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The Role of Neoantigens in Naturally Occurring and Therapeutically Induced Immune Responses to Cancer

Jeffrey P. Ward[#], **Matthew M. Gubin**[#], and **Robert D. Schreiber**[2]

Washington University School of Medicine, St. Louis, MO, United States

[#] These authors contributed equally to this work.

Abstract

Definitive experimental evidence from mouse cancer models and strong correlative clinical data gave rise to the Cancer Immunoediting concept that explains the dual host-protective and tumor-promoting actions of immunity on developing cancers. Tumor-specific neoantigens can serve as targets of spontaneously arising adaptive immunity to cancer and thereby determine the ultimate fate of developing tumors. Tumor-specific neoantigens can also function as optimal targets of cancer immunotherapy against established tumors. These antigens are derived from nonsynonymous mutations that occur during cellular transformation and, because they are foreign to the host genome, are not subject to central tolerance. In this review, we summarize the experimental evidence indicating that cancer neoantigens are the source of both spontaneously occurring and therapeutically induced immune responses against cancer. We also review the advances in genomics, bioinformatics, and cancer immunotherapy that have facilitated identification of neoantigens and have moved personalized cancer immunotherapies into clinical trials, with the promise of providing more specific, safer, more effective, and perhaps even more generalizable treatments to cancer patients than current immunotherapies.

1. INTRODUCTION

After decades of controversy, the ability of the immune system to influence cancer development and progression has now become apparent (Grivennikov, Greten, & Karin, 2010; Mantovani, Allavena, Sica, & Balkwill, 2008; Schreiber, Old, & Smyth, 2011; Shankaran et al., 2001). Two parallel lines of investigation, one focused on assessing naturally occurring immune responses to developing cancers and the other focused on immunotherapy-induced durable responses to established tumors have ultimately led to unequivocal resolution of this long-standing argument. These independent approaches have demonstrated the importance of tumor-specific neoantigens as critical targets of antitumor immune responses (Schumacher & Schreiber, 2015). Immune recognition of neoantigens has the potential to destroy developing cancers before they become clinically apparent, shape the immunogenicities of cancer cells rendering them more fit to grow progressively in an immunocompetent environment, and ultimately to facilitate the immune elimination of growing tumors when manipulated in the appropriate therapeutic manner. The concept that

[2] Corresponding author: schreiber@immunology.wustl.edu.

neoantigens may be optimal targets for cancer immunotherapy is a very old one dating back to the 1940s and steadily evolving since that time (Table 1). The evolution of this idea has undergone a dramatic acceleration with the advent and employment of next generation sequencing and computational approaches which have made it possible to predict cancer specific mutations that function as neoantigens for adaptive immunity (Gubin, Artyomov, Mardis, & Schreiber, 2015). The analyses of therapeutically active neoantigens has also led to the realization that both major histocompatibility complex (MHC) class I (MHC I) and MHC class II (MHC II) epitopes are required for effective antitumor immune responses. These developments now leave cancer immunologists and clinical oncologists poised to develop truly personalized treatment approaches against established cancers with the goal of increasing specificity and eliminating toxicity compared to the current therapies. The focus of this review is to summarize the key experimental evidence that has led to a paradigm shift in thinking about immune system–cancer interactions resulting in the current excitement over using neoantigens as tumor-specific targets for immune control of cancer.

2. CANCER IMMUNOEDITING AS AN ENCOMPASSING MODEL OF IMMUNE SYSTEM–TUMOR INTERACTIONS

The dual host-protective and tumor-promoting actions of the immune system on developing cancers have been codified as a process termed “Cancer Immunoediting” (Fig. 1; Schreiber et al., 2011; Shankaran et al., 2001). Cancer Immunoediting initiates after cellular transformation has occurred and intrinsic tumor suppressor mechanisms have been circumvented. In its most complex form, Cancer Immunoediting is comprised of three phases: Elimination, Equilibrium, and Escape. In the Elimination phase, developing tumors are recognized and destroyed by the cooperative actions of innate and adaptive immunity long before they become clinically apparent. If the immune system fails to eliminate the entire tumor, the surviving cells may enter the Equilibrium phase where their overall expansion is immunologically restrained but where net tumor cell destruction does not occur. It is in Equilibrium that immunological sculpting occurs and if the “edited” tumor cells are altered to such an extent that they can no longer be identified as foreign by the host immune system, they begin to grow progressively, establish an immunosuppressive tumor microenvironment and emerge as the clinically apparent disease we know as cancer.

These naturally occurring immune system–tumor interactions were not always accepted and, in fact, were the subject of much scientific debate for most of the 20th century. In 1909, Paul Ehrlich first suggested that the immune system repressed cancer development in long-lived mammals (Ehrlich, 1909). However, this hypothesis could not be stringently tested because so little was known about the composition and function of the immune system at the time and tractable experimental systems to objectively evaluate the cell-extrinsic processes that controlled cancer development had not yet been developed. Five decades later, after a deeper understanding of the immune system had been obtained and inbred strains of mice had been developed that permitted studies of the immune system’s role in cancer development, F. MacFarlane Burnet and Lewis Thomas proposed the term “cancer immunosurveillance” to describe a process in which they envisaged that the immune system, and particularly T cells, could recognize and destroy transformed cells early in their development thereby protecting

the host against cancer outgrowth (Burnet, 1957, 1970; Thomas, 1959). If the immune system was indeed capable of detecting and eliminating newly transformed tumor cells, then cancer would be expected to occur with higher frequencies in immunodeficient compared to immunocompetent individuals. However, when this hypothesis was put to the experimental test in the 1970s by Osias Stutman, no evidence was found to support its validity (Stutman, 1974, 1979). Specifically, nude mice on a CBA/N genetic background (the only immunodeficient mouse strain available at that time) did not display higher tumor rates of spontaneous cancers or cancers induced by the chemical carcinogen 3^0-methylcholanthrene (MCA) than their wild-type counterparts. At the time, these experiments were considered so definitive that the concept of cancer immunosurveillance was summarily abandoned and the field developed arguments why the immune system could never see a developing tumor.

However, in the mid 1990s, it became clear that there were caveats to the Stutman conclusions that he could not have known at the time. Specifically, nude mice were subsequently found to possess some basal T cell function and thus were recognized as imperfect models of immunodeficiency (Hunig, 1983; Ikehara, Pahwa, Fernandes, Hansen, & Good, 1984; Maleckar & Sherman, 1987). The existence and antitumor functions of natural killer (NK) cells and other innate lymphocytes were also not known at the time (Herberman & Holden, 1978). The role of aryl hydroxylase isoforms in the bioconversion of MCA to its carcinogenic form was only appreciated two decades later together with the fact that CBA/N nude mice expressed the highest specific activity isoform of the enzyme (Heidelberger, 1975). The latter raised the possibility that carcinogenesis in the mice used by Stutman may have been too efficient for the immune system to control.

As a variety of better-characterized immunodeficient mouse strains became available, we and others subsequently showed that immunodeficient mice indeed develop more chemically induced and spontaneous tumors than their genetically matched immunocompetent counterparts. For example, *Rag2*$^{-/-}$ mice (which lack T, B, and natural killer T (NKT) cells), IFN-γ receptor-deficient mice (*IFNGR1*$^{-/-}$ mice), and mice lacking perforin (*pfp*$^{-/-}$ mice) treated with MCA develop tumors both more rapidly and with a higher frequency than wild-type mice (Kaplan et al., 1998; Shankaran et al., 2001; Smyth et al., 2000; Street, Cretney, & Smyth, 2001). The incidence of MCA sarcoma generation was lowest in wild-type mice, higher in *Rag2*$^{-/-}$ mice, and highest in *Rag2*$^{-/-}$ × γ*c*$^{-/-}$ mice (which lack all lymphocytes, including NK cells) implicating the innate immune system in the control of the outgrowth of developing tumors (O'Sullivan et al., 2012). In addition, when tumors derived from immunodeficient and immunocompetent mice were compared to one another, the former are more immunogenic and less tumorigenic than the latter (Shankaran et al., 2001). Thus the intact immune system not only protected against cancer development but also sculpted the immunogenicity of tumor cells that eventually formed, leading to cancers that were more fit to grow in an immunocompetent host. Tumors derived in immunodeficient mice were therefore highly immunogenic and were therefore called "unedited." In contrast, tumors derived from immunocompetent mice displayed reduced immunogenicity and were therefore called "edited" (Schreiber et al.,2011). Consequently, we introduced the term "Cancer Immunoediting" to stress the fact that immunity manifests both host-protective and tumor-promoting effects on developing cancers. This conclusion thereby significantly

broadened the concept of cancer immunosurveillance and better reflected the physiologic function of immunity in its interaction with cancer.

The concept of Cancer Immunoediting was solidified by clinical observations demonstrating that a similar process also occurred in humans. Based on historical data, it was long recognized that individuals with congenital immunodeficiencies displayed higher cancer rates, but many of these cancers were of infectious origins and therefore did not allow for unequivocal conclusions to be made (Penn, 1999). However, meta-analyses of clinical data revealed that organ transplant patients who were immunosuppressed as adults indeed displayed higher incidences of cancers with no known viral etiologies. For example, renal transplant patients from multiple institutions displayed higher incidences of colon, pancreas, lung, and endocrine cancers and melanoma compared to nontransplanted, non-immunosuppressed normal individuals (Birkeland et al., 1995) and reviewed in Dunn, Bruce, Ikeda, Old, and Schreiber (2002). In addition, cancer patients were often found to express T cells and antibodies specific for the tumors that they harbored (Dunn, Old, & Schreiber, 2004b). Some of the best-characterized cases were those involving paraneoplastic neurologic degenerations where patients presented with neurologic symptoms which were subsequently found to be the result of natural immune responses to cryptic neoplasia (Roberts, Perera, Lang, Vincent, & Newsom-Davis, 1985). Perhaps the best correlative evidence comes from the finding that cancer patients frequently show immune infiltrates into their tumors that are tumor specific and that the quantity, quality, and location of memory $CD8^+$ T cells in a patient's tumor can have prognostic value in determining the course of treatment for that patient (Galon et al., 2006). This approach has become known as the "Immunoscore" and, in the case of colorectal cancer, has been shown to have better predictive value than conventional tumor staging.

2.1 Elimination

Elimination, the first phase of Cancer Immunoediting, thus represents a modernized and expanded view of cancer immunosurveillance, where the molecules and cells of innate and adaptive immunity work together to recognize and destroy a developing tumor. The key components involved in the Elimination phase of Cancer Immunoediting include cells of both innate immunity [eg, NK, macrophages and dendritic cells (DCs)] and adaptive immunity (eg, NKT, $CD4^+$, and $CD8^+$ T cells; Smyth, Godfrey, & Trapani, 2001; Teng, Galon, Fridman, & Smyth, 2015). Similarly, host effector molecules such as tumor necrosis factor (TNF)-α, Fas/FasL, granzyme, perforin, TNF-related apoptosis-inducing ligand (TRAIL), as well as recognition molecules such as NKG2D in protective antitumor immunity have been shown to play critical roles in the Elimination Phase (Diefenbach, Jensen, Jamieson, & Raulet, 2001; Smyth, Cretney, et al., 2001) and reviewed in (Mittal, Gubin, Schreiber, & Smyth, 2014). Both type I interferons (IFN-α/β) and IFN-γ are required for the development of protective antitumor immune responses but play distinct roles in this phase of the process. Whereas IFN-γ targets both tumor and hematopoietic cells, IFN-α/β acts primarily on host cells (Diamond et al., 2011). Specifi-cally, in the mouse, type I IFNs enhance cross-presentation activity of tumor antigens by $CD8α^+$/$CD103^+$ DCs while IFN-γ promotes induction of $CD4^+$ T helper I (Th1) cells and $CD8^+$ cytotoxic T lymphocytes (CTL) and is the critical interferon for enhancing MHC I

expression on tumor cells (Diamond et al., 2011; Fuertes et al., 2011). If all cancer cells are eliminated, then the Elimination phase represents the full extent of the Cancer Immunoediting process.

