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Breaking tolerance in cancer immunotherapy: time to ACT

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The discovery of defined tumor antigens and their application in therapeutic cancer vaccines has not yet resulted in a successful therapy for cancer patients. Recent data suggest that this might be because most current clinical immunotherapeutic strategies rely on a tolerized tumor-reactive T-cell repertoire, resulting in a weak T-cell response that cannot induce tumor regression in the face of a multitude of normal and tumor-induced immunoregulatory mechanisms. New insights from animal models and clinical trials suggest a rationale for combination approaches in which the ineffective endogenous anti-tumor immune response is enhanced through a combination of adoptive cell transfer (ACT), specific vaccination and cytokine help for the reliable induction of a robust anti-tumor immune response and tumor regression.

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Introduction

The molecular identification of defined human tumor antigens in the early 1990s invigorated the field of cancer immunotherapy: scientific rationale predicted that vaccine-induced T-cell immunity to these antigens would destroy tumors and provide long-term protection against tumor recurrence. However, one decade of clinical experience with cancer vaccines has not resulted in immunotherapy that consistently induces robust T-cell responses and long-lasting tumor regressions [1–3]. It appears that a major hurdle in the induction of strong, curative anti-tumor immunity is immunological tolerance to cancer cells. This review will summarize some recent developments in the field of immunological tolerance to cancer and discuss their ramifications for possible strategies towards more effective cancer immunotherapy.

Mechanisms of immunological tolerance to cancer

It is now clear that there are several potential bottlenecks in the immune response to cancer cells:

1. Lack of high-avidity tumor-specific T cells.
2. Inefficient priming of tumor-specific T cells.
3. Physical and functional deletion of primed tumor-specific T cells.
4. Tumor evasion and counterattack.

Lack of high-avidity tumor-specific T cells

Most tumor antigens, particularly the shared self-antigens which are the most useful for off-the-shelf vaccine development, are expressed in the thymus during thymic T-cell selection and thus induce profound central immunological tolerance through clonal T-cell deletion. This notion has been reinforced by the discovery that even (tumor) antigens that were previously thought to be exclusively expressed in the periphery can be expressed in the thymus under control of proteins such as the autoimmune regulator (AIRE) gene product [4]. In addition, dendritic cells (DCs) can cross-present peripheral antigens in the thymus and induce T-cell deletion [5]. The result is a tolerized repertoire of T cells with only low to intermediate avidity for self-tumor antigens. The relatively high activation threshold of these T cells results in poor proliferation and effector function upon antigenic stimulation [6,7•]. Despite possible exceptions, such as the apparent high thymic output of functional MART-1 melanoma antigen-specific T cells, the majority of evidence points to the presence of only low numbers of high-avidity self-tumor-antigen-specific T cells in both healthy individuals and cancer patients [8].

Inefficient priming of tumor-specific T cells

T cells that have survived thymic selection and enter the periphery remain under the control of an elaborate system of checks and balances that prevent autoimmune disease [4,7•]. These mechanisms of peripheral tolerance also limit the activation and proliferation of tumor-specific T cells [9]. First, T cells with proven specificity for tumor antigens tend to largely ignore tumor cells. There is ample evidence in mouse and man that most solid cancers, similar to normal somatic cells, do not directly prime self-tumor-antigen-specific T cells very efficiently [10,11•,12]. The most straightforward explanation for this phenomenon is that any self-reactive T cell of sufficiently low avidity to escape thymic deletion will also be of too low avidity to respond to that antigen on a tumor cell. Tumor-specific T cell ignorance is compounded by the

absence on most tumor cells of co-stimulatory molecules that promote optimal T-cell activation by professional antigen-presenting cells such as dendritic cells (DCs) [13].

Tumor antigen cross-presentation

Whether tumor antigens can be productively cross-presented to T cells has been the subject of considerable debate [14–16]; it now appears that the degree of cross-presentation depends greatly on the individual antigen and experimental system studied [15,17]. Tumor antigens can be cross-presented, but the resulting T cell response is usually weak due to the typically poor avidity of responding T cells. In addition, DCs in tumor-draining lymph nodes are often incompletely activated, favoring the induction of cross-tolerance instead of cross-priming [16,18]. Still, strong overexpression or alternative processing of tumor antigens in tumor cells can sometimes break tolerance (Y Ono *et al*., unpublished; [19]). Human cancer vaccines may also act through cross-priming; Thomas *et al*. [20] detected mesothelin-specific $CD8^+$ T cells restricted by patient HLA molecules after vaccination with an HLA-mismatched pancreatic whole tumor cell vaccine. It remains to be determined exactly which conditions favor tumor-specific T-cell cross-priming [20,21] or cross-tolerance [18].

