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Brief Reviews

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Cellular immune responses that protect against tumors typically have been attributed to CD8 T cells. However, CD4 T cells also play a central role. It was shown recently that, in a patient with metastatic cholangiocarcinoma, CD4 T cells specific for a peptide from a mutated region of ERBB2IP could arrest tumor progression. This and other recent findings highlight new opportunities for CD4 T cells in cancer immunotherapy. In this article, I discuss the role and regulation of CD4 T cells in response to tumor Ags. Emphasis is placed on the types of Ags and mechanisms that elicit tumor-protective responses. I discuss the advantages and drawbacks of cancer immunotherapy through personalized genomics. These considerations should help to guide the design of next-generation therapeutic cancer vaccines. *The Journal of Immunology*, **2015, 194: 2049–2056.**

Antitumor immune defenses include Abs, T cells, and NK cells. Abs are effective against surface-exposed, tumor-specific Ags. The use of monoclonal, bifunctional, or multispecific Abs to treat cancer requires multiple injections and is expensive (1–4). T cells express polymorphic AgRs for specific Ag recognition, possess effector functions, and develop memory characteristics. T cells are at the core of "immune surveillance" theories and are the best candidates to exact a toll on cancer cells in an Ag-specific manner (5, 6). NK cells and related cell types (NKT cells and cytokine-induced killer cells) express nonpolymorphic cell surface receptors that target cells for destruction in an Ag-independent manner. These cells lack the ability to acquire functional memory characteristics (7–9).

Typically, cellular immune responses that protect against tumors have been attributed to CD8 T cells. One main reason is that, similar to most normal tissues, tumor cells express little, if any, MHC class II molecules (10, 11). When MHC class II molecules are expressed, the invariant chain is often highly expressed, resulting in the generation of class II–associated invariant chain peptides that prevent the presentation of endogenous peptides in tumor cells (12). Consequently, pursuits of T cell–based therapies have focused primarily on CD8 T cells. In experimental animals, tumor-specific CD8 T cells are highly protective (reviewed in Ref. 13). In humans, tumor-specific CD8 T cells are present in patients with hematologic malignancies and solid tumors and within the pool of tumor-infiltrating lymphocytes, but they express high levels of PD-1 or exhibit suppressive characteristics (14–17). However, therapeutic vaccines designed to induce CD8 T cell responses have been largely disappointing (18–20).

In recent years, there has been increased interest in adoptive T cell therapies (21). In one approach, a patient's T cells are genetically engineered to express a chimeric TCR, which consists of an Ag-binding domain of an Ab fused to the signaling components of a TCR and other signaling domains (22). This targeted treatment has shown great promise for treating B cell malignancies and is poised for success in other types of cancer (23, 24). In a second approach, tumor-infiltrating lymphocytes are isolated, expanded, and, in some cases, selected for TCR specificity before being reinfused into the same patient. The goal of this approach is to kill cancer cells through the recognition of MHC/peptide complexes (reviewed in Refs. 25, 26).

Despite the clinical successes of adoptive T cell therapies and Abs, cancer vaccines could be a more effective and potentially less toxic approach (27). In this article, I discuss strategies to pursue this objective with a focus on CD4 T cells and their role in antitumor immunity and tumor protection. Particular emphasis is placed on the process of peptide selection for inclusion in cancer vaccines comparing peptides from unmutated self-tumor Ags and mutated gene products.

CD4 T cells in immunity

CD4 T cells typically recognize peptides 12–16 aa in length presented by MHC class II molecules. These cells play a central role in the beginning and maintenance of adaptive immune responses. Their contribution to antitumor immunity is complex and reflects the diverse functions of various types of CD4 T cells (reviewed in Ref. 28). Almost 50 y ago, it was observed that, during the generation of Ab responses against thymus-dependent Ags, T cells provide help to B cells, facilitating isotype switching and affinity maturation (29). In 1971, Mitchison (30) showed that these effects require that both T and B cells recognize and respond to two different regions of the same protein. It was later shown that activation of CD4 T cells requires processing and presentation of the T cell determinant by the B cell (31). Thus, the helper

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Abbreviations used in this article: FDA, U.S. Food and Drug Administration; MaF, mutated genes and gene fusion regions.

