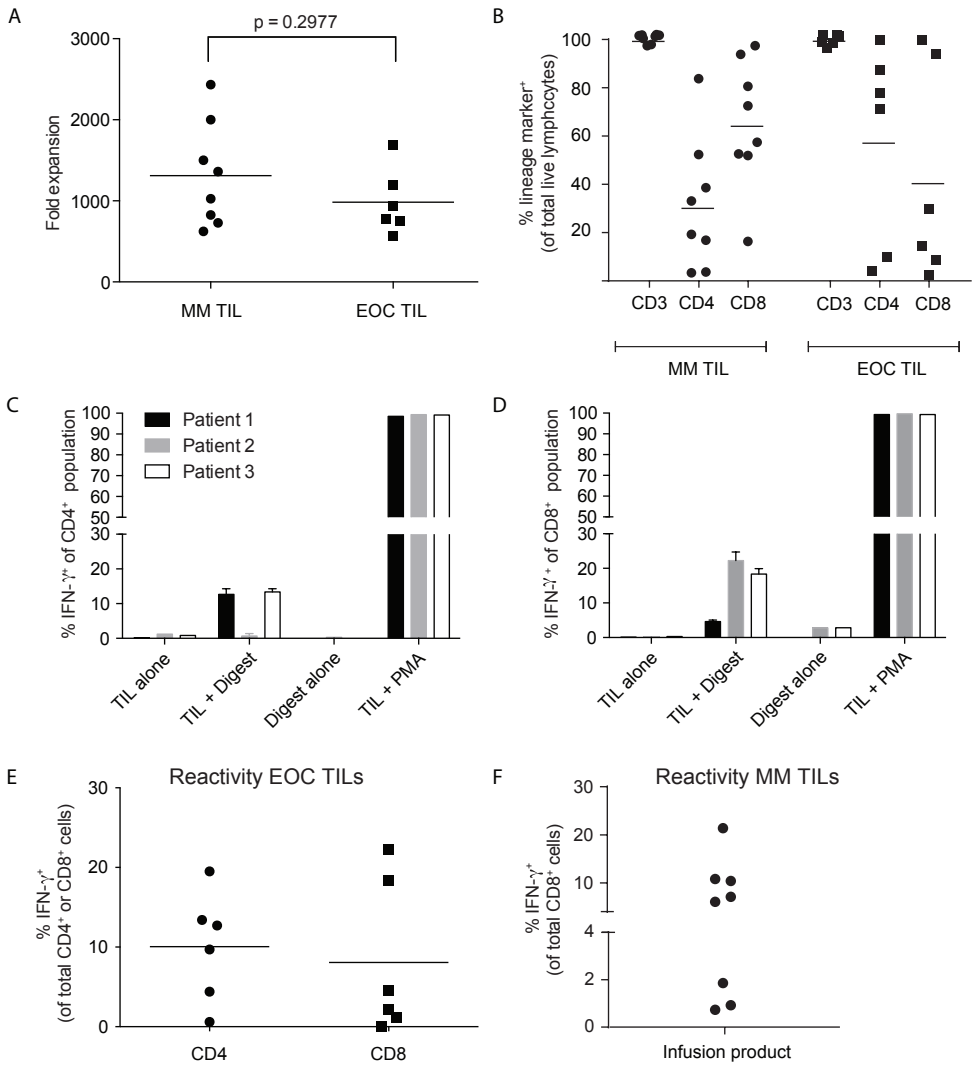


Figure 1. Analysis of EOC TIL cultures. (a) Fold expansion of eight MM TIL (circles) cultures is shown in comparison with six EOC TIL cultures (squares). Black bars indicate mean fold expansion. A 2-sided unpaired t-test showed no significant difference in expansion rates. **(b)** Phenotypic analysis of MM and EOC TIL by flow cytometry. Horizontal bars indicate mean frequencies of $CD3^+$, $CD4^+$, and $CD8^+$ cells of total live lymphocytes. **(c)** Intracellular IFN-γ staining of $CD4^+$ T cells upon coculture of established TIL cultures with autologous tumor cells is shown for three EOC patients. Tumor digests containing a mixture of tumor cells and lymphocytes were used as target cells. Unstimulated T cells and T cells stimulated with PMA/ionomycin served as negative and positive control samples, respectively. **(d)** Intracellular cytokine staining of $CD8^+$ T cells upon coculture of established TIL cultures with autologous tumor cells is shown for the same three EOC patients as in Figure 1C. **(e)** Percentage of cells with detectable intracellular IFN-γ expression are shown for all six EOC TIL cultures for both the $CD4^+$ and $CD8^+$ T-cell compartment. Horizontal bars indicate mean levels of cytokine expression. **(f)** Reactivity of eight clinical-grade MM TIL infusion products is shown.

DISCUSSION

Metastatic EOC has a poor prognosis and few systemic treatment options exist for patients with chemotherapy refractory disease. Cancer immunotherapy might provide a new lead for treatment, as in primary EOC the presence of TIL has been positively correlated with survival[7]. Several studies have reported responsiveness of EOC patients to different forms of immunotherapy such as DC based vaccination strategies and immune checkpoint blockade[8,16]. However, the majority of patients still succumb to progressive disease after immunomodulation. Early TIL trials in the '90s demonstrated a potential clinical benefit in a therapeutic and prophylactic setting but treatment protocols have changed significantly since then, which justifies a renewed analysis of the potential of TIL therapy for EOC. Here, we show in six preclinical laboratory-scale expanded TIL cultures that tumor-reactivity is present in $CD4^+$ and $CD8^+$ T-cell compartments. In several TIL trials, the presence of $CD8^+$ T cells was found to be a predictive marker for response to therapy, and in this respect the bias towards $CD4^+$ T cells observed in EOC TIL cultures may form some concern[17,18]. However, recent data suggest a relevant role for $CD4^+$ tumor-reactive T cells as well. In particular, one case study demonstrated a clinical response in a cholangiocarcinoma patient treated with a TIL product that was highly enriched (>95%) for $CD4^+$ T-cells reactive against an MHC class II restricted neo-antigen[19]. In addition, a study from our group indicated that reactivity against neo-antigens is commonly observed in the $CD4^+$ T-cell compartment in melanoma patients, including patients that respond to TIL therapy[20]. Thus, the high frequency of $CD4^+$ T cells in our EOC TIL cultures could be of clinical relevance.

Our current approach has two caveats that need to be taken into account when planning to conduct a clinical trial. First, TIL expansions in the current setting have been performed at a laboratory-scale in which we did not reach cell numbers usually obtained in clinical-scale protocols. However, experience with melanoma-derived TIL showed that these small-scale expansions are a good predictor for expansion at clinical scale[18,21]. Second, we observed tumor-reactivity in TIL cultures derived from untreated patients with newly diagnosed EOC. Whether these findings can be extrapolated to the metastatic setting in which patients have been heavily pretreated still needs to be assessed.

Nonetheless, the current data are encouraging with respect to the feasibility of establishing tumor-reactive TIL cultures in EOC. Therefore, we plan to perform a phase I/II trial in ten patients with stage III/IV EOC that have failed at least two lines of therapy. These patients are expected to progress rapidly and have few treatment options left. Primary endpoints will be feasibility and safety as well as clinical efficacy. To our knowledge, two other centers are currently conducting a similar trial (NCT01883297 and NCT02482090), with slightly different protocols. The combined data of these studies will hopefully act synergystically to reveal the potential of TIL therapy in EOC patients.

