

Molecular Pathways: Comparing the Effects of Drugs and T Cells to Effectively Target Oncogenes

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Abstract

Mutant cancer-driving oncogenes are the best therapeutic targets, both with drugs like small-molecule inhibitors (SMI) and adoptive T-cell therapy (ATT), the most effective form of immunotherapy. Cancer cell survival often depends on oncogenes, which implies that they are homogeneously expressed by all cancer cells and are difficult to select against. Mutant oncogene-directed therapy is relatively selective, as it targets preferentially the oncogene-expressing cancer cells. Both SMI and ATT can be highly effective in relevant preclinical models as well as selected clinical situations, and both share the risk of therapy resistance, facilitated by the frequent genetic instability of cancer cells. Recently, both therapies were compared in the same experimental model targeting the same oncogene. It showed that the oncogene-inactivating drug selected resistant clones, leading eventually to tumor relapse, whereas ATT eradicated large established tumors completely. The mode of tumor destruction likely explained the different outcome with only ATT destroying the tumor vasculature. Elucidating the cellular and molecular mechanisms responsible for tumor regression and relapse will define optimal conditions for the clinic. We argue that the ideal conditions of ATT in the experimental cancer model can be translated to individuals with cancer. *Clin Cancer Res*; 19(2); 320–6. ©2012 AACR.

Background

The survival of cancer cells often remains long-term dependent on a single oncogene, a phenomenon termed as "oncogene addiction" (1, 2). This was shown in mice transgenic for an oncogene controlled by the tetracycline (Tet) system, which allowed to switch the oncogene on and off in a cell type-specific fashion with doxycycline (3). Doxycycline mimicked small-molecule inhibitors (SMI) blocking oncogene activity. The downregulation of cancer-driving oncogenes, for example, *RAS*, *MYC*, *BCR-ABL*, *HER-2*, or *SV40* large T antigen (Tag), led to sustained tumor regression in different models including lymphoma, leukemia, adenoma, glioblastoma, sarcoma, and several different carcinomas (4–13). Drug resistance and tumor recurrence was observed in most models, albeit to different extent (2). The phenomenon of oncogene addiction led to the first SMI, imatinib mesylate, which was designed to inactivate the BCR-ABL fusion protein. BCR-ABL is an overactive tyrosine kinase causing chronic myeloid leukemia (CML) by a chromosomal translocation. The clinical effectiveness of imatinib against BCR-ABL-associated CML (14) led to

further successful SMIs, for example, in the treatment of patients with melanoma and non-small cell lung cancer (NSCLC; ref. 15). Yet, therapeutic effects of SMIs are transient in most patients, and drug resistance is common with tumor burden being one critical factor. For example, in most imatinib-treated CML patients in the blast crisis, tumor recurred after 3 to 6 months, whereas those in the chronic phase remained tumor free for years (16). Many reasons can account for drug failure. The effectiveness of SMI in inhibiting oncogene activity in clinical situations is less clear than that of the Tet system in the experimental model. Currently, it is also difficult to assess which cancers in the clinic are long-term dependent on a single oncogene.

ATT experiments have been carried out for almost 50 years. Improvements such as the grafting of T cells selectively in lymphodepleted hosts have improved its success (17, 18). However, outcomes were variable because experimental models varied with regard to T-cell phenotype, target antigen, host conditions, and tumor burden. Under optimal conditions, ATT can lead to eradication of large established tumors in preclinical models distinguishing ATT from other immunotherapies. Herein we define a tumor as established when it grew for several weeks and reached a volume of at least 500 mm³, which is equivalent to approximately 10⁹ cells and the least clinically detectable tumor size (19). It is critical to use models of such tumor burden, because only then tumors have built up a stroma, which is essential for progression and a decisive target of ATT (20). Furthermore, genetic instability, a hallmark of human cancer and primary cause of therapy resistance, becomes apparent with increasing tumor burden. T cells

