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Scope of the thesis
Chapter 1
The human immune system has the ability to recognize a large variety of pathogens. However, not only viruses and bacteria can be efficiently cleared from the body, as the immune system is also involved in the recognition and elimination of cancer cells. As early as the 1950’s, immunologists were interested in understanding if and how lymphocytes could combat cancer (Burnet, 1957) and throughout the years it has become apparent that – at least in some cases – T lymphocytes can specifically recognize and eliminate tumor cells (Dunn et al., 2002). Within this introduction I will discuss four aspects of cancer immunology of which our basic understanding is key to the research presented in this thesis: i) T cell immunity; ii) cancer antigens; iii) regulators of the immune response and finally; iv) current cancer immunotherapies. This is followed by a brief description of each chapter, together outlining the scope of this thesis.

**T cell immunity**

T cells are an important cell subset of the adaptive immune system. CD8 cytotoxic T cells are the main T cell subset to eliminate target cells. They can directly kill these target cells by the release of cytotoxic granules or they secrete cytokines to recruit CD4 helper T cells. The main function of CD4 T cells is to support the antibody responses, but are also known to kill their target cells (Medzhitov and Janeway, 1997). T cells can exert these functions because they harbor a specific T cell receptor (TCR) on their cell surface, which recognizes peptides in the context of a MHC molecule (pMHC) on their target. The collection of different TCRs among T cells is defined as a TCR repertoire. A diverse repertoire is generated and maintained to cope with a variety of antigens, since the immune system is unable to predict which pathogen derived antigens or cancerous antigens will be encountered. The repertoire of TCRs that can theoretically be formed is in the order of $10^{13}$. However, actual TCR diversity is sculpted by i) positive and negative selection in the thymus and ii) by a finite amount of T cells. The reduction in the number of possible TCRs – estimates are that approximate $2.5 \times 10^7$ TCRs are present in a human body – is overcome by the expression of TCRs that are promiscuous and can thus cope with a large number of antigens, while still remaining specific (Nikolich-Zugich et al., 2004).

**Cancer antigens that can be recognized by T cells**

It is now well established that T cells recognize tumors and do so through the TCR–pMHC interaction described above. The antigenic space of human tumors – that is defined as the collection of tumor specific antigens that can be recognized by T cells – can roughly
be divided into two classes of antigens, each with distinct characteristics; i) non-mutated self-antigens, which are peptides derived from self proteins that are either not expressed in normal cells or are overexpressed in tumor cells, and ii) viral and mutated antigens (or “neo-antigens”), which are peptides derived from mutated proteins.

For long it was thought that the non-mutated differentially expressed self-antigens could be ideal targets for off-the-shelf immunotherapies, because they can possibly be shared among many different patients. This is in sharp contrast with the group of neo-antigens that arise as the consequence of tumor specific mutations; these are in large part patient specific and at the same time truly foreign to the immune system. However, the targeting of non-mutated self antigens has not proven to be very successful as a treatment option. Accumulating evidence indicates that the recognition of neo-antigens contributes significantly to the anti-tumor response. Data showing that melanoma and lung cancer patients with a high mutational load show a significantly better clinical response to immune checkpoint therapy as compared to patients with a low mutational load, is in support of this hypothesis (Rizvi et al., 2015; Snyder et al., 2014). The analysis of neo-antigen specific T cell responses relies heavily on the use of exome sequencing data and computational algorithms that predict the likelihood of an epitope being presented to T cells (Kesmir et al., 2002; Lundegaard et al., 2008).

**Regulators of the immune response**

Despite the presentation of a suitable antigenic determinant, it is important to realize that presentation alone does not necessarily result in an anti-tumor response. Multiple strategies are employed by tumors to attenuate the effectiveness of T-cell mediated attack. One such strategy is the engagement of inhibitory signaling pathways to block an effective anti-tumor response. For example, PD-1 is a co-inhibitory receptor that is expressed on activated T cells. Tumor cells – but also tumor stroma – can express its ligands (PD-L1/PD-L2) and signaling through this inhibitory pathway actively inhibits tumor cell killing (Sharma and Allison, 2015). Efforts to intervene with this inhibitory axis to improve tumor cell killing have shown therapeutic effects and will be discussed later. A second potential example of a strategy to evade T cell mediated immune attack is by a process called immunoediting, where under pressure of the immune system non-immunogenic tumor cells are selected which do not express epitopes that are recognized by the immune system. The evidence supporting this concept is most convincing in the murine setting (Dunn et al., 2002; Matsushita et al., 2012; Mittal et al., 2014; Shankaran et al., 2001), but a bioinformatics approach provided first evidence that such immunoediting can also occur in human tumor development (Rooney et al., 2015). In this thesis, I have analyzed the presence of this process in patients with melanoma.
Current cancer immunotherapies