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function of CD4 T cells requires Ag processing and presentation on MHC class II molecules, both of which can occur in the B cell serving as an APC (32–34). A similar form of T cell cooperation was demonstrated for CD4 T cells helping the activation and expansion of CD8 T cells (35). CD4 T cells also play a pivotal role in the generation and maintenance of memory CD8 T cells (36–39). In addition to their helper role for B cells and CD8 T cells, CD4 T cells can be distinguished on the basis of the cytokines that they produce (IFN-γ is produced by Th1 cells, IL-4 is produced by Th2 cells, and IL-17 is produced by Th17 cells) or by their ability to downregulate the function of other T cells (regulatory T cells) (40, 41). The final type of CD4 T cells, T follicular helper cells, selects high-affinity, Ab-producing B cells for clonal expansion in germinal centers (42). The complex array of functions by the different classes of CD4 T cells in relation to the antitumor immune response was reviewed recently (28).

CD4 T cells mediate tumor protection

Early studies using tumor-bearing mice treated with an adoptive transfer of tumor-reactive CD4 T cells or by selectively depleting CD4 T cells demonstrated that these cells were needed in the effector phase of a protective antitumor immune response against tumors lacking MHC class II (43–49). These experiments also found that activated CD4 T cells induced delayed-type hypersensitivity–like reactions and attracted inflammatory cells (macrophages, granulocytes, eosinophils, and NK cells) in or around the tumor (47, 50). Protection was thought to be mediated by CD4 T cells secreting IFN-γ, which would mediate cytotoxicity of tumor cells synergistically with TNF-α while also inducing reactive oxygen species and NO, inhibiting angiogenesis and stimulating cytotoxic macrophages (48, 51–57). More recently, protection from tumors lacking MHC class II was studied after a low-dose adoptive transfer of Th1-like CD4 T cells specific for the melanoma-associated Ag Trp1 (58, 59). Both studies found that these Th1-like CD4 T cells acquired cytotoxicity in vivo and secreted IFN-γ, leading to the upregulation and expression of MHC class II molecules on the surface of tumor cells. This mechanism enabled MHC class II–restricted killing and protection independent of B, NK, or other T cells in the host (58, 59). For tumors expressing MHC class II molecules, CD4 T cells kill target cells directly via conventional MHC-dependent recognition (46, 58, 60, 61). This includes CD4 T cell responses against carbohydrate epitopes (62). A recent report found that conventional Th1 CD4 T cells can be converted into cytolytic CD4 T cells by reducing the expression of the transcription factor ThPOK (63).

In cancer patients, MHC class II–restricted CD4 T cell responses against self-Ags have been detected in the circulation and at the tumor site (64–73). Consistent with the finding that human CD4 T cells against pathogens are cytotoxic—lysing target cells—human CD4 T cells were reported to lyse tumor cells in a MHC class II–restricted manner by predominantly perforin- or granzyme-mediated killing (74–80). Collectively, human CD4 T cells can suppress tumor growth through the secretion of IFN-γ or directly kill tumor cells expressing adequate levels of MHC class II and self-Ags on their surface (79, 81). Activated CD4 T cells also could lead to the expression of MHC class II molecules through the secretion of IFN-γ or by blocking the inhibitory receptor CTLA-4, as demonstrated in the mouse (59). Whichever scenario applies, if a comparison with MHC class I–restricted CD8 T cell activation and function is warranted, one would expect that few (<100) complexes would suffice to mediate both intratumor activation and tumor cell killing by CD4 T cells (82, 83).

T cell tolerance

The induction and maintenance of tumor-specific T cells are regulated by mechanisms that, alone or in combination, diminish the ability of the immune system to control tumor growth and spread. These mechanisms include the following: central and peripheral tolerance, ignorance, the size of the repertoire and the hierarchical order with which T cell determinants are used and become immunogenic, regulatory T cells, myeloid suppressor cells, immunosuppression generated in the tumor microenvironment through inflammation, and endoplasmic reticulum stress and its influence on phagocytic cells and Ag presentation (84–91).

