Review

The cancer antigenome

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Cancer cells deviate from normal body cells in two immunologically important ways. First, tumour cells carry tens to hundreds of protein-changing mutations that are either responsible for cellular transformation or that have accumulated as mere passengers. Second, as a consequence of genetic and epigenetic alterations, tumour cells express a series of proteins that are normally not present or present at lower levels. These changes lead to the presentation of an altered repertoire of MHC class I-associated peptides. Importantly, while there is now strong clinical evidence that cytotoxic T-cell activity against such tumour-associated antigens can lead to cancer regression, at present we fail to understand which tumour-associated antigens form the prime targets in effective immunotherapies. Here, we describe how recent developments in cancer genomics will make it feasible to establish the repertoire of tumour-associated epitopes on a patient-specific basis. The elucidation of this ‘cancer antigenome’ will be valuable to reveal how clinically successful immunotherapies mediate their effect. Furthermore, the description of the cancer antigenome should form the basis of novel forms of personalized cancer immunotherapy.

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Introduction

It has long been known that T cells have the capacity to eliminate human cancer cells, as shown by the anti-leukaemic effects of allogeneic haematopoietic stem cell transplantation and donor lymphocyte infusions (Barnes *et al.*, 1956; Sprangers *et al.*, 2007). In this setting, the presence of polymorphic antigens on the surface of tumour cells that are foreign to the infused T cells forms the basis of tumour recognition. However, in recent years, it has become apparent that also autologous T cells can—at least in some cases—

show profound anti-tumour reactivity in cancer patients. In particular in melanoma, the clinical activity of tumour-reactive T cells has now been documented in a large number of clinical trials. Specifically, treatment of patients with the antibody ipilimumab, which interferes with the function of the T-cell inhibitory receptor CTLA4, has been shown to result in a significant effect on patient survival (Hodi *et al.*, 2010). More recently, treatment of patients with antibodies that block signalling through PD-1, a second inhibitory receptor on T cells, has also shown considerable clinical promise (Brahmer *et al.*, 2012; Topalian *et al.*, 2012). As an alternative to these antibody-based strategies, regression of even large tumour masses has been observed upon the adoptive transfer of autologous, *ex vivo* expanded, tumour-infiltrating lymphocytes (TILs) obtained from metastatic melanoma lesions. Objective response rates for anti-CTLA4 therapy and TIL treatment are ~10–15% in randomized phase III studies and ~50% in multiple non-randomized phase I–II studies, respectively, and both treatments can induce complete tumour regressions in a subset of treated patients (Rosenberg *et al.*, 2008; Hodi *et al.*, 2010; Robert *et al.*, 2011). These clinical data provide clear evidence that human tumour cells can express antigenic determinants—epitopes—that can be the target of autologous T cells, and that enhancement of such reactivity can lead to cancer regression. Importantly, in the majority of patients responding to these immunotherapeutic strategies, we do not know which antigens are the target(s) in the observed tumour regression. Such knowledge would be of obvious value, as it could allow one to steer reactivity towards antigens of interest.

Within this review, we will distinguish two main classes of tumour-specific antigens that together make up the cancer antigenome, the class of ‘neo-antigens’ and the class of non-mutated ‘self-antigens’ (Figure 1). The presentation of neo-antigens by tumour cells is a direct consequence of the large number of somatic mutations that are found in human tumours. Such neo-antigens may be newly displayed at the surface of tumour cells because a mutation increases the efficiency with which a peptide is presented by MHC molecules, for instance by increasing its binding affinity. Alternatively, generation of neo-antigens that can be recognized by T cells may occur when a mutation alters the T-cell receptor (TCR)-exposed area of a peptide that is also presented by MHC molecules in its non-mutated form.

Presentation of the second class of tumour-associated epitopes, the non-mutated ‘self-antigens’, involves the display of epitopes from gene products that are normally only expressed in a restricted set of cell types. Thus, rather than being a direct consequence of mutations, presentation of self-antigens is a consequence of the tissue-specific or transformation-induced gene expression profile of tumour cells.

An important distinction between these two classes of antigens is that T-cell reactivity against self-antigens can

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Figure 1 Distribution of self- versus neo-antigens and their tolerance and toxicity profile. Schematic overview of the different types of antigens that can be potentially targeted by T cells: self-antigens and neo-antigens. Blue denotes the shared antigens between different patients and red represents unique, patient-specific antigens. At present, there is little evidence that shared antigens can be truly patient specific, although some of the shared antigens are only expressed in a fraction of a given human tumour type.

only occur when T-cell tolerance towards a given antigen is incomplete, and there is strong data to suggest that for at least part of the tumour-associated self-antigens, the T-cell repertoire available for tumour recognition is of a lower avidity. In contrast, as neo-antigens are fully tumour specific, central T-cell tolerance does not form a concern. By the same token, T-cell responses against neo-antigens are not expected to result in autoimmune toxicity against healthy tissues, making immunotherapeutic manipulation highly attractive from a theoretical point of view (Figure 1).