Strategies to exploit the human immune system to fight cancer have greatly advanced over the recent years. Here, I will discuss two examples of such therapies to treat cancer patients; i) immune checkpoint therapy and ii) cellular immunotherapy.

The initial success of immune checkpoint therapy was accomplished by the treatment of a cohort of metastatic melanoma patients with a CTLA4 blocking antibody (Hodi et al., 2010). Persistent responses were observed in approximately 20% of melanoma patients treated with anti–CTLA-4 in a combined analysis of phase II and phase III studies (Schadendorf et al., 2015). Treatment of patients with non-small cell lung cancer (NSCLC), melanoma or renal cell cancer (RCC) using an antibody blocking the PD1–PDL1 axis showed an impressive response in 18%, 28% and 27% of the patients, respectively (Topalian et al., 2012). As of today, patients with different tumor types – melanoma, lung cancer, stomach cancer, bladder cancer, head and neck cancer, renal cell cancer and Hodgkin’s lymphoma – have shown clinical responses in early clinical trials (Sharma and Allison, 2015). Finally, a recent phase III study showed that in untreated patients with metastatic melanoma, anti-PD1 alone or anti-PD1 in combination with anti–CTLA-4 showed a significantly longer progression-free survival than anti–CTLA-4 alone (6.9%, 11.5% and 2.9%, respectively)(Larkin et al., 2015).

An alternative type of immunotherapy is adoptive transfer of Tumor Infiltrating Lymphocytes (TIL). In this treatment protocol, TIL from a patient are expanded into very large numbers \textit{ex vivo} and then infused back into the patient together with a high dose of IL-2. This way of treating (mainly melanoma) patients was pioneered by the group of Steve Rosenberg from the NIH and has shown response rates of approximately 50% of patients treated (Hinrichs and Rosenberg, 2014). It was shown that – at least part of – the efficacy of the treatment was mediated by CD8$^+$ T cells (Dudley et al., 2013). Whereas the efficacy of both checkpoint inhibitors and cellular mediated cancer-immunotherapies is now well established, it is at the moment largely unclear which antigenic determinants are mediating the clinical regression that is seen in a subset of the patients. A better understanding of this will not only help us understand basic tumor immunology, but it will also help us improving current immunotherapies.

The scope of this thesis is to explore the importance of neo-antigens in the anti-tumor responses. In particular, I have developed technologies to systematically analyze the contribution of neo-antigen specific reactivity in the CD4$^+$ and CD8$^+$ T cell compartments. Additionally, I discuss what the expected value of neo-antigens is for immunotherapy.
Description of the chapters in this thesis

In chapters 2 and 3 we review the two main candidate antigen classes for anti-tumor immune responses; i) non-mutated self-antigens, which are peptides derived from self-proteins that are either normally not expressed in normal cells or are overexpressed on a tumor cell, and ii) viral and mutated self-antigens (or “neo-antigens”). For these different types of antigens three questions are needed to be addressed before deciding whether or not an antigen is an attractive immunotherapy target. Firstly, is the antigen shared among a large group of patients? Secondly, is the expression pattern of the antigen sufficiently tumor specific? And finally, how likely is it that such an antigen is lost when immune pressure is applied? Results presented in Chapters 2 and 3 can be used as a guide in addressing these questions, and therefore choosing an appropriate target for immunotherapy.

Peptide-MHC (pMHC) multimers have been developed to monitor antigen specific T cell responses in humans and have become one of the most widely used tools in a variety of disease settings (Altman and Davis, 2003). In chapter 4 we assess the influence of minor polymorphic differences between HLA subtypes on the ability to detect antigen specific T cells for a given peptide. The results reveal that limited polymorphisms in HLA molecules significantly affect the ability to detect antigen specific T cell responses. I provide tools to prevent this obstacle for common variants within the HLA-A*02 allele group.