METHODS

Patient material. EOC samples were obtained from six patients undergoing primary surgery for suspected ovarian cancer. MM samples were obtained from eight patients undergoing a palliative metastasectomy and eight additional patients that were treated with clinical-grade TIL. Informed consent had been given prior to the surgical procedure and the study protocols (N12-INT for EOC TIL, N03-LAM for preclinical MM TIL and N10-TIL for clinical MM TIL) were approved by the local medical ethical committee. Tumor samples were enzymatically digested to obtain single-cell suspensions, containing both lymphocytes and tumor cells.

Cell culture. TIL cultures were initiated from both frozen and fresh single cell digests. Cells were seeded at $1x10^6$ cells/ well in a 24-well plate in complete medium (RPMI, 10% human AB serum), supplemented with IL-2 (6000IU/ml, Novartis). TIL density was maintained at $0.5e5\text{-}075x10^5$ cells/ ml of medium over the following weeks. After 10-14 days, TIL cultures were analyzed by flow cytometry for the expression of T-cell markers, using anti-CD3 FITC (clone SK7, BD Biosciences), anti-CD4 PE (clone SK3, BD Biosciences) and anti-CD8 APC (clone SK1, BD Biosciences) antibodies. Finally, each TIL culture was subjected to a 14-day rapid expansion protocol (REP) in the presence of allogeneic irradiated feeder cells (Sanquin, The Netherlands), anti-CD3 and IL-2[18].

Tumor reactivity assay. Upon completion of the REP, T-cell cultures were exposed to autologous tumor cells at a 1:1 ratio in a multi-well format. Brefeldin A and Monensin were added to allow intracellular cytokine accumulation. After overnight coculture, samples were fixed and permeabilized, and stained for IFN-γ (clone B27, BD Biosciences). Unstimulated T cells and T cells stimulated with PMA and ionomycin (Sigma-Aldrich) were used as negative and positive controls for T-cell activation, respectively. Samples were analyzed on a BD Calibur (BD Biosciences).

REFERENCES

1. Marszalek, A.*, et al.* Outcome in Advanced Ovarian Cancer following an Appropriate and Comprehensive Effort at Upfront Cytoreduction: A Twenty-Year Experience in a Single Cancer Institute. *Int J Surg Oncol* **2010**, 214919 (2010).
2. Baldwin, L.A.*, et al.* Ten-year relative survival for epithelial ovarian cancer. *Obstet Gynecol* **120**, 612-618 (2012).
3. Naumann, R.W. & Coleman, R.L. Management strategies for recurrent platinum-resistant ovarian cancer. *Drugs* **71**, 1397-1412 (2011).
4. Zhang, L.*, et al.* Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* **348**, 203-213 (2003).
5. Sato, E.*, et al.* Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A* **102**, 18538-18543 (2005).
6. Stumpf, M.*, et al.* Intraepithelial CD8-positive T lymphocytes predict survival for patients with serous stage III ovarian carcinomas: relevance of clonal selection of T lymphocytes. *Br J Cancer* **101**, 1513-1521 (2009).
7. Hwang, W.T.*, et al.* Prognostic significance of tumor-infiltrating T cells in ovarian cancer: a meta-analysis. *Gynecol Oncol* **124**, 192-198 (2012).
8. Hamanishi, J.*, et al.* Safety and Antitumor Activity of Anti-PD-1 Antibody, Nivolumab, in Patients With Platinum-Resistant Ovarian Cancer. *J Clin Oncol* **33**, 4015-4022 (2015).
9. Disis, M.L.*, et al.* Avelumab (MSB0010718C), an anti-PD-L1 antibody, in patients with previously treated, recurrent or refractory ovarian cancer: A phase Ib, open-label expansion trial. *J clin oncol (Meeting Abstracts)* **33**, 5509 (2015).
10. Varga, A.*, et al.* Antitumor activity and safety of pembrolizumab in patients (pts) with PD-L1 positive advanced ovarian cancer: Interim results from a phase Ib study. *J clin oncol (Meeting Abstracts)* **33**, 5510 (2015).
11. Aoki, Y.*, et al.* Use of adoptive transfer of tumor-infiltrating lymphocytes alone or in combination with cisplatin-containing chemotherapy in patients with epithelial ovarian cancer. *Cancer Res* **51**, 1934-1939 (1991).
12. Fujita, K.*, et al.* Prolonged disease-free period in patients with advanced epithelial ovarian cancer after adoptive transfer of tumor-infiltrating lymphocytes. *Clin Cancer Res* **1**, 501-507 (1995).
13. Rosenberg, S.A.*, et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* **17**, 4550-4557 (2011).
14. Dudley, M.E.*, et al.* CD8+ enriched "young" tumor infiltrating lymphocytes can mediate regression of metastatic melanoma. *Clin Cancer Res* **16**, 6122-6131 (2010).
15. Dudley, M.E.*, et al.* Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* **26**, 5233-5239 (2008).
16. Kandalaft, L.E.*, et al.* Autologous lysate-pulsed dendritic cell vaccination followed by adoptive transfer of vaccine-primed ex vivo co-stimulated T cells in recurrent ovarian cancer. *Oncoimmunology* **2**, e22664 (2013).
17. Besser, M.J.*, et al.* Clinical responses in a phase II study using adoptive transfer of short-term cultured tumor infiltration lymphocytes in metastatic melanoma patients. *Clin Cancer Res* **16**, 2646-2655 (2010).

18. Radvanyi, L.G.*, et al.* Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* **18**, 6758-6770 (2012).
19. Tran, E.*, et al.* Cancer Immunotherapy Based on Mutation-Specific CD4+ T Cells in a Patient with Epithelial Cancer. *Science* **344**, 641-645 (2014).
20. Linnemann, C.*, et al.* High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nat Med* **21**, 81-85 (2015).
21. Besser, M.J.*, et al.* Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. *Clin Cancer Res* **19**, 4792-4800 (2013).

Chapter 11

Discussion

The future challenges in cancer immunotherapy

In recent years, cancer immunotherapy has proven itself as a valuable addition to the therapeutic arsenal of medical oncologists. The current stage of cancer immunotherapy development, as discussed in **Chapter 1**, is characterized by numerous phase III trials showcasing significant improvements in overall survival that have led to the approval of immune checkpoint inhibitors as therapy for metastatic melanoma, renal cell cancer, and NSCLC. Encouragingly, melanoma patients that respond to therapy appear to benefit long-term, as 3-year survival rates of ipilimumab treated patients plateaus at 22%, which is a doubling in overall survival for stage IV melanoma[1]. This stands in stark contrast with targeted therapies, to which the majority of patients respond but of whom few seem to benefit long-term[2,3]. This potentially "deep" responsiveness is likely a characteristic of immunotherapeutic intervention as a similar effect is witnessed in melanoma patients treated with autologous tumor infiltrating lymphocytes (TIL). One study demonstrated 3- and 5-year survival rates of 36% and 29%, respectively, in a group of 93 TIL patients of which 19 (20%) were free of disease 10 years after treatment[4]. It is tempting to speculate that activation of the endogenous T-cell pool by immunotherapy causes the formation of immunological memory against tumor-associated antigens, thereby conferring long-lasting protection (discussed in **Chapter 5**), which is unique in the treatment of cancer.

Now that survival rates for metastatic melanoma have doubled and the promise of immunotherapy seems to hold true for other cancer types as well, does this mean that we have reached the zenith of cancer immunotherapy development? All clinical successes aside, there are still many challenges ahead and, based on the findings in this thesis, I highlight the top three that in my opinion deserve most attention in the coming years. These are: 1) overcoming resistance to cancer immunotherapy, 2) identifying biomarkers for clinical responsiveness and, 3) designing patient-specific therapies.