Without going into the details of each of these mechanisms, it is noteworthy that sporadic tumors in mice are immunogenic, yet tolerance is induced by the expansion of nonfunctional T cells (92). Likewise, CD8 T cells generated by vaccination (peptide-in-adjuvant) in melanoma patients predominantly have a quiescent phenotype (93). In mice, CD8 T cells against self-Ags become tolerant through epigenetic mechanisms that are independent of the tolerogen (94). Together, these examples suggest that at least one main reason for the inefficient control of cancer by T cells is the T cells themselves. One possibility is that this is the result of immunosuppressive signals within the tumor microenvironment (85). Not surprisingly, the reactivation of T cells with agonistic Abs against inhibitory receptors on T cells (immune checkpoints) has been associated with clinical remission in some forms of cancer (95, 96).

Cooperation between CD4 Th cells

Although it is undisputed that CD4 T cells provide help to B and CD8 T lymphocytes, who helps them? Years ago, my colleagues and I proposed that cooperation between CD4 T cells enables the activation and expansion of CD4 T cells specific for poorly immunogenic determinants and/or tolerized CD4 T cells, which would otherwise be unable to expand or expand only to a limited extent (97). We named this process Th–Th cooperation or "help for helpers" (98). Th–Th cooperation enables the activation of CD4 T cells specific for a self-tumor Ag, providing complete, durable, and specific protection against s.c. tumor implants and tumor rechallenge (99). The mechanism of Th–Th cooperation is based on associative recognition of Ag, where self and nonself Th cell determinants are presented by the same APC (32, 34, 100, 101). It also was found that the activation of anti-nonself T cells precedes the activation of anti-self T cells by 48 h. This provides a cytokine environment to further activate APCs, enabling the activation of otherwise unresponsive anti-self CD4 T cells (97). Furthermore, the help received through this "immunological switch" is as effective as the signals imparted using agonistic Abs to CD40 and OX40, alone or in combination (97).

The Th–Th cooperation model postulates that the anti-nonself response precedes and drives the anti-self CD4 T cell

response (Fig. 1, *left panel*) based on a sequential three-cell interaction (Fig. 1, *right panel*) where the same APC processes and presents the nonself determinant and activates the corresponding anti-self CD4 T cells. Upon activation, cytokines produced by anti-nonself CD4 T cells heighten the expression of costimulatory molecules on the APC, enabling the presentation of the self determinant to CD4 T cells specific for self (102). In this model, the anti-nonself response is anticipatory of the anti-self response. A similar three-cell model was proposed for a CD4 T cell–dependent activation of CD8 T cells in which CD4 T cell activation by the APC is crucial (103). The collective value of this model is 2-fold. It provides a mechanism for otherwise subimmunogenic CD4 T cell determinants of a self-tumor Ag to break self-tolerance (102). It also points to the possibility of directly immunizing against weak CD4 T cell determinants, such as self-Ags, needed to induce protective antitumor responses in vivo by exploiting the ability of CD4 T cells to activate APCs, which, in turn, primes other (anti-self) CD4 T cells through enhanced costimulation (104).

It appears that the balance between tolerance and immunity depends on an inherent property of the immune system: the productive cooperation between two Th cells with different specificity, one for a nonself determinant and one for a self determinant, engaged by the same APC. One prediction of this model is that attempts to activate CD4 T cells against "weak" self-tumor antigenic peptides without enabling the "immunological switch" could lead either to their inactivation (34) or to immunity without clinical response (discussed in Ref. 105). At the turn of the twentieth century, German pathologist Georg Schone noted that "the degree of immunity which develops against tumor depends on the foreignness of the immunizing cell with respect to the organism into which it is introduced. The more foreign cells accordingly serve as the more effective and the more closely related cells as the less effective antigens" (106). Th–Th cooperation through associative recognition of Ag incorporates this idea and enables the generation of protective cellular responses against self-tumor Ags. This has been applied successfully in several systems in mice and humans (107–110). Thus, Th–Th cooperation can be viewed as an archetypal form of immune regulation that provides a mechanistic solution to how class determination of effector CD4 T cells with anti-self specificity is generated in vivo.