On a more general note, the analysis of neo-antigen specific T cell responses relies heavily on next generation sequencing technologies combined with the use of computational algorithms – for instance those that predict the likelihood of an epitope being processed and presented to T cells (Kesmir et al., 2002; Lundegaard et al., 2008). The aim of chapter 5 is to evaluate which fraction of cancer neo-antigens that can be recognized by CD8+ T cells is successfully identified with current exome-based epitope prediction strategies. By the use of a dataset that includes neo-antigens that were previously identified through the use of unbiased, computational-independent strategies we show that neo-antigen predictions can be done with a high sensitivity of approximately 70%.

With a highly sensitive exome-based epitope prediction pipeline in place and tools to follow CD8+ neo-antigen specific T cell responses in a patient specific manner, we show in chapter 6 for the first time that this can be used to dissect the effects of immunotherapy in a melanoma patient. In this specific patient – who had >1500 somatic mutations in the tumor – we detected a cytotoxic neo-antigen specific response towards the mutated gene product of ATR in the TIL compartment. We also were able to follow this response over time in the blood of this patient and remarkably, the magnitude of this response increased fivefold within weeks after start of anti-CTLA4 therapy. By now we have screened a total of 11 patients and detected neo-antigen specific CD8 T cell reactivity in approximately 80% of patients.
On the basis of the evidence that neo-antigen specific CD8+ T cell reactivity is frequently observed in patients with melanoma (van Rooij et al., 2013, van Buuren et al., 2015, unpublished) and the recent report that neo-antigen specific CD4+ T cells can mediate tumor regression in a metastatic cholangiocarcinoma (Tran et al., 2014), we decided to assess whether neo-antigen specific CD4 T cell reactivity is a common phenomenon in human cancers. In chapter 7 we describe the development of technology to analyze neo-antigen reactivity in the CD4 compartment of TIL. Results show that neo-antigen reactivity by CD4+ T cells is frequently present in patients with melanoma and that these T cells can directly recognize the tumor. This is in support of the hypothesis that the recognition of neo-antigens contributes significantly to the anti-tumor response and that development of neo-antigen specific immunotherapies is therefore attractive.

The recent successes of immune checkpoint therapy (anti-CTLA4 and anti-PD-1/PD-L1) have resulted in a significantly improved overall survival in patients with different cancer types (Sharma and Allison, 2015). However, since only a fraction of the patients respond to therapy there is a need to identify biomarkers that can discriminate which patients are likely to respond to therapy. In chapter 8 I discuss an intriguing analysis in which the authors describe a genetic basis with which it is possible to distinguish between melanoma patients who do or do not respond to anti-CTLA4 therapy (Snyder et al., 2014). Specifically, patients that do respond to anti-CTLA4 therapy are proposed to be distinguished from the non-responding patients by the use of a set of 101 tetrapeptide amino acid signatures within the predicted neo-antigens for each patient. In chapter 8 I discuss i) why these results are difficult to understand from an immunological perspective and that we cannot confirm such signature in the patient cohort we studied and ii) which other aspects of the cancer immunity cycle could provide appropriate biomarkers to help decide which patients should receive a specific type of cancer immunotherapy.

Immuoediting is a process where tumor cells that do not express epitopes that can be recognized by the immune system are selected. These tumor cells are then ‘invisible’ for immune recognition and are allowed to grow out. The evidence supporting this concept has been collected in murine models mostly (Matsushita et al., 2012; Shankaran et al., 2001). Chapter 9 describes a bioinformatics approach in which we aimed to address whether or not immunoediting is a process that occurs in human cancer. This analysis was performed using two independent cohorts of melanoma patients. The number of predicted neo-antigens in settings in which immunoediting could potentially occur was compared to the number of predicted neo-antigens in settings in which immunoediting is not expected to occur (for example because the relevant HLA allele was absent in these patients). A recent report was able to show that immunoediting does not seem to be a common feature of many different cancer types; while evidence in favor of immunoediting was observed in patients with colorectal-, clear kidney- and stomach cancer, melanoma
patients did not show signs of immunoediting (Rooney et al., 2015). In line with these results, our analysis showed that large-scale immunoediting is absent in patients with melanoma.

Chapter 10 summarizes the main results and discusses the implications for immunotherapy, particularly in context of the findings of this thesis.
References


Chapter 1