In spite of the significant appeal of neo-antigen-specific T-cell reactivity, our current understanding of tumour-specific T-cell immunity is in large part restricted to the class of non-mutated self-antigens. This strong bias is a consequence of the fact that the majority of the mutations in human tumours that could lead to neo-antigens are unique to that tumour (see below). Thus, contrary to the non-mutated self-antigens that are to a substantial extent shared between patients, most neo-antigens are patient specific (Figure 1). Importantly, with recent advances in cancer genomics and immunomonitoring, the analysis of the full repertoire of tumour-associated antigens on a patient-specific basis has now become a realistic goal.

Current knowledge of the repertoire of human tumour antigens

Our first understanding of the molecular determinants on tumour cells that can be recognized by human T cells came in 1991 when Boon and co-workers isolated the MAGE-1 antigen via cDNA expression cloning, using recognition by tumour-reactive cytotoxic T lymphocytes (CTLs) from a melanoma patient as a readout system (van der Bruggen *et al*, 1991). In the following decades, a large number of self-antigens that are aberrantly expressed in human tumours has been

discovered, either using tumour-reactive CTL or using patient sera (serological expression cloning, SEREX) as a readout system. In addition, a large set of tumour-associated epitopes has been identified by analysing which peptides from candidate tumour antigens (i.e., proteins that are highly/aberrantly expressed in tumours) could lead to the induction of a tumour-reactive T-cell response, an approach termed as ‘reverse immunology’ (Kawakami *et al*, 2004).

The self-antigens that have been discovered—predominantly in melanoma—by these different approaches can be divided into several subclasses: A first class represents epitopes for which expression is normally largely restricted to male germline cells. These cancer-germline (C/G) antigens are frequently overexpressed in tumours due to demethylation events, as has been shown for the prototypic MAGE antigens (A, B and C) (Chomez *et al*, 2001). A second class of self-antigens consists of the tissue differentiation antigens, antigens that are shared by tumour cells and the tissue it originated from. A well-known example of this class is the Melan-A/MART-1 antigen that is expressed in melanoma but also in healthy melanocytes (Coulie *et al*, 1994; Kawakami *et al*, 1994). A third class of epitopes is derived from proteins that are overexpressed in tumours, but that are also expressed in healthy tissues, such as the Her-2/Neu and PRAME antigens (Fisk *et al*, 1995; Kessler *et al*, 2001).

While we now know the identity of a large number of non-mutated self-antigens that are expressed in human cancer (e.g., for HLA-A2, some 150 epitopes from non-mutated self-antigens have been identified; (Andersen *et al*, 2012b), it is not immediately clear whether recognition of these antigens is associated with cancer regression, and arguments can be made both in favour and against their importance. Specifically, a large series of clinical trials has evaluated whether induction of T-cell responses against non-mutated self-antigens was associated with clinical efficacy. While occasional clinical responses have been observed in these trials, clinical response rates have on general been disappointingly low (around 3–5%, reviewed in Rosenberg *et al*, 2004 and Boon *et al*, 2006), in some cases even when high numbers of circulating peptide-reactive T cells could be obtained (Rosenberg *et al*, 2005). In more recent years, the potential of targeting non-mutated self-antigens has also been evaluated in clinical studies in which T cells were infused that had been genetically engineered to express a tumour-reactive TCR. In trials that utilized TCRs specific for the melanocyte differentiation antigens MART-1 and gp100, a modest clinical efficacy was observed (response rates of ~15–20%; Morgan *et al*, 2006; Johnson *et al*, 2009) and T-cell infusion was accompanied by significant toxicity due to recognition of healthy tissues expressing the same antigen. More encouraging, in a trial in which a TCR specific for the NY-ESO-1 C/G antigen was used in patients with metastatic melanoma and synovial sarcoma, clinical response rates were higher (~50%), although these responses were not always durable (Robbins *et al*, 2011).

