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Author: Buuren, Marit M. van
Title: Analysis of the neo-antigen specific T cell response: and consequences for personalized immunotherapy
Issue Date: 2016-06-01
Human Cancer Regression Antigens

Pia Kvistborg¹, Marit M. van Buuren¹, and Ton N.M. Schumacher¹

¹ Division of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

*Current Opinion in Immunology* 25(2): 284-90 (2013)
Abstract

Cytotoxic T-cells can recognize antigens that are presented on the surface of human tumor cells and thereby mediate cancer regression. Importantly, those immune interventions that have thus far proven most successful in the clinic – that is checkpoint blockade and tumor-infiltrating lymphocyte (TIL) therapy – enhance T-cell activity without a deliberate focus on specific antigens. Thus, one major question remains unsolved: what is the nature of the antigens that need to be recognized on human cancer to result in tumor control?

Here we discuss the repertoire of human tumor antigens by three main parameters. Firstly, the extent to which these antigens are shared by larger patient groups; secondly, the degree of tumor-selective expression; and finally, the likelihood of antigen loss the moment selection pressure is applied. Using this framework, we describe those classes of antigens that can be considered preferable targets in both active and passive T-cell-based cancer immunotherapy.
General intro and outline

With the EMA and FDA registration of the anti-CTLA4 antibody Ipilimumab for the treatment of metastatic melanoma [1], and with the highly encouraging clinical data of anti-PD1 antibodies in a number of human malignancies [2], cancer immunotherapy has reached mainstream oncology. Both anti-PD1 and anti-CTLA4 antibodies block inhibitory receptors on T lymphocytes and, based on data in mouse models, an involvement of the CD8+ subset of cytotoxic T cells in their clinical activity is plausible. Direct evidence for the ability of cytotoxic T cells to mediate human cancer regression has been obtained in clinical trials of adoptive T-cell therapy. Specifically, recent trials with genetically engineered T cells rendered reactive towards human MHC class I restricted tumor antigens have shown clear anti-tumor effects, albeit sometimes accompanied by significant toxicity (see further below). Furthermore, infusion of CD8-enriched TIL has shown clinical activity in patients with metastatic melanoma [3].

Collectively, these data indicate that at least part of the clinical activity of cancer immunotherapies is due to the recognition of MHC class I-restricted antigens that are expressed by human cancer cells. Interestingly though, the clinically most advanced form of immunotherapy, the blockade of T-cell checkpoint molecules, does not in any way aim to enhance T-cell reactivity towards specific tumor-associated antigens. Rather, T-cell checkpoint blockade ‘merely’ leads to an increase in the size and/ or activity of the T-cell compartment towards a diverse pool of antigens. The observation that such antigen non-specific interventions can lead to cancer regression – and in the case of anti-PD1 with relatively little evidence for autoimmune toxicity – suggests that the T-cell compartment does have a baseline activity towards tumors that is up and above that towards healthy tissues. Nevertheless, should it become feasible to steer T-cell activity towards defined tumor-associated antigens, this would likely be of value.

The first human tumor antigen recognized by autologous T cells, MAGE-A1, was identified by van der Bruggen and coworkers some 20 years ago [4], and in the subsequent decades, literally hundreds of tumor-associated T-cell epitopes have been described [5,6]. However, as already pointed out in a landmark review by Gilboa in 1999 (well worth rereading) [7], not all tumor-associated antigens will be equally attractive targets, both with respect to potential to induce cancer regression, with regard to safety, and with regard to the hurdles faced in clinical implementation.
A cube with tumor-associated antigens

The repertoire of human tumor-associated antigens can be evaluated with respect to three main characteristics: firstly, the extent to which antigens are shared between tumors of different patients; secondly, the degree to which antigens are selectively expressed by tumor cells, and finally, the ease with which tumor cells will lose expression of these antigens when immune pressure is applied. In this conceptual framework, each tumor antigen is a ‘dot’ in a three dimensional space, depending on how it scores with respect to these three characteristics. The ‘sweet spot’ in this cube is easy to define, antigens that are fully tumor restricted, that are shared between patients and that remain expressed even when T cell pressure is exerted. Alas, the sweet spot is relatively empty and we expect that further antigen discovery efforts will not change this. As such, it is essential to determine which characteristics we consider more or less important when aiming to enhance T-cell reactivity towards defined tumor antigens (Figure 1).