2.2 Equilibrium

However, if some cancer cells survive, then the process can progress to the second phase—Equilibrium—a period when immunity is able to control the net outgrowth of cancer cells and thereby keep them clinically unapparent without completely eliminating them. Anecdotal evidence for the Equilibrium phase came from observations of cancer transfer following organ trans-plantation. In a particularly well-documented case, two patients who received kidney transplants from the same cadaver donor both subsequently developed malignant melanoma (MacKie, Reid, & Junor, 2003). The origins of the cancer were traced back to the donor who had been diagnosed with melanoma that had been successfully treated 16 years before death and who had been presumed to be cancer free. However, by transfer of a kidney from this donor into “na¨ıve” recipients who were then immunosuppressed to protect against graft rejection, it is presumed that tumor cells held in equilibrium by the donor’s immune system were then released from their dormant state and began to grow in a progressive manner. This clinical scenario was recapitulated in a defined preclinical model in 2007 that provided the first experimental validation of the postulated Equilibrium phase (Koebel et al., 2007). In that study, 80% of mice treated with low doses of MCA remained free of clinically apparent cancers for greater than 200 d. However, if these mice were treated on day 200 with a cocktail of monoclonal antibodies that eliminated $CD4^+$ and $CD8^+$ T cells and blocked IFN-γ, they showed a rapid appearance of sarcomas at the original site of MCA injection. Subsequent studies showed that adaptive immunity was the driver of the Equilibrium phase since antibodies that inhibited adaptive immunity (specifically anti-CD4, or anti-CD8 or anti-IFN-γ or anti-IL-12) released the dormant tumor cells from their equilibrium state while mAb that inhibit innate immunity [such as those that deplete NK cells (anti-NK1.1), inhibit NK cell recognition (anti-NKG2D), or block NK cell effector function (anti-TRAIL)] did not. Interestingly, dormant cancer cells were found in lesions that contained actively proliferating lymphocytes. Tumor cells held in Equilibrium retained their highly im-munogenic phenotype and thus remained unedited. In contrast, the rare dormant cancers that spontaneously progressed to actively growing tumors displayed reduced immunogenicity and thus had undergone editing. These results have been expanded to other tumor models including the use of mice lacking p53 (Teng et al., 2012) as well as a Tag-induced pancreatic cancer model where T cells arrested the growth of tumors via a mechanism dependent on IFN-γ and TNF (Braumuller et al., 2013). Equilibrium can represent an end stage of Cancer Immunoediting where cancer cells remain in a durable state of immunity-induced dormancy throughout the remaining lifespan of the host without progressing to clinically apparent cancer.

2.3 Escape

If editing results in a reduction of tumor immunogenicity such that the immune system can no longer control tumor cell outgrowth, an immuno-suppressive tumor microenvironment develops resulting in the outgrowth of tumor cell variants that eventually become clinically apparent tumors (ie, Escape). Thus Escape from immune control (the third phase of Cancer

Immunoediting) is now acknowledged to be one of the “Hallmarks of Cancer” (Hanahan & Weinberg, 2011).

Immune Escape can occur through many different mechanisms involving both changes in tumor cells and/or the microenvironment. Tumors may avoid immune recognition through loss of NKG2D ligands, down-regulation of MHC I, beta 2 microglobulin and calreticulin, reduced expression of costimulatory molecules, and/or antigen loss (extensively reviewed in Dunn, Old, & Schreiber, 2004a; Vesely et al., 2011). Tumor cells also upregulate proteins that allow increased resistance to apoptosis and promotion of survival (such as STAT-3 or the antiapoptotic molecule Bcl2; Yu, Pardoll, & Jove, 2009). Development of an immunosuppressive tumor microenvironment through recruitment of suppressive cells such as myeloid-derived suppressor cells and regulatory T cells (Tregs), production of immunosuppressive cytokines such as IL-10 and transforming growth factor beta (TGFβ) or expression of immune checkpoints of the B7 family such as programmed death ligand 1 (PD-L1)/PD-1, cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), T cell immunoglobulin and mucin domain 3 (TIM-3) by either tumor cells, immune cells, or both also promote immune escape (Mellman, Coukos, & Dranoff, 2011). Additionally a growing list of new moieties that contribute to tumor-induced immunosuppression such as T cell Immuno-globulin and ITIM Domain (TIGIT), CD73, V-domain Ig suppressor of T cell activation (VISTA), and B and T lymphocyte attenuator have been identified (Chauvin et al., 2015; Jin et al., 2010; Wang et al., 2011; Watanabe et al., 2003). Of these negative regulatory molecules, CTLA-4 was the first to be identified as a target to enhance T cell immunity in tumor-bearing mice (Leach et al., 1996) and was also the first to be targeted therapeutically in tumor-bearing patients (Hodi et al., 2003). CTLA-4 is a negative costimulatory receptor that is critical for maintaining immune homeostasis and preventing autoimmunity. Mice lacking CTLA-4 develop spontaneous lethal lymphoproliferative disease (Waterhouse et al., 1995) and humans treated with high-dose anti-CTLA-4 develop life-threatening immune complications (Gangadhar & Vonderheide, 2014). Importantly, landmark work by James Allison and colleagues revealed that CTLA-4 is responsible for the absence of reactivity of T cells against tumor antigens in tumor-bearing mice and patients and that T cell immunity to tumors can be enhanced following treatment with anti-CTLA-4 (Sharma & Allison, 2015; van Elsas, Hurwitz, & Allison, 1999; van Elsas et al., 2001). Allison’s work revealed a clinical benefit to manipulating the complex balance between therapeutic enhancements of antitumor immunity while maintaining control over autoimmunity. CTLA-4 expression on $CD4^+$ and $CD8^+$ T cells is temporally delayed compared to expression of its activating counterpart CD28 (Pardoll, 2012). During normal T cell activation, CD28 interacts with CD80/86 (B7.1/ B7.2) expressed on antigen presenting cells (APCs) and delivers a positive costimulatory signal to the responding T cell. However, this response is naturally regulated by expression of CTLA-4 that subsequently translocates to the T cell surface. CTLA-4 displays higher affinity to CD80/86 than CD28 and thus preferentially engages CD80/86 on target cells generating a negative costimulatory signal that shuts down T cell activation via mechanisms involving the protein phosphatases, SHP2 (PTPN11) and PP2A. Based on its mechanism of action, CTLA-4 is thought to primarily inhibit T cell priming. Thus in the context of a tumor-bearing individual, CTLA-4 expression on T cells blocks generation of new antitumor T cell specificities and thereby

contributes significantly to the immunosuppressive nature of the microenvironment of edited, progressively growing tumors.

Subsequent work by others has revealed that a second inhibitory receptor, PD-1, is also involved in limiting the activity of activated T cells in tumor-bearing individuals (Dong et al., 2002; Dong, Zhu, Tamada, & Chen, 1999; Freeman et al., 2000). Rather than blocking T cell priming as affected by CTLA-4, it functions to dampen T cell effector functions. PD-1-dependent T cell inhibition results following engagement with its ligands, PD-L1 (B7-H1) or PD-L2 (B7-H2) that can be expressed on tumor cells as well as host cells in the tumor microenvironment (Latchman et al., 2001). PD-1 is upregulated upon antigen stimulation and becomes highly expressed upon continuous or chronic T cell receptor (TCR) signaling (Barber et al., 2006). In contrast, PD-L1 is constitutively expressed by a wide variety of immune and nonimmune cells (such as T cells, NK cells, monocytes, macrophages, DC, B cells, epithelial cells, murine hepatocytes, and vascular endothelial cells) and many other cells upregulate PD-L1 in the presence of strong inflammatory signals (such as IFN-γ), presumably to limit tissue damage induced by potent but potentially destructive T cell responses (Loke & Allison, 2003). Additionally, some human and mouse tumors constitutively express high levels of PD-L1 and this appears to be a mechanism by which tumors evade immune Elimination (Iwai et al., 2002). Thus like CTLA-4, PD-1 contributes significantly to the immunosuppressive nature of the tumor microenvironment and thus facilitates outgrowth of edited tumors despite the fact that they may still possess some degree of immunogenicity. It is this characteristic that has permits for the success of checkpoint blockade cancer immunotherapy.

3. ANTIGENIC TARGETS OF CANCER IMMUNOEDITING

A central tenet of Cancer Immunoediting is that recognition of tumor antigens by T cells drives the immunological sculpting of cancers. Tumor antigens can be divided into three broad categories: (a) tumor-associated antigens (TAA), (b) cancer-germline/cancer testis antigens (CTA), and (c) tumor-specific antigens (TSAs) (Coulie, Van den Eynde, van der Bruggen, & Boon, 2014; Heemskerk, Kvistborg, & Schumacher, 2013).

TAA are comprised of proteins encoded by genes encoded in the normal genome that may represent either normal differentiation antigens (such as rearranged Ig and TCR genes expressed in B and T lymphomas, respectively) or aberrantly expressed normal proteins [eg, melanosomal proteins such as tyrosinase, gp100, and melanoma antigen recognized by T cells 1 (MART-1)]. In the mid 1990s multiple groups identified a number of shared melanocyte differentiation antigens (Bakker et al., 1994; Kawakami et al., 1994; Wang, Robbins, Kawakami, Kang, & Rosenberg, 1995). A common feature of these melanoma antigens is their expression by normal melanocytes in the skin and eye as well as their overexpression in malignant melanoma cells. Overexpressed normal proteins that possess growth/ survival-promoting functions [such as Wilms tumor 1 (WT1), a transcriptional regulator (Ohminami, Yasukawa, & Fujita, 2000); Survivin (an apoptosis inhibitor); Her2/neu (a growth factor receptor component) (Fisk, Blevins, Wharton, & Ioannides, 1995); or Telomerase (a senescence inhibitor)] represent TAA that directly participate in oncogenesis.

CTA is the second category of tumor antigens which are normally expressed in germ cells (testis and ovary) and trophoblast tissues as well as in cancer cells. Because of their relatively restricted tissue distribution, these antigens have represented attractive targets for immunotherapy. The first human CTA was identified using cDNA expression cloning in 1991 by Thierry Boon and colleagues. In this study, van der Bruggen et al. isolated a gene shared by a panel of melanoma cell lines that could be recognized by CTL in an HLA-A*01 restricted manner, and is now known as melanoma antigen family A1 (MAGE-A1; van der Bruggen et al., 1991) NY-ESO-1, subsequently identified by Lloyd Old, Ugur Sahin, and colleagues using serological analysis of recombinant cDNA expression libraries (SEREX), was cloned from an esophageal tumor and is one of the best-characterized human CTA with respect to its immunology (Chen et al., 1997). In addition to esophageal cancers, NY-ESO-1 is expressed in a wide range of tumors including hematopoietic cancers (eg, acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), and myeloma) and solid tumors (eg, breast, lung, melanoma, ovarian, sarcoma, urinary bladder, and uterine cancers). Both natural and therapeutically induced humoral and cellular immune responses against NY-ESO-1 have been well documented in cancer patients. In humans, CTA are particularly diverse where over 100 family members have been identified (Simpson, Caballero, Jungbluth, Chen, & Old, 2005). In contrast, CTA in mice are much less polydisperse (De Backer et al., 1995).