Based on these data, but also based on recent analyses of T-cell reactivity in patients that received ipilimumab or TIL therapy (see below), it seems possible that a significant part of the clinically relevant T-cell activity in human cancers does not involve recognition of self-antigens, but could involve recognition of patient-specific neo-antigens. A small number of studies have aimed to address the potential importance of

neo-antigen recognition by (highly involved) expression cloning approaches, and these studies led to the identification of a number of tumour-specific neo-antigens that are recognized by autologous T cells. Furthermore, in a seminal study by Lennerz *et al* (2005) an unbiased analysis was performed for antigens recognized by different T-cell cultures from a single melanoma patient. This analysis led to the identification of a series of neo-antigens, and T-cell reactivity against these neo-antigens dominated the tumour-specific T-cell response in this patient (Lennerz *et al*, 2005). Based on these early studies, it is clear that recognition of human neo-antigens can occur in patients, even in the absence of immunotherapy. However, data have been lacking to reveal whether such responses are enhanced by therapies. Likewise, whether such responses are a crucial component of therapeutic efficacy or can be selectively enhanced has not been established.

The cancer genome

The development of second-generation sequencing technology has made it feasible to describe the full mutation load (i.e., the 'genetic landscape') of human tumours (Meyerson *et al*, 2010; Zhao and Grant, 2011). Specifically, comparison of the genome sequence of cancer tissue to that of non-transformed tissue from the same patient has been used to reveal the full range of genomic alterations within a tumour—including nucleotide substitutions, structural rearrangements and copy number alterations (Meyerson *et al*, 2010). In early studies that described cancer genomes, analysis was still restricted to subsets of the genome (e.g., all kinase genes; the kinome), because of high costs and limited sequencing capacity. However, with technology advancing and costs dropping, analysis of the entire protein-encoding part of the genome (the exome) or the entire cancer genome has become feasible and will soon become routine.

Exome sequencing and whole genome sequencing each have their specific advantages and disadvantages in the detection of mutations that could potentially be relevant for the immune system. Exome sequencing (which covers only the ~1% coding region of the genome) has the clear advantage that it can provide a higher sequence coverage and consequently a higher ability to detect mutations, including mutations that are only expressed by part of the tumour cells. As a downside, this method is still somewhat limited by our knowledge of the protein-coding parts of the genome and by uneven capture efficiency across exons and, as a consequence, some mutations may be missed. In one study, a hepatocellular cancer was analysed by both whole genome and exome sequencing. The results obtained showed that a significant fraction (25 of the 63) of the mutations that were identified by whole genome sequencing could not be detected by exome sequencing, with the missed mutations primarily being present in areas with low coverage (Totoki *et al*, 2011). On the other hand, data from the Sanger Institute have demonstrated that of a set of mutations that had previously been identified in cell lines by conventional sequence analysis, the vast majority (313 out of 326 mutations, 97%) was also identified by whole exome sequencing (S Behjati and M Stratton, personal communication).

As a third alternative to whole genome and exome sequencing, tumour-specific mutations within the set of expressed

genes (the transcriptome) may be identified by RNA sequencing. This approach has the advantage over exome sequencing that it is not limited to known genes, and thereby has the potential to also detect novel transcripts, for instance formed by intragenic fusions or, in case of pathogen-induced cancers, non-human genes. As a downside, it is difficult to identify a matched control, as mRNA expression profiles of tumour and normal tissue will not be identical, and this makes it challenging to distinguish tumour-specific mutations from polymorphisms. As a second concern, the ability to reliably call mutations within RNA species that are only present at a low level, either because of low level gene expression or because of low mRNA stability (for instance due to non-sense-mediated RNA decay) will be limited.

Mutation rates and patterns in human cancers

Within a period of only a few years, second-generation sequencing has allowed a description of the genomic changes within thousands of human cancer exomes and genomes (Stratton, 2011; (<http://www.sanger.ac.uk/genetics/CGP/cosmic>)). The genome changes encountered include single-base substitutions, small insertions and deletions (together referred to as indels), copy number changes, DNA rearrangements, but also structural variants at the level of the chromosome (e.g., amplifications, deletions and fusion/translocation events). Single-base substitutions (single-nucleotide variants, SNVs) comprise the vast majority of mutations that are encountered in human cancers and range in number from ~1000 to 100 000 in the entire genome (Stratton, 2011). However, the number of mutations that is relevant to immune recognition—mutations that lead to the production of a protein sequence that is absent in the germline—is much smaller. This type of mutations includes indels that alter the open reading frame of proteins, intragenic fusions that lead to the production of a fusion sequence, splice site mutations and, in particular, non-synonymous SNVs within exons.