![A cube with tumor-associated antigens](image)

**Figure 1.** A cube with tumor antigens: 3-D representation of human tumor-associated antigens: Axes represent the likelihood of antigen retention upon T-cell pressure (X), degree of tumor restricted expression (Y), and degree of sharing between patients (Z). Each colored sphere shows a simplified representation of a group of antigens, defined within the text. Within each sphere a representative example is given. Yellow: neo-antigens (ATR$\_L$ [44]), green: differentiation antigens (MART-1 [9 and 10]), brown: C/G antigens (NY-ESO-1 [46]), orange: driver oncogene antigens (HPV16 E6 and E7 [47]), purple: overexpressed antigens (WT1 [48]).
Axis I: From shared antigens to patient-specific antigens

Historically, many research groups have focused on the identification of antigens that are expressed by tumors of large groups of patients, for the simple reason that it offered the promise of off-the-shelf therapeutic vaccines that could be used broadly. Because of this preference and because of experimental bias (‘who wants to work on T cells that only recognize one tumor’), a large majority of the epitopes that we currently know are derived from such shared antigens.

Shared antigens may either be restricted to a specific tumor type, or may be expressed by many different tumor types. An example of the latter class is formed by the cancer/germline (C/G) antigens, with the added note that expression is generally only seen in a rather small fraction of tumors of a given type (see for instance [8]). A second group of shared antigens is formed by proteins that display a cell lineage-specific expression pattern, such as the melanocyte differentiation antigens that are expressed in the majority of melanomas (for instance [9,10]). Likewise, antigens derived from viruses that are associated with cellular transformation, such as the HPV antigens in for instance cervical cancer and head and neck cancer, can also be considered ‘shared’. Finally, a series of epitopes has been described that is derived from proteins that are overexpressed within tumor cells, such as the WT1 antigen in leukemia [11].

While the shared antigens thus form a relatively heterogeneous group, at the other end of this axis we find only one specific group of antigens, those antigens that arise as a consequence of somatic mutations. Most neo-antigens are due to random mutations that are unrelated to cellular transformation and these antigens can therefore be considered highly patient-specific [12]. It should be noted though that a subgroup of the mutated neo-antigens is to some extent shared between patients. Specifically, those neo-antigens that are formed as a consequence of mutations that are associated with cellular transformation (and therefore the subject of Darwinian positive selection) such as the BRAFV600E and CDK4_R24C mutations in melanoma can be considered shared, and in particular for the CDK4_R24C epitope, evidence for T-cell recognition is strong [13,14]. Likewise, a small group of shared neo-antigens is observed in microsatellite instability-positive colorectal cancer, in which, for instance, the TGF-βRII gene contains frequent frameshift mutations in mononucleotide repeats [15,16].

As noted above, at least in part for trivial reasons, the majority of currently known antigens belong to the class of shared antigens. A key question that we feel should be addressed in the coming years is the relative contribution of shared versus non-shared antigens to the MHC-associated peptide repertoire on individual human tumors. In particular, for tumors with a high mutation load such as melanoma and non-small cell lung cancer, we consider it plausible that this repertoire will be dominated by patient-specific neo-antigens, something that would justify an increased focus on this group of antigens.
Axis II: From stable antigens to instable antigens
The moment immune pressure develops, selection of tumor cells that do not express a particular T-cell antigen can start to occur. Here, we will refer to those antigens that are either likely to be retained or likely to be lost under immune pressure as ‘stable’ and ‘instable’ antigens, respectively. The probability that tumors will escape a given immune pressure depends on only two fundamental factors: the likelihood that tumor cells that do not express a given antigen are formed or already present, and the net selective advantage that such antigen loss confers. Traditionally, the field has primarily focused on one specific aspect of the latter factor, by searching for epitopes in proteins considered essential for tumor cell survival or proliferation. Such (potential) epitopes include mutations in driver oncogenes such as the BRAFV600E mutation found in 40% of melanomas [17], and the CDK4R24C mutation that is an infrequent somatic mutation in melanoma [14]. In addition, also proteins of the MAGE family have been found to influence tumor cell survival [18,19] and expression of the PRAME antigen likewise confers a growth/survival advantage to melanoma cells [20].