Human CD4 T cells recognize tumor Ags

Human CD4 T cells can recognize MHC class II–restricted self-tumor Ags, such as tissue-specific Ags, common tumor Ags (i.e., Ags present in the vast majority of tumors, irrespective of their histological origin), and viral Ags causative of tumor transformation (98). In some instances, peptides from unmutated tumor Ags bind different MHC class II alleles, so-called "promiscuous" peptides (79, 111–115). These peptides are therapeutically useful because they could be used to immunize a large segment of the human population.

Of particular interest, however, are those instances in which CD4 T cells are specific for MHC class II–restricted peptides corresponding to mutated genes (nonsynonymous mutations) or gene fusion (translocation) regions (MaFs) (Fig. 2). Early examples include the triosephosphate isomerase, the LDPF fusion gene product between the low-density lipid receptor and the GDP-L-fucose:b-D-galactoside-2-a-L-fucosyltransferase, and CDC27, a component of the anaphase-promoting complex involved in cell cycle regulation (116–118). A recent publication reported that scleroderma patients who also developed cancer and carry a mutation in the *POLR3A* gene spontaneously expand MHC class II–restricted CD4 T cells specific for peptides from the mutant POLR3A gene product (119). Various other peptides were described in the past decade (120). The peptide recently identified by Tran et al. (121) corresponds to a mutation in the ERBB2IP gene. Inoculation of the patient with autologous CD4 T cells reactive against this mutated peptide caused a dramatic decrease in target lesions and prolonged stabilization of disease, possibly through direct cytotoxic activity (121).

Recognition of MaF-derived peptides is not unique to CD4 T cells; MHC class I–restricted CD8 T cells also were reported to target peptides from the BCR-ABL and TMPRSS2-ERG fusion gene products (122, 123). A complete listing of MHC class II–restricted peptides was published recently (124). Recent findings suggest that solid tumors have an average of 33–66 genes with somatic mutations that are expected to alter their protein products (i.e., synonymous mutations) (125). As more human tumors are analyzed by exon and whole-genome sequencing, it is likely that the number of MaF-derived peptides will increase.

CD4 T cell immunotherapy: Neoantigens versus unmutated tumor Ags

Are there particular properties of Ags and peptides that one should use to induce anti-tumor CD4 T cell responses in a clinical setting? MaF gene products might initially appear advantageous because MaF-derived peptides are neoantigens with CD4 T cell precursors that exist in a nontolerized form within the repertoire of the nonimmunized individual. With

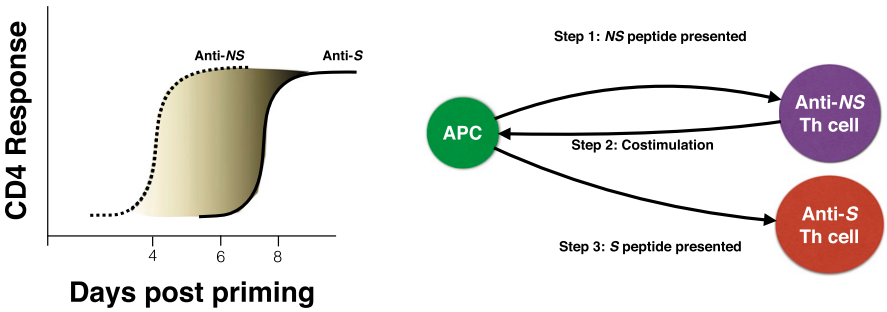

FIGURE 1. Temporal dynamics in the activation of CD4 T cell responses against NS and S determinants through Th–Th cooperation. NS and S determinants presented in linked association by the APC (a B lymphocyte) instruct the response of the corresponding CD4 T cells through a biphasic, sequential process. The anti-NS response is anticipatory (*left panel*) of the activation of anti-S CD4 T cells and is a prerequisite for their subsequent expansion through a three-cell model of dynamic interactions (*right panel*). NS, nonself; S, self.