Some animal data suggest that growing tumors efficiently prime naïve T cells; however, this is often attributable to the use of tumors expressing foreign antigens selected for their potent immunogenicity and to which the mouse T-cell repertoire has not undergone thymic or prolonged peripheral tolerization. Indeed, the large majority of animal studies that employ true self or neo-self antigens, and more importantly most human studies, reveal detectable yet very poor endogenous priming of tumor-specific T-cells[19,22,23], often followed by their rapid deletion or dysfunction [24•,25••,26••].

Physical deletion and functional suppression of primed tumor-specific T cells

Low-avidity T cells are typically poorly activated by endogenous, tumor-derived antigen but can sometimes respond to a cancer vaccine that induces strong antigen presentation in an inflammatory setting or to antigenic stimulation *ex vivo*. This activation can dramatically lower the threshold for antigen recognition, in some cases enabling recognition of the tumor cells that were previously ignored [11,27]. Yet these activated T cells also become immediately susceptible to the normal homeostatic immune mechanisms that prevent excessive immune reactivity as well as to tumor-specific immune deviations that limit T-cell proliferation, function and survival.

T_{reg} cells in cancer

Activated T cells can be silenced through the action of various types of suppressor T cells, now renamed regulatory T (T_{reg}) cells. T_{reg} cells can strongly suppress IL-2 production and proliferation of antigen-specific T cells and, in animals, can prevent tumor regression [28,29]. In an elegant study by Turk *et al*. [29] the depletion of T_{reg} cells enabled the induction of specific T cells by a primary tumor, preventing the growth of a second tumor inoculum. Still, these T cells failed to stop primary tumor growth, suggesting that depletion of T_{reg} cells can enhance tumor immunity but might not be sufficient to allow the induction of curative immunity.

Suppressive T_{reg} cells, some of which are tumor-antigen-specific, have recently been found in human breast, ovarian and lung cancer, and in melanoma, indicating that they might keep a cancer patient's immune system from rejecting 'self-like' cancer cells [30•,31,32]. It is unknown whether the eradication of T_{reg} cells contributes to the high success rate of allogeneic hematopoietic stem cell transplantation in lymphodepleted patients with hematological malignancies, or to the 50% response rate in melanoma patients receiving non-myeloablative lymphodepletion followed by adoptive cell transfer (ACT) with *ex vivo* expanded tumor-specific T cells ([33,34]; see also the review by Wrzesinski and Restifo in this issue [35]).There is ongoing debate as to whether the T cell inhibitory molecule CTLA-4 mediates the suppressive effect of some types of T_{reg} cells. What is clear is that *in vivo* CTLA-4 blockade can facilitate T-cell dependent tumor regression in mice [28,36], and to some extent in melanoma patients [37,38], suggesting that CTLA-4 mediates a significant inhibition of anti-tumor immunity.

Physical deletion of tumor-specific T cells

Successful priming can expose tumor-specific T cells to direct deletion by tumor-derived antigen [9,13]. For example, the absence of high-avidity $CD8^+$ T cells against the self-antigen proteinase-3 correlated with high tumor burden in patients with chronic myelogenous leukemia. *In vitro* stimulation with high specific peptide concentrations likewise induced apoptosis of high-avidity proteinase-3 specific T cells, suggesting it was high tumor (antigenic) burden that induced the selective deletion of high-avidity T cells *in vivo* [9,13,18,24].

Inhibition of tumor-specific T cell effector function

In addition to being physically deleted, tumor-specific T cells can be functionally silenced [9,13,39]. A recent study found a profound inhibition of IFN-γ production in tumor-derived but not blood-derived MART-1 melanoma-antigen-specific $CD8^+$ T cells. Importantly, tumor-derived cytomegalovirus-specific T cells were fully functional [26]. A different study found that tumor-infiltrating T cells secreted normal IFN-γ levels but were incompletely differentiated and expressed abnormally low amounts of perforin and granzyme [25]. Apparently there is an antigen-specific functional

impairment of T cells at the tumor site, possibly due to chronic stimulation by tumor-derived antigen [39,40], local T_{reg} cell activity [30•,31,32] or direct T-cell inhibition by tumor cells and their products, as discussed below.