While a sizable group of epitopes is thus derived from gene products that are or appear to be associated with tumor outgrowth, we wish to stress that this does not imply that tumor cell escape through antigen loss will not occur. Specifically, even known driver mutations have recently been shown to sometimes display a heterogeneous distribution pattern in human tumors [21]. By the same token, the rapid emergence of tumor resistance upon treatment of melanoma patients with small molecule BRAFV600E inhibitors [22,23] also suggests that human tumors may perhaps rely less on specific mutant proteins than previously appreciated. When aiming to target ‘stable epitopes’, it may therefore turn out to be more efficient to focus on those antigens that are cross-presented efficiently, rather than epitopes within oncogenes, as the resulting destruction of tumor stroma should make outgrowth of individual tumor cells that have lost these antigen less likely [24,25].

With respect to the likelihood that tumor cells that do not express a particular antigen are formed, for some antigens heterogeneous expression can already be observed without any immunotherapeutic intervention. For example, for reasons that to our knowledge have not been elucidated, C/G antigens often show heterogeneous expression patterns in human tumors. It is possible that such heterogeneous expression reflects an ongoing or prior T-cell-based selection. However, as naturally occurring immune responses against C/G antigens are often weak, we consider this less likely. As alternative explanations, heterogeneity in expression may potentially reflect multiple differentiation states of a single population of tumor cells, or could signify the presence of truly independent subclones with stochastic (in)activation of C/G antigen expression. In particular the second possibility would reduce the attractiveness of this antigen class, and it may be possible to resolve this issue by lineage tracing [26]. With regard to the possibility that the presence of tumor cells with various differentiation states could impact antigen recognition, recent exciting
work from the Tüting lab indicates that T cell activity can in fact be a causal factor in this process. Specifically, this work demonstrates that targeting of tumor-associated antigens can result in loss of expression of melanocyte differentiation antigens through TNF-alpha induced de-differentiation [27]. This observation provides one potential explanation for the finding that targeting of these antigens by gene-modified T cells has thus far led to only transient clinical responses [28].

**Axis III: From tumor-restricted to broadly expressed antigens**

At one extreme on this axis lie the neo-antigens that arise as a consequence of somatic mutations, but also virus-derived antigens such as the HPV oncoproteins. In the past, C/G antigens have often also been regarded as fully tumor-restricted, with expression only occurring within tumors and at immune privileged sites. However, more recent efforts to map the expression pattern of C/G genes in greater detail have revealed that at least a number of these antigens are expressed at varying levels in healthy tissue that is accessible to the immune system [29]. It is furthermore noted that the approaches with which expression of C/G antigens – and other antigens – have been analyzed may potentially miss expression within small subsets of cells. Thus, some of the C/G antigens that are currently considered safe targets may turn out not to be. Importantly though, a recent trial that utilized T cells modified with an NY-ESO-1-specific TCR indicates that at least some C/G antigens can be targeted by a high affinity T-cell compartment without noticeable toxicity [30].

Two additional groups of antigens can be distinguished that show a lower level of tumor selective expression. First, lineage differentiation antigens such as the melanocyte differentiation antigens are not only expressed in melanomas but also in healthy melanocytes in skin and eye, and in the inner ear. A recent TCR gene therapy trial by the Rosenberg group indicates that toxicity due to the targeting of such lineage differentiation antigens can be substantial [28]. An even broader expression pattern is observed for most proteins in the group of overexpressed self-antigens. The main argument for the targeting of this group of antigens is that ‘a window of opportunity’ may exist that allows T cells to distinguish between healthy tissue and tumor tissue on the basis of relative expression levels. Thus far, vaccine trials that have targeted these antigens have not shown any evidence of toxicity. However, in the absence of clear evidence for cancer regression in these trials, this may simply reflect the lack of induction of a robust high affinity T-cell response. In contrast, very severe toxicity has been observed in a clinical trial in which one of the overexpressed antigens, Her2neu, was targeted by T cells modified within a chimeric antigen receptor [31]. Likewise, targeting of CEA, another of the overexpressed antigens, by TCR-modified T cells has been shown to lead to severe colitis [32], mirroring prior observations by Offringa in a murine model [33].
What is recognized?

In the above section we have described the parameters that make different groups of antigens more or less attractive targets. Below, we describe how often these different groups of antigens are actually recognized by the endogenous T-cell repertoire, with a specific focus on melanoma, as data for other tumor types are still highly limited.