The third antigen category includes genes that are uniquely expressed in tumor cells and may represent either oncogenic viral proteins or abnormal proteins that arise as a consequence of somatic mutations or posttranslational modifications. In the former case, gene products of oncogenic viruses can represent TSA such as EBNA1 and LMP1/2 found in Hodgkin’s lymphoma and nasopharyngeal carcinoma; or Human papillomavirus (HPV) E6 and E7 expressed in cervical cancers. Spontaneously arising mutations, from exposure to carcinogens and/or from the genomic instability that is characteristic of neoplastic cells, can produce mutated proteins that function as TSA. These neoantigenic peptides can result from point mutations (missense mutations), alterations in the reading frame, extending the coding sequence beyond the normal stop codon (nonstop mutations), DNA insertions and deletions (Indels), or by chromosomal translocations (Heemskerk et al.). In contrast to TAA, TSA are almost exclusively unique to an individual. Support for this idea was originally documented in the first half of the 20th century by Foley, Gross, Prehn, Old, and colleagues using carcinogen-induced mouse tumors (Foley, 1953; Gross, 1943 Old, 1982; Prehn & Main, 1957). When mice were cured of their tumors by surgical resection and rechallenged with the same tumor cells, they were protected against rechallenge but not against challenge with independent tumors. Mice that were immunized with irradiated tumor cells were also protected against challenge with the same nonirradiated tumor, whereas mice pretreated with normal donor tissue were not protected. Other groups subsequently confirmed these results, leading to the widespread acceptance that mouse tumors and potentially human tumors could be specifically recognized by the immune system, at least under certain conditions. Additionally, it was found that tumor challenge and resection or tumor immunization was usually effective only when the immunizing tumor was the same as that used for the challenge, thus providing compelling evidence that the response was indeed tumor specific. Additional experiments performed in the 1970s by Thierry Boon and colleagues supported

the notion that the immune system could recognize TSA and provided some of the first experimental evidence that the response in part could be directed at mutant antigens (De Plaen et al., 1988). Upon treatment of a mouse carcinoma cell line in vitro with a strong mutagen, some tumor cell line variants from the treated population could not form progressively growing tumors when injected into naïve syngeneic mice. Strikingly, when the same mice that had rejected the tumors were rechallenged with the parental carcinoma line, those mice were protected against tumor growth, even though the parental carcinoma line was seemingly nonimmunogenic. These results were confirmed and extended by Hans Schreiber and colleagues using preclinical models of ultra-violet (UV)-induced mouse tumors with paired normal tissue from the same mouse in which the tumor originated, unequivocally demonstrating that somatic mutations could form TSA (Dubey et al., 1997; Monach, Meredith, Siegel, & Schreiber, 1995). The first human TSAs were discovered in 1995 when Wo€lfel et al. identified an R24C mutation in CDK4 by screening a cDNA library isolated from cultured melanoma cells while Coulie *et al.* isolated a mutation at an intron/exon boundary, both of which formed immunogenic peptides that could be recognized by autologous CTL (Coulie et al., 1995; Wolfel et al., 1995). While most cancer mutations are private, a fraction of mutations are indeed shared between different cancers and different patients. In some cases, driver mutations can be immunogenic including those formed from mutant RAS (Linard et al., 2002) or BRAF (Somasundaram et al., 2006), as well as chromosomal fusions such as BCR-ABL or TEL-AML (Greco et al., 1996).

Additional neoantigens that may be shared between different cancers can result from aberrant phosphorylation. During transformation, protein kinase activity becomes dysregulated, leading to hyperphosphorylation of signaling proteins and changes in proliferation, differentiation, and cell growth. Phosphorylated residues can enhance the stability of individual peptides for both MHC I (Mohammed et al., 2008; Zarling et al., 2000; Zarling et al., 2006) and MHC II (Li et al., 2010). This observation suggests that phosphoproteins may be a particularly desirable shared target for cancer immunotherapy, as aberrantly phosphorylated residues may not have been subject to central tolerance. Cobbold et al. identified 10 phosphopeptides presented by HLA-A*02:01 and 85 presented by HLA-B*07:02 from a panel of hematologic malignancies including both leukemia and lymphoma specimens using a mass spectrometry approach (Cobbold et al., 2013). Many of these phosphopeptides were derived from signaling molecules with well-established roles as drivers of transformation. Interestingly, there were more than two-fold more phosphopeptides detected from aggressive malignancies (AML and ALL) compared with more indolent cancers [chronic lymphocytic leukemia (CLL) and hairy cell leukemia (HCL)] or from healthy tissue. In addition, CTL lines could be derived from healthy individuals specific for only the phosphorylated form of LSP-1, a lymphoma marker, which were capable of recognizing HLA-A*02:01 positive cell lines derived from AML and CLL patients. Phosphopeptide-specific T cells predominantly had the phenotype of the central memory compartment, suggesting that most healthy individuals had mounted responses to tumor-associated phosphopeptides during their lifetimes, at frequencies similar to those responding to nonper-sistent viruses. However, responses against a panel of phosphopeptides were reduced or absent in patients with active CLL, suggesting that patients with intact responses against phosphopeptides had improved survival. However, due to the small sample

size available, this analysis did not reach statistical significance. Perhaps most significantly, a profound recovery in responses against phosphopeptides was observed in several patients with AML after undergoing an allogeneic stem cell transplant, and a phosphoprotein-specific CTL line from a patient isolate was able to kill an AML cell line in vitro. These findings suggest that the selection of transplant donors by the presence of phosphoprotein-specific responses in addition to standard criteria may be an attractive option to prevent disease relapse. Phosphopeptides expressed by solid tumors have also been identified, and TCRs specific for phospho-proteins may serve as additional epitope determinants for transgenic T cells (Zarling et al., 2014). Techniques allowing detection of potential phosphoneoantigens with mass spectrometry approaches using small amounts of starting material are available (Abelin et al., 2015).

In additional to aberrant phosphorylation, dysregulated glycosylation of proteins can lead to formation of tumor neoantigens. As normal tissues trans-form, changes in glycosylation in proteins integral to cell adhesion, motility, invasiveness, and signaling occur with the potential to form antigens for immune detection (Ono & Hakomori, 2004). Therapeutic antibodies can strongly bind to glycan epitopes that are uniquely expressed on the target tumor cell population as compared to normal tissues to disrupt molecules required for neoplastic cell growth, as well as to mediate antibody-dependent cellular cytotoxicity (Dingjan et al., 2015).

4. SETTING THE GROUNDWORK: GENOMIC APPROACHES TO CANCER ANTIGEN IDENTIFICATION

Advances in next generation sequencing allowed for whole genome sequencing of cancers and a better understanding of the mutational landscape present in many cancers (Koboldt, Steinberg, Larson, Wilson, & Mardis, 2013). In 2008, James Allison and Bert Vogelstein performed in silico analysis combining breast and colorectal cancer-sequencing data with epitope prediction algorithms and hypothesized that breast and colorectal cancers accumulate unique HLA epitopes (Segal et al., 2008). They proposed that as cancer is a process where transformed tissues accumulate genetic changes over time, all cancers would contain mutations with a potential to form epitopes recognizable by the immune system. Subsequent studies have further demonstrated that cancers over a broad spectrum contain a remarkable number of mutations that could form epitopes (Alexandrov et al., 2013). Lessons from preclinical models have helped shape our understanding of the mutational landscape that is surveyed by the immune system.

Two independent reports in 2012 used genomic sequencing and epitope prediction algorithms to identify mutant neoantigens responsible for rejection of a highly immunogenic unedited tumor (Matsushita et al., 2012) or an edited progressively growing tumor in a mouse prophylactically vaccinated with neoantigen-specific synthetic long peptides (SLP) (Castle et al., 2012). The highly immunogenic unedited MCA sarcoma line (d42m1) derived from immunodeficient *Rag2*$^{-/-}$ mice was subjected to cDNA capture sequencing to identify expressed missense mutations. These mutations were then computationally translated into corresponding proteins and pipelined into MHC I binding algorithms to

predict strong MHC I binders. A mutation in the highly expressed protein spectrin-β2 was predicted and subsequently validated as a major rejection antigen responsible for the spontaneous rejection of the d42m1 tumor when transplanted into syngeneic wild-type immunocompetent mice. Importantly, when d42m1 was passaged through immunocompetent mice, it underwent cancer immunoediting, leading to outgrowth of preexisting tumor cells that lacked mutant spectrin-β2. A complementary study from Jacks and colleagues reached similar conclusions about Cancer Immunoediting using a genetically engineered mouse model of cancer (DuPage et al., 2012). Both of these studies demonstrated that selection for tumor variants that do not express strong antigens is one mechanism of Cancer Immunoediting. Importantly, although edited tumors from both mice and human may lack strong antigens required for *spontaneous* rejection, some still retain antigens that confer residual immunogenicity to the tumor that can be accessed by the proper type of cancer immunotherapy.

Using a weakly immunogenic melanoma tumor line derived from an immunocompetent mouse (B16-F10), Sahin and colleagues developed prophylactic personalized neoantigen-specific SLP vaccines based on predicted MHC I binding scores of mutant peptides identified by genomic sequencing. Analysis of 50 peptides revealed 16 to be immunogenic. Furthermore, two of the peptides provided prophylactic protection against tumor growth when incorporated into an SLP vaccine. These studies were the first to experimentally demonstrate that genomic sequencing and epitope prediction algorithms could identify mutant rejection antigens. These studies were soon to be followed by an explosion in genomic analyses to inform antitumor immune responses.

5. DEVELOPING CANCER IMMUNOTHERAPIES BASED ON GENOMIC IDENTIFICATION OF TUMOR-SPECIFIC NEOANTIGENS

Using neoantigens for therapeutic benefit has significant conceptual advantages over the use of TAA. The former are expressed exclusively by transformed cells and therefore are similar to foreign proteins in that they are not subject to central immunological tolerance. Perhaps equally important, neoantigens are tumor specific and therefore targeting them obviates concerns about cytotoxicity toward healthy tissue. Indeed, accumulating data suggest that neoantigens are important components of cancer immunotherapy. In 2005 a seminal study by Lennerz et al. used a cDNA library from a patient-derived melanoma line to screen autologous T cells and identified several neoantigens that induced T cell responses as assessed by IFN-γ ELISPOT (Lennerz et al., 2005). Reactivity against these neoantigens dominated the tumor-specific T cell response in the patient. That same year, Rosenberg and Robbins identified multiple neoantigens recognized by adoptively transferred tumor infiltrating lymphocytes (TIL) in a single patient with metastatic melanoma treated with adoptive T cell immunotherapy (Zhou et al., 2005). Importantly the neoantigen-specific T cells persisted in the patient. These data were also some of the first to suggest that the relevant T cell clones existed before ex vivo expansion and that the autologous T cell therapy was amplifying preexisting T cell responses.