Tumor evasion and counterattack

Tumor-specific T cells are subject not only to normal immunoregulatory mechanisms but also to tumor-induced inhibition of their priming, proliferation, effector function and survival. The ever-increasing number of molecularly defined mechanisms by which tumors escape immune destruction have been thoroughly reviewed elsewhere [9,41]. Although a systematic inventory of the presence or absence of all these mechanisms in individual patients' tumors has never been reported, such an inventory can be compiled from the literature for the malignant mouse melanoma, B16. Table 1 lists a series of immunoevasive and counterattack mechanisms that have all been identified in a variety of human tumors and are present in B16 melanoma. With the caveat that B16 is a long-term established melanoma cell line, this exemplifies how tumors can escape immune destruction through multiple simultaneous mechanisms of immune evasion and counterattack.

Tumor immunotherapy: only for the hopelessly optimistic?

The sheer number and variety of possible mechanisms underlying the remarkable resistance of tumors to immunotherapy makes it difficult to envision a therapeutic strategy that could effectively overcome each obstacle with a specific countermeasure. Such a strategy is further complicated because different mechanisms may play a more or less dominant role depending on tumor histology and the genetics of individual cancer patients and their tumors. Unless one particular mechanism is identified as a consistent and dominant inhibitory factor, successful immunotherapy by reversing tumor-specific T-cell tolerance and tumor evasion/counterattack might prove a lengthy and rocky road with few significant milestones in sight.

One way to circumvent the normal and tumor-induced inhibition of tumor-specific T cells is to remove them from the tumor-bearing host and expand them *ex vivo* before re-infusion. ACT with allogeneic stem cells and donor lymphocytes has shown the potential of adoptive transfer in the treatment of hematological malignancies, whereas a clinical trial of ACT with autologous, *ex vivo* expanded tumor-specific T cells after non-myeloablative

Table 1

Barriers to immunotherapy of B16 melanoma.

Barrier	Effect on T cell-mediated anti-tumor immunity	References
Very low expression of MHC class I and class II molecules	Poor recognition/killing by $CD8^+$ and $CD4^+$ T cells	[58,59]
No TAP-1 expression	Poor peptide-loading and surface stability of MHC class I molecules	[60,61]
Very low expression of CD1d non-classical MHC molecule	Poor recognition/killing by NK cells	[58]
No expression of known co-stimulatory molecules	Poor direct priming of tumor-specific $CD8^+$ T cells	[62]
Low affinity of antigenic $gp100_{25\text{-}33}$ peptide for MHC class I molecule	Poor recognition by gp100-specific $CD8^+$ T cells; poor cross-priming by DCs	[11•,63]
T_{reg} cell-mediated suppression of anti-tumor immunity	Suppression of tumor-specific $CD4^+$ and $CD8^+$ T-cell proliferation and function	[28,29]
CTLA-4-mediated suppression of anti-tumor immunity	Inhibitory signaling in tumor-specific T cells	[64]
Tumor cells express PD-L1/B7H-1	Inhibition of tumor-specific T-cell proliferation, cytokine production and cytotoxicity	[65]
Tumor cells express constitutively active STAT-3	Suppression of proinflammatory cytokine and chemokine production; inhibition of DC maturation and T-cell priming	[66]
Tumor cells secrete TGF-β	Suppression of tumor-specific T-cell proliferation	[59]
Tumor cells express CD95/FasL	Induction of apoptosis of tumor-infiltrating T cells	[59]
Tumor cells express galectin-1	Induction of T-cell growth arrest and apoptosis, and blocking of inflammatory cytokine production	[67]
Tumor cells express arginase	Local arginine depletion and inhibition of TCR expression and T-cell proliferation	[68]
Tumors induce protective stromal barrier	Poor tumor invasion by tumor-specific lymphocytes	[12]
DCs in tumor-draining lymph node express IDO	Local tryptophan depletion and inhibition of T-cell proliferation	[69]

lymphodepletion showed remarkable efficacy in metastatic melanoma patients [33,34,35]. The latter study demonstrates that T cells that have failed to control tumor growth can be expanded *ex vivo* under non-tolerogenic conditions and can, upon re-introduction into the conditioned patient, mediate strong tumor regression.