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transferred in the course of allogeneic bone marrow (stem cell) transplantation can cure patients with leukemia (21) and virus-associated cancer (22). With regard to solid tumors, ATT in the clinic was hampered by the difficulty in generating T cells *in vitro*. Nevertheless, tumor-infiltrating lymphocytes (TIL) from melanomas, expanded *in vitro* and reinfused, achieved regression in a high proportion of patients (23). As was the case with SMIs, tumor recurrence frequently occurred. Thus, both SMI and ATT have been successfully used in the clinic but are impeded by therapy resistance. Because both therapies compete with each other, with clinical efficacy being the only measure, we recently established cancer models that allowed for the first time direct comparison of SMI with ATT targeting the same oncogene to treat large established tumors (9). Here, the molecular and cellular mechanisms leading to tumor regression and, possibly, recurrence are reviewed. We argue that ATT, by newly developed techniques, has become feasible and offers opportunities for improvement that at least in some indications it will successfully compete with SMI.

Mechanisms of Tumor Regression by Oncogene Inactivation and Development of Drug Resistance

Depending on the cell type and the involved oncogene, various mechanisms have been associated with tumor regression following oncogene inactivation in mouse models. These include proliferative arrest, apoptosis, cell differentiation, autophagy, senescence, tumor dormancy, and modulation of the tumor microenvironment (Fig. 1). In the Tet-TagLuc model that uses sarcoma cells transformed by a Tet-controlled fusion protein of Tag and luciferase (TagLuc), noninvasive bioluminescence imaging visualized oncogene downregulation concomitant with proliferative arrest (loss of Ki-67 expression) as early as 1 day after doxycycline application (9). Tag inactivates tumor suppressors p53 and retinoblastoma (Rb), which are likely released upon its inactivation. In other models, for example, downregulation of BCR-ABL, RAS, MYC, and HER-2 in different cancers, apoptosis was observed (2). In the Tet-TagLuc model, we failed to see induction of apoptosis (cleaved caspase-3 expression). Instead, switching off TagLuc induced autophagy (lipidation of light chain 3 microtubule-associated protein). Autophagy, the degradation of proteins and damaged organelles in cytoplasmic vesicles, is induced under stress conditions such as starvation, hypoxia, and drug treatment. Autophagy was associated with tumor regression.

Different mechanisms associated with oncogene inactivation-induced tumor regression are not mutually exclusive. TagLuc downregulation additionally led to rapid upregulation of fibronectin, a differentiation marker, which has been implicated in cellular senescence (24). In MYC-driven cancer models, oncogene inactivation similarly induced terminal differentiation (5, 10, 25), which ultimately may lead to senescence, characterized

by irreversible cell-cycle arrest (26). Senescent cells remain metabolically active and produce such molecules as metalloproteinase, growth factors, proinflammatory cytokines, and chemokines, termed senescence-associated secretory phenotype (SASP), which may reinforce senescence in an autocrine manner and in a p16^{INK4a}/Rb-dependent fashion (24, 26–29). Conversely, growth factors secreted by senescent cells may support tumor growth and drug resistance by acting on neighboring cells, which allows selection of variants that activate alternative oncogenic pathways (28, 30). Recent clinical data with BRAF inhibitors support this assumption (31, 32). Whether tumor dormancy, observed upon MYC-inactivation (5), is related to cellular differentiation or senescence is not known.

The role of the tumor microenvironment for tumor regression upon oncogene inactivation is poorly understood. Tumor regression by MYC inactivation in experimental models was accompanied by regression of blood vessels, compatible with the activity of MYC to repress the angiogenesis inhibitor thrombospondin-1 and induce VEGF (25, 33–35). Reduced numbers of endothelial cells were also found within RAS-deprived melanomas (11) and TagLuc-deprived sarcomas (9), even though in the latter model most endothelial cells persisted in regressing tumors. It is currently not clear whether reduction of the tumor vasculature is a cause of tumor regression or a consequence of cancer cell death and subsequent remodeling of the tumor microenvironment. Together, a variety of mechanisms are associated with oncogene inactivation-induced tumor regression. They may occur simultaneously, they may differ depending on the transformed cell type, and they may occur, at least partially, in a similar fashion regardless of the inactivated oncogene.