The reverse immunology approaches for mutant tumor antigen identification used in the Matsushita et al. and Castle et al. studies made the possibility of designing patient-specific treatments exploiting the full repertoire of a patient's antigenome a reality. For this purpose, mutations are identified through deep sequencing, the region surrounding the mutation are virtually "translated," and then input into epitope prediction algorithms. Critical to this endeavor is the need to be able to correctly identify somatic mutations using next generation deep sequencing and accurately predict those mutations that form immunogenic neoantigens.

In order for an antigen to be immunogenic, it must be presented by MHC and recognized by T cells through their TCR. Whereas MHC I binds antigens of 8–11 amino acids in length and presents them to cytotoxic $CD8^+$ T cells, MHC II presents antigens of 11–20 amino acids to $CD4^+$ T cells (Babbitt et al., 1985; Bjorkman et al., 1987). The MHC alleles are remarkably diverse and the number of potential peptides processed from a given pathogen or tumor is also large, with a small minority actually binding to the MHC. This makes predicting which peptides will bind MHC challenging. Fortunately, multiple computational algorithms for prediction of antigen processing, presentation, and immunogenicity exist.

5.1 Epitope Prediction Algorithms

Multiple prediction tools for MHC I binding exist, with SYFPEITHI developed by Hans-Georg Rammensee being the first widely used and validated method (Rammensee et al., 1999). Subsequently, other prediction algorithms have been developed including those available from the Immune Epitope Database and Analysis Resource (IEDB; www.iedb.org; Vita et al., 2015). The IEDB is an online comprehensive database of T cell epitopes and tools for predicting MHC binding with the most commonly used prediction tools for MHC I tumor antigens being: (a) artificial neural networks (ANN)/NetMHC (Lundegaard et al., 2008; Nielsen et al., 2003), (b) NetMHCpan (Nielsen et al., 2007), and (c) SMMPMBEC and SMM (Kim, Sidney, Pinilla, Sette, & Peters, 2009; Peters & Sette, 2005). ANNs, like NetMHC, are algorithms modeled after the neural connections in the brain, learning from a set of input training data. NetMHC is one of the most commonly used and best validated epitope prediction programs available. While common allele predictions are often quite accurate, rare alleles are trained and validated on fewer data and thus are usually less accurate. To address this issue, pan-specific programs, such as NetMHCpan, were created to extrapolate from existing data to less common alleles. SMM and SMMPMBEC, as described by Sette and Peters, calculate matrices from affinity data of peptides binding to MHC. This allows for suppression of noise caused by the inevitable experimental error as well as limited data points present in the training data. Other prediction tools available from IEDB include ARB, Comblib_Sidney2008, Pickpocket, and Consensus.

For most studies, the primary selection criterion for predicting epitopes is the binding affinity of the peptide epitope for MHC I. However, whether the peptide is even available to bind MHC I, that is how efficiently the antigen is processed, also needs to be considered. Antigen processing involves the degradation of proteins within the cytoplasm by the proteasome (a specialized proteasomal complex induced by IFN-γ called the immunoproteasome is primarily responsible for the degradation of proteins into peptides that

are optimal in size for MHC binding), followed by transportation of the peptides to the endoplasmic reticulum via the transporter associated with antigen processing (TAP) proteins (Blum, Wearsch, & Cresswell, 2013; Rock et al., 1994). Algorithms exist to predict both proteasomal cleavage and TAP transport. NetChop uses a neural network to predict proteasomal processing (Nielsen, Lundegaard, Lund, & Kesmir, 2005), whereas NetCTL and NetCTLpan also use a neural network to predict T cell epitopes but they combine predicted MHC binding, proteasomal cleavage, and TAP transport to generate a score (Peters, Bulik, Tampe, Van Endert, & Holzhutter, 2003). The MHC-NP algorithm assesses the probability that a peptide is naturally processed and binds to a given MHC based on data obtained from MHC elution experiments (Giguere et al., 2013).

In contrast to MHC I, predicting MHC II epitopes has remained a more difficult challenge. MHC II prediction methods have consistently underperformed MHC I, in part due to a paucity of data sets for MHC II training. Nevertheless, multiple MHC II binding algorithms are available with the most commonly used being NetMHCII (Nielsen & Lund, 2009) and TEPITOPEpan (Hammer et al., 1994).

There are also tools available that predict the relative ability of a peptide– MHC complex to elicit a T cell response, taking into account the amino acid properties as well as position in the peptide sequence. Peters and Sette have developed a model of peptide–MHC properties that enhance immunogenicity and this prediction tool is available from IEDB (Calis et al., 2013). Other T cell reactivity predictors include POPI, iMatrix (models the TCR-peptide and peptide–MHC interface), and CTLPred. However, these algorithms have limitations, as they have not been extensively validated. Combining both antigen processing and MHC binding should result in an increased accuracy of predicted epitopes. However, these algorithms are limited by the available data and are only as good as the data used to generate them. Since these models are currently based on restricted data sets from either in vitro studies or data sets based on previously identified T cell epitopes, the accuracy of the results should continue to improve as more antigens are identified.

5.2 Retrospective Bioinformatic Analyses of Previously Identified Cancer Neoantigens

The ability to identify mutant neoantigens permits a deeper understanding of the immune responses to cancer and raises the promise of therapeutic use of these antigens. Insights into the immunogenic mutant neoantigen landscape of many human cancers have come from recent studies from Wu and Hacohen (Fritsch et al., 2014). They determined that a large majority of known mutated neoantigens in multiple tumor types from patients experiencing long-term survival or tumor regression had strong or moderate predicted MHC I binding affinity for their respective alleles. In a separate report, they used bioinformatic and experimental approaches to explore the epitope landscape of 91 CLLs and predicted an average of 22 mutated HLA-binding peptides per CLL (Rajasagi et al., 2014). Further analysis of two patients that achieved long-term remission revealed CTL responses against predicted neoantigens could be detected. Application of their epitope prediction approach to sequencing data from many different cancer types revealed a range of predicted neoantigens per individual tumor, providing evidence that neoantigens are frequent in most human cancers.

A similar retrospective analysis was performed by Schumacher and colleagues, where data sets of known human cancer neoantigens were analyzed to determine whether they would have been identified using genomic approaches (van Buuren, Calis, & Schumacher, 2014). Specifically the following criteria were assessed: (1) sequencing coverage to allow confident calling of the mutant base, (2) NetChop cleavage probability of 0.5 or greater, (3) predicted binding affinity [predicted half-maximal inhibitory concentration (IC_{50})] of less than 500 nM using the NetMHCpan algorithm, and (4) low "similarity-to-self" of the mutant compared to wild-type epitope. The authors describe this "similarity-to-self" test as one that determines the likelihood that the mutant and wild-type epitopes can be distinguished by the T cells, either by altered levels of mutant antigen presentation or by an altered structure of the MHC-presented mutant antigen. Here again, algorithms were used to assess whether the potential mutant neoepitope would be presented at higher levels than the wild-type epitope. If the wild-type parental sequence was not predicted to be presented by MHC either because it was not processed or did not bind MHC, the peptide was considered different than self. Additionally, they explored whether the mutations altered the peptide–MHC/TCR interaction. In general, the TCR exposed surface lies in the core region of the epitope, which is the peptide sequence between the two anchor residues. If the core region of the mutant epitope is different than that of the parental sequence using a peptide:MHC binding energy covariance (PMBEC) value of ≤0.05, the mutant peptide is considered different than self. This analysis suggested that the current available methods for neoantigen prediction are relatively accurate and these methods would identify most of the previously known neoantigens.

5.3 Experimental Evidence from Preclinical Cancer Models That Neoantigens Form the Basis for Effective Personalized Cancer Immunotherapy

Experimental validation that mutant neoantigens identified by genomic and bioinformatics approaches can function in a therapeutic setting came from three studies that were published in 2014–2015. One study, stemming from work in our laboratory, employed a MCA sarcoma line (T3) that forms progressively growing tumors when transplanted into na¨ıve syngeneic immuno-competent mice but is rejected in tumor-bearing mice following treatment with monoclonal antibodies blocking CTLA-4 or PD-1 (Gubin et al., 2014). Genomic sequencing analysis of T3, as illustrated in Fig. 2, followed by epitope prediction revealed two predominant H-2K^b epitopes [a G1254V mutation in Laminin α subunit 4 (mLama4) and a A506T mutation in Asparagine-linked glycosylation 8 (α−1,3-glucosyltransferase) (mAlg8)] as being the most likely targets of T cells activated by checkpoint blockade therapy. This prediction was validated by ex vivo screening of TIL isolated directly from tumors using either a panel of H-2K^b MHC I tetramers carrying one of the top 62 predicted H-2K^b epitopes or four top predicted H-2D^b epitopes as well as testing the eluted T cells for antigen-specific stimulation as detected by intracellular cytokine staining following coincubation with irradiated splenocyte feeder cells pulsed with the individual predicted peptides (Fig. 3). The validity of these findings were further confirmed by the following criteria: (a) the same epitopes were identified when tested on CTL lines generated from mice that had rejected T3 tumors following anti-PD-1 treatment, (b) the mutant epitopes were identified by mass spectrometry on IFN-γ-treated T3 tumors propagated in vitro, (c) tetramer positive staining T cells accumulated temporally in progressively growing tumors in

vivo in mice treated with anti-PD-1 and reached maximal levels just prior to tumor rejection, and (d) prophylactic vaccination of mice with a combination of mLama4 and mAlg8 peptides protected the mice from subsequent challenge with T3 tumor cells. Perhaps most importantly, growing T3 tumors were rejected in mice treated with a therapeutic vaccine comprised of SLP encompassing the mLama4 and mAlg8 mutations together with the adjuvant Poly I:C. Rejection induced by the therapeutic vaccine was nearly as effective as treatment of tumor-bearing mice with checkpoint antibodies. Rejection was observed only rarely with Poly I:C alone or with an irrelevant SLP vaccine plus Poly I:C.

Similar results were obtained in a contemporary study by Yadav et al. who used mass spectrometry in combination with whole-exome and transcriptome sequencing to predict immunogenic TSA expressed by the carcinogen-induced colon adenocarcinoma MC-38 and the model prostate cancer TRAMP-C1 (Yadav et al., 2014). Of the 1290 and 67 expressed mutations found in MC-38 and TRAMP-C1, respectively, 7 were found to be presented on MHC I in MC-38 and none by TRAMP-C1 MHC I. All but one of the identified neoepitopes were predicted by the NetMHC algorithm to bind MHC I ($IC_{50} < 500$ nM). Of these identified mutant neoantigens, mutant forms of Reps1, Adpgk, and Dpagt1 protected mice from subsequent tumor challenge, achieving therapeutic tumor protection when administered together with agonist anti-CD40 antibody.

