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Human tumor-specific T lymphocytes: does function matter more than number?

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In recent years, several clinical trials have involved the vaccination of cancer patients with tumor-specific antigens that are recognized by T lymphocytes. Anti-vaccine T-cell responses in these patients have been monitored on the assumption that their magnitude would correlate with clinical efficacy. Although analysis of these data show that such a correlation is emerging, detailed analyses of the few patients who benefit clinically from the vaccinations suggest that the function of the anti-vaccine T cells might be more important than their number. Recent studies show that in cancer patients numerous tumor-specific T cells appear to be quiescent in the presence of the tumor. Understanding how an efficient vaccine interferes with this coexistence is one of the current challenges of cancer immunotherapy.

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Introduction

Human T lymphocytes that recognize tumor-specific antigens have become amenable to precise immunological analysis as a result of the identification of a wide variety of tumor-specific antigens and the development of tetramer technology. Clinical trials using vaccines comprising well-defined tumor antigens are usually followed by an analysis of the anti-vaccine T-cell response in search of a meaningful surrogate marker of clinical efficacy. But even though several methods can be used to estimate the frequencies of human anti-vaccine T cells, only few studies have tried to establish a correlation between the detection of T-cell responses and clinical outcomes in vaccinated patients. The first part of this review summarizes these studies; the second part of this review focuses on the notion that high frequencies of tumor-specific T cells do not guarantee therapeutic efficacy, and that the function of these lymphocytes could matter more than their number.

Before discussing the numbers and functions of tumor-specific T cells in the context of therapeutic vaccination, it is important to remember that an optimal T-cell response will not lead to rejection of a tumor that has become resistant to immune attack because of loss of antigen expression or other mechanisms. In a recent clinical trial combining conditioning chemotherapy, transfer of anti-tumor T cells and IL-2 therapy, 18 out of 35 melanoma patients experienced a clinical response [1•]. This observation indicates that at least 50% of melanoma tumors cannot completely resist immune attack.

Frequency of tumor-specific anti-vaccine T cells: does number count?

Clinical studies of anti-tumoral vaccination have been monitored on the premise that massive anti-vaccine T-cell responses are required for tumor rejection. In mice vaccinated with tumor antigens, the intensity of anti-vaccine T-cell responses appears to correlate with clinical efficacy [2,3]. Is this also observed in patients?

Estimating the frequency of human tumor-specific T cells

Only a few methods are available to estimate the frequencies of T cells that recognize particular defined tumor antigens. A direct estimation of this frequency can be obtained using *ex vivo* assays such as tetramer labeling or cytokine secretion measured by Elispot, provided that the frequency exceeds a threshold of about 5×10^{-4} of the $CD4^+$ or $CD8^+$ T cells present. To detect T cells at lower frequencies the lymphocytes have to be amplified first by re-stimulation *in vitro* with the antigen. This results in two difficulties. Firstly, a frequency can only be estimated if re-stimulation is carried out in limiting dilution conditions, which involves a heavy workload. Secondly, you can only measure the frequency of those precursors that proliferated enough to generate a detectable clonal progeny. This leads to underestimated frequencies.

It is noteworthy that the absence of detectable T cells in the *ex vivo* assays does not exclude the possibility that a response occurred *in vivo*. The reason for this is that the detection threshold of 5×10^{-4} necessary for the *ex vivo* assays is 1 000-fold higher than the frequency of naïve T cells. For T cells recognizing peptide MAGE-$A3_{168–176}$ on HLA-A1 this frequency is 4×10^{-7} of $CD8^+$ cells [4], and

we observed similar frequencies for T cells that recognize gp100, NA17, LAGE-1, or MAGE-A10 antigens. The Melan-$A^{MART\text{-}1}{}_{28–36}$ peptide is a remarkable exception, with a very high naïve T cell frequency of about 5×10^{-4} of $CD8^+$ cells [5].