The critical question is which mechanisms confer drug resistance. Tumor burden is of pivotal importance. If comparably small Tet-TagLuc tumors were treated by oncogene inactivation, most completely regressed without recurrence (9). If large tumors were treated, drug-resistant clones emerged without exception. Each tumor had a different genetically acquired mutation in the transactivator gene preventing doxycycline binding and TagLuc downregulation, resembling secondary mutations in the target oncogenes selected by SMI in the clinic (36–38). These data suggest that genetic instability, likely caused by the p53/Rb-inactivating activity of TagLuc, together with tumor burden, caused drug resistance. Because TagLuc inactivation resulted in rapid growth arrest but acquisition of mutations requires cell division, resistant clones likely preexisted *in vivo* before therapy. Similarly, recurrent tumors in patients may often be derived from preexisting variants insensitive to the drug due to secondary mutations, oncogene amplification, activation of upstream receptor tyrosine kinases, or pursuing alternative survival pathways (37, 39, 40), and hence, were never amenable to respond to the drug. However, provided that the majority of cancer cells are

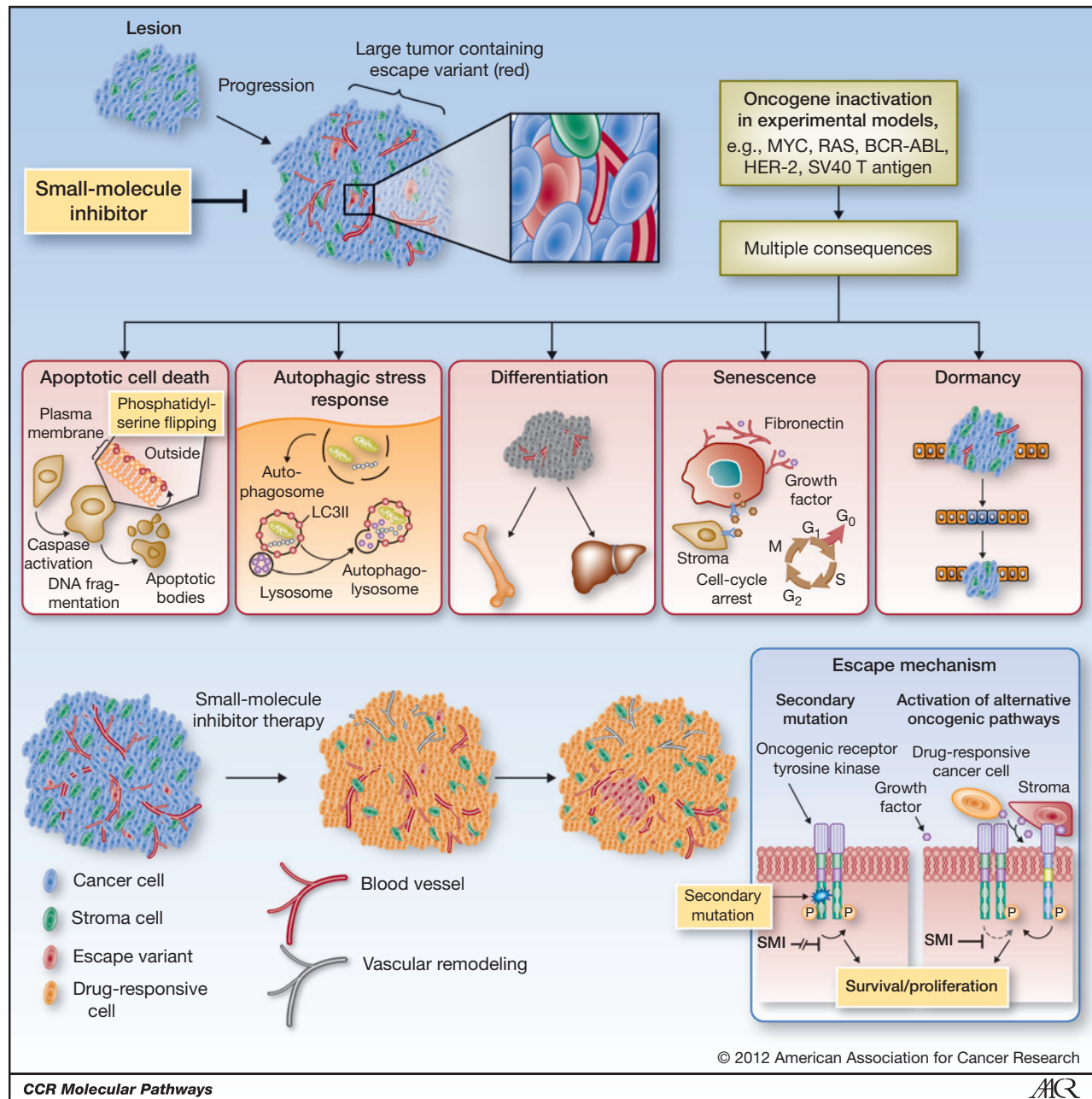


Figure 1. Anticancer therapy against large established tumors using small-molecule inhibitor (SMI) therapy. Top, due to inherent genetic instability, cancer cells acquire additional mutation during progression from small malignant lesions to established tumors. Before starting therapy, the majority of clinically detectable tumors ($\geq 500 \text{ mm}^3$) contain cancer cell variants already that are resistant to the intended drug treatment (red cell). Several transgenic models with tetracycline controllable oncogenes (as indicated) proved the concept of "oncogene addiction." Middle, treatment with oncogene inactivation drugs (SMI) can have multiple consequences on drug-responsive cancer cells, including apoptotic cell death, autophagic stress response that can culminate in cell death, differentiation, senescence, and tumor dormancy (as shown from left to right). These effects are