Interestingly, sequencing studies have revealed a substantial variability between the number of non-synonymous changes between different tumour types (Greenman *et al*, 2007; Stratton, 2011). For instance, the description of the first cancer genome sequence in 2008 revealed a total of 10 non-synonymous mutations in the coding regions of an acute myeloid leukaemia (Ley *et al*, 2008). Somewhat higher numbers of non-synonymous mutations within exonic regions have been reported for a basal-like breast cancer (28, $n=1$) (Ding *et al*, 2010), glioblastoma multiforme (average of 36, $n=21$) (Parsons *et al*, 2008), pancreatic tumours (average of 48, $n=24$) (Jones *et al*, 2008b), hepatocellular cancer (63, $n=1$) (Totoki *et al*, 2011), and colon and breast tumours (average of 76 and 84, respectively, $n=11$ each) (Sjoberg *et al*, 2006; Wood *et al*, 2007). Furthermore, very high mutation rates have been observed in cancers with substantial exogenous mutagenic exposures, such as ultraviolet light in the case of melanoma (187 non-synonymous mutations in a case report and an average of 201 mutations in 14 tumours) (Pleasant *et al*, 2010a; Wei *et al*, 2011), or exposure to tobacco smoke carcinogens in lung cancers (94 non-synonymous mutations in a lung cancer cell line and >300 mutations in one primary tumour) (Lee *et al*, 2010; Pleasant *et al*, 2010b). Finally, as expected, tumours with mismatch repair deficiencies also carry large numbers of

mutations (Greenman *et al*, 2007). As an example, whereas microsatellite stable (MSS) colon cancers carried on average ~100 mutations, the number of mutations in two microsatellite unstable (MSI) colon cancers was 532 and 915 (Timmermann *et al*, 2010).

The above data provide ballpark figures on the number of mutations that are present in a high proportion of the cells within different types of human tumours at a given point in time. However, tumour cells continue to accumulate mutations throughout the stages of tumour progression. Furthermore, metastasis can involve the distal outgrowth of tumour cells that were derived from a subclone that was small within the primary tumour. Because of this potential for genetic heterogeneity, it is important to also understand the degree of kinship between different areas within the same tumour, and between different metastatic lesions within a patient.

Heterogeneity within primary tumours and between tumour metastases

Comparative sequence analysis of multiple inpatient lesions has now been performed for a number of human tumour types. In a study by Jones *et al* (2008 a,b), it was evaluated to what extent mutations found in an index metastatic lesion were also present in the matched primary tumours and at other metastatic sites within the same patient. Sanger sequencing revealed that only a minor fraction (around 3%) of the mutations that were identified within the index lesions were not present in the primary tumour or other metastases (Jones *et al*, 2008a). More recently, whole genome sequencing has been employed to compare the mutation profile of a primary basal-like breast cancer and a brain metastasis (Ding *et al*, 2010). In these tumours, a total of 50 mutations (including SNV and indels) were found, of which 48 were shared between the two. Furthermore, similar results were obtained in a hepatocellular cancer, in which 205 out of 214 mutations (96%) were present in both the primary tumour and two metastases (Tao *et al*, 2011).

In contrast to these data that show a high degree of kinship between different tumour sites, one report documented that 19 out of 32 mutations within a metastasized lobular breast cancer were not present in the primary tumour that was resected 9 years earlier, prior to radiotherapy. Furthermore, of the 11 mutations that were shared, only 5 were abundant with the primary tumour, whereas the remainder had a very low allele frequency, in between 1 and 13% (Shah *et al*, 2009). Likewise, a substantial fraction (around 35%) of mutations observed in different metastatic lesions of patients with pancreatic cancer was not shared between individual metastases (Yachida *et al*, 2010). Finally, a recent study that analysed heterogeneity between different metastases and also between different regions of the primary tumour in patients with renal cell carcinoma demonstrated that, in these cases, the fraction of mutations that were detected in only some of the lesions was very high (~65%). Furthermore, substantial heterogeneity was even observed within the primary tumour mass. Thus, for a randomly identified mutation, the likelihood that this mutation would be present at all tumour sites within these patients was only one in three (Gerlinger *et al*, 2012).