In the most recent studies, the Sahin group demonstrated using three separate preclinical cancer models that the majority of the predicted TSA in fact elicit $CD4^+$ T cells responses upon vaccination, even when vaccine epitopes are predicted based on MHC I prediction algorithms (Kreiter et al., 2015). Strikingly, elicitation of $CD4^+$ T cell responses using either peptide or RNA vaccination mediated protection from established tumors and was also shown to induce responses against additional MHC I epitopes through epitope spreading. In a separate study, Platten and colleagues used an SLP containing a MHC II epitope corresponding to mutated isocitrate dehydrogenase type 1 (IDH1), a mutation commonly found in a subgroup of gliomas, to demonstrate immune control of preestablished syngeneic IDH1 (R132H)-expressing tumor cells transplanted into mice devoid of mouse MHC and transgenic for human MHC I and MHC II (Schumacheret al., 2014). Together these studies demonstrate that neoantigen vaccines can be highly effective in therapeutically controlling established tumors and even inducing their immune elimination when the antigens included in the vaccine include both MHC I and MHC II epitopes. While it is clear that $CD8^+$ T cells can directly kill tumors expressing MHC I and produce antitumor effector cytokines, the role of MHC II antigens is less obvious. $CD4^+$ T cells may exert antitumor effects through the production of antitumor effector cytokines, licensing of DCs, or direct effects on tumors expressing MHC II. More work is needed to delineate the mechanism behind the antitumor effects of $CD4^+$ T cells. This work has encouraged renewed enthusiasm for development of tumor-specific vaccines as a method to treat cancer that may be more specific, safer, and potentially more effective than the methodologies that are available to us today.

6. NEOANTIGENS AS THERAPEUTIC TARGETS IN HUMAN CANCER

As our understanding of the dual functions of the immune system to both eliminate and sculpt the development of progressively growing tumors evolved, so too did the capacity to

use the immune system as a therapeutic tool to control cancer. The recognition that tumor antigens were key to the immune system's capacity to discriminate between cancer cells and normal self formed the basis for many early clinical vaccine trials targeting TAA and subsequently CTA as antigens. While occasional successes were observed in these approaches, the overall response rates were disappointing and at best were very tumor-type specific (Rosenberg, Yang, & Restifo, 2004).

However, two more recent immunotherapeutic modalities (adoptive T cell therapy and checkpoint blockade) are displaying significantly higher response rates and display efficacy toward a much wider range of tumor types. It is of great significance that these more successful new therapies are directed, at least in part, against tumor-specific mutant neoantigens and based on this finding, clinical trials are now ongoing in many institutions that are exploring the use of personalized cancer immunotherapies based on targeting cancer-specific neoantigens (Table 2).

Support for this latter concept has come, in part, from correlative studies of the mutational load in various cancers and the response of a patient bearing these cancers to immunotherapy. Despite the potential for durable responses with the newer types of cancer immunotherapy, only a percentage of patients achieve objective responses to cancer immunotherapy (Hodiet al., 2010; Rosenberg et al., 2011). Because of the stochastic process by which mutations that form neoantigens are generated during cellular transformation, and because cancer immunotherapy relies on expression of antigens for both $CD4^+$ and $CD8^+$ T cells, genomics approaches are being investigated to develop a predictive biomarker of response to therapy. The genomic landscape of some tumors such as melanoma is characterized by a high mutational load (Alexandrov et al., 2013) as a consequence of exposure to UV light, which results in expression of a significant number of aberrant proteins products never before seen by the immune system capable of functioning as antigenic targets of a tumor-specific immune response. Using next generation sequencing, Snyder et al. demonstrated a correlation between clinical benefit from CTLA-4 blockade and the mutational load in metastatic melanoma (Snyder et al., 2014). This finding was subsequently validated by Van Allen et al. who used larger patient cohorts (Van Allen et al., 2015). This finding is not limited to melanoma, as a similar analysis in patients with non-small cell lung cancer also found that a correlation between a tumor's nonsynonymous mutation burden and objective patient response to PD-1 blockade exists (Rizvi et al., 2015). Many other histologies that result in a sizable fraction of human malignancies have mutation ranges that fall between 1 and 10 somatic mutations per megabase and thus are likely to express sufficient neoantigenicity to render them immunogenic. It remains an open question if immunotherapy approaches can be designed to induce therapeutic responses against tumors that express lower antigen burdens.

In a 515 patient study, RNA-sequencing analysis revealed increased numbers of mutational epitopes were associated with increased patient survival, higher intratumoral CTL content, and upregulation of genes encoding the immune checkpoints PD-1 and CTLA-4 (Brown et al., 2014). Little evidence of CTL infiltration was present in tumors with few mutational epitopes. This study provided the foundation for an extensive genomic analysis by Hacohen and colleagues using TCGA data sets of solid tumor biopsies. Rooney et al. derived a

cytolytic index matrix based on expression of perforin and granzyme B (Rooney et al., 2015). When compared across 18 tumor types, this cytolytic score correlated with neoantigen load, as well as expression of viral transcripts. In addition, fewer neoantigens were present in colorectal tumors (CRC) than would be expected based on their mutation rate, implying that strong immune pressure had exerted a sculpting effect on the tumors as they developed. Interestingly, despite their restricted expression, CTA did not correlate with cytolytic function. Mutations in genes with clearly established immune functions, such as beta 2 microglobulin (Restifo et al., 1996), MHC I heavy chains (Shukla et al., 2015), and caspase 8 were also enriched in tumor tissues, which would be expected to be selected for in tumors that escape immune control.

Attempts have also been made to correlate neoantigen load and the like-lihood of response to immunotherapies in gastrointestinal malignancies. Using a cohort of 103 colorectal cancers with microsatellite instability, Maby et al. showed that $CD8^+$ TIL density correlates with the total number of frameshift mutations (Maby et al., 2015). Peripheral $CD8^+$ T cells derived from patients with microsatellite unstable colon cancer could lyse target cells pulsed with predicted neoepitopes derived from frameshift mutations after in vitro culture. Taken together, these results suggest that immunogenic neoantigens are more likely to arise in genetically unstable tumors and drive the T cell-dependent cytolytic activity that is critical to effect Cancer Immunoediting and immunotherapy. Interestingly, microsatellite instability $(MSI)^{hi}$ colorectal cancers represent the only CRC subset that is susceptible to checkpoint blockade immunotherapy (Le et al., 2015), a result that once again supports the hypothesis that cancer-specific mutant neoantigens are the favored targets of T cells that can be reactivated by this type of immunotherapy.

Finally, transcriptomic analysis on a subset of tumors from melanoma patients demonstrated that a cytolytic gene signature, along with elevated transcript expression of PD-L2, correlated with neoantigen load and response to ipilimumab (Van Allen et al., 2015). Interestingly the expression of CTLA-4 itself was an indicator of response. These findings may reflect the ongoing preexisting T cell responses possibly against mutant neoantigens, especially considering the presence of subsets of melanoma patients with inflamed tumor microenvironments that are a result of $CD8^+$ T cell reactivity as demonstrated by the Gajewski laboratory (Spranger et al., 2013) as well as findings suggesting that increased numbers of PD-L1 positive $CD8^+$ T cells correlates with response to PD-1 blockade (Tumeh et al., 2014). This concept is not limited to prediction of response in melanoma, as a correlation between antigen load and response to pembrolizumab also exists in NSCLC (Rizvi et al., 2015).

6.1 Neoantigens in Adoptive Cellular Therapy in Humans

As cancer immunoediting of developing tumors progresses from equilibrium to escape, the balance shifts toward cancer progression as adaptive immunity loses its ability to control tumor growth. By removing tumor-specific T cells from the inhibitory tumor microenvironment and allowing them to regain their cytotoxic function ex vivo prior to transfer back into the patient, adoptive cellular therapy (ACT) with TIL attempts to reverse this transition and achieve tumor elimination. In a description of their recent experience, the

Steven Rosenberg group at the Surgery Branch of the National Cancer Institute treated 93 metastatic melanoma patients with infusion of autologous T cells in conjunction with IL-2 and different lymphodepleting regimens (Rosenberg et al., 2011). Response rates in this patient cohort varied between 49% and 72%. More impressively, 19 of the 20 patients who displayed a complete remission had responses that were durable beyond 3 years. Similar results have been reported in smaller series from other centers (Radvanyi et al., 2012).

Whether TAA can serve as the targets of the immune response during ACT has been intensively investigated. Initial studies on TILs from melanoma patients focused on the identification of T cell populations specific for shared TAA such as gp100, MART-1, and tyrosinase-related protein 1 (reviewed by Coulie et al., 2014). Despite their presence in normal tissues, CTL targeting these TAA rarely caused severe autoimmune toxicities, but their frequencies in TILs were usually quite low (Kvistborg et al., 2012). In two more recent studies, transfer of T cells highly selected for the melanocyte differentiation antigens gp100 and MART-1 led to clonal engraftment and autoimmune dermatitis in the majority of patients, but no objective responses (Chandran et al., 2015). Experiences such as these led to attempts to design transgenic TCRs with higher affinity for TAAs in the hope that this would increase the efficacy of tumor cell killing by effector cells. The Rosenberg group developed approaches to isolate high-affinity TCRs against gp100 and MART-1 using immunization of mice transgenic for HLA-A*02 or selection for high-affinity TCRs from T cell clones, respectively (Johnson et al., 2009). The genes encoding these TCRs were then transduced into autologous peripheral blood T cells that were subsequently used for adoptive transfer. This approach successfully led to objective responses in nearly a third of patients with metastatic melanoma. However, vitiligo and loss of vision and hearing were frequently seen in this study as a consequence of the destruction of normal melanocytes present in the skin, eye, and ear, respectively. In another example, despite extensive preclinical testing without an indication of off-target effects, an affinity-enhanced TCR specific for MAGE A3 caused fatal toxicity in two patients due to cross-recognition of the muscle protein Titin that was only detectable when a beating myocyte culture was tested as a target (Cameron et al., 2013; Linette et al., 2013). These results indicate that whereas strong T cell-dependent responses against tumor cells can be therapeutically effective, the utility of this approach may be limited by the associated devastating off-target destruction of normal tissues.

However, recent work has revealed that the ACT approach may be made more specific if it employs TIL that display specificity for tumor-specific neoantigens. In 2013 the groups of Robbins and Rosenberg identified seven unique mutant MHC I epitopes that were presented by autologous tumor cells and recognized by in vitro expanded TIL from metastatic lesions of three patients who had shown objective responses to TIL therapy (Robbins et al., 2013). In a followup study they showed that therapeutically effective TIL not only consists of $CD8^+$ T cells specific for MHC I-restricted neoantigens but also $CD4^+$ T cells specific for MHC II-restricted tumor neoantigens from gastrointestinal malignancies (Tran et al., 2014; Tran et al., 2015). In this second study, TIL derived from a metastatic lung lesion from a cholangiocarcinoma patient were found to contain $CD4^+$ T cells specific for an epitope derived from a mutation in erbb2 inter-acting protein (ERBB2IP). When these tumor-specific $CD4^+$ T cells were expanded ex vivo, the expanded population was found to contain approximately 25% mutant ERBB2IP-specific $CD4^+$ Th1 cells. Adoptive transfer of the

expanded cell population back into the patient led to a transient objective tumor response before subsequent disease progression. The same patient then received another infusion of TIL containing more than 95% ERBB2IP-specific $CD4^+$ T cells and again experienced a response with a reduction in tumor burden that was durable after 20 months of follow up. No adverse events were reported by the therapeutic use of neoantigen-specific T cells. Similar studies on different patients bearing different tumors are ongoing at NCI and elsewhere to explore whether tumor neoantigen-specific T cells represent a preferred approach to ACT that will lead to improved efficacy and reduced toxicity. Certainly, if this approach is successful, one could envisage engineering neoantigen-specific T cells that express intracellular domains containing signaling cassettes that promote T cell survival and effector functions.