In early work by the Jotereau group, reactivity of melanoma TIL against target cells transfected with a large number of different melanocyte differentiation antigens or C/G antigens was compared [34]. This analysis demonstrated that reactivity against the melanocyte differentiation antigens MART-I and gp100, was common relative to that against C/G antigens. More recently, high-throughput MHC multimer-based technologies have been developed [35-38] that allow one to measure T-cell reactivity against large collections of antigens with a much higher sensitivity and independent of T-cell function. These technologies have now been utilized to assess reactivity of melanoma TIL against the entire collection of known melanoma associated epitopes, including epitopes from overexpressed antigens [6]. Echoing the Jotereau data, these experiments revealed a frequent recognition of the melanocyte differentiation antigens and only occasional reactivity against C/G antigens, with highly patient specific patterns of recognition. Analysis of expression of a limited number of C/G antigens and T-cell responses in matched samples suggests that the infrequent detection of T-cell responses against C/G antigens may in large part reflect the relatively rare expression of these antigens [13]. However, more data will be required to test this hypothesis.

An interesting observation in these MHC-multimer based analyses was that reactivity against the very large group of overexpressed antigens was highly uncommon. Intriguingly, of those five epitopes for which recognition was observed, four are atypical as they are formed through alternative splicing events or derived from alternative ORFs. Furthermore, most epitopes for which recognition was observed were identified through the use of patient-derived T-cell populations, rather than by the ‘reverse immunology’ approach that has been used for the majority of epitopes in overexpressed antigens. On the basis of these data we consider it plausible that many of the overexpressed antigens that have previously been identified for melanoma are likely to have little clinical relevance. We consider it possible though that this in part reflects the method of identification that has predominantly been used, an issue that can be overcome by the direct and unbiased identification of (overexpressed) antigens from the MHC-associated peptide repertoire on tumor cells [39].

Even though the recent high-throughput immunomonitoring studies looked at T-cell reactivity against very large collections of shared epitopes, the cumulative fraction of tumor-infiltrating T cells that were reactive with these epitopes was shown to be very
low [6,13]. One possible explanation for this observation could be that reactivity against patient-specific neo-antigens forms a substantial component. Prior work has clearly established that patient-specific neo-antigens can be recognized in human cancers, and in a heroic study it was shown for one patient that reactivity against patient-specific antigens can be stronger than that against shared antigens [12]. Furthermore, data from Coulie and colleagues have likewise suggested that T cell reactivity against neo-antigens may be prominent in melanoma patients [40]. However, for lack of suitable technology, systematic analyses of T-cell responses have not been possible.

Importantly, with the development of next-generation sequencing, it has recently become feasible to describe the repertoire of tumor-specific mutations within individual tumors with relative ease, and this information may subsequently be utilized to predict patient-specific mutated antigens [41]. In two recent studies, proof of principle for this approach has been provided in transplantable mouse tumor models. In a study by Sahin and colleagues, exome sequencing was used to identify mutations in the B16 melanoma, and 3 of 50 neo-epitopes investigated were subsequently shown to be recognized by T cells [42]. In a study by Robert Schreiber and coworkers, whole exome sequencing also led to the identification of a mutated epitope that was recognized by T cells. Furthermore, subsequent experiments showed how this epitope could be lost from the tumor cell population upon T-cell mediated pressure [43].

To determine whether exome-guided dissection of antigen specific T-cell responses can be used to understand tumor-specific T-cell reactivity in human cancer, we have recently performed analyses of autologous T-cell reactivity on the basis of melanoma whole exome sequencing data. These data reveal that dominant T-cell reactivities against patient-specific neo-antigens can be identified in humans using this strategy. Furthermore, such analyses can also be utilized to assess the effects of immunotherapeutic interventions, as revealed by a marked change in a neo-antigen specific T-cell response upon anti-CTLA4 treatment (van Rooij et al [44] and unpublished observations). As a side note, with still only very limited data available, it appears that T-cell responses against neo-antigens may often be of a higher magnitude than those against for instance the C/G antigens, a hypothesis that needs to be investigated further.

It is apparent that the new cancer genome driven methods of immunomonitoring will in some cases miss neo-epitopes, such as mutant epitopes that are subjected to post-translational modification or shuffled through protein splicing. Nevertheless, if these technologies can provide a reasonable overview of neo-antigen specific T-cell responses in cancer patients on a routine basis, their value will likely become large. For instance, it will be interesting to reveal whether neo-antigen specific T-cell reactivity is proportional to mutation load [41], and whether the presence of strong neo-antigen specific T-cell reactivity predict the effects of cancer immunotherapy.
In summary, for the one tumor type for which substantial data is available, the data suggest that firstly, T-cell responses against the currently known overexpressed antigens are uncommon relative to those against antigens with a more limited or fully tumor-restricted expression pattern. Secondly, T-cell responses are both observed against antigens that are likely to be retained by tumor cells and antigens for which no evidence for positive selection is available. Finally, T-cell responses against patient-specific antigens may – at least for melanoma – be as numerous (and conceivably more efficacious) than T-cell responses against shared antigens.