Frequencies of anti-vaccine T cells

Reported frequencies of anti-vaccine T cells following immunization with tumor antigens vary from $>10^{-2}$ to 10^{-6} of the total T-cell population. Anti-Melan-$A^{MART\text{-}1}$ T cells were found at frequencies of up to 2×10^{-2} of $CD8^+$ cells in melanoma patients vaccinated with peptide either in incomplete Freund's adjuvant (IFA) [6], or in IFA with CpG [7]. Anti-$gp100_{209–217}$ cytotoxic T lymphocytes (CTLs) were found at $>10^{-2}$ of $CD8^+$ cells in tumor-free melanoma patients vaccinated with peptide in IFA [8,9]. Anti-MAGE-$A3_{168–176}$ CTLs were present at 3×10^{-3} and 10^{-3} in patients vaccinated with ALVAC-MAGE or peptide-pulsed dendritic cells (DCs), respectively [10,11]. HLA-DP4-restricted $CD4^+$ cells recognizing peptide MAGE-$A3_{243–258}$ were found at 7×10^{-4} of $CD4^+$ cells after vaccination with peptide-pulsed DCs [12]. Finally, several patients vaccinated with peptide or ALVAC-MAGE had monoclonal anti-MAGE-$A3_{168–176}$ CTL responses at low frequencies of about 10^{-6} of $CD8^+$ cells [4,10].

T-cell responses and clinical outcomes

Very few studies have analyzed whether these anti-vaccine T-cell responses correlate with the observed tumor regressions [4,6,13,14] (Table 1). A correlation seems to emerge from two studies [4,6], compatible with the hypothesis that the anti-vaccine T-cell response is necessary, but not in itself sufficient, to initiate tumor rejection. A tight correlation is unlikely to be found for two reasons. First, some patients have strong anti-vaccine T-cell responses without detectable clinical benefit. It is certain that a limiting factor for clinical efficacy, in addition to the frequency of anti-vaccine T cells, is tumor resistance to immune attack. Second, and perhaps more surprisingly, some patients display tumor regression with no or very few detectable anti-vaccine T cells [4]. In such patients, tumor-specific CTLs that recognized antigens absent from the vaccine were primed or amplified after vaccination [15•]. In regressing metastases, these anti-tumor CTLs were 10 000 times more frequent than the anti-vaccine T cells and, therefore, probably effected tumor rejection [16•]. These results are in line with those of other groups that described post-vaccination T cells which recognized tumor antigens that were absent from the vaccine [17–19]. A plausible model is that anti-vaccine T cells, even at very low frequencies, modify an immunosuppressive environment within the tumor, opening a permissive window for the priming or re-stimulation of other anti-tumor T cells.

Functions of anti-tumor T cells: what is involved?

High frequencies of anti-tumor T cells, present either after vaccination [6] or after spontaneous anti-tumor responses [15•], do not secure tumor regression. The coexistence of tumor cells and primed tumor-specific T

Table 1

Studies addressing the correlation between immunological and clinical responses in metastatic melanoma patients with detectable disease and vaccinated with defined tumor antigens.

Vaccines	Antigenic peptides	Patients displaying regression of ≥1 metastasis	Method of monitoring anti-vaccine T cells	Reported T cell responses in patients with:		Ref.
				Evidence of tumor regression	No evidence of tumor regression	
ALVAC-MAGE[a]	MAGE-$A3_{168–176}$ (HLA-A*0101)	4/15	MLPC-tetramer cloning[d]	3/4	1/11	[4]
Peptide + IFA	Melan-$A_{28–36}$ (HLA-A*0201)	2/21	*ex vivo* tetramer/ *ex vivo* elispot IFNγ	2/2	4/19	[6]
Mono-DC[b] + peptide	MAGE-$A3_{168–176}$ (HLA-A*0101)	6/11	*ex vivo* elispot IFNγ	5/6	4/5	[13]
CD34-DC[c] + peptide	Melan-$A_{28–36}$, $tyrosinase_{368–376}$, $gp100_{g209–2\ M}$, MAGE-$A3_{271–279}$ (HLA-A*0201)	7/18	*ex vivo* elispot IFNγ	7/7[e]	9/11[5]	[14]

[a]ALVAC-MAGE is a recombinant canarypox virus of the ALVAC type carrying a minigene coding for two antigenic peptides: MAGE-A3168–176 and MAGE-A1161–169. [b]Dendritic cells derived from adherent blood mononuclear cells cultured with GM-CSF and IL-4 and matured by monocyte-conditioned medium. [c]Dendritic cells derived from circulating CD34+ precursor cells mobilized by G-CSF, cultured with GM-CSF, FLT3-L and TNF. [d]Mixed lymphocyte–peptide cultures in which blood mononuclear cells are stimulated with peptide over two weeks, followed by labeling with tetramer. Anti-vaccine CTL clones are cloned from the tetramer-positive cells. [e]In this study, 6/7 clinical regressors and 3/11 clinical progressors responded to at least three of the four antigens. Abbreviations: FLT3-L, fms-related tyrosine kinase 3 ligand; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor.