Overcoming resistance

As mentioned earlier, cancer immunotherapy can be quite effective in some patients but certainly does not benefit all patients with the same type of malignancy (**Chapter 2** and **3**). Therefore, one important question that is emerging from our growing experience with cancer immunotherapy is how to deal with such therapy resistance. In **Chapter 5**, three levels of resistance to immune attack are described: 1) intrinsic resistance, 2) naturally acquired resistance and 3) therapy-induced acquired resistance, with an increasing focus on the latter in recent years. In cancer immunotherapy, disease benefit is often defined as stable disease (SD) >24 weeks, partial response (PR) or complete response (CR), with the majority of patients experiencing SD and PR. The latter group is more at risk of subsequent disease progression than the group of CR patients. Apparently, the immune system can only keep a temporary check on tumors in these patients before they eventually grow out. While our understanding of the mechanisms that result in such acquired resistance is presently

limited, a number of observations can be made. First, sequential treatment with different forms of immunotherapy (e.g. immune checkpoint blockade and TIL therapy) appears to be feasible, with patients failing one regime sometimes responding to the next[5-7], indicating that the mechanism of resistance can be therapy line-specific. Second, cytotoxic T cells are regarded as an important (if not the main) mediator of anti-tumor immunity and therefore the extent to which these immunotherapeutic interventions alter the function and behavior of antigen specific T cells is of more than academic interest. Transcriptome analysis of neo-antigen specific T cells in a mouse sarcoma model treated with anti-CTLA-4 or anti-PD-1 antibodies have revealed profound differences in gene-expression profiles[8]. How these findings relate to the human situation is presently unknown but could be determined by mining RNA sequence data from (neo-) antigen reactive T-cell populations before and after the administration of cancer immunotherapy. Such datasets will improve our understanding on how immunotherapy shapes the anti-tumor immune response and may conceivably also reveal T-cell based mechanisms of therapy resistance. Finally, the other side of therapy-induced acquired resistance is the effect of immune pressure on the tumor cells themselves and the mechanisms they employ to evade T-cell mediated killing. Obvious candidates for immune escape are loss of molecules involved in the antigen-presentation machinery (e.g. beta-2-microglobulin), or the selection of epitope negative variants[9-11]. Development of *in vitro* or *in vivo* screening platforms, using for example short-hairpin or CRISPR/Cas9 guiding RNA libraries, could reveal additional mechanisms of immune escape unrelated to antigen presentation and thereby provide novel targets for treatment refractory tumors.

The two other forms of resistance (intrinsic and naturally acquired resistance) discussed in **Chapter 5** are also of significant importance; as many patients do not derive benefit whatsoever from immunotherapeutic intervention. In some disease histologies, such as ovarian and colorectal cancer, this is particularly surprising as there is a strong correlation between immune infiltration and clinical prognosis. Tools to determine the degree of tumor reactivity of the intratumoral T cells directly *ex vivo*, as developed in **Chapter 9**, will provide important information on the usefulness of stimulating the endogenous T-cell repertoire in these malignancies.

Biomarker assessment

As the number of immunotherapies available for clinical use is rapidly increasing, so does our need for biomarkers that can predict responsiveness towards them. The importance of biomarker development can be viewed from a number of perspectives.

First, physicians prefer to treat patients within a given tumor type that are most likely to respond, in order to avoid unnecessary side effects and delay other, potentially more effective, treatment options. Second, although perhaps an unpopular view from a medical ethical point of view, the current costs of cancer immunotherapy are putting a tremendous

pressure on health care resources, and therefore need to be controlled. A large variety of biomarkers in the context of cancer immunotherapy have been identified, mostly in melanoma. Importantly, none of these markers is able to provide a black-and-white cut-off for responsiveness to immunotherapy[12]. Arguably, such a perfect biomarker is unlikely to exist, as the cancer immunity cycle is a complex process[13], which can fail at multiple steps and that can therefore not be captured by a single parameter. In **Chapter 2**, we identify lactate dehydrogenase (LDH) and to a lesser extent erythrocyte sedimentation rate (ESR), both measurable in patient serum, as correlates of survival upon ipilimumab treatment. Whether the observed correlation is of prognostic or predictive nature is unclear as it is unknown whether LDH influences lymphocyte function upon CTLA-4 blockade. Notably, LDH has recently been shown to also serve as a biomarker for outcome in anti-PD-1 treated patients[14], suggesting a broader value.

Third, biomarker development can lead to the identification of disease histologies that were previously not considered targetable by cancer immunotherapy. While responsiveness was in first instance primarily observed in melanoma patients, it is now clear that many other cancer types can be responsive as well. Molecular profiling of human cancer in combination with high-throughput immunomonitoring has led to the realization that T cells specifically targeting mutated gene products that are presented on the cell surface of tumor cells in the context of MHC class I or II form an important contributor to the establishment of tumor control (**Chapter 6** and **Chapter 8**). Screening technologies aimed at identifying such T-cell populations are elaborate and few labs have the resources or material to approach this issue in a systematic manner for both responding and non-responding patients. Therefore, several groups have assessed mutational load, as an indirect measure of 'tumor foreignness', in patients treated with immunotherapy and found a positive correlation with clinical benefit[15-17]. By this reasoning, other "non-melanoma" cancer types with a similar mutational load could as well be responsive to immune modulatory therapy[18-20]. Indeed, this appears to be the case in particular for PD-1/PD-L1 blockade in patients with tobacco-induced non-small cell lung cancer (NSCLC) and bladder cancer[21-24].

Supporting evidence that links mutational load to immunotherapy-derived disease control comes from a phase II trial in colorectal cancer patients with either mismatch repair proficient or deficient tumors in which clinical activity was exclusively observed in the latter cohort[25]. Additionally, clinical responses were observed in a small cohort of mismatch repair deficient non-colorectal cancer patients. Although factors unrelated to neo-antigen specific T-cell reactivity also remain a possible explanation for the observed correlation (**Chapter 4**), these findings warrant the investigation of immunotherapy effectiveness in other cancers with large amounts of DNA damage. Interestingly, a large fraction of patients with Hodgkin's lymphoma, a disease known to have a low mutational burden, is responsive to anti-PD-1 therapy. Amplification of the PD-L1 locus is a characteristic of these tumors[26], and perhaps

interference with reverse signaling through PD-L1 explains the effect of PD1 blockade in this malignancy. Alternatively, non-mutant antigens or neo-antigens that are encoded by somatically hypermutated immunoglobulin genes or induced by first-line chemotherapy may explain the effects of therapy, an area for future study.

In order to identify novel biomarkers, clinical trials in all phases need to be designed in such a way that they permit the collection of tumor biopsies and blood at least before treatment and upon development of resistance, but preferably also during treatment. Analysis of the impact of immunotherapy on the tumor microenvironment will without doubt provide important mechanistic insights that will guide the development of future (combination) therapies (**Chapter 6**). It is important to bear in mind though that tissue-based immunomonitoring has its disadvantages, as only a small fragment of tumor can be sampled each time. Due to inter- and intratumor heterogeneity, type II errors can be expected to occur regularly, perhaps explaining the clinical responses that are occasionally seen in PD-L1 negative tumors in several anti-PD-L1/ anti-PD-1 trials[24].