3. What would be preferred targets?

On the basis of the data discussed in the prior two sections, it seems reasonable to describe broad classes of preferred and less-preferred antigens for both active and passive immunotherapy, with the caveat that for most human tumors, our understanding of the tumor-expressed epitope repertoire is still piecemeal.

Broadly expressed versus tumor-restricted: This we consider by far the most important parameter. As based on data in mouse models, it seems plausible that the effect of antigen non-specific interventions can be enhanced by co-administration of antigen. We consider it less likely that such combined treatment will be effective when using many of the currently known overexpressed antigens, as T-cell responses against this group of antigens thus far appear very rare, presumably because of T-cell tolerance. Possibly, identification of overexpressed antigens by more advanced strategies may change this situation [39], but at present we would favor the use of antigens with a more restricted expression pattern.

With respect to antigen expression patterns and passive immunotherapy using T cells expressing high affinity TCRs, as based on the recent preclinical and clinical data, the requirement for a high level of tumor-specificity is without doubt very high here because of toxicity concerns. This excludes most of the broadly expressed antigens and likely even many of the C/G antigens as potential targets. We do note that for some overexpressed antigens, such as perhaps WT1, a sufficient level of tumor selectivity may still be reached by the sheer amount of overexpression, but we expect these examples to be exceptions.

Stable versus instable: This we consider the least important parameter. Certainly, all else being equal, it would appear attractive to target epitopes that are retained by tumor cells in spite of immune pressure, and the recent data from the Schreiber group indicate that immune editing can occur, at least in mouse models [43]. However, the dependence of tumors on individual oncogenes - the most commonly investigated category of presumed stable antigens - may be less than previously expected, and the bulk of the epitope repertoire on tumor cells may just not be derived from stable antigens. As such,
it may be more pragmatic to simply utilize a few antigens for which targeting in isolation would lead to rapid tumor escape, a concept validated by for instance the development of combination therapies for the management of AIDS.

*Shared versus patient specific:* This is where we feel that the field has most to gain, in particular with respect to active immunotherapy. For some human tumor types, highly tumor-restricted epitopes are expressed that are shared between patients, a case in point being the HPV antigens that have been targeted by for instance the Melief group [45]. However, for other human tumors, highly tumor-restricted antigens that are shared between patients may often not be found. Because of this, it would seem worthwhile to set up infrastructure for personalized active immunotherapies that would allow the targeting of antigens that are only infrequently expressed. As an example, non-specific active immunotherapies such as CTLA4 or PD-1 blockade may be combined with vaccines that specifically contain those C/G antigens for which expression has been shown in patient biopsy material. By the same token, on the basis of the recent data [42-44], it may in the longer run prove feasible to develop personalized immunotherapies that target patient-specific neo-antigens. While this brings us a long way from the off-the-shelf therapeutic vaccines once hoped for, the fact that this would allow targeting of truly tumor-specific antigens for which a high affinity T cell repertoire is present may make it well worth the effort.
References and recommended reading

Papers of particular interest have been highlighted as:

* of special interest
** of outstanding interest


* This study demonstrates the marked clinical potential of blockade of the PD2441 - PDL2441 axis in human cancer.


* This work shows that only a small fraction of tumor-infiltrating T-cells in melanoma are reactive with the known shared antigens.


Data in this study demonstrate that individual TIL cell products from melanoma patients contain unique patterns of reactivity against shared melanoma-associated antigens, and that the combined magnitude of these responses is surprisingly low.


mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood* 2009, **114**:535-546.


31. Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA: *Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2*. *Mol Ther* 2010, **18**:843-851.


* A sophisticated clinical study that uses a set of naturally presented epitopes on RCC for vaccination and demonstrates an association of vaccine-induced immune responses with clinical course.


** This is the first study describing how exome data can be used to identify neo-antigens in a mouse tumor model.


** This study demonstrates how a mutated epitope recognized by T cells, can be identified by whole exome sequencing, but also demonstrates how such an epitope can be lost from the tumor cell population upon T cell pressure.


** This is the first report to show how autologous cancer exome data can be employed to reveal T cell responses against patient-specific neo-antigens in humans.