thought not to be mutually exclusive. Bottom, left, SMI therapy targets drug-responsive cancer cell but leaves the tumor stroma, including tumor vasculature, largely intact. In addition, drug-responsive cells that do not respond with apoptotic cell death, but, for instance, undergo senescence, become part of the tumor microenvironment, which can suppress but also support survival and proliferation of escape variants, for example, by releasing growth factors. Bottom, right, drug resistance is caused by secondary mutation within the oncogene, for example, receptor tyrosine kinase (RTK), (blue star) that in most cases prevents drug binding (left) or by activation of alternative oncogenic pathways, for example, by growth factors released by stroma or drug-responsive tumor cells (right).

drug responsive, the various mechanisms outlined above likely create a microenvironment that influences the resistant clone. Which mechanism (apoptosis, differenti-

ation, autophagy, senescence, dormancy, or microenvironment) positively or negatively affects the resistant clone is still poorly understood.

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Mechanisms of Tumor Eradication by ATT: How to Prevent Relapse

In the same model of large established Tet-TagLuc tumors, in which drug resistance was the rule, ATT was used with CD8⁺ effector T (T_E) cells, recognizing a single peptide epitope of Tag. Escape variants appeared likely, as *in vitro* T cells could select variants with mutation in the peptide epitope (41). However, without exception, the T_E cells eradicated large established tumors and no escape variants were ever selected (9), which raises 2 questions. First, how did the rejection response by the T_E cells differ from the regression/recurrence response induced by the drug, and second, what distinguishes the experimental cancer model from the clinical ATT trials, mainly in melanoma? Here, objective responses were frequent, but long-term responses rare (23). In most patients, it remained unclear whether tumor recurrence was due to immune

escape, for example, loss of the target antigen or the peptide presenting MHC I molecules (42, 43), or loss of T-cell function.