Patient-specific immunotherapy

In the sections above, I have primarily focused on antibody-based cancer immunotherapies. At this moment, these therapies certainly form the most advanced branch of cancer immunotherapy, a success that, in addition to its clinical potency, is also due to its relative ease of generation and uniform clinical application. There are, however, other more patient-tailored branches that are worth exploring, which may particularly benefit those patients that fail standard treatment regimens. Two of the most investigated patient-specific approaches to date are adoptive cellular therapy (comprising TIL, TCR and CAR therapy) and therapeutic cancer vaccinations. Although intensively studied and potentially highly effective, it is still unknown which therapy is best applied in which situation. To answer this question, it will be important to better understand the status of the cancer immune response in individual patient, and in the following section I sketch how such knowledge could conceivably be used to guide treatment choice. A first step to achieve this is to identify the expression of potential tumor-rejection antigens by means of next-generation sequencing as they form the prime targets in personalized cancer immunotherapy (discussed in **Chapter 4**). In a second step, these data should be combined with multiplexed immunomonitoring assays that allow one to assess the presence, number and frequency of antigen-specific T-cell populations on a per patient basis[27-29]. Although perhaps a gross oversimplification of the entire cancer-immunity cycle, these two steps should provide at least a fundamental understanding of the immunogenicity of each tumor. Additional information on the general immunocompetence of the cancer-bearing patient or the presence of local immune suppressive mechanisms may be used to subsequently stratify patients more accurately into different treatment categories[30].

With such information, one could distinguish four scenarios in which different patient-specific immunotherapies could be applied: when an endogenous immune response has occurred against 1) a tumor with multiple antigens or 2) a tumor with single to a few antigens, or when an immune response has not occurred against 3) a tumor with multiple antigens, or 4) a tumor with few antigens.

In the first case, a setting where a variety of antigens is expressed (either neo-antigens, shared antigens or a combination) to which the immune system has mounted an endogenous immune response, TIL therapy is likely a good option. The *ex vivo* expansion and adoptive transfer of autologous TIL has already shown to durably benefit a substantial percentage of melanoma patients that underwent treatment[7,31,32].

The second setting, in which only few relevant antigens are expressed or few have elicited an immune response, offers even more space for a patient-specific approach. This is the case in cancers with a moderate mutational load, such as micro-satellite stable colorectal cancer or ovarian cancer (**Chapter 9**). Low-frequency tumor-specific T-cell populations may conceivably be isolated or stimulated from bulk TIL (**Chapter 7** and **8**) or peripheral blood and subsequently reinfused into a lymphodepleted host[33]. As an example, clinical responses have now been observed in HPV-induced cervical cancer (3/9 patients) by selecting TIL cultures that showed *in vitro* reactivity against E6 and E7 loaded target cells[34]. In gastro-intestinal tract malignancies, phase I/II trials are currently underway in which TIL cultures reactive against autologous mutated epitopes are selected for infusion with notable success in at least one published case[35]. The benefit of such an approach is that a predefined set of antigens can be targeted simultaneously, although selection for reactivity against a given antigen automatically entails losing other, potentially more relevant, cell populations (**Chapter 7** and **8**).

In the third setting, multiple antigens are expressed but the host fails to induce an adequate immune response. In these cases, there is likely a problem in antigen-presentation, either at the tumor site or in peripheral lymph nodes where matured professional APCs need to prime T-cell responses (discussed in **Chapter 5**). Cancer epitope vaccinations that induce de novo immune responses could overcome this problem. For years, much effort has been put in optimizing strategies (e.g. RNA, DNA or peptide-based) resulting in potent vaccines with the ability to raise immune responses against several antigens simultaneously that are detectable in peripheral blood[36]. However, aside from anecdotal clinical responses, a significant benefit over standard treatment has not yet been observed[37]. In recent years, the focus has shifted from targeting shared antigens towards mutated antigens for which there is expected to be only low-level, if any, tolerance and thus an increased likelihood of inducing clinically relevant immune responses. Results from immunotherapy trials that utilize the patient autologous mutanome in a vaccination setting are therefore eagerly awaited[38].

In the fourth setting, only a single or few antigens form relevant targets to which the host immune system fails to induce a strong immune response. In this case, TCR gene therapy, in which immune-reactivity is imposed by genetically transferring T-cell receptor genes into healthy naïve PBMCs with ample tumor-killing capacity, would be the preferred clinical choice. Treatment with autologous PBMCs redirected to target the NY-ESO-1 epitope has shown clinical effectiveness in melanoma, synovial cell sarcoma, and multiple myeloma[39,40]. In cases where the target antigen is not expressed on MHC molecules one may turn to the use of CAR therapy. Clinical effectiveness has been observed in leukemia patients using CARs that target CD19, an antigen that is expressed exclusively on B cells[41,42].

In summary, as patient-specific immunotherapy is largely driven by the upfront choice of potential tumor rejection antigens, there is a need for ongoing characterization of antigenic profiles in human cancer and the identification and prioritization of the most relevant targets (**Chapter 6**). Additional components, such as the presence of MDSCs or other non-checkpoint related inhibitory mechanisms that shape the cancer-immunity cycle would obviously need to be taken into account when making a final stratification into the different therapeutic branches of personalized cancer immunotherapy.

Concluding remarks

In this thesis, I have shown how cancer immunotherapy has taken up its place in oncological practice, have demonstrated several technologies than can broaden its application, and have provided insights on how to improve overall anti-tumor efficacy. Furthermore, I have discussed how the focus of immunologists and clinicians is shifting, as the field is moving towards the management of presently unknown resistance mechanisms and treatment of additional cancer types. Currently, immunotherapeutic options are abound, ranging from one size fits all antibodies to highly patient-specific cellular therapies that (for now) can only be fabricated in specialized centers, and combination (immuno-) therapies are expected to diversify the therapeutic arsenal even further. Ongoing research aimed at elucidating factors that dictate outcome to specific cancer immunotherapeutics should ultimately help clinicians decide the best therapeutic option for each individual cancer patient.

REFERENCES

1. Schadendorf, D*., et al.* Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J Clin Oncol* **33**, 1889-1894 (2015).
2. Chapman, P.B*., et al.* Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* **364**, 2507-2516 (2011).
3. Larkin, J*., et al.* Vemurafenib in patients with BRAFV600 mutated metastatic melanoma: an open-label, multicentre, safety study. *The Lancet Oncology* **15**, 436-444 (2014).
4. Rosenberg, S.A*., et al.* Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. *Clin Cancer Res* **17**, 4550-4557 (2011).
5. Hamid, O*., et al.* Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med* **369**, 134-144 (2013).
6. Weber, J.S*., et al.* Safety, efficacy, and biomarkers of nivolumab with vaccine in ipilimumab-refractory or -naive melanoma. *J Clin Oncol* **31**, 4311-4318 (2013).
7. Besser, M.J*., et al.* Adoptive transfer of tumor-infiltrating lymphocytes in patients with metastatic melanoma: intent-to-treat analysis and efficacy after failure to prior immunotherapies. *Clin Cancer Res* **19**, 4792-4800 (2013).
8. Gubin, M.M*., et al.* Checkpoint blockade cancer immunotherapy targets tumour-specific mutant antigens. *Nature* **515**, 577-581 (2014).
9. Dierssen, J.W*., et al.* HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. *BMC Cancer* **7**, 33 (2007).
10. del Campo, A.B*., et al.* Immune escape of cancer cells with beta2-microglobulin loss over the course of metastatic melanoma. *Int J Cancer* **134**, 102-113 (2014).
11. Khong, H.T. & Restifo, N.P. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* **3**, 999-1005 (2002).
12. Mahoney, K.M. & Atkins, M.B. Prognostic and Predictive Markers for the New Immunotherapies. *Oncology Journal* **28**, 39-48 (2014).
13. Chen, D.S. & Mellman, I. Oncology meets immunology: the cancer-immunity cycle. *Immunity* **39**, 1-10 (2013).
14. Diem, S*., et al.* Serum lactate dehydrogenase as an early marker for outcome in patients treated with anti-PD-1 therapy in metastatic melanoma. *Br J Cancer* **114**, 256-261 (2016).
15. Rizvi, N.A., *et al.* Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. *Science* **348**, 124-128 (2015).
16. Snyder, A*., et al.* Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N Engl J Med* **371**, 2189-2199 (2014).
17. Van Allen, E.M*., et al.* Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. *Science* **350**, 207-211 (2015).
18. Schumacher, T.N. & Schreiber, R.D. Neoantigens in cancer immunotherapy. *Science* **348**, 69-74 (2015).
19. Alexandrov, L.B*., et al.* Signatures of mutational processes in human cancer. *Nature* **500**, 415-421 (2013).
20. Vogelstein, B*., et al.* Cancer genome landscapes. *Science* **339**, 1546-1558 (2013).
21. Brahmer, J*., et al.* Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N Engl J Med* **373**, 123-135 (2015).
22. Topalian, S.L*., et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* **366**, 2443-2454 (2012).