Of course hope remains that a set of shared tumor neoantigens will eventually be found that can be used globally for cancer immunotherapy. However, at this point in time, shared mutant neoantigens are extremely rare and even when identified are restricted to a limited number of HLA alleles. Nevertheless, viral antigens can function as a specific type of shared tumor neoantigen and have found use as the targets for ACT. TIL products selected for high numbers of $CD8^+$ T cells specific for HPV can lead to tumor regression in patients with metastatic cervical cancer (Stevanovic´ et al., 2015). Using TIL derived from an excised metastatic anal carcinoma, Draper et al. isolated an HLA-A*02:01-restricted TCR specific for HPV-16 E6, which upon transduction of the TCR genes into peripheral blood T cells enabled the recognition and killing of HPV-16 positive cervical and head and neck cell lines (Draper et al., 2015). This result suggests that adoptive transfer of T cells engineered to recognize HPV-associated malignancies may be an effective off-the-shelf tumor neoantigen-specific treatment.

6.2 Neoantigens as Targets of T cells Activated by Checkpoint Blockade in Humans

Seminal studies done in the Allison laboratory using preclinical models of murine cancers identified the potential of antibody blockade of the immune checkpoint molecule CTLA-4 as a potentially curative treatment strategy by “unleashing” endogenous tumor-specific T cells to destroy cancer cells (Hurwitz, Yu, Leach, & Allison, 1998; Leach et al., 1996; van Elsas et al., 1999). These findings were rapidly translated into the clinic with ipilimumab, a humanized monoclonal antibody against CTLA-4, which became the first immunotherapy agent to improve survival in metastatic melanoma, gaining FDA approval in 2011 (Hodi et al., 2010). The success of ipilimumab spurred the development of monoclonal antibodies against other immunoinhibitory molecules. Two monoclonal antibodies that block PD-1, nivolumab and pembrolizumab, have been FDA approved for use in metastatic melanoma and NSCLC, and nivolumab also has an indication for renal cell carcinoma (Borghaei et al., 2015; Garon et al., 2015). In addition, antibodies targeting both PD-1 and PD-L1 are currently being evaluated in clinical trials for a wide range of other malignancies. Early phase clinical trials also suggest that checkpoint blockade is efficacious in other solid and hema-topoietic malignancies, including bladder, stomach, head and neck carcinoma, and Hodgkin lymphoma (Sharma & Allison, 2015).

The presence of $CD8^+$ T cells at the invasive margin prior to anti-PD-1 therapy is a predictor of response, raising the question of the nature of the tumor antigens recognized by this cell subset (Tumeh et al., 2014). The role of TAA as the targets of the therapeutic immune response induced by ipilimumab was investigated by Kvistborg et al. who screened a cohort of 40 melanoma patients treated with ipilimumab for the presence of $CD8^+$ T cells specific for a panel of 145 HLA-A*02 restricted shared antigens (Kvistborg et al., 2014). As a control, the frequency of $CD8^+$ T cells specific for cytomegalovirus (CMV), influenza, or Epstein-Barr virus (EBV) viral epitopes was also assessed. The latter remained stable before and after therapy. In contrast, the number of melanoma-specific responses increased in many patients, with as many as six new specificities recognized. However, the magnitude of preexisting melanoma-specific responses that was detectable before therapy did not change after ipilimumab therapy. Thus it was not possible to correlate changes in the magnitude of preexisting antitumor responses to that of clinical responses.

In contrast to these results, a positive correlation has been observed in T cell responses to tumor neoantigens in patients who respond to checkpoint blockade. In the first case report to describe the identification of neoantigen-specific CTL in a patient after ipilimumab therapy, van Rooij et al. used exome sequencing, RNA sequencing (RNASeq), and epitope prediction algorithms together with a screening approach utilizing MHC I tetramers to identify T cells specific for a mutant form of ataxia telangiectasia and Rad3-related gene (ATR) from a culture of TIL in a patient with melanoma (van Rooij et al., 2013). Although mutant antigen-specific CTL could be identified at low frequencies more than a year preceding ipilimumab therapy, their frequency increased more than fivefold within 5 wks after treatment, coinciding with reduction in tumor burden. Similar findings were seen in a patient with NSCLC who had a prolonged response to pembrolizumab, with neoantigen-specific $CD8^+$ T cells identifiable in the peripheral blood within 3 wks of treatment initiation and reaching their zenith within 6 wks, before decreasing (Rizvi et al., 2015). Again, the increase in antigen-specific effector cells correlated with a response in overall tumor burden as measured radiographically. These neoantigen-specific CTL were characterized as displaying a polyfunctional phenotype expressing IFN-γ, TNF-α, the degranulation marker CD107a, and the chemokine CCL4 after stimulation with mutant, but not the wild-type peptide. Both of these studies indicate that blockade of both CTLA-4 and PD-1 induces the proliferation of $CD8^+$ T cells specific for neoantigens.

6.3 The Use of Neoantigen Cancer Vaccines in Humans

The introduction of vaccines against common infectious diseases is the crowning achievement of the field of immunology. Unfortunately, thus far, this success has not translated into therapeutic benefits for patients with cancer. It has proven difficult to raise robust immune responses capable of overcoming the inhibitory environment present in tumors that have escaped immune control. For successful immune responses to develop in tumor-bearing individuals, a series of complex molecular and cellular events must take place in a highly coordinated fashion (Chen & Mellman, 2013). Tumor antigens must be taken up, processed, and presented by APC that also must express the appropriate costimulatory signals. Responding T cells must express TCRs specific for tumor antigen/MHC and must come into contact with a suitably activated APC in appropriate topographical locations

within the host. T cells that receive the appropriate signals must then expand in sufficient numbers to destroy progressively growing tumor cells despite the myriad of inhibitory molecules and barriers that are present in a developing tumor. A separate point to note is that peripheral T cells specific for tumor-associated self-peptides have been subjected to negative thymic selection, and their TCRs often exhibit lower mean binding affinities than typical foreign antigens (Stone, Harris, & Kranz, 2015). Therefore the affinities of TCRs for self-peptide MHC tumor-associated epitopes may be too low for optimal $CD4^+$ and $CD8^+$ T cell effector function.

Numerous platforms geared at inducing immune responses to vaccination with TAA have been developed, including those that employ whole tumor cells, peptides together with adjuvants, DNA and RNA constructs, or cellular vaccines using APCs pulsed with tumor antigens among others. As a whole, clinical trials using TAAs as targets have been disappointing, and this topic has been reviewed extensively elsewhere (Rosenberg et al., 2004). This is not to say that vaccines targeting TAAs have not been thoroughly investigated in clinical trials. In one of the largest studies of this kind, although a vaccine targeting MAGE-A3 demonstrated a trend toward a clinical benefit preventing relapse after resection of early stage non-small cell lung cancer NSCLC in a phase II trial (Vansteenkiste et al., 2013), a subsequent phase III study with a goal enrollment of more than 2000 patients using this platform was recently terminated due to lack of efficacy during a midtrial preplanned data analysis. It is instructive that only one cellular vaccine has gained FDA approval, a preparation consisting of autologous peripheral blood mononuclear cells exposed ex vivo to a recombinant fusion protein of prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor (GM-CSF; sipuleucel-T). In a randomized phase III study, an overall survival benefit of 4.1 months was seen among patients with castration-resistant prostate cancer, despite no change in the time to disease progression (Kantoff et al., 2010). Due to this limited benefit, the need for leukapheresis, and high cost, adoption of sipuleucel-T therapy has been limited.

Given the limitations of TAA vaccines and clear preclinical and clinical data suggesting that endogenous antitumor responses can be targeted to TSA, considerable interest has been stoked in developing personalized, tumor-specific vaccination approaches. As previously mentioned, we demonstrated in our preclinical MCA sarcoma model that vaccination against TSA could be as therapeutically effective as checkpoint blockade immuno-therapy. These results have been generalized to other mouse tumor models by other groups. But what about the use of neoantigen vaccines in human cancer patients?

Given the difficulty in the past with the identification of truly private tumor-specific mutant antigens, the first trial of a personalized neoantigen vaccine targeted shared mutations in oncogenic drivers. In a 1995 study, five patients with pancreatic adenocarcinoma were vaccinated with autologous DC pulsed with an RAS peptide containing their tumor-specific mutation (Gjertsen et al., 1995). Two of the five patients mounted a transient proliferative response against the vaccine, although in one patient a response against wild-type RAS was also detectable, but both patients eventually succumbed to their disease. Two later studies reported vaccine responses in 58% and 85% of patients, with the later study reporting 20% survival at 10 years in a cohort of patients vaccinated after complete resection of their

pancreatic cancer, compared to no long-term survivals in a matched cohort treated without vaccine (Wede´n et al., 2011). Given their incidence, approaches to target viral antigens associated with malignant transformation have reached clinical trials. SLP vaccines consisting of HPV E6 and E7 were effective at inducing an immune response that caused regression of vaginal intraepithelial neoplasia (Kenter et al., 2009) and similar vaccines are under study for more advanced cervical cancers.

The first study to use a genomics approach for neoantigen prediction and vaccination to generate neoantigen-specific $CD8^+$ T cell responses has been recently reported in three patients with previously resected melanoma. Carreno et al. used exome sequencing and in silico prediction to identify missense mutations that formed HLA-A*02:01 binding peptides which were confirmed biochemically (Carreno et al., 2015). Autologous DCs were pulsed with seven separate potential patient-specific neoantigens together with two known gp100 shared epitopes and infused into each patient. Each patient had evidence of a $CD8^+$ T cell response against one neoantigen, which could be identified in the peripheral blood prior to vaccination, and after vaccination each patient showed immune responses against two additional vaccine epitopes. At the time of this report, two of the patients had stable disease, with the remaining patient having no evidence of recurrence, without any evidence of autoimmune effects. This study suggests that vaccination with a panel of neoantigens can induce or enhance immune responses against tumor neoantigens. It is not possible at this point to determine whether the vaccine induced a de novo novel immune response against novel tumor antigens or boosted preexisting but undetectable immune responses to tumor antigens. It also remains unclear whether the antivaccine responses that appear after vaccination are capable of reacting with tumor cells since the analysis of T cell specificities in peripheral blood was performed using the vaccinating peptide and not tumor. Nevertheless, this study certainly indicates that a personalized cancer neoantigen vaccine approach is feasible, and multiple clinical trials have either begun enrollment or are planned at multiple institutions using different vaccine approaches to target neoantigens.

7. CONCLUDING REMARKS

Two parallel lines of investigation, one focused on the identification of endogenous immune responses to cancer, and the other on defining antigens that serve as therapeutically useful targets for immunotherapies, have both led to the same conclusion that tumor-specific neoantigens are ideal targets for immunotherapy. Where does the field go from here? The currently available bioinformatics approaches to identify neoantigens are clearly successful, but it is now apparent that the majority of in silico predictions do not induce tumor-reactive T cell responses which has necessitated the development of complex screening methods for their validation. Much work remains to be done in order to develop epitope prediction pipelines that are capable of predicting the TSAs that are recognized by endogenous and therapeutically induced immune responses to cancer with a high level of accuracy, which will be a necessary step forward before widespread translation of vaccine approaches into the clinic can occur.