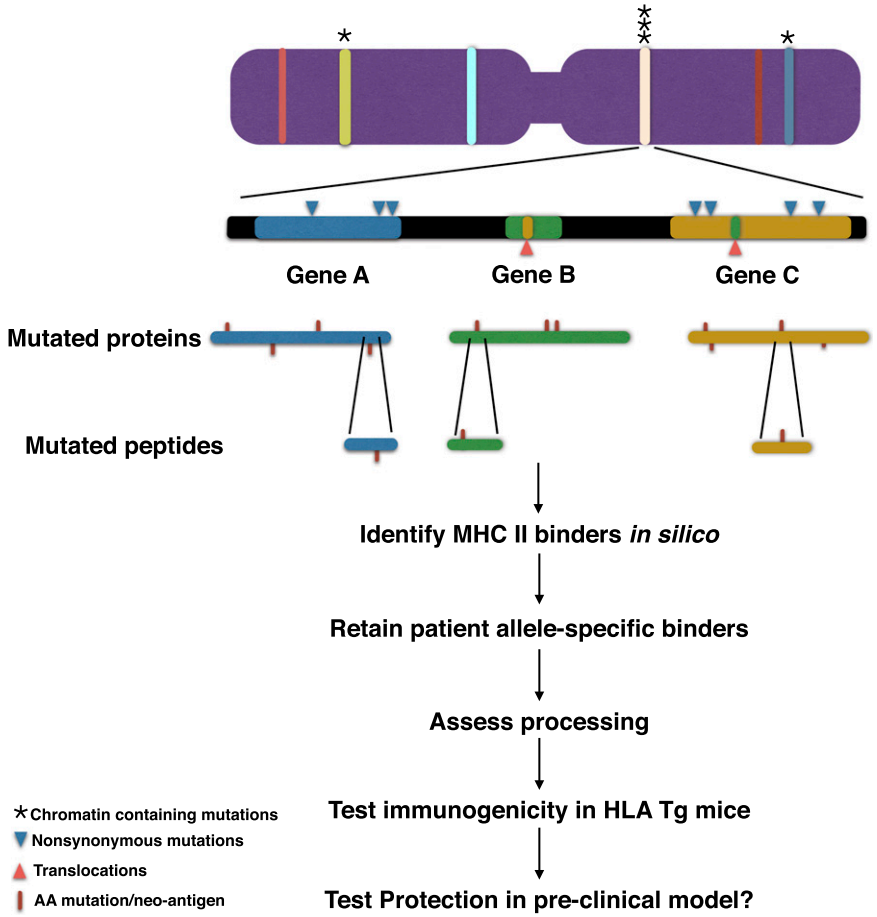

FIGURE 2. Process for the identification of CD4 T cell determinants from MaF gene products. Chromosomal areas containing nonsynonymous mutations or translocations, as well as attendant genes, are identified by next-generation exome sequencing. From the corresponding amino acid sequences comprising MaF gene products, discrete length peptide (~15 aa) sequences centered in or around MaFs are then identified and cataloged. These peptides are then subject to in silico prediction for MHC class II binding (~2800 MHC class II alleles) using bioinformatics tools (e.g., IEDB) followed by patient haplotype matching. Next, one must determine whether the identified peptides are naturally processed and presented. Ideally, this should be done in a patient-specific manner using the patient's own tumor cells. Because this may be not be feasible, established tumor cell lines transfected with a minigene coding for the selected MaF peptide(s) should be used. At this time, no predictive criteria exist for either in vivo immunogenicity or protective value. MHC binding affinity (percentile rank) correlates with immunogenicity but does not guarantee immunogenicity. Preclinical studies in HLA-DR/DQ/DP–transgenic mice remain the only possibility before human experimentation, but this again is no guarantee that these responses are relevant to protection in vivo. As discussed in the text, it is important to interrogate the specificities of tumor-infiltrating T cells, but this may be a difficult hurdle to overcome based on tissue availability and logistics. An alternative could be to interrogate circulating T cells using, for example, MaF-specific tetramers to determine whether such T cells exist in the patient. However, detection of T cells specific for selected MaF peptides in blood cannot be considered a proxy of tumor-infiltrating lymphocytes and/or protective CD4 T lymphocytes. AA, amino acid.

exon and whole-genome sequencing becoming increasingly common, algorithms already exist to rapidly match MaF regions with MHC alleles, thereby facilitating the identification of peptides for personalized cancer medicine. Yet this approach is fraught with concerns based both on the biology of the immune system and of cancer, particularly intratumor heterogeneity (126).