Collectively, these data show a striking degree of variability between patients with respect to the genetic kinship between

primary and metastasized tumours, with in some cases different lesions being nearly identical, whereas in other cases the majority of mutations being private. It seems plausible that kinship between different tumour sites will in part differ in a systematic way between tumour types, for instance depending on whether metastasis is likely to occur early or late in the disease process. In addition, disparity between different lesions is likely to be influenced by therapy, both by the direct DNA damaging effect of for instance radiation therapy or alkylating agents, but also by the genetic bottleneck that an efficient therapy will form. Finally, the kinship between different lesions will in part be governed by chance; whether the cell that forms a given metastasis happens to derive from a dominant or minor clone within the primary tumour. More data will be required to understand which of the above factors is most dominant. However, even with the limited data currently available, it is already apparent that genetic heterogeneity with human tumours will be an important factor to take into account when targeting tumour-specific neo-antigens.

Driver and passenger mutations

Classically, the mutations that are found in cancer cells are divided into two categories, 'Drivers' and 'Passengers', according to their role in cancer development. Driver mutations are those mutations that confer a selective advantage to the cells that carry them, and these include inactivating mutations in tumour suppressor genes and activating mutations in oncogenes. All other mutations, which are neutral with respect to cell division or death, are considered as 'passengers'. Such passengers were either already present in the ancestor cancer cell at the moment it acquired one of its driver mutations, or were acquired (by a subclone of tumour cells) during subsequent tumour growth (Stratton *et al*, 2009).

The number of human genes for which a role in tumour development has been shown or is suspected is substantial. To date, ~400 (2%) of the ~22 000 protein-coding genes have been reported to have recurrent mutations in human cancer and are therefore likely to confer a selective advantage (Lee *et al*, 2010; Stratton, 2011).

Furthermore, as many of these driver mutations only occur in a low fraction of tumours (Wood *et al*, 2007), our knowledge of recurring—and hence presumed to be driver—mutations in human cancer is likely to still be incomplete. Nevertheless, even though the number of driver mutations that can occur in human tumours is substantial, it is important to realize that most of the mutations that are found in a given tumour are likely to be passengers that are neutral to tumour growth (estimated at 85% in a study by Wood *et al*, 2007). This implies that most of the potential T-cell reactivity towards neo-antigens will be directed against mutated gene products that are dispensable for tumour growth. The consequence of T-cell targeting of drivers and passengers is described below. In addition, we will introduce a third class of mutations, called 'essential passengers'.

From cancer genomes to cancer antigenomes

The mutational landscapes described above indicate that there is a clear opportunity for the immune system to

distinguish tumour cells from healthy tissue. How can genomic information on human cancers be utilized to understand which mutations may result in T-cell recognition? As discussed above, only those mutations that result in the expression of a non-germline protein sequence can lead to the formation of neo-antigens. As for most tumours the bulk of such mutations are formed by non-synonymous SNV we will here focus on this category of mutations, but the same analysis pipeline applies to for instance indels or gene fusions that alter germline protein sequences.

The majority of MHC class I binding peptides is nine amino acids long. Thus, when we for now ignore the contribution of longer peptides, nine neo-peptides can be formed for each non-synonymous SNV, in which the mutated residue is present at either one of the positions within the peptide. However, of those neo-peptides, only a small fraction (roughly a few %) will bind with high affinity to a given HLA allele. Importantly, through seminal work of Rammensee and colleagues in the early nineties (Falk *et al*, 1991) and work by many groups since then, we have obtained a very detailed understanding of the ligand preference of human MHC class I alleles. Therefore, with the repertoire of protein-changing mutations in a cancer genome identified, it is straightforward to predict with reasonable accuracy which encoded peptides are likely to bind to the MHC class I alleles expressed by that patient, using algorithms such as NetMHC (Buus *et al*, 2003).

As an example of such an *in silico* epitope prediction, Segal *et al* (2008) have analysed the potential for neo-antigen formation using a set of 1152 mutations that were found in 11 colon and 11 breast cancers. The results obtained showed that, on average, 7–10 mutated peptides were predicted as ligands for the human MHC class I alleles (HLA-A*0201) for these 2 tumour types. Extrapolation of these data to the 6 different HLA class I alleles (-A, -B and -C, 2 each) that can be expressed results in a total number of ~40 to ~60 potential targets for T-cell recognition per tumour. However, a number of factors that will determine which of these predicted MHC ligands can actually be seen by T cells should be taken into account.