Given the large numbers of identified TAA and evidence that immune responses against shared antigens can have therapeutic benefit, it would be unwise to dismiss this class of

antigens entirely in the future design of cancer immunotherapies. More work needs to be done to directly compare situations where immune responses can be directed against TSA and TAA simultaneously in both preclinical models and clinically in order to define which type of antigen, if any, is more efficacious for a given situation. Another key point is that it is likely that combinations of vaccine, ACT, and/or checkpoint blockade approaches will be the most effective way to focus the immune system to eliminate cancer. Clinical trials attempting to induce new immune responses against neoantigens with vaccines while simultaneously modulating the tumor microenvironment with checkpoint blockade to foster the development of a robust neoantigen-specific immune response are already being planned at multiple cancer centers. In addition, combining neoantigen-based therapies with standard of care therapies (eg, chemotherapy or radiotherapy) may also find therapeutic usefulness especially in the case of tumors with low mutational loads where standard-of-care therapies could give rise to additional mutations that could be targeted by cancer-specific vaccines.

Decades of careful studies in preclinical model systems and clinical investigation has led the field of cancer immunology to the day where nearly all patients with melanoma and NSCLC will receive some form of immuno-therapy during their course of treatment. The identification of neoantigens as the optimal targets of cancer immunotherapy promises to enter clinical practice to guide the diagnosis, prognosis, and treatment options for patients with a wide range of tumor histologies. We now sit on the brink of the introduction of standard of care immunotherapy approaches for nearly all patients with cancer.

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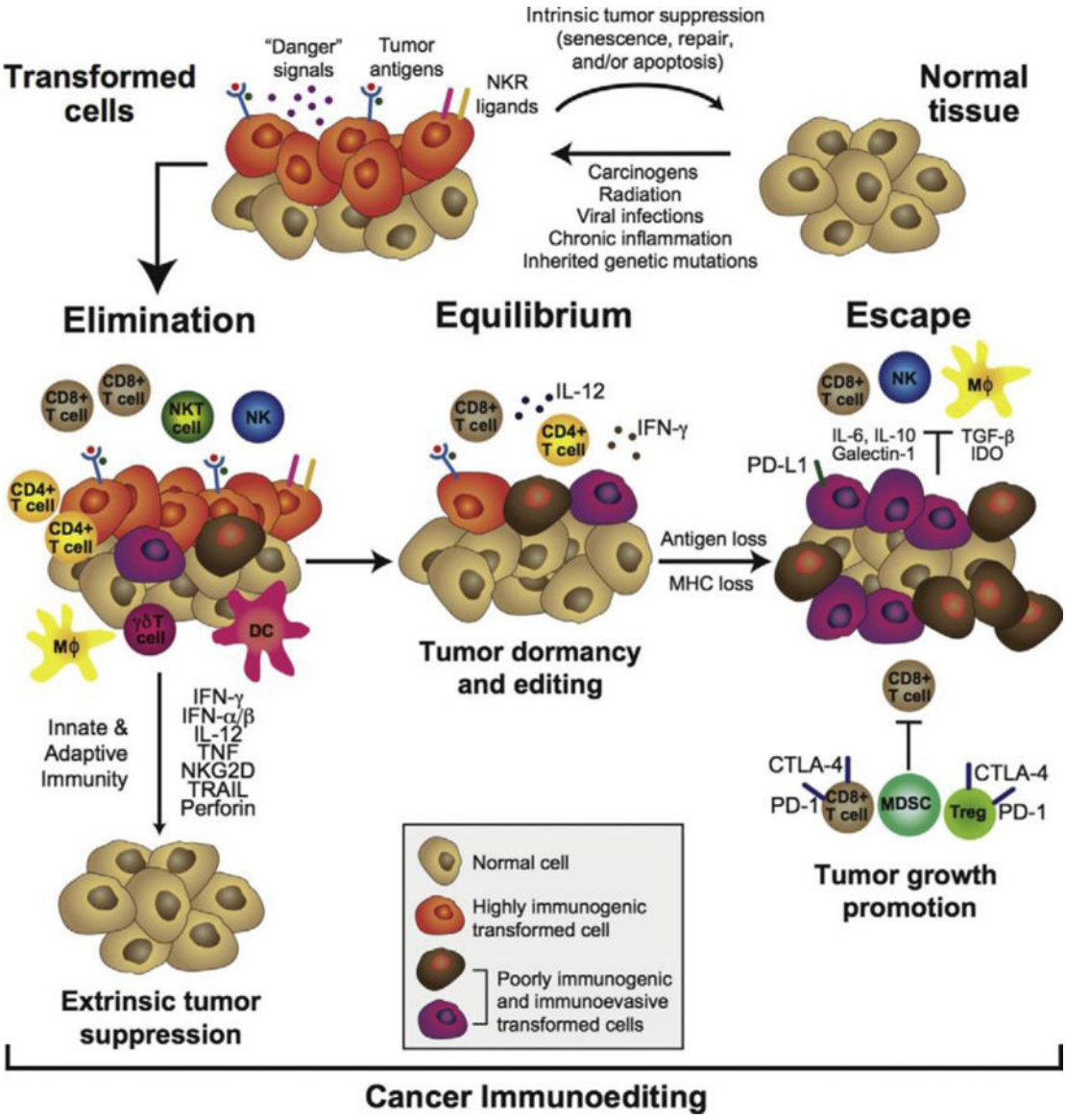

Figure 1.

Cancer Immunoediting is an extrinsic tumor-suppressor mechanism that engages after cellular transformation has occurred and intrinsic tumor-suppressor mechanisms have failed. In its most complex form, Cancer Immunoediting consists of three phases: Elimination, Equilibrium, and Escape. In the Elimination phase, innate and adaptive immunity work in concert to destroy emerging tumors before they become clinically apparent. This phase may represent the full extent of the process upon complete tumor elimination, whereby the host remains cancer free. If, however, a cancer cell variant resists elimination, it may then enter the Equilibrium phase, in which its outgrowth is immunologically constrained. Editing of tumor immunogenicity occurs in the Equilibrium phase. Equilibrium may curb outgrowth of occult cancers for the lifetime of the host. However, as a consequence of immune selection pressure, tumor cell variants may arise that are no longer recognized by adaptive immunity, become insensitive to immune effector mechanisms, and/or induce an immunosup-pressive

tumor microenvironment. These tumor cells may then enter the Escape phase, in which their outgrowth is no longer impeded by immunity and thus manifest as clin-ically apparent cancer. *Figure adapted from Vesely, M. D., Kershaw, M. H., Schreiber, R. D., & Smyth, M. J. (2011). Natural innate and adaptive immunity to cancer.* Annual Review of Immunology, 29*, 235–271.* http://dx.doi.org/10.1146/annurev-immunol-031210–101324 *and Schreiber, R. D., Old, L. J., & Smyth, M. J. (2011). Cancer immunoediting: Integrating immunity's roles in cancer suppression and promotion.* Science, 331*(6024), 1565–1570.* http://dx.doi.org/10.1126/science.1203486.

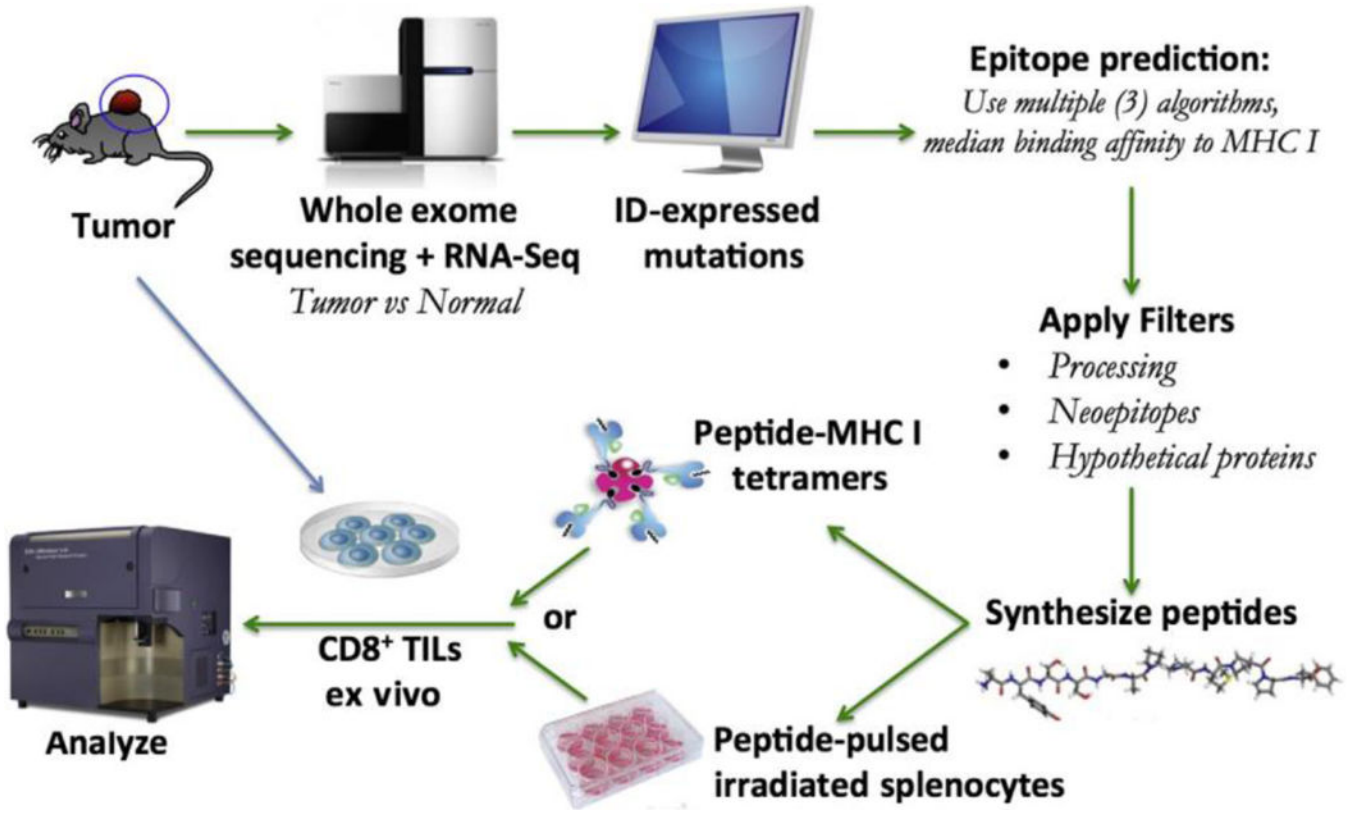

Figure 2.

Genomics-and bioinformatics-based identification of mutant neoantigens. Tumor cells and normal tissue are subjected to whole exome and RNA-sequencing to identify expressed nonsynonymous somatic mutations. Corresponding mutant epi-topes are then analyzed in silico for MHC class I binding. Filters are then applied for anti-gen processing, whether the mutant epitope has a stronger predicted binding affinity than the corresponding wild-type peptide, and deprioritization of hypothetical proteins. Peptides corresponding to predicted epitopes are then synthesized and used to identify mutant neoantigen-specific T cells in freshly explanted TIL using MHC I multimer-based screens or functional assays (eg, cytokine release, ELISPOT, or intracellular cytokine staining) by peptide stimulation.