Combination therapy: a three-stranded cord is not easily broken

Animal models of melanoma have been instructive in defining new immunotherapeutic strategies and the mechanisms through which they function. As an example, B16 melanoma is notoriously resistant to treatment even with strategies that induce the complete regression of other, more immunogenic, murine tumors. Indeed, studies on the efficacy of an immunotherapeutic approach in different murine tumors usually identify B16 as the most difficult to treat [42–44], possibly due to its many immunoevasive and counterattack mechanisms (Table 1). From this viewpoint, B16 might be a suitable model for most human cancers that are also remarkably resistant to immunointerventions that are highly successful against many murine tumors.

B16 naturally expresses mouse gp100, the homologue of the human melanoma antigen gp100, which has been targeted in a variety of vaccine trials. ACT with large numbers of naïve or *in vitro* activated $CD8^+$ T cells bearing a mouse gp100-specific TCR did not slow the growth of even undetectably small three-day established subcutaneous B16 tumors [11]. Likewise, vaccination with mouse gp100 peptide and agonistic anti-CD40 monoclonal antibody or recombinant vaccinia or fowlpox virus encoding mouse gp100 did not induce tumor regression. Even high-dose IL-2 therapy, effective in some melanoma patients, did not slow aggressive tumor growth. Any dual combination of ACT, vaccination and IL-2 was also ineffective, all of which mimics the general experience with mono- or dual-combination therapies using ACT and/or vaccination and/or IL-2 for the treatment of cancer patients [1,3].

Despite their ineffectiveness as single or dual immunological intervention, ACT, vaccination and IL-2 in a triple combination reliably induced the complete and long-lasting regression of large, 1 cm subcutaneous B16 tumors. There was a strong quantitative aspect to tumor regression; complete cure was dependent on large numbers of T cells, powerful vaccination and medium to high doses of IL-2. Prior lymphodepletion reduced the required number of infused T cells, reminiscent of the clinical trial of ACT after lymphodepletion [33,34]. IL-2 not only boosted $CD8^+$ T cell numbers, but also dramatically reversed their lack of effector function at the tumor site. When thus activated, previously ineffective gp100-specific T cells induced the complete regression of large B16 tumors in a host lymphocyte independent fashion and without the frequent induction of tumor escape variants [11].

It is somewhat surprising that complete tumor regression relied only on the administration of tumor-specific T cells, vaccination and cytokine help to counter the multitude of known suppressive immune deviations induced by B16 melanoma (Table 1). This suggests that it might be possible to overcome the multiple normal and tumor-induced tolerogenic and immune escape mechanisms that prevent immune-mediated destruction of tumors by overwhelming them with large numbers of vaccine-activated, cytokine-driven tumor-specific T cells (Table 2; [45]). Such an approach is reminiscent of the sometimes massive endogenous immune responses mounted by healthy individuals against immunoevasive pathogens such as Epstein-Barr virus [46]. At the present time this strategy might be more readily applied than an approach that aims to overcome each individual barrier to

Table 2

General barriers to the anti-tumor immunity and potential countermeasures.

Barrier	Countermeasure	References
The endogenous repertoire to self or neoantigens on tumors generally consists of low numbers of T cells with poor avidity	Adoptive transfer of *ex vivo* expanded autologous tumor-reactive T cells	[11,33,34,70]
	Adoptive transfer of TCR-transduced autologous T cells	[47•,48–50]
Tumor-reactive T cells can be ignorant due to low affinity of the peptide for MHC molecules or low affinity of the TCR for the MHC-peptide complex	Vaccinate with altered peptide ligand: increased binding of peptide to MHC molecule and/or	[11,51,71]
	increased binding of MHC-peptide to TCR	[72,73]
Tumor-reactive T cells can be anergized by antigen on normal cells, tumor cells or on immature DCs presenting captured tumor antigen	Adoptively transfer non-anergic, *ex vivo* activated tumor-reactive lymphocytes	[11,33,34,70]
	Support transferred T cell proliferation and effector function by conditioning lymphodepletion and common γ-chain cytokine help	[11,33,34,53,54,74,75]
Tumor cells can escape destruction by directly or indirectly inhibiting or killing tumor-specific T cells	Deplete T_{reg} cells through lymphodepletion	[11,33,34]
	Overwhelm inhibition with more T cells	[34,45]

therapeutic tumor immunity with a specific pharmacological or immunological intervention.