(i) Mutations in genes that are not expressed within a given tumour is a non-event from an immunological point of view. Thus, the number of mutations that can be expected to be of immunological interest can be obtained by correcting for the fraction of genes expressed in the average tumour, and perhaps even somewhat more, as DNA repair is more efficient for transcribed genes. (ii) While binding of mutated peptides to MHC molecules is probably the most significant bottleneck in epitope presentation, it is not the only one. Specifically, the protein that contains the mutated residue needs to be processed—primarily by the proteasome—such that the peptide that has the potential to bind to MHC class I molecules is actually produced. Furthermore, this peptide then has to be transported into the ER lumen by the TAP1/TAP2 transporter to allow assembly with MHC class I, providing another—albeit minor—bottleneck. (iii) Even if a given mutated peptide is presented by MHC class I at the cell surface, this does not guarantee T-cell recognition. Specifically, T-cell recognition can only occur when TCRs that have the ability to recognize the mutant epitope but not the parental peptide exist within the T-cell repertoire. While prior data indicate that the immune system has a high

ability to distinguish even minor variations in MHC-bound peptides, certain types of mutation, such as alterations at the N-terminal peptide residue or conservative substitutions at other positions can be missed (Kessels *et al*, 2004).

Because of the above factors, the number of mutated epitopes that will actually be presented at the cell surface of tumour cells and can also be recognized by T cells will be substantially lower than the 40–60 predicted MHC ligands, perhaps by an order of magnitude. Thus, the number of neo-epitopes that can be recognized by T cells may be a handful for tumours such as breast and colorectal cancer, and tumours with a lower mutation load such as medulloblastoma (Parsons *et al*, 2011) may often lack such determinants entirely. Vice versa, tumours that generally have a high mutation load, such as melanoma, smoking-associated lung cancer or MSI colon cancers (Lee *et al*, 2010; Timmermann *et al*, 2010; Wei *et al*, 2011) can be expected to present an array of neo-antigens to which T cells could react.

Is there clinical data that would suggest a correlation between tumour mutation load and immunogenicity? Melanoma has long been known to be a relatively ‘immunogenic’ tumour, with occasional spontaneous regressions, and with strong evidence for clinical activity of tumour-specific T cells. Likewise, MSI colon cancers—which have a high mutation load due to DNA repair deficiencies—have been shown to have a better prognosis and an increased CD8⁺ T-cell infiltrate relative to MSS tumours (Dolcetti *et al*, 1999). Thus, even though the evidence is highly indirect, these data provide some indication for a causal relationship between mutation load and T-cell recognition. Because of the possible correlation between mutation load and T-cell recognition, analyses of T-cell reactivity against human neo-antigens may in first instance perhaps best focused on tumours such as melanoma and smoking-associated lung cancer.

While cancer genome information has not yet been utilized to analyse autologous T-cell reactivity in humans, proof-of-principle for the ability to identify tumour-specific neo-antigens by cancer genome-based approaches has been obtained in mouse models in two recent studies. In work by Sahin and colleagues, whole exome sequencing of the B16 melanoma cell line—the most widely used melanoma model in mice for many years—revealed the presence of around 500 non-synonymous mutations, a number that is within the range that is found within human melanoma. Importantly, when 50 of these mutations were analysed in more detail, 3 of these could be shown to encode endogenously processed immunogenic epitopes. Furthermore, when for two of these three epitopes the potential value of vaccination was tested, inhibition of tumour growth could be demonstrated for both of these (Castle *et al*, 2012). In work by Schreiber and colleagues, exome sequencing was used to identify a dominant neo-antigen in a mouse tumour cell line, and this knowledge was subsequently exploited to demonstrate that immune pressure could lead to epitope loss (see below) (Matsushita *et al*, 2012). Based on the successful identification of novel neo-antigens in the still only partially characterized sets of mutations in these two recent studies, it seems plausible that whole genome/exome-based analyses of human tumours will likewise be informative.

Analysis of T-cell reactivity against the cancer antigenome

The above sections describe the number of potential neo-antigens that is present in different tumour genomes and how such neo-epitopes can be predicted with relative ease. In addition, RNAseq data obtained on tumour material can in parallel be used to determine which of the non-mutated self-antigens (e.g., cancer-germline antigens) are expressed within an individual tumour, thereby making it possible to examine the potential role of both classes of antigens. An important question is how this knowledge can subsequently be used to evaluate spontaneous or therapy-induced autologous T-cell reactivity against such antigens. As such an analysis would involve very large numbers of potential epitopes (dozens of potential neo-antigens for tumours such as breast and colorectal cancer, hundreds for tumours such as melanoma), such analyses should be able to measure T-cell responses against large collections of antigens in limited amounts of clinical material. Furthermore, as tumour-specific T-cell responses are often of a very low magnitude, the sensitivity of such analyses should be high.