Box 1 Possible reasons for the coexistence of tumor cells with primed tumor-specific T cells.

- **Tumor resistance: when lymphocytes are competentex vivo**.
- environment not permissive to T-cell infiltration
- decrease or loss of antigen expression
- resistance to lysis, or to TRAIL- or Fas-induced apoptosis
- contact inhibition of T cells (NK inhibitory receptors)
- **Lymphocyte quiescence: when lymphocytes are deficientex vivo**.
- shortage of soluble factors (tryptophan, arginine, IL-2, etc.)
- inappropriate co-stimulation
- immunosuppressive soluble factors (TGF-β, galectin-1, IL-10, prostaglandins, etc.)
- Treg cells
- **Too low lymphocyte:tumor cell ratio**.
- insufficient expansion of anti-tumor T cell clones
- T cell apoptosis within the tumor

TRAIL, TNF-related apoptosis-inducing ligand.

cells can result from at least two mechanisms: tumor resistance and lymphocyte quiescence (Box 1).

Tumor resistance

The mechanisms of tumor resistance to fully competent lymphocytes display a diversity beyond the scope of this review. We have selected two recent observations to discuss here. The first deals with a function of the anti-tumor T cells themselves; namely the production of IFN-γ. This cytokine induces the change of standard proteasomes into immunoproteasomes, which differ by the three catalytic subunits and therefore produce a different spectrum of peptides. If a tumor antigen can be processed by the standard proteasome, but not by the immunoproteasome, the presence of IFN-γ around tumor cells will inhibit this processing. This might apply to the Melan-A^{MART-1} peptide [20]. At first, T-cells proliferate after recognizing the peptide on tumor cells. But then the IFN-γ that they produce downmodulates antigen expression. This scenario might explain the coexistence of melanoma cells with high numbers of anti-Melan-A^{MART-1} CTLs [21].

A second mechanism of tumor resistance involves the barriers put up by the tumor vasculature that do not permit lymphocyte infiltration [22]. In mice, the systemic application of CpG greatly increases the infiltration of transferred T cells into tumors [23•]. This strategy could be combined with vaccination in patients.

Lymphocyte quiescence

A few years ago, Lee *et al*. [24] reported that anti-tyrosinase blood $CD8^+$ T lymphocytes were functionally deficient; that is, they did not lyse when analyzed directly *ex vivo*. A recent study by the Romero group [25••] showed that anti-Melan-A^{MART-1} T cells present in metastatic lymph nodes of melanoma patients did not produce IFN-γ after *ex vivo* re-stimulation with peptide. Interestingly, this defect was reversible after culturing the cells for two days with IL-2 and IL-7. Anti-Melan-A^{MART-1} T cells from the blood of the same patients were not deficient in IFN-γ production when re-stimulated *ex vivo*. Finally, anti-cytomegalovirus (CMV) T cells found in metastatic lymph nodes were also not deficient in IFN-γ [25••]. These results define a state of primed T lymphocyte quiescence that is reversible, localized and antigen specific.

Causes of lymphocyte quiescence

There are certainly several causes of such a quiescence, which is sometimes referred to as functional tolerance or paralysis. One possibility is the lack of a soluble factor necessary for complete T-cell function; for example, tryptophan shortage prevents T-cell proliferation [26]. Tryptophan is degraded by indoleamine 2,3-dioxygenase (IDO), which is expressed in many tumors and provides them with a mechanism to resist immune attack, even in an immunized animal [27•]. IDO is also expressed by murine plasmacytoid DCs in tumor-draining lymph nodes [28], where it could be responsible for local immunosuppression.

Another candidate for the cause of quiescence is arginine, as arginine starvation decreases the half-life of CD3ζ mRNA and inhibits T-cell proliferation *in vitro* [29,30]. Arginase I, which metabolizes arginine, was detected in suspensions of human non-small-cell lung cancer cells, which also contained T cells with a decreased expression of CD3ζ compared to allogenic lymphocytes [31].