23. Powles, T.*, et al.* MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. *Nature* **515**, 558-562 (2014).
24. Herbst, R.S.*, et al.* Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* **515**, 563-567 (2014).
25. Le, D.T.*, et al.* PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *N Engl J Med* **372**, 2509-2520 (2015).
26. Ansell, S.M.*, et al.* PD-1 Blockade with Nivolumab in Relapsed or Refractory Hodgkin's Lymphoma. *N Engl J Med* **372**, 311-319 (2014).
27. Kvistborg, P.*, et al.* TIL therapy broadens the tumor-reactive CD8(+) T cell compartment in melanoma patients. *Oncoimmunology* **1**, 409-418 (2012).
28. van Rooij, N.*, et al.* Tumor exome analysis reveals neoantigen-specific T-cell reactivity in an ipilimumab-responsive melanoma. *J Clin Oncol* **31**, e439-442 (2013).
29. Kvistborg, P.*, et al.* Anti-CTLA-4 therapy broadens the melanoma-reactive CD8+ T cell response. *Sci Transl Med* **6**, 254ra128 (2014).
30. Motz, G.T. & Coukos, G. Deciphering and reversing tumor immune suppression. *Immunity* **39**, 61-73 (2013).
31. Dudley, M.E.*, et al.* Randomized selection design trial evaluating CD8+-enriched versus unselected tumor-infiltrating lymphocytes for adoptive cell therapy for patients with melanoma. *J Clin Oncol* **31**, 2152-2159 (2013).
32. Radvanyi, L.G.*, et al.* Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* **18**, 6758-6770 (2012).
33. Cohen, C.J.*, et al.* Isolation of neoantigen-specific T cells from tumor and peripheral lymphocytes. *J Clin Invest* **125**, 3981-3991 (2015).
34. Stevanovic, S.*, et al.* Complete Regression of Metastatic Cervical Cancer After Treatment With Human Papillomavirus-Targeted Tumor-Infiltrating T Cells. *J Clin Oncol* **14**, 1543-1550 (2015).
35. Tran, E.*, et al.* Cancer Immunotherapy Based on Mutation-Specific CD4+ T Cells in a Patient with Epithelial Cancer. *Science* **344**, 641-645 (2014).
36. Schreibelt, G.*, et al.* Effective Clinical Responses in Metastatic Melanoma Patients after Vaccination with Primary Myeloid Dendritic Cells. *Clin Cancer Res* **22**, 2155-2166 (2016).
37. Klebanoff, C.A.*, et al.* Therapeutic cancer vaccines: are we there yet? *Immunol Rev* **239**, 27-44 (2011).
38. Castle, J.C.*, et al.* Exploiting the mutanome for tumor vaccination. *Cancer Res* **72**, 1081-1091 (2012).
39. Robbins, P.F.*, et al.* Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* **29**, 917-924 (2011).
40. Rapoport, A.P.*, et al.* NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med* **21**, 914-921 (2015).
41. Kochenderfer, J.N.*, et al.* Adoptive transfer of syngeneic T cells transduced with a chimeric antigen receptor that recognizes murine CD19 can eradicate lymphoma and normal B cells. *Blood* **116**, 3875-3886 (2010).
42. Maude, S.L.*, et al.* Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* **371**, 1507-1517 (2014).

Chapter 12

English summary

Nederlandse samenvatting

Dankwoord

Curriculum Vitae

List of publications

ENGLISH SUMMARY

In recent years, immunotherapy has taken up a place alongside surgery, chemotherapy and radiotherapy in the therapeutic arsenal against cancer. The scientific development of cancer immunotherapy has gone through three distinct stages, outlined in **Chapter 1**, which are 1) discovery and compilation of knowledge on the immune system and cancer, mainly by murine modeling, 2) understanding tumor-specific immune response in humans and the first translational efforts, 3) the release of clinically effective immunotherapeutic drugs on the market. Since the start of the last stage, which began in 2010 with FDA and EMA approval of ipilimumab for metastatic melanoma, cancer immunotherapy has developed into a stand-alone treatment modality alongside classical approaches such as chemotherapy and radiotherapy. A unique characteristic of immunotherapy is that cancer cells are targeted indirectly as its primary effect is the (re-) activation of cells of the adaptive immune system. Although a growing number of patients are now benefitting from immunotherapy, there are still many more that do not or only do so temporarily.

The scope of this thesis is to identify factors that could predict responsiveness, or lack thereof, to cancer immunotherapy, and develop tools for personalized treatment approaches.

From bedside

In **Chapter 2** and **3**, we retrospectively investigate the efficacy of an anti-CTLA-4 blocking antibody, ipilimumab, in patients with metastatic uveal and cutaneous melanoma. While we find that for uveal melanoma only few patients benefit, in cutaneous melanoma survival rates are much higher and, importantly, that they are comparable to the initial phase III trials. In the cohort of cutaneous metastatic melanoma patients we furthermore identified elevated baseline levels of serum ESR and LDH to negatively correlate with survival. The latter was confirmed in an independent cohort of patients thus suggesting its usefulness as a selection marker for treatment initiation. We continue on the topic of immune-checkpoint blockade in **Chapter 4** by discussing the findings derived from a phase II trial in which an anti-PD-1 blocking antibody is used to effectively treat colorectal cancers with defects in their DNA-mismatch repair machinery. We hypothesize that other tumor types may be targeted as well, depending on their mismatch repair status, rather than their site of origin. Although immune-checkpoint blockade is potentially highly effective, as observed in the first chapters, not all patients with the same cancer type benefit in a durable manner. In **Chapter 5**, we discuss potential mechanisms of resistance to cancer immunotherapy in its entirety and highlight the need for personalized approaches. One such a personalized approach would be to target cancers in an antigen-specific manner. In **Chapter 6**, we discuss our current knowledge on the classes of C/G antigens and mutated antigens and elaborate on several factors that determine their suitability as tumor-rejection antigens.