The detection of antigen-specific T cells independent of their functional capacities first became possible when Altman *et al* (1996) demonstrated that fluorescently labelled multimers of MHC molecules containing a peptide of interest could be used to specifically detect T cells that recognize this epitope. This MHC multimer technology has become a widely used technology for immunological monitoring, and has been used to detect even low frequencies of antigen-specific T cells in patient samples. While the original technology for production of MHC multimers did not allow the generation of the large sets of MHC multimers that are required for personalized immunomonitoring, this issue has been overcome by the development of a 'peptide exchange technology'. In this strategy, large quantities of MHC complexes are refolded in the presence of a conditional peptide ligand that cleaves itself upon UV light exposure. This allows one to produce collections of hundreds or thousands of different peptide-MHC complexes for T-cell staining in a straightforward manner (Rodenko *et al*, 2006; Toebes *et al*, 2006). This peptide exchange technology has been developed for most of the common HLA A and B alleles (Toebes *et al*, 2006; Bakker *et al*, 2008; Brackenridge *et al*, 2011). Furthermore, the group of G Grotenbreg has also developed this technology for a series of HLA-C alleles (G Grotenbreg, personal communication).

UV-induced peptide exchange allows one to produce the large collections of peptide-MHC complexes required to evaluate T-cell reactivity against potential neo-antigens. However, the amount of clinical material that can be obtained is generally insufficient to measure T-cell reactivity against all these potential antigens when analysed by conventional MHC multimer flow cytometry (e.g., to analyse T-cell reactivity against 100 potential antigens, some 500 ml of peripheral blood would be required). To address this second issue, we have developed a strategy, termed as 'combinatorial coding', in which each peptide-HLA complex is conjugated to a unique two (or multi-) colour code. As a consequence, each individual fluorochrome can be used in many colour combinations, and antigen-specific T cells are identified by the colour code that they carry. We have developed this strategy

for the use of 8 different fluorochromes, thereby allowing one to visualize 28 different T-cell populations in a single sample (Hadrup *et al*, 2009; Andersen *et al*, 2012a). In parallel to this, Davis and colleagues have developed a similar combinatorial staining approach using a smaller number of colours (Newell *et al*, 2009). As an alternative to the large-scale MHC multimer-based screens made possible by these technologies, it should also be possible to evaluate T-cell reactivity by functional assays. Advantage of such functional screens would be that these can be performed independent of the HLA type of the patient. However, sensitivity of these assays is often lower and sample availability may in many cases be a limiting factor. A general pipeline that can be used to describe the patient-specific cancer antigenome and T-cell reactivity towards it is outlined in Figure 2.

In a proof-of-concept study to evaluate the potential of large-scale MHC multimer based analysis of human T-cell reactivity, we recently evaluated antigen recognition of tumour-infiltrating T cells from melanoma patients, using a panel of all the known shared (i.e., primarily non-mutated) HLA-A2 antigens (Kvistborg *et al*, 2012). In this study, most tumour-derived T-cell populations could be shown to contain reactivity against one or multiple epitopes within this set of 145 shared antigens. However, in the vast majority of cases, these T-cell responses were of a surprisingly low magnitude (<0.1%), and on average they made up <1% of the tumour-resident cytotoxic T-cell population. These data demonstrate that high-throughput analysis of T-cell reactivity in clinical samples is feasible. At the same time, the observation that >99% of T cells in these samples is not reactive with any of the shared antigens tested is consistent with the notion that reactivity against neo-antigens may be prominent in human T-cell infiltrates in melanoma.

How to exploit an understanding of the cancer antigenome?

The above-described pipeline for analysis of the tumour-specific T-cell response on a patient-specific basis should in the coming years reveal the balance between T-cell reactivity against shared and patient-specific antigens and how such T-cell reactivity is influenced by therapy. If reactivity against neo-antigens can be shown to be prevalent in certain human cancers, then it will be interesting to consider how to exploit them.

Characteristics of the neo-antigens

An important consideration when aiming to exploit the expression of neo-antigens to drive T-cell recognition of tumour cells is whether immune pressure is likely to lead to rapid epitope loss (Figure 3). Induction of T-cell reactivity against oncogenic mutations within cancer genes at first glance appears to be the most attractive with respect to this issue. However, such mutations only form a relatively small subset of all mutations present, reducing the likelihood of T-cell recognition. In addition, genetic heterogeneity within tumours may render part of the tumour cells less dependent on a given oncogenic mutation than may a priori be expected (Gerlinger *et al*, 2012). A second class of neo-antigens of interest is formed by mutations within essential genes in cases in which the wild-type copy is lost. Cancer cells frequently lose heterozygosity at large chromosomal areas.