Drug therapy quite selectively eliminated cancer cells. Endothelial cells and probably the whole microenvironment remained largely alive. Therefore, we argue that a vital stroma facilitated the survival and outgrowth of resistant clones. In sharp contrast, during ATT, cancer cells and the tumor vasculature were simultaneously destroyed, so that the entire tumor tissue became necrotic (9). Under such hostile conditions, potential escape variants are unlikely to survive, a phenomenon termed bystander elimination (20). T_E cell-mediated rejection of large established tumors depended on such cytokines as IFN- γ and TNF- α and such cytotoxic molecules as perforin (refs. 20, 44; Fig. 2). In one model of established tumors, tumor stroma cells needed to respond to both cytokines, which prevented selection of

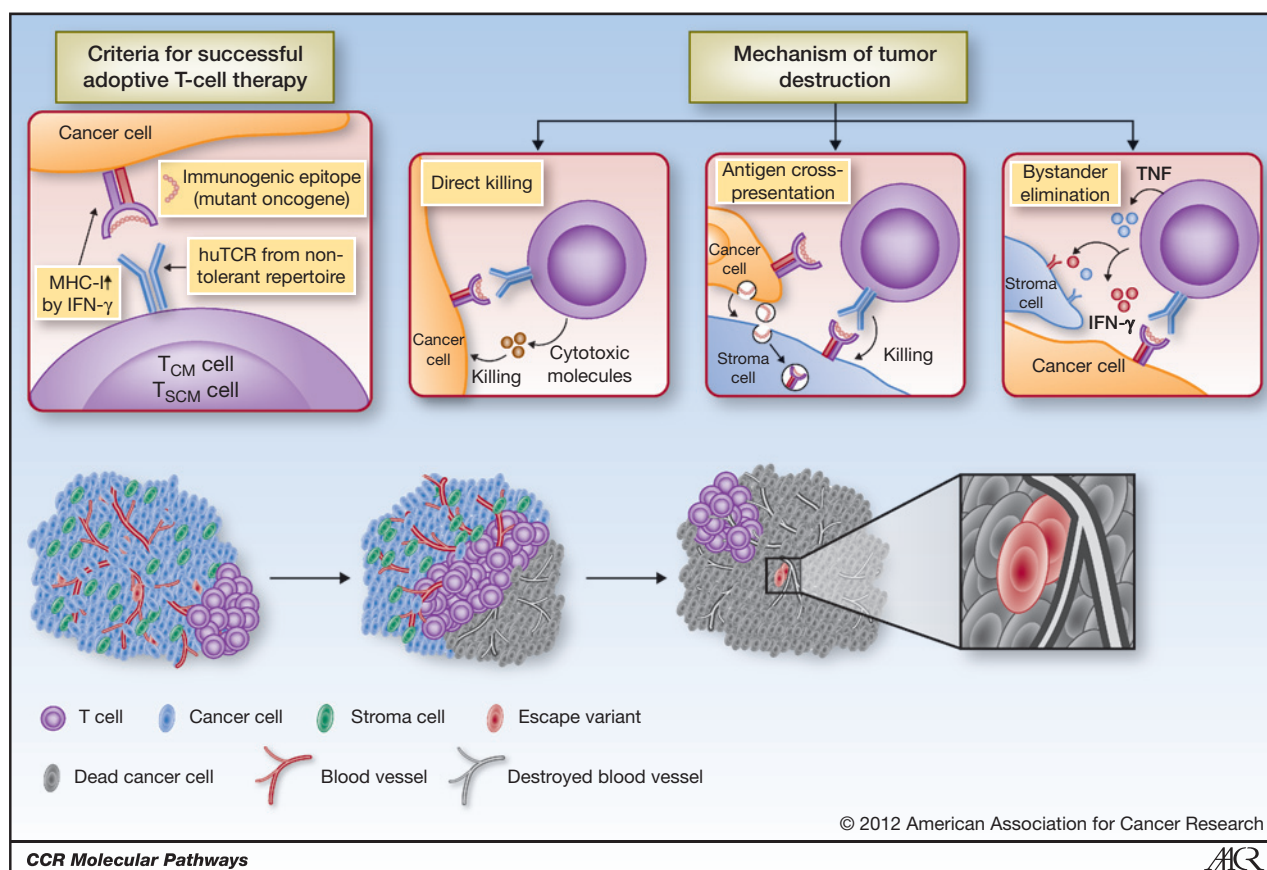


Figure 2. Anticancer therapy against large established tumors using adoptive T-cell therapy (ATT). Top left, at least 4 reasons may account for successful TCR gene therapy: 1, cancer cells respond to IFN- γ by MHC I upregulation. 2, TCR is isolated from the nontolerant repertoire of humanized TCR gene loci/MHC I transgenic mice. 3, TCR is introduced into patients' T_{CM}/T_{SCM} cells. 4, TCR is specific for mutant immunogenic epitope of cancer-driving oncogene. Top, right, tumor destruction by ATT involves 3 main mechanisms: First, direct killing of cancer cells by T_E cells occurs upon recognition of antigenic epitopes presented on MHC I molecules by cancer cells. TCR engagement leads to expression of cytotoxic molecules by T_E cells that lyse the antigen-presenting cancer cells. Second, under certain conditions, tumor antigens are transferred to noncancer cells, for example, via vesicle transport, and are presented by MHC I molecules on stroma cells. Those cross-presenting stroma cells become targets for T_E cells and will be killed upon recognition. Finally, molecules (TNF- α and IFN- γ) released upon cancer cell recognition by T_E cells can have destructive effects on neighboring