Towards improved immunotherapy

A clinical test of a combination immunotherapy as outlined above is not trivial to implement. Most significantly, tumor-specific T cells can only be isolated and grown from roughly half of metastatic melanoma patients, and from virtually none of the patients with non-melanoma cancers. This major limitation could be relieved by the use of autologous lymphocytes retrovirally transduced with tumor-specific TCR genes, as recently demonstrated by several groups [47•,48–50]. Initial concerns regarding the oncogenicity of retroviral transduction have diminished by the recognition that observed adverse events were directly linked to the treatment-specific genes, transduced cell types and disease characteristics [51]. TCR gene transfer enables the rapid engineering of T cells that efficiently recognize any of the growing number of tumor antigen–MHC combinations for which TCR genes have been isolated, thus providing a reliable source of tumor-specific T cells for ACT.

TCR gene-transduced T cells can be activated *in vivo* by vaccination, as their target antigens and target TCRs are defined [48]. A multitude of cancer vaccine trials have identified a variety of vaccine formulations that are safe and can induce measurable T-cell expansion [3]. As the third component of combination immunotherapy, IL-2 appears to enhance the efficacy of both vaccine-induced and adoptively transferred T cells in mouse and man [11•,36,52–55]. Recent data suggest that other common γ-chain cytokines, such as IL-7, IL-15 and IL-21, might be even more effective, possibly by preventing activation-induced cell death and/or promoting the differentiation of adoptively transferred T cells to a more persisting, central memory phenotype [56,57].

The continuing optimization of immunological techniques will facilitate the design of immunotherapy of patients with advanced, otherwise incurable cancer; this could lead to strategies that facilitate and broaden the applicability of ACT for cancer patients in a scenario such as that summarized in Box 1.

Box 1 An approach to the immunotherapy of cancer.

1. Patient leukapheresis, HLA typing and tumor antigen expression analysis.
2. Selection of therapeutic TCRs.
3. Retroviral TCR gene transduction of patient PBLs.
4. Patient DC preparation.
5. Patient lymphodepletion.
4. Short-term TCR gene-transduced T cell expansion and adoptive transfer.
5. Vaccination with antigen-loaded DCs.
6. Administration of common γ-chain cytokine.
7. Repeat if necessary.

Such a futuristic yet feasible approach would bypass known bottlenecks in the anti-tumor immune response and facilitate the generation of large numbers of tumor-specific T cells, their subsequent *in vivo* activation and expansion through vaccination, and further enhanced expansion, effector function and survival by cytokine help. Each component of this regimen has already demonstrated safety and limited efficacy in mice and, with the exception of TCR gene-transduced T cells currently in clinical trials, in cancer patients. Although logistically challenging, a combination approach is possible. Most importantly, by no longer relying on the endogenous T-cell repertoire, the success rate of generating large numbers of tumor-specific T cells *ex vivo* should only be limited by the growing availability of antigen-specific TCRs [47•,48–50]. The use of TCR gene-transduced autologous T cells would also extend the availability of antigen-specific immunotherapy by ACT to non-melanoma cancers.

Conclusions

Preclinical and clinical evidence suggests that the anti-tumor immune response, even when stimulated by vaccination, is weak due to the poor quantity and quality of the tolerized endogenous anti-tumor T-cell repertoire as well as a multitude of tumor-specific immune deviations. Our currently incomplete knowledge and understanding of these multiple pathways and mechanisms makes it difficult to design rational therapies that will release the brakes on the endogenous anti-tumor immune response through combinations of specific countermeasures. There is an opportunity to combine ACT to supply high numbers of *ex vivo* generated tumor-specific T cells, which can then be activated *in vivo* with currently available, validated vaccines and supported with cytokine help. Combination strategies might yield new insights into which elements of the immune response to cancer are limiting and increase the clinical benefit of cancer immunotherapy.

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