Figure 2 Unravelling the cancer antigenome. First, mutations (red symbols) are determined within the genomic DNA (black lines—either by whole exome or whole genome sequencing) and comparison with normal tissue DNA. Using RNA (blue line) sequencing, the expression of these mutations can be evaluated prior to the prediction phase (optionally) in order to focus on those genes that are expressed within the tumour. Subsequently, epitopes are predicted using algorithms for peptide cleavage and MHC binding, resulting in the description of the antigenome of that tumour. Finally, T-cell assays can be performed, for example, using multiplexed MHC multimer staining, to determine whether T cells recognizing these neo-antigens are present within the patients' repertoire or whether they can be induced from the naïve T-cell repertoire.

If a mutation is present within an essential (household) gene of which the wild-type copy is absent, then T-cell reactivity against the neo-epitope can only lead to immune escape by mutation reversal. We suggest to refer to this class of mutations as 'essential passengers'. Coulie and coworkers have to our knowledge provided the first description of such an essential passenger, by identification of a mutant malic enzyme epitope that was recognized by autologous T cells on a tumour that had lost the wild-type copy of this essential

Figure 3 Mutation types within the cancer antigenome. Mutations (red symbols) present in human tumours can be subdivided into three groups. Drivers, mutations that are essential for tumour transformation or growth. 'Essential passengers', mutations in genes that cannot easily be deleted by the tumour as it is critical for tumour cell survival and no wild-type copy is present. Mere passengers, mutations that have no impact on tumour growth or survival but can generate T-cell neo-antigens. For the latter mutation type, the likelihood of tumour escape is high compared to driver and essential passenger mutations.

enzyme (Karanikas *et al*, 2001). The third and final class of mutations is formed by the true passengers, mutations that are irrelevant for cellular transformation and in gene copies that the cell does not need to maintain. Selective pressure by the immune system may well lead to the loss of such mutated genes. In line with this, intriguing work by Schreiber and colleagues in a murine model has demonstrated that loss of expression of a passenger mutation can be observed upon T-cell pressure (Matsushita *et al*, 2012). At present, it is unknown whether the T-cell pressure during human cancer development is sufficient to lead to a similar immune selection, an important question for future research. We note that the comparison of the frequency of neo-epitopes in human cancers that for instance lack or express a certain HLA allele could be utilized to answer this question. With regard to the induction of immune reactivity against passenger mutations by immunotherapy, it does seem plausible that this would lead to Darwinian selection of epitope loss variants. However, the simultaneous targeting of a handful of passenger mutations may well be enough to substantially reduce this risk, and we consider it possible that the simultaneous targeting of a number of passengers could turn out to be more effective than the targeting of a single driver or essential passenger.

Manipulation of T-cell reactivity against neo-antigens

How can the description of the repertoire of tumour-associated antigens, and in particular neo-antigens, on a patient-specific basis be exploited therapeutically? At present two different strategies can be envisioned. First, one could focus on those neo-antigens for which T-cell reactivity can be demonstrated within the tumour or peripheral blood of that patient. A significant advantage of this T cell-centered strategy is that it focuses the immunotherapeutic intervention on

those mutations for which there is evidence that the mutant peptide is expressed by tumour cells and can be recognized by T cells. As a downside, if for many neo-antigens that are present on tumour cells spontaneous T-cell responses do not occur, such a focus would be unnecessarily restrictive. As an alternative strategy, potential neo-antigens could be selected solely on the basis of genomic information/RNA expression data and subsequent epitope predictions. Such an approach would include neo-antigens for which spontaneous T-cell recognition is absent but would likely also include many mutant sequences that are not presented by HLA, or for which a T-cell repertoire is lacking.

The clinical use of such epitopes to induce or enhance tumour-specific T-cell reactivity should be considered as a form of personalized medicine ('personalized cancer immunotherapy'), and the regulatory framework to guide the clinical development of such personalised therapies is

currently a topic of discussion (C Britten, personal communication). Perhaps the most straightforward use of such neo-antigens will be their administration as vaccines, for instance as synthetic peptides or recombinant DNA or RNA vaccines. The limited data on such an approach in mouse models (Castle *et al*, 2012; Matsushita *et al*, 2012) make one cautiously optimistic; it will now be important to obtain proof-of-principle in humans.

Note added in proof

The first evidence that patient-specific neo-epitopes can be identified through the use of cancer exome data has recently been provided (van Rooij *et al*, 2013).

Conflict of interest

The authors declare that they have no conflict of interest.

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