To bench

Personalized treatment approaches are the way forward in tumors that have become resistant to immunotherapy. Such an approach is already applied in patients receiving TIL therapy. However, TIL infusion products often exhibit only low frequency antigen-specific T-cell responses. In **Chapter 7**, we have developed a technology platform to isolate defined T-cell populations of interest, whether they are targeting non-mutated or mutated antigens, and show that this can enhance anti-tumor reactivity in *in vitro* assays. In **Chapter 8**, we show in a mouse model of human melanoma that a TIL product enriched for reactivity against mutated antigens is of superior quality than a non-enriched TIL product. We also demonstrate that there is likely some degree of hierarchy between T-cell responses targeting mutated antigens.

In **Chapter 9**, we describe the development of a technology that allows us to isolate and reconstruct TCR gene sequences from single T cells without the need for extensive culturing. We apply this method to a small set of ovarian and colorectal cancer samples and find that the majority of the T cells infiltrating these tumors are non tumor-specific. An interesting finding that requires further exploration and should be taken into consideration when choosing the treatment strategy for these tumors.

And back

The presence of intratumoral T cells in ovarian cancer has been linked in many studies to prolonged overall survival. In **Chapter 10**, we demonstrate that TIL cultures can be established according to standard clinical protocols and, importantly, that they harbor *in vitro* tumor-reactivity against MHC class I and II molecules. With these preclinical data we aim to initiate a phase I/II clinical study for metastatic epithelial ovarian cancer at the Netherlands Cancer Institute.

Finally, we discuss the findings described in this thesis in **Chapter 11** and highlight some of the future prospects of cancer immunotherapy development. In the coming years the field will focus on mechanisms of resistance, development of biomarkers for response and the design and implementation of patient-specific approaches. A major challenge for the future is that a growing number of immunotherapeutic drugs are now entering the final stages of development, and physicians will need to make evidence-based choices on which drug is the best option for each individual patient.

NEDERLANDSE SAMENVATTING

Immuuntherapie is naast chirurgie, radiotherapie en chemotherapie een nieuwe optie in het behandelarsenaal tegen kanker. De wetenschappelijke ontwikkeling van immuuntherapie kan worden onderverdeeld in drie verschillende periodes, zoals besproken in **Hoofdstuk 1**, te weten 1) het verzamelen van kennis omtrent het immuunsysteem en kanker, veelal aan de hand van muismodellen, 2) de analyse van tumor-specifieke immuniteit in mensen en enkele eerste translationele toepassingen van deze kennis, 3) het beschikbaar komen van medicijnen voor patiënten. Sinds de aanvang van deze laatste periode, die begon in 2010 toen de FDA en EMA hun goedkeuring gaven voor het gebruik van ipilimumab tegen gemetastaseerd melanoom, heeft immuuntherapie zich ontwikkeld tot een op zichzelf staande behandelmodaliteit naast de meer klassieke behandelingen zoals chemotherapie en radiotherapie. Een uniek kenmerk van immuuntherapie is dat de kankercellen niet rechtstreeks worden aangevallen, maar op een indirecte manier via de cellen van het adaptieve immuunsysteem die zich op hun beurt weer richten op antigenen die tot expressie komen op het oppervlak van de tumorcel. Ondanks dat een groeiende groep patiënten baat heeft bij behandeling met immuuntherapie zijn er nog velen die dat niet, of slechts in beperkte mate, hebben.

Het doel van dit proefschrift is om factoren te identificeren die kunnen voorspellen welke patiënten wel en welke patiënten niet reageren op immuuntherapie. Daarnaast richt dit proefschrift zich op de ontwikkeling van technologieën die het mogelijk maken om patiënt-specifieke therapieën verder te ontwikkelen.

Van het bed

In **Hoofdstuk 2** en **3** onderzoeken we in een retrospectief cohort patiënten met uveaal en cutaan melanoom de effectiviteit van ipilimumab, een antilichaam dat de werking van CTLA-4 tegengaat. In de groep patiënten met uveaal melanoom wordt slechts een enkele respons gezien, terwijl de overleving in de groep met cutaan melanoom veel hoger is. Daarnaast werd in de laatste groep serum LDH als marker voor overleving geïdentificeerd waarmee deze mogelijk kan worden ingezet als selectiecriterium voor behandeling. Voortbordurend op het onderwerp van immuun checkpoint blokkering bespreken we in **Hoofdstuk 4** de bevindingen uit een fase II studie waarin een antilichaam tegen PD-1 wordt ingezet om patiënten met colorectaal carcinoom te behandelen. Dit middel blijkt in het bijzonder goed te werken bij een subgroep van tumoren die gekenmerkt wordt door defecten in het mechanisme dat DNA-schade repareert. Onze hypothese is dat andere tumorsoorten met grote hoeveelheden DNA schade mogelijk ook gevoelig kunnen zijn voor deze behandeling, onafhankelijk van het celtype waaruit de kanker ooit is ontstaan. Ondanks dat immuuntherapie zeer effectief kan zijn, zoals uit bovenstaande hoofdstukken is gebleken, zullen niet alle patiënten met

dezelfde tumorsoort evenveel baat hebben bij behandeling. In **Hoofdstuk 5** bespreken we de mogelijke mechanismen waarmee kanker zich kan onttrekken aan de invloeden van immuuntherapie en bespreken we de noodzaak van de ontwikkeling van patiënt-specifieke behandelingen. Zo'n individueel patiëntgerichte aanpak kan bestaan uit een behandeling die specifieke T cel populaties activeert om vooraf gedefinieerde (groepen) tumor antigenen te bestrijden. Aangezien de antigeendiversiteit groot is, is het belangrijk een onderscheid te kunnen maken tussen relevante en minder relevante doelwitten. In **Hoofdstuk 6** bespreken we de huidige kennis omtrent twee groepen antigenen, namelijk de C/G antigenen en de gemuteerde antigenen, en de factoren die bepalend zijn voor hun geschiktheid als doelwitten voor antigeen-specifieke immuuntherapie.

Naar de labtafel

Een individuele aanpak biedt waarschijnlijk de beste kansen om tumoren te behandelen die resistent zijn geworden tegen immuuntherapie. Een dergelijke individuele aanpak wordt reeds toegepast in de behandeling met autologe TIL. In **Hoofdstuk 7** laten we een methode zien waarmee antigeen-specifieke T cel populaties kunnen worden geïsoleerd, gericht tegen zowel niet-gemuteerde als gemuteerde antigenen, en tonen we aan dat doormiddel van isolatie van tumor-specifieke T cel populaties de *in vitro* anti-tumor reactiviteit van TIL producten toeneemt. In **Hoofdstuk 8** demonstreren we vervolgens dat een TIL product, verrijkt voor reactiviteit tegen gemuteerde antigenen, betere tumorcontrole geeft dan een niet-verrijkt cel product in een muismodel voor humaan melanoom. Daarnaast laten we zien dat er kwalitatieve verschillen bestaan tussen afzonderlijke T cel responsen en de mate waarin deze gemuteerde antigen weten uit te schakelen.

Van tumorsoorten als ovarium en colorectaal carcinoom is inmiddels bekend dat de aanwezigheid van intratumorale T cellen van prognostische waarde is voor de overleving. Echter in welke mate deze T cel populaties gericht zijn tegen tumor specifieke antigen is tot op heden onbekend. In **Hoofdstuk 9** beschrijven we een technologie die ons in staat stelt om TCR genen te isoleren uit afzonderlijke T cellen, zonder dat daar uitvoerige celkweken voor hoeven te worden verricht. We hebben deze methode toegepast op een kleine collectie materiaal afkomstig van patiënten met ovarium en colorectaal carcinoom, waarbij we zien dat de meerderheid van de T cellen die zich in de tumor bevinden niet specifiek gericht zijn tegen tumor-specifieke antigenen. Dit is een interessante bevinding waar meer onderzoek naar gedaan moet worden en waar rekening mee moet worden gehouden wanneer voor deze groep tumoren een behandelstrategie wordt gekozen.