The first concern is that not all mutations and gene fusion products may code for peptides that bind MHC class II alleles of the individual with sufficient affinity to be immunogenic. If one can identify peptides that do bind to MHC class II, it is not clear how to determine whether these peptides can be processed and presented by the patient's own tumor cells. These concerns can only be answered experimentally (Fig. 2). Likewise, it also would be important to identify MaF peptides that bind to different alleles and demonstrate their immunogenicity in HLA-transgenic mice (Fig. 2) (127). Provided that these steps are satisfied, what criteria will be used to predict which MaF peptides are tumor protective? Tran et al. (121) demonstrated that CD4 T cell determinants of clinical value can be identified by characterizing tumor-infiltrating lymphocytes using a library of minigenes coding for all of the possible mutations in multiple genes found in the patient's tumor. This challenging approach is hardly applicable on a large scale. Thus, verification that putatively selected MaF peptides are recognized by tumor-infiltrating lymphocytes appears to be an unavoidable step.

In addition to the difficulties in identifying immunologically and clinically relevant MaF-derived peptides, there are concerns about intratumor heterogeneity (126). Data from genome sequencing, single-cell analyses, and multiregion sequencing point to a surprising genetic heterogeneity, including subclonal differences in driver mutations (128–132). Accordingly, mutations in one cell may not be representative of mutations present in another that has grown aggressively and spread (i.e., metastasis) or in a cell residing in spatially distinct areas of the tumor. The findings suggest that MaF peptide–based immunotherapy against the MaF peptide se-

lected as the neoantigen and tumor target could be of limited value.

Other potential concerns when vaccinating using a CD4 MaF peptide are the expression level and penetrance of the mutated protein in the tumor. For instance, Sampson et al. (133) showed that vaccination against variant III of the epidermal growth factor receptor, which is expressed in glioblastoma cells, is associated with the elimination of *EGFRvIII*-expressing cells at recurrence, but it did not prevent recurrence. A recent study on preclinical vaccination in mice implanted with tumors, in which not all of the cells were transduced with the IDH1R132H target gene, also found evidence of immunological escape (127). This suggests that immunological escape constitutes both a conceptual and practical obstacle. It would be of interest to know whether MaF peptides recognized by CD4 T cells can initiate epitope spreading. This concept was described originally for autoimmune diseases, but there is little evidence for this phenomenon in response to mutated cancer Ags (134, 135).

One last concern about the usefulness of vaccinating with MaF peptides is that focusing exclusively on the genomic landscape of a cancer patient ignores emerging evidence that cell nonautonomous processes condition tumor growth, tumor progression, and clonal diversity. A recent study on the dynamics of clonal repopulation using colorectal cancer cell xenografts in SCID mice showed that genetically stable clones differ with regard to proliferation and response to chemotherapy (136). Similar conclusions were reached by two other studies, both favoring the idea that tumor growth and malignant phenotypes are driven by a subpopulation of cells that can stimulate the growth of other cells in a cell-nonautonomous way (137, 138). Thus, it is increasingly likely that tumor growth and acceleration during cancer progression are independent of genetic mutations, calling for careful assessment of the cost/benefit ratio of MaF-based CD4 T cell immunotherapy.