cells, for example, endothelial cells, that do not present antigenic epitopes on MHC I molecules. Those cells are killed by bystander elimination. Bottom, ideally, T cells invade tumors in large numbers, leaving dead tumor cells, including stroma cells, behind. Potential antigen loss variants that escaped T-cell destruction fail to survive in the harsh tumor microenvironment created by those destructive T_E cells. huTCR, human T-cell receptor; T_{SCM}, T cells with stem cell-like characteristics.

antigen loss variants (20, 44). In addition, tumor antigen cross-presenting stroma cells were lysed by T_E cells *in vitro*. On the basis of the Tet-TagLuc model, we argue that antigen recognition on the cancer cells was sufficient for T_E cells to produce IFN- γ , which acted on and destroyed the tumor vasculature without being directly recognized by the T_E cells (9, 45, 46). The efficacy of ATT to reject large established Tet-TagLuc tumors can be explained by the optimal conditions of the experimental setting that relied on factors such as the phenotype of the T_E cells, the cancer cells, the target antigen, and the host. T cells were derived from immunized T-cell receptor (TCR) transgenic mice, whose T cells were specific for Tag (9). They had not been cultured *in vitro* for prolonged time, which may render T cells ineffective (47), and they may have contained central memory T (T_{CM}) cells or T cells with stem cell-like characteristics (T_{SCM}) that showed improved efficacy *in vivo* (48, 49). The T cells were derived from the nontolerant repertoire, which excludes skewing toward low-avidity T cells by tolerance mechanisms, and recognized the target antigen as foreign. The cancer cells presumably upregulated MHC I molecules in response to IFN- γ , comparable with "soft" lesions in patients (50). The hosts, Rag-1^{-/-} mice, were lymphopenic. Most importantly, the target antigen was the cancer-driving oncogene, expressed by all cancer cells, but not normal cells. How can these features be translated into the clinic?