En weer terug

In meerdere studies is de aanwezigheid van intratumorale T cellen in ovarium carcinoom positief gecorreleerd aan langere overleving. In **Hoofdstuk 10** laten we zien dat TIL kweken afkomstig uit ovariumcarcinoom materiaal opgezet kunnen worden volgens de geldende klinische protocollen voor TIL productie. Bovendien laten we zien dat deze kweken reactief zijn tegen MHC klasse I en II antigenen die tot expressie komen op deze tumoren. Deze verkregen preklinische data worden nu gebruikt om een fase I/II klinische trial in het Antoni van Leeuwenhoek ziekenhuis op te zetten voor gemetastaseerd epitheliaal ovarium carcinoom.

Tot slot bespreken we in **Hoofdstuk 11** de bevindingen uit dit proefschrift en komen de toekomstige perspectieven voor de ontwikkeling van kankerimmuuntherapie aan bod. De komende jaren zullen gekenmerkt worden door een toenemende aandacht voor resistentiemechanismen, de ontwikkeling van voorspellende biomarkers en het ontwerpen en implementeren van patiënt-specifieke behandelmethoden. De grote hoeveelheid immuuntherapieën die in de komende jaren op de markt zullen verschijnen, vormen een grote uitdaging voor artsen die een evidence-based keuze dienen te maken over de vorm van behandeling die het meest geschikt is voor iedere individuele patiënt met kanker.

DANKWOORD

Het duurt niet lang of de zon komt straks weer op. In het zusterhok naast mij hoor ik een baby huilen, maar verder is het nu rustig. Verlicht door de TL buizen op de verloskamers en lichtelijk vermoeid na een nachtelijk weekje werken, begin ik uiteindelijk aan het laatste onderdeel van mijn proefschrift. Het resultaat daarvan heb je nu in handen, het dankwoord! De mensen die hebben bijgedragen aan de totstandkoming van dit proefschrift ben ik namelijk heel wat dank verschuldigd.

Allereerst wil ik mijn promotor en co-promotor, **Ton** en **Christian**, bedanken voor hun steun en begeleiding de afgelopen jaren. **Ton**, ik ben je dankbaar dat je mij een plek hebt gegeven binnen je onderzoeksgroep waarin ik me onder jouw supervisie de afgelopen jaren heb kunnen ontwikkelen. De uitdagende onderzoekslijnen die je voor mij wist uit te stippelen en je vertrouwen dat het uiteindelijk allemaal wel zou gaan werken hebben dit proefschrift gemaakt tot wat het is. **Christian**, met jou heb ik mijn eerste publicaties geschreven en ik heb van dichtbij meegemaakt met hoeveel enthousiasme en passie jij kliniek en onderzoek combineert. Iets wat niet altijd even makkelijk is en waar ik, nu ik zelf weer in de kliniek zit, des te meer bewondering voor heb gekregen.

Ook de overige groepsleiders van de afdeling, **Jannie**, **John** en **Karin** wil ik graag bedanken voor hun kritische blik op mijn werk en alle waardevolle discussies die we de afgelopen jaren hebben gevoerd. Daarnaast wil ik de leden van mijn promotiecommissie bedanken voor het kritisch lezen van dit proefschrift. In het bijzonder bedank ik **Gemma**, die mij na vier jaar onderzoek opnieuw heeft geïntroduceerd bij de gynaecologie.

Grote dank gaat uit naar mijn twee paranimfen, **Pia** en **Sascha**. To start with **Minipigtje**, I admire your passion for science, your fun-loving character, your crazy sense of humor and your ability to chit-chat with the high rollers in the field without blinking an eye. So many memorable and glorious moments on both scientific and personal level we shared the last couple of years. **Sascha**, hoe cool dat jij straks tijdens mijn verdediging naast mij staat! Niet alleen sta je daar als een hele waardevolle vriend waar ik alles mee kan bespreken, je bent ook in bredere zin de vertegenwoordiger van een club mensen waar ik stapelgek op ben. Dankzij jullie kan ik geregeld even stoom afblazen, alle hectiek in mijn hoofd relativeren en beseffen dat het leven niet alleen om werken en presteren draait.

Mijn lieve (oud-)collega's uit het NKI die me zo op mijn gemak hebben doen voelen in een wereld die aanvankelijk niet de mijne was. **Silvia'tje**, there are so many things to thank you for that I don't know where to start, and I probably shouldn't even try because you already know all of them. You are one of the most thoughtful, energetic and loyal friends that I have and I thank you for passing some of it on to me. **Joost**, fijne collega, fijne vriend, het was heerlijk om een kamer met je te mogen delen. **Raquel**, de toewijding en passie die jij hebt voor je vak is ongekend. Bedankt voor je altijd kritische blik en onze leuke momenten

samen. **Buurtje**, zonder jou waren een hoop van mijn projecten nooit gelukt, mooi hoe we ons er samen doorheen hebben geslagen. **Tomasz**, thanks for designing that beautiful cover, you already said it before and I see it now, you are truly an artist. Mijn kamergenoten van de afgelopen jaren a.k.a. het TIL/TCR team: **Bianca, Nienke, Samira, Maaike, Noor, Renate** en **Wouter,** bedankt voor al jullie hulp, ondersteuning en gezelligheid.

Alle overige **B3 collega's** van de afgelopen jaren, wat zijn jullie een heerlijke groep om mee samen te werken. Zoveel kennis en kunde op één afdeling en tegelijkertijd zoveel gezelligheid, wat heb ik er van genoten! Speciale dank ben ik verschuldigd aan **Laura** en **Lorenzo**, voor al die oneindige muisproeven. En mijn studenten, **Lois** en **Marije**, enorm bedankt voor al het harde werk en de goeie inzet tijdens jullie stage.

Alle **mede-auteurs** op mijn publicaties en **collaborators** binnen en buiten het NKI, bedankt voor jullie steun en de prettige samenwerking de afgelopen jaren. Special thanks to **Dan Powell** and **Evros (leventimou!)** from UPenn, my internship at your department has been a decisive moment in my career and I am forever grateful that you accepted an inexperienced med student, who had never held a pipette in his hand before, into your lab.

Mijn **fijne vrienden uit Groningen** en alles wat daar in de loop van de jaren bij is aangehaakt. Ik kan niet vaak genoeg benadrukken hoeveel waarde ik aan onze vriendschappen hecht. Jullie hebben mij de afgelopen jaren, vaak zonder dat jullie het door hadden, door de lastige momenten heen gesleept en vormen een vertrouwde basis waar ik altijd op terug kan vallen en helemaal mezelf kan zijn. Speciale dank aan **Thomas** (mijn zelfbenoemde schaduwparanimf), **Jytte** en **Gemma** voor alle ontspannende momenten en goeie gesprekken verspreid over de hele stad, gewoon om even stoom af te blazen en elkaar waar nodig een hart onder de riem te steken.

Lieve **Frieda** en **Hannah**, onze maandelijkse etentjes waren en zijn zo ontzettend waardevol voor mij. **Frieda**, jouw scherpe blik verbaast me elke keer weer en ik kan geen genoeg van je indrukwekkende verhalen krijgen. Ik ben ontzettend dankbaar met zo'n bijzondere vriendin als jij in mijn leven. **Hannah**, het ene hoofdstuk gesloten, een nieuw hoofdstuk begint. Ik spreek voor twee als ik zeg hoe trots de boys op jou zijn en wat je bereikt hebt en dankbaar dat we deel van die reis hebben mogen uitmaken.