A reasonable alternative is to focus on MHC class II–binding peptides from unmutated sequences of already identified Ags, such as telomerase, survivin, MUC.1, and HER-2, which are widely overexpressed in human cancers. These frequently overexpressed proteins could lend themselves to inclusion in vaccines designed to exploit Th–Th cooperation (139–143). Presentation of unmutated peptides by the APC, along with a suitably selected nonself CD4 T cell determinant, may prove sufficient for immunological and clinical effects. In addition, there is evidence for epitope spreading following immunization of cancer patients with unmutated tumor Ag peptides (144–149). Although no single MHC class II determinant can cover the entire human population (there are 2870 MHC class II alleles in the Immuno Polymorphism Database-ImMunoGeneTics-Human Leukocyte Antigen release as of November 2014), epitopes can be selected on the basis of the frequency of the MHC class II alleles binding to MHC class II supertypes or binding multiple alleles (promiscuous peptides) (79, 150, 151). This represents a simpler and more economical approach compared with those centered around the use of unique peptides from MaF gene products. Advantages and disadvantages exist: one advantage is that the expansion of CD4 T cells that specifically recognize tumor self-Ags could be a source of help for antitumor CD8 T cells locally in the tumor microenvironment (152). The activation of self-reactive T cells must not pass a critical threshold to avoid the clinical manifestations of autoimmunity against normal tissues expressing the same Ag or tissues expressing other self-Ags via bystander help. However, it was argued and demonstrated that a tolerable degree of autoimmunity is a key aspect of successful cancer immunotherapy (153, 154).

Regulatory issues

As with all new therapeutic modalities, it is important to consider the regulatory process that lies ahead. Although the U.S. Food and Drug Administration (FDA) has approved new biologic therapeutics for cancer at an unprecedented and surprisingly expedited pace (e.g., the mAb pembrolizumab for melanoma), the future of therapeutic vaccines that vary from patient to patient as a result of the nature of the immunogens and the MHC polymorphism remains unclear. Designer personalized cancer vaccines may not be immediately embraced by the FDA but will require a slow process of adaptation and adoption of new measures as we transition into a new regulatory era. Currently, the most expedited approach might be to seek approval for the methods needed to prepare the delivery of MaF-based new therapies (i.e., approval could be granted to the process rather than to the end product). Furthermore, the approval of investigational new drug applications could be expedited if these were limited to one specific mutation, such as KRAS (which is relevant for colon, lung, and pancreatic cancers) or epidermal growth factor receptor (lung cancer and glioblastoma), because these are regarded as cancer-driving mutations (125). The identification of a promiscuous peptide within a mutated region, as recently demonstrated for IDH1 (127), also would simplify the process. Perhaps these and other considerations together with anecdotal successes, such as the one reported recently by Tran et al. (121), would encourage a dialogue between regulatory agencies and proponents of the new approach to find acceptable solutions, shape a new policy, and avoid tempering the current enthusiasm for genomic-based interventions that target the immune system. This appears to be the spirit of "*Paving the Way for Personalized Medicine: FDA's Role in a New Era of Medical Product Development*" released by the FDA in October 2013 (155).

Conclusions

There exists little doubt that CD4 T cell–based therapies, and vaccination in particular, will play a relevant role in tumor control and patient management in the future. One key issue is whether to focus on MaF neoantigens on an individual basis at a cost that may not be affordable and without a guarantee of durable success or to focus on therapeutic vaccines using MHC class II–binding peptides from unmutated sequences of already-characterized tumor Ags along the principle of Th–Th cooperation. Focus on MaF gene products appears to be a logical solution to an immunological quandary with promise for clinical benefit. However, as discussed in this article, there exist many conceptual and practical hurdles. This approach may not be a viable option for all tumors; perhaps only tumors carrying specific translocations may be suited for this approach. In addition, the existing evidence is limited to mostly anecdotal reports, and the long-term success of this approach remains uncertain. There are also financial considerations, including high costs, uncertainties about FDA approval, and likely little

return on investment given the small size of patient population, which may hinder the development of such personalized cancer vaccines. In contrast, peptides from the unmutated sequence of cancer-relevant Ags could simplify vaccine production, thereby benefitting a large fraction of cancer patients at a much lower cost. The answer to this timely question may influence the direction of future efforts for effective cancer immunotherapies.

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