Clinical-Translational Advances

For ATT with the best possible outcome, we envisage the following scenario. The use of TCR gene therapy overcomes a number of current hurdles (51). It allows equipping patients' own T cells with desired specificities and generation of sufficient numbers of T cells in a short period of time, avoiding their exhaustion (47). The TCR will be transduced into T_{CM} or T_{SCM} cells, which may ensure better persistence and function upon transfer (48, 49). TCR-engineered T cells will be infused into patients with cancer rendered lymphopenic by chemotherapy or irradiation, allowing efficient engraftment but inhibiting immune suppression (17, 18, 23, 52). Transgenic mice expressing human MHC molecules and a diverse human TCR repertoire serve as a tool to rapidly analyze whether peptide antigens are immunogenic, that is, if they are processed and presented by MHC molecules and if they efficiently induce T-cell responses following immunization (53). With the use of the human TCR transgenic mouse, any human peptide sequence not encoded by the mouse genome is suitable for immunization and might yield TCRs with optimal affinity. Optimal affinity means that the T cells are restricted to human self-MHC molecules and recognize the peptide antigen as foreign, for example, as representatives of the nontolerant repertoire. By using peptide/MHC multimers, specific T cells of the transgenic mice can be sorted, then, human TCRs isolated, for example, by single cell PCR, the TCRs optimized for efficient expression while avoiding mispairing with endogenous TCR and are used for transduction of patients' T cells with viral vectors (54, 55). We anticipate that such engineered T cells, comparable with the Tet-TagLuc tumor model, will

exert optimal effector function within the tumor microenvironment and not only eliminate the cancer cells but also destroy the tumor vasculature by release of large amounts of IFN- γ and TNF- α , thereby indirectly eliminating potential escape variants.

The key problem of ATT is to target the right antigen to prevent tumor recurrence and toxic side effects (56). This sounds simple, given the large number of putative tumor antigens. However, most are tumor-associated (self) antigens (TAA). TAAs are also expressed by normal cells. Expression by rare but vital cells has usually not been analyzed. Moreover, TAA expression may be heterogeneous within the tumor/metastases of a given individual. Thus, targeting TAAs bears the risk of ineffective long-term responses and destruction of normal tissues. Clinical trials with TCR [or chimeric antibody receptor (CAR)]-engineered T cells, for example, directed against Melan-A/MART-1, gp100, HER-2 and carcinoembryonic antigen, support this assumption (57–59). Some differentiation antigens, such as CD19, expressed by B cells and B-cell lymphomas, represent exceptions. CAR-engineered T cells, specific for CD19, effectively eliminated B-cell lymphomas and normal B cells as an acceptable side effect (60). However, for many, if not most, TAA's substantial toxicity by effective ATT is predictable.

If, in contrast, mutant cancer-driving oncogenes are targeted by ATT, many of the problems with TAAs will resolve. Except for antigens encoded by cancer viruses, mutated antigens are the only tumor-specific antigens (TSA). Mutant cancer-driving antigens are expressed by all cancer cells, exclusively by cancer cells (with the possible exception of cross-presenting stroma cells; ref. 20), and difficult to select against. Of note, target antigen expression on normal cells can prevent tumor rejection by ATT (61). Some TSAs are shared between tumors, for example, RAS, BRAF, p53, or fusion regions of chromosomal translocations. However, despite continuous claims of their immunogenicity, often analyzed by reverse immunology, firm evidence that T cells raised against shared TSAs reject tumors is lacking. For example, the TEL-AML chromosomal breakpoint region was claimed to generate an immunogenic HLA-A0201-restricted epitope (62), but subsequently, it was shown that it is not processed and presented (63). Thus, raising T cells against shared TSAs is difficult and likely possible only for a few HLA restriction molecules. The solution to this problem is whole cancer genome analysis and identification of patient-specific somatic mutations, leading to immunogenic epitopes and being putatively cancer driving. Some cancers, such as melanoma, acquired more than 33,000 mutations, of which around 0.9% were point mutations in coding regions (64). In fact, patient-specific immunogenic TSAs have been detected in melanomas (65).

In conclusion, under ideal conditions ATT eradicates large tumors, whereas SMI treatment eventually leads to drug resistance. We think ideal ATT can be pursued clinically through the following process: sequence the cancer genome, identify recurrent somatic mutations in coding

regions, use computer algorithms to predict immunogenic epitopes, analyze peptide/MHC stability, verify immunogenicity in and isolate optimal affinity TCRs from MHC/TCR humanized mice, construct TCR retroviral vectors, transduce patients' T cells, and transfer TCR-redirectioned T cells back into lymphopenic patients. We are aware that clinically additional problems have to be resolved, which could not be discussed here.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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