Mijn **Haarlemse collega's**. Ik weet niet of jullie het gemerkt hebben, maar ik vond het nogal een heftige overgang van lab naar kliniek. Jullie hebben er in korte tijd voor gezorgd dat ik me helemaal op mijn plek voel. Speciale shout-out naar **Lot** en **Eef**: ANIOS for life! **Gerda**, bedankt voor je verhelderende inzichten, dankzij jou durf ik nu met een gerust hart de volgende grote stap op mijn carrièrepad te maken.

Pap en **mam**, ik heb zoveel van jullie geleerd en ik weet dat jullie altijd voor mij klaar zullen staan. Ik kan jullie hier niet vaak genoeg voor bedanken. Lieve **zusjes**, ik ben er ongelofelijk trots op dat ik me jullie grote broer mag noemen. **Famylje Veenema,** wat bin ik bliid mei jimme. Fryslân fielt foar my as thúskommen en ik sjoch út nei noch in hiel soad gesellige jûnen yn 'e tún en yn 'e snie.

Tjibbe, met jou is het leven zo leuk! Samen ontdekken we de wereld en alles wat het te bieden heeft. Jij weet wanneer ik er doorheen zit, ver voordat ik het zelf in de gaten krijg en je hebt inmiddels voldoende trucs bedacht om mij tot rust te krijgen als ik weer eens doordraaf. Je geeft me de ruimte die ik nodig heb, maar houdt me tegelijkertijd met de benen op de grond. Bedankt voor alles wat we al samen hebben beleefd en voor de mooie momenten die nog gaan komen.

CURRICULUM VITAE

Sander Kelderman was born on the 17th of February 1986 in Heemskerk (The Netherlands). In 2004 he completed his secondary education at the Arentheem College in Arnhem. In the same year he began his medical studies at the University of Groningen where he received his bachelor's degree in 2007. During his master's he followed one year of medical internships at the University Medical Center in Groningen and one and a half year at the Medisch Spectrum Twente in Enschede as well as several clinical internships abroad. For his research internship he went to the lab of dr. Dan Powell Jr. at the University of Pennsylvania in Philadelphia (USA). There, he received his first hands-on training in biomedical research and became fascinated by the field of immunology and oncology. He finished his master's *cum laude* and obtained his medical license in 2011. Initially, he had planned to pursue a clinical career in Obstetrics and Gynecology but upon returning to The Netherlands he decided to continue with biomedical research instead. In the summer of 2011 he started his PhD research in the lab of prof. Ton Schumacher at The Netherlands Cancer Institute in Amsterdam, of which the results are presented in this thesis. In September 2015 he returned to the clinic as a junior resident in the department of Obstetrics and Gynecology at the Spaarne Gasthuis in Haarlem. In 2017 he will start the Obstetrics and Gynecology residency program at the Academic Medical Center in Amsterdam.

LIST OF PUBLICATIONS

Strønen E., Toebes M., Kelderman S., van Buuren M.M., Yang W., van Rooij N., Donia M., Böschen M.L., Lund-Johansen F., Olweus J., Schumacher T.N. Targeting of cancer neoantigens with donor-derives T cell receptor repertoires. ***Science.*** **352: 1337-41 (2016).**

Kelderman S., Heemskerk B., Fanchi L., Philips D., Toebes M., Kvistborg P., van Buuren M.M., van Rooij N., Michels S., Germeroth L., Haanen J.B. & Schumacher T.N. Antigen-specific TIL therapy for melanoma: A flexible platform for personalized cancer immunotherapy. ***Eur J Immunol.*** **46: 1351-60 (2016).**

Kelderman S. & Kvistborg P. Tumor antigens in human cancer control. ***Biochim Biophys Acta.*** **1865: 83-89 (2016).**

Becattini S., Latorre D., Mele F., Foglierini M., De Gregorio C., Cassotta A., Fernandez B., Kelderman S., Schumacher T.N., Corti D., Lanzavecchia A. & Sallusto F. T cell immunity. Functional heterogeneity of human memory CD4(+) T cell clones primed by pathogens or vaccines. ***Science.*** **347: 400-406 (2015).**

Schreibelt G., Bol K.F., Westdorp H., Wimmers F., Aarntzen E.H., Duiveman-de Boer T., van de Rakt M.W., Scharenborg N.M., de Boer A.J., Pots J.M., Olde Nordkamp M.A., van Oorschot T.G., Tel J., Winkels G., Petry K., Blokx W.A., van Rossum M.M., Welzen M.E., Mus R.D., Croockewit S.A., Koornstra R.H., Jacobs J.F., Kelderman S., Blank C.U., Gerritsen W.R., Punt C.J., Figdor C.G. & de Vries I.J. Effective Clinical Responses in Metastatic Melanoma Patients after Vaccination with Primary Myeloid Dendritic Cells. ***Clin Cancer Res.*** **22: 2155-66 (2015).**

Kelderman S., Schumacher T.N. & Kvistborg P. Mismatch Repair-Deficient Cancers Are Targets for Anti-PD-1 Therapy. ***Cancer Cell*****. 28: 11-13 (2015).**

Kvistborg P., Philips D., Kelderman S., Hageman L., Ottensmeier C., Joseph-Pietras D., Welters M.J., van der Burg S., Kapiteijn E., Michielin O., Romano E., Linnemann C., Speiser D., Blank C., Haanen J.B. & Schumacher T.N. Anti-CTLA-4 therapy broadens the melanoma-reactive $CD8^+$ T cell response. ***Sci Transl Med.*** **6: 254ra128 (2014).**

Kelderman S., Schumacher T.N. & Haanen J.B. Acquired and intrinsic resistance in cancer immunotherapy. ***Mol Oncol.*** **8: 1132-1139 (2014).**

Kelderman S., Heemskerk B., van Tinteren H., van den Brom R.R., Hospers G.A., van den Eertwegh A.J., Kapiteijn E.W., de Groot J.W., Soetekouw P., Jansen R.L., Fiets E., Furness A.J., Renn A., Krzystanek M., Szallasi Z., Lorigan P., Gore M.E., Schumacher T.N., Haanen J.B., Larkin J.M. & Blank C.U. Lactate dehydrogenase as a selection criterion for ipilimumab treatment in metastatic melanoma. ***Cancer Immunol Immunother.*** **63: 449-458 (2014).**

Kelderman S., van der Kooij M.K., van den Eertwegh A.J., Soetekouw P.M., Jansen R.L., van den Brom R.R., Hospers G.A., Haanen J.B., Kapiteijn E. & Blank C.U. Ipilimumab in pretreated metastastic uveal melanoma patients. Results of the Dutch Working group on Immunotherapy of Oncology (WIN-O). ***Acta Oncol.*** **52: 1786-1788 (2013).**

Urbanska K., Lanitis E., Poussin M., Lynn R.C., Gavin B.P., Kelderman S., Yu J., Scholler N. & Powell D.J., Jr. A universal strategy for adoptive immunotherapy of cancer through use of a novel T-cell antigen receptor. ***Cancer Res.*** **72, 1844-1852 (2012).**

Kelderman S., Steenvoorde P. & van der Valk P.D. Sternoclavicular joint tuberculosis. ***Ned Tijdschr Geneeskd.*** **156: A3315 (2012).**