Molecularly defined vaccines for cancer immunotherapy, and protective T cell immunity

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ABSTRACT
Malignant cells are frequently recognized and destroyed by T cells, hence the development of T cell vaccines against established tumors. The challenge is to induce protective type 1 immune responses, with efficient Th1 and CTL activation, and long-term immunological memory. These goals are similar as in many infectious diseases, where successful immune protection is ideally induced with live vaccines. However, large-scale development of live vaccines is prevented by their very limited availability and vector immunogenicity. Synthetic vaccines have multiple advantages. Each of their components (antigens, adjuvants, delivery systems) contributes specifically to induction and maintenance of T cell responses. Here we summarize current experience with vaccines based on proteins and peptide antigens, and discuss approaches for the molecular characterization of clonotypic T cell responses. With carefully designed step-by-step modifications of innovative vaccine formulations, T cell vaccination can be optimized towards the goal of inducing therapeutic immune responses in humans.

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1. Introduction

The concept of using vaccination in the treatment of cancer has been tied to the history of vaccines themselves. However, major interest in it has grown recently with the increasing understanding of the role that the immune system plays in shaping the biological behavior of cancer. Landmark advances that have promoted the interest in cancer vaccine development have been (i) the mounting evidence supporting the general concept of immune surveillance both in experimental mouse models [1] and in cancer patients [2]; (ii) the identification of T cell defined tumor antigens at the molecular level [3] and (iii) the demonstration in both mouse and humans that T cells of well defined specificity can mediate the complete elimination of large solid tumors [4–6].

The path to cancer vaccine development goes from the choice of the target antigen(s), the formulation, and the regimen of immunization to the assessment of its impact. In contrast to prophylactic vaccines, which generally aim at generating high levels of protective antibodies, therapeutic vaccines aim at eliciting potent T cell responses that can mediate the destruction of tumor cells. The first major decision down the therapeutic vaccine development path concerns the type of antigen to be used. Hundreds of T cell defined tumor antigens have been identified so far (http://www.cancerimmunity.org/peptidedatabase/Tcellepitopes.htm). The second decision concerns the choice of the vehicle to deliver the antigen to the immune system. A relatively large variety of possibilities exist including naked DNA, mRNA, recombinant protein, synthetic peptide, recombinant viral or bacterial vectors or dendritic cells loaded ex vivo with antigen. In this review, we will focus on the state of the art of peptide- and protein-based cancer vaccines. We will also cover salient aspects of current immunomonitoring of human T cell responses, and biological parameters of T cells that can protect from tumors, in the context of clinical trials of therapeutic vaccination, chiefly in metastatic melanoma patients. Because there are hundreds of relevant reports, this review is based on a non-exhaustive selection.

2. Antigens

The first human T cell defined tumor antigen was molecularly cloned in 1991 [3]. Since, hundreds of tumor antigens have been identified for virtually every tumor type. Such antigens are recognized by αβ T cells, both MHC class II restricted CD4 and MHC class I restricted CD8. The patterns of expression of these antigens are extremely heterogeneous. A major surprise soon after the discovery of molecularly defined tumor antigens was that many were encoded by non-mutated and non-viral genes. From a safety standpoint, the use of such antigens in vaccination poses the risk of autoimmune side effects. Ideal tumor antigens are those known as shared tumor-specific antigens because they are selec-
tively expressed in tumor cells, of various histotypes, and not in MHC-expressing normal tissues. Examples of such type of antigens are the MAGE-A or NY-ESO-1 antigens. The various members in this category of antigens are expressed at variable proportions depending on the tumor type and the disease stage. Thus, vaccines based on these antigens require selection of patients bearing tumors that express the target antigen.

Another category of tumor antigens that are deemed valuable for vaccine development are those derived from oncogenic proteins which are overexpressed in tumors. It is thought that such targets offer not only a reasonable safety profile but they would also minimize the event of tumor escape upon immunological pressure. A representative example of such a target is the WT1 protein [7].

Bona fide non-self tumor antigens are derived from two major sources: viral antigens in the case of tumors of oncogenic viral origin such as cervical carcinomas caused by HPV infection, and somatic mutations. In this regard, high throughput sequencing of the genome of a growing number of human tumors have revealed that large numbers of somatic mutations accumulate in all of them, even chromosomal rearrangements, so that thousands of such mutations can occur in a given individual tumor [8,9]. T cells are uniquely able to detect the mutated peptides derived from such mutated sequences. These peptides are likely to be strongly immunogenic because of the absence of central tolerance. However, these generally are unique tumor antigens thus calling for individualized vaccine design, which could be prohibitively expensive with the current technologies.

Recently, interest has focused on antigens that may be selectively expressed by tumor initiating cells, or cancer stem cells. Targeting such cells would increase the odds of achieving effective and durable tumor control by the immune system.