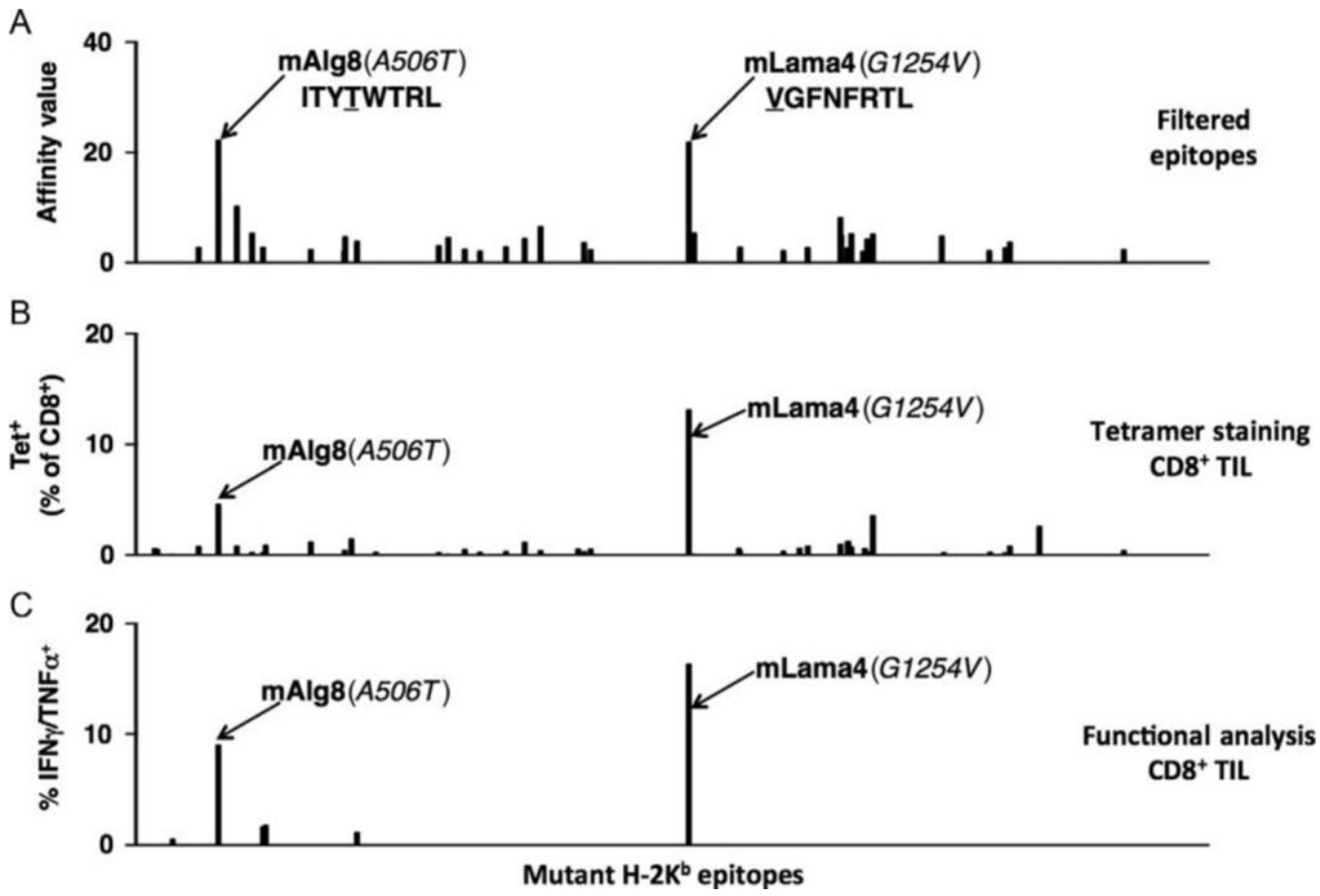

Figure 3.

(A) Predicted MHC I binding affinity of filtered epitopes predicted by in silico analysis of missense mutations in the T3 tumor line. (B) Screening for specificities of $CD8^+$ TIL from anti-PD-1-treated, T3 tumor-bearing mice using MHC I tetramers loaded with top predicted peptides. (C). IFN-γ and TNF-α induction in $CD8^+$ TIL from anti-PD-1-treated, T3 tumor-bearing mice following culture with irradiated splenocytes pulsed with the top predicted peptides. *Figure adapted from Gubin, M. M., Zhang, X., Schuster, H., Caron, E., Ward, J. P., Noguchi, T.,… Schreiber, R. D. (2014). Checkpoint block-ade cancer immunotherapy targets tumour-specific mutant antigens.* Nature, 515*(7528), 577–58.* http://dx.doi.org/110.1038/nature13988.

Table 1

Pioneering Studies Revealing the Importance of Cancer Neoantigens

Year	Discovery	Reference
1943	Mice with carcinogen-induced tumors are protected against rechallenge with the same tumor line, indicating tumors have tumor-specific antigens	Gross (1943)
1977	Generation of CTL clones against tumor antigen of nonviral origin	Gillis and Smith (1977)
1985	Antigens recognized by T cells are presented on MHC	Babbitt, Allen, Matsueda, Haber, and Unanue (1985) and Bjorkman et al. (1987)
1987	T cells from human melanoma patients react with autologous tumor but not normal tissue	Herin et al. (1987) and Van den Eynde et al. (1989)
1988	Identification of a tumor-specific mutant antigen in an in vitro mutagenized mouse tumor	De Plaen et al. (1988)
	Use of ACT therapy in patients with metastatic melanoma	Rosenberg et al. (1988)
1991	Identification of the first human tumor antigen, the CTA antigen MAGEA1	van der Bruggen et al. (1991)
1994	Identification of melanoma antigens using mass spectrometry	Cox et al. (1994)
1995	First tumor-specific mutant antigen in human tumors identified	Coulie et al. (1995) and Wolfel et al. (1995)
1996	Use of peptide–MHC tetramers to analyze antigen-specific T cells	Altman et al. (1996)
	Antitumor activity of anti-CTLA-4 demonstrated in mice	Leach, Krummel, and Allison (1996)
1997	Use of SEREX to identify the CTA antigen NY-ESO-1	Chen et al. (1997)
1999	Development of the MHC class I epitope database and prediction algorithm SYFPEITHI	Rammensee, Bachmann, Emmerich, Bachor, and Stevanovic (1999)
2001	Demonstration that the immune system can protect against cancer and shape tumor immunogenicity Proposal of Cancer Immunoediting, thus unifying the dual host-protective and tumor promoting and sculpting ability of the immune system	Shankaran et al. (2001)
2003	Development of the NetMHC epitope prediction algorithm	Nielsen et al. (2003)
2004	Establishment of IEDB	Vita et al. (2015)
2005	T cells specific for tumor-specific mutant antigens persist in the blood and tumor of a melanoma patient after ACT	Zhou, Dudley, Rosenberg, and Robbins (2005)
	Autologous T cells to a human melanoma is dominated by responses to tumor-specific mutant antigens	Lennerz et al. (2005)
2007	Experimental demonstration of cancer immune equilibrium	Koebel et al. (2007)
	First cancer whole exome sequencing	Wood et al. (2007)

Year	Discovery	Reference
2008	First cancer whole genome sequencing	Ley et al. (2008)
	Burt Vogelstein and Jim Allison propose that all cancers have mutations that could form neoantigens	Segal et al. (2008)
2009	Detection of antigen-specific T cells by combinatorial encoding of MHC multimers	Hadrup et al. (2009)
2011	FDA approval of the immune checkpoint inhibitor ipilumumab (anti-CTLA-4)	Hodi et al. (2010)
2012	First use of genomic sequencing and epitope prediction algorithms to identify tumor-specific mutant antigens	Castle et al. (2012) and Matsushita et al. (2012)
	Demonstration that tumor-specific mutant antigens can drive Cancer Immunoediting	DuPage, Mazumdar, Schmidt, Cheung, and Jacks (2012) and Matsushita et al. (2012)
2013	Use of genome sequencing and epitope prediction to identify human mutant neoantigens recognized by adoptively transferred T cells	Robbins et al. (2013)
	In vivo expansion of mutant antigen-specific T cells in a human melanoma patient following anti-CTLA-4 treatment	van Rooij et al. (2013)
2014	Predicted mutant neoantigens correlate with increased CTL cytotoxicity and patient survival	Brown et al. (2014)
	Autologous $CD4^+$ T cells largely specific for a tumor-specific mutant antigen leads to tumor regression when adoptively transferred into a cancer patient	Tran et al. (2014)
	Demonstration that tumor-specific mutant antigens are targets of checkpoint blockade cancer immunotherapy	Gubin et al. (2014)
	Tumor-specific mutant antigen SLP vaccines provide therapeutic tumor protection in preclinical models	Gubin et al. (2014) and Yadav et al. (2014)
	Mutational load and neoantigen landscape may predict patients who benefit from checkpoint blockade cancer immunotherapy	Snyder et al. (2014)
2015	Genetic analysis reveals CTL activity correlates with mutant neoantigens load and provides evidence of immunoediting for some human tumors	Rooney, Shukla, Wu, Getz, and Hacohen (2015)
	Identification of neoantigen-specific $CD4^+$ T cells that infiltrate melanoma metastases	Linnemann et al. (2015)
	Demonstration that vaccination with MHC II epitopes induces therapeutic antitumor responses in preclinical models	Kreiter et al. (2015)

Table 2

Ongoing or Planned Clinical Studies of Neoantigen Vaccines

Tumor Type	Phase	Vaccine Platform	Institution	Start Date	ClinicalTrial.gov Identifier
Melanoma	1	Neoantigen polyepitope coding RNA vaccine	Biontech AG	December 2013	NCT02035956
Melanoma	1	Synthetic long neoantigen peptides plus poly-ICLC	Dana-Farber Cancer Institute	January 2014	NCT01970358
Glioblastoma	1	Neoantigen peptide plus poly-ICLC + GM-CSF	Immatics Biotechnologies	October 2014	NCT02149225
MGMT-unmethylated Glioblastoma, Glioblastoma Multiforme	1	Synthetic long neoantigen peptides plus poly-ICLC	Dana-Farber Cancer Institute	November 2014	NCT02287428
Triple-negative breast cancer	1	Neoantigen polyepitope DNA vaccine	Washington University School of Medicine	June 2015	NCT02348320
Triple-negative breast cancer	1	Synthetic long neoantigen peptides plus poly-ICLC	Washington University School of Medicine	September 2015	NCT02427581
Triple-negative breast cancer	1	Neoantigen polyepitope coding RNA vaccine	Biontech AG	September 2015	NCT02316457
Glioblastoma multiforme astrocytoma, Grade IV	0	Synthetic long neoantigen peptides plus poly-ICLC	Washington University School of Medicine	November 2015	NCT02510950
Non-small cell lung cancer	0	Neoantigen dendritic cell vaccine	Washington University School of Medicine	January 2016	NCT02419170
Pancreatic, colorectal	1	Peptide vaccine plus IFA	MD Anderson Cancer Center	March 2016	NCT02600949