Finally, a local shortage of IL-2 could be another important factor in inducing lymphocyte quiescence. There are several recent additions to the numerous studies on the role of IL-2 for anti-tumor T-cell function. In mice, exogenous IL-2 is a crucial factor for treating established tumors by T-cell adoptive transfer [32]. In patients, low-dose IL-2 prolongs the persistence of transferred anti-tumor T-cell clones [33]. In patients chronically infected with HIV-1, virus-specific $CD8^+$ T cells appeared to be quiescent; that is, they fail to proliferate *in vitro* after stimulation with the antigen. Addition of autologous anti-HIV-1 $CD4^+$ cells restored this proliferation, but not in the presence of neutralizing anti-IL-2 antibodies [34•]. If, indeed, IL-2 is a relevant limiting factor for the activity of tumor-specific T cells, an interesting adjunct to existing vaccines could be the recombinant adenovirus-IL-2 currently being investigated by direct injection into solid tumors [35].

Soluble factors produced by tumor or stromal cells could induce a state of T-cell quiescence. TGF-β is widely expressed and regulates T-cell homeostasis through multiple mechanisms. Its role in regulating anti-tumor T cells has been documented in transgenic mice whose T cells are insensitive to TGF-β signaling. These animals resisted challenge to two types of tumors, with enhanced

$CD8^+$-mediated tumor-specific CTL responses [36]. Another soluble factor that might be involved in T-cell quiescence is the lectin galectin-1, which was reported to inhibit the production of Th1 cytokines and to induce apoptosis (reviewed in [37]). It was also shown to protect murine melanoma cells from attack by T cells [38].

Regulatory T cells in lymphocyte quiescence

Regulatory T cells might play an important part in anti-tumor lymphocyte quiescence. Wang and colleagues [39••] demonstrated the existence of tumor-specific human regulatory cells. They showed that anti-LAGE-1 $CD4^+$ T-cell clones, derived from lymphocytes infiltrating into a melanoma tumor, had potent suppressive activities that were triggered by an antigenic peptide presented on HLA-DR13. Both proliferation of and cytokine secretion by autologous indicator $CD4^+$ clones were strongly inhibited. This is the first description of a human suppressive $CD4^+$ clone with a defined specificity. From other recent papers, we note that Tregs were shown to be recruited into ovarian tumors through CCL*22* [40•]. There was also a twofold enrichment of Tregs in metastatic melanoma lymph nodes in comparison to the proportions found in tumor-free nodes or blood [41]. Coming back to the shortage in soluble factors mentioned above, it is worth noting that Tregs, which depend on IL-2 without producing it themselves, compete for this growth factor with responder T cells [42]. In addition, they were also shown to induce the expression of IDO by DCs, thereby depleting tryptophan supplies [43•].

Concluding remarks

So far, the therapeutic vaccination of cancer patients with defined tumor antigens has resulted in objective clinical efficacy in only a minority of patients: some evidence of tumor regression in about 20%, and clinical responses assessed with RECIST criteria in about 3% [4,44].

In the majority of vaccinated patients who do not show tumor regression, but who nevertheless have numerous tumor-specific T cells, we need to better understand the rules governing this coexistence. A promising approach to address this is gene expression profiling, not only of the tumor but also of anti-tumor T cells. One study has managed to distinguish blood mononuclear cells obtained from renal carcinoma patients from blood mononuclear cells from normal donors or even patients with other types of cancer [45]. Subtle and intriguing differences in gene expression have been observed between circulating $CD8^+$ T cells from melanoma patients and those from healthy donors, and several of the discriminating genes are related to apoptosis [46].

Through detailed analyses we should make every effort to clarify the tumor rejection process in the few patients who benefit from vaccination. Such a study has already shown that the simple model of rejection by numerous anti-vaccine CTLs does not account for what happens in all regressing tumors [16•]. Unforeseen effector mechanisms might prove to be important; for example, IFN-γ produced by murine $CD8^+$ T cells, which interferes with tumor angiogenesis [47•,48].

There is still good reason to believe that such projects may lead to more efficient, simple and non toxic procedures for therapeutic vaccination against cancer.

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