3. Adjuvants

Both peptides and recombinant proteins are poorly immunogenic by themselves, hence the need to administer them in conjunction with adjuvants. The role of adjuvants is at least two-fold. On one hand, adjuvants deliver antigens to the immune system during a period of time long enough to allow for efficient priming of the T cell response. This period has been determined to be of at least 24 h in certain systems. On the other hand, adjuvants trigger the activation and maturation of dendritic cells. As a consequence, DCs, loaded with antigen, migrate to the proximal lymph nodes and acquire the ability to optimally present antigens for initiation of de novo T cell responses. The former adjuvant function is fulfilled by agents such as mineral oils for emulsion formation (incomplete Freund's adjuvant), liposomes or biodegradable micospheres. The latter is the property of defined microbial derived molecular patterns which are sensed by receptors of the Toll-like receptor (TLR) family and by endogenous ligands which can be sensed by damage associated molecular patterns (DAMPs) receptors, such as NOD-like receptors (NLRs) [10–12].

Relatively few adjuvants are currently available for human use. These include alum and MF59. However, a growing number of molecularly defined adjuvants are in clinical development and used in clinical trials of cancer vaccination. These include various synthetic or recombinant TLR ligands, mineral oils such as Montanides, saponins and liposomes. Table 1 summarizes the most commonly used immunological adjuvants in the context of clinical trials.

4. Clinical trials

4.1. Vaccines based on synthetic peptides

Numerous vaccines have been designed based on the use of short synthetic peptides which mimic the exact epitope recognized by cytolytic CD8 T lymphocytes (CTL) when associated with the restricting MHC molecule. This limits the applicability of the vaccine to cancer patients of the appropriate MHC haplotype. Since HLA alleles are extremely polymorphic, the practical approach to this type of vaccination has focused the efforts on those peptides presented by the most frequent HLA alleles. Thus, HLA-A2 and to a lesser extent other alleles such as -A1, -A3, -B7, -B35, are the alleles relevant for individuals of Caucasian origin. HLA-A24 is for Asian individuals and HLA-A30 for African individuals. Despite this drawback, short synthetic peptides do present significant advantages for vaccine development. These are the straightforward production and pharmacological formulation, their relative low cost and the possibilities offered for accurate monitoring of the specific T cell responses in vaccinated subjects. Virtually hundreds of small clinical trials have been reported using short synthetic peptide vaccines. There is a handful of peptides which have been widely used, most of them HLA-A2 restricted, and therefore are relatively well characterized in terms of their safety profile and of the characteristics of the naturally acquired and vaccine induced specific T cell responses. These are: Melan-A/MART-1 [13], one of the gp100 epitopes [14,15] and tyrosinase [16,17] for the melanocyte differentiation antigens; prostate surface antigen and PSAP for prostate [18]; carcinoembryonic antigen and MUC-1 for mucosal tumors [19–21]; HER-2/neu for breast carcinoma [22]; G250 for renal cell carcinoma [23]; the PR1 shared by two myeloid leukemia associated antigens, PR3 and neutrophil elastase which are normally expressed in granulocytes and overexpressed in myeloid leukemia cells [24] (this peptide is in phase III trial testing in leukemia); the shared tumor-specific antigens MAGE-A and NY-ESO-1 for various tumor types [25,26]; and the overexpressed proteins survivin [27] and telomerase [28] for many tumor types.

Natural exact length peptides, recognized by tumor reactive CTL, have been used as vaccines in cancer patients. One such peptide is the one derived from MAGE-A3 and recognized by HLA-A1 restricted CTL. Responses to this peptide are relatively weak regardless of the delivery vehicle used to vaccinate. Detailed studies have been reported concerning the immunogenicity of this antigen and the clinical impact of MAGE-A3 based vaccines [29–31]. However, typically, a substantial proportion of T cell defined tumor-derived antigenic peptides are suboptimal for binding to HLA, with consequent fast dissociation from MHC and weak immunogenicity. Introducing single amino acid substitutions at MHC anchor residues can increase the peptide binding to MHC resulting in highly stable complexes. It has been observed that the half-life of MHC/peptide complexes is directly correlated to immunogenicity. However, even subtle conformational changes in the complex may generally recruit T cells with limited cross-reactivity with the naturally processed antigen. The extent to which this happens is variable and very much analogue peptide-dependent.

As mentioned above, any pure synthetic peptide requires formulation with an appropriate adjuvant to be immunogenic upon vaccination. Many of the clinical trials with peptides have used emulsification of the peptide solution with a mineral oil, a procedure first described several decades ago by Freund. Hundreds of melanoma patients have been vaccinated with the gp100 209 T210M peptide analogue emulsified in Montanide™. Three major lessons can be drawn from the corresponding clinical trials. First, there is a certain dose effect in terms of the proportion of responding patients and also of the frequency of induced specific CD8 T cells. The recommended peptide dose appears to be 1 mg of peptide analogue per injection, although the majority of patients are efficiently vaccinated with 100 µg. Second, a randomized phase II trial allowed concluding that a regimen of injections every three weeks elicited responses comparable to one of biweekly injections [32]. And third, the proportion of cross-reactive T cells elicited by the peptide analogue that can efficiently recognize and kill tumor
cells is lower than one third [33]. The clinical benefit observed upon vaccination with this analogue has been a matter of debate. The initial trial in which emulsion injections were accompanied by high dose IL-2 (HDI) suggested a high objective tumor response rate (approximately 40%) [34]. Subsequent trials have confirmed this although the response rate observed is lower (approximately 20–25%). A recent randomized trial comparing HDI alone to HDI and gp100 peptide analogue vaccination showed significant clinical efficacy both in tumor response rate and overall survival [35].

The first clinical study of vaccination with a Melan-A/Mart-1 derived antigen was performed with the synthetic nonapeptide 27–35, the initially identified immunodominant HLA-A2 restricted CTL epitope, emulsified in mineral oil and injected every three weeks by the subcutaneous route [36]. In a pre-clinical study of the fine specificity of Melan-A/Mart-1 antigen recognition by TILs, we identified the decapeptide 26–35 (the original nonapeptide extended by one amino acid residue at the NH2 terminus) as the optimal length antigenic peptide. Moreover, we found that while the fine specificity of Melan-A/Mart-1 antigen recognition by TILs, induced strong T cell responses even when using the less antigenic natural (wild type) Melan-A decapeptide [40]. An analysis, at the clonal level, of the fine specificity of antigen recognition by vaccine induced CD8 T cells showed that immunization with the peptide bearing the wild type sequence recruited cells with fully tumor reactive TCR, and resulted in stronger induction of effector cell function. In contrast, the T cells recruited by vaccination with the peptide analogue were less activated and contained close to 50% of clones that only poorly recognized tumor cells. The same vaccine formulation was also shown to confer immunogenicity to NY-ESO-1 157–165 peptide analogues [41,42] and possibly extended survival in vaccinated patients [42].

Since it is difficult to predict which peptide analogues may faithfully recapitulate the naturally processed and presented antigen, a vaccination strategy that minimizes the risk of inducing nontumor reactive T cells is desirable. Taking into account the experience with these peptides, one possibility opened by the availability of potent adjuvants is to prime with the wild type and subimmunogenic peptides and boost with the peptide analogues. In such a way, priming would lock the CD8 T cell response onto the highest avidity and possibly tumor reactive clones and the boosting with the enhanced peptide analogues would efficiently drive the expansion and differentiation of the primed clones.

One of the predictable events upon vaccination with single peptide epitopes is tumor escape. Targeting of multiple antigens is likely to reduce this risk. We have shown that a combination of three peptides in the same Montanide emulsion is immunogenic and suggests that in vivo competition among peptides binding to the same HLA allelic product does not greatly impair immunogenicity [43]. Importantly, the practical issues associated with formulation have been recently addressed [44]. A comparison of a four peptide- and a twelve-peptide vaccine was performed in a randomized clinical trial. The results suggested the superiority of the more complex vaccine composition [17]. The same approach has been used to vaccinate with naturally processed tumor peptides. The latter have been identified via a novel approach combining biochemical isolation of MHC class I peptide ligands and gene expression arrays to sort the genes that are selectively overexpressed in the primary tumor, relative to normal tissue sampled at the same time of surgical removal of the tumor [45–47].

Peptide vaccines have also been tested in the clinics using other delivery modes. Peptides have been administered as mixtures with the cytokine GM-CSF by the intradermal route. To increase the immunogenicity of the formulation, the peptide GM-CSF mixture has been emulsified in Montanide. In a recent trial using class II restricted peptides from various tumor-associated antigens using this adjuvant preparation, the majority of patients had measurable helper T cell responses and some even objective clinical benefit [48]. Single peptides or mixtures of peptides have also been delivered using ex vivo loading onto autologous monocyte-derived dendritic cells [49–66].

4.2. Vaccines based on recombinant proteins

Some of the major tumor-associated antigens have been generated as recombinant proteins for clinical testing in cancer patients. In fact, the most advanced vaccine candidates in terms of efficacy measurement are in this category. One such compound is the

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**Table 1**

Some adjuvants currently used in clinical trials involving human subjects.

<table>
<thead>
<tr>
<th>Name of the adjuvant</th>
<th>Composition</th>
<th>Vaccines in use</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alum</td>
<td>Aluminum salts</td>
<td>Diphtheria-pertussis-tetanus, diphtheria-tetanus (DT), DT combined with hepatitis B (HBV), haemophilus influenza B or inactivated polio virus, HAV, Streptococcus pneumonia, meningococcal and human papilloma virus (HPV)</td>
<td>[180]</td>
</tr>
<tr>
<td>MF59</td>
<td>Oil (squalene)-in-water emulsion</td>
<td>Fluid, Flucreta, candidate vaccines against malaria, HBV and HIV</td>
<td>[181]</td>
</tr>
<tr>
<td>MPL</td>
<td>Non-toxic derivative from Salmonella minnesota</td>
<td>Fludrix, HBV, cervix, HPV combined with aluminium salts in the proprietary AS04 formulation</td>
<td>[182]</td>
</tr>
<tr>
<td>Montanides (ISA51, ISA720)</td>
<td>Water-in-oil emulsions containing squalene and mannide-monooleate as an emulsifier</td>
<td>Malaria, HIV, and cancer vaccine clinical trials</td>
<td>[183]</td>
</tr>
<tr>
<td>Saponins (Quil-A, ISCOM, QS-21, AS02 and AS01)</td>
<td>Triterpene glycosides isolated from plants</td>
<td>Clinical trials with QS21 as vaccine adjuvant in progress, against malaria, influenza and cancers</td>
<td>[184,185]</td>
</tr>
</tbody>
</table>
recombinant prostatic acid phosphatase (PAP) fused to the human cytokine GM-CSF. The product formulated as a vaccine by loading onto autologous antigen presenting cells, by incubation with isolated peripheral blood lymphocytes, is known as Sipuleucel-T. Intravenous delivery of the cellular vaccine preparation at two weeks intervals has been associated with a statistically significant extension of survival of hormone refractory prostate carcinoma patients, as compared to placebo treated control groups [67,68]. Of interest, it appears that the use of CD54 expression levels on APCs as a potency indicator is also associated with survival in patients receiving the vaccine. A pivotal randomized placebo-controlled phase III trial with more than 500 patients (ImPACT; Immunotherapy Prostate) was very recently reported to have met its end point of extended survival (http://files.shareholder.com/ImmunotherapyProstate). Of interest, it appears that the use of CD54 expression levels on patients, as compared to placebo treated control groups[67,68].

The primary antigen KLH have been used as vaccines together with GM-CSF. Encouraging immunological and clinical results have been reported. Both humoral and cellular specific responses in vaccinated patients have been associated with objective tumor responses, molecular remission and increased progression free survival [71]. Recently, the results of a pivotal, randomized, multicenter, double-blind, controlled clinical study showed that the vaccine significantly prolonged disease-free survival in follicular non-Hodgkin’s lymphoma [72].

The MAGE-A3 tumor antigen has been prepared as a recombinant fusion protein with lipoprotein D derived from H. influenza at its N-terminus and a polyhistidine tail at its C-terminus. An initial clinical study of vaccination with this protein alone in advanced metastatic melanoma elicited specific immunity in few patients as well as encouraging clinical activity [73]. Immunization with a combination of recombinant protein and an adjuvant containing the TLR-4 ligand monophosphoryl lipid A, the saponin QS21 and an oil in water emulsion significantly increased immunogenicity as most lung carcinoma patients had specific antibody responses and CD4 T cells specific for MAGE-A3 could be detected in some patients [74]. In contrast, only some of the patients receiving the recombinant protein alone had immune responses that could not be efficiently boosted, suggesting tolerance induction. Vaccination was immunogenic and associated with clinical benefit in a small proportion of metastatic cancer patients [75]. Importantly, a randomized, placebo-controlled phase II trial in resected non-small cell lung carcinoma showed a 27% relative reduction of recurrence risk when compared with placebo [76]. Another randomized phase II clinical trial was performed in metastatic melanoma patients to determine the relative efficacy of the adjuvant composition. One arm consisted of the same vaccine as the one used in the lung carcinoma trial. The second arm included an adjuvant containing liposomal CpG-ODNs, a TLR-9 ligand, in addition to the monophosphoryl lipid A (TLR-4 ligand), oil in water emulsion and QS21. The latter proved to be clearly more potent in terms of both clinical benefit and specific immune responses [77]. A large pivotal phase III clinical trial of immunization with recombinant MAGE-A3 protein in combination with the latter adjuvant combination is being conducted worldwide in lung carcinoma patients.

One of the most immunogenic tumor antigens is NY-ESO-1, a 180 amino acid long polypeptide expressed selectively in tumor cells and not in normal cells with the exception of germinal cells in the gonads [78]. The full-length recombinant protein has been used in vaccination trials in combination with a variety of adjuvants. These include ISCOMatrix, microspheres of around 40 nm formed by the mixing of cholesterol, phospholipids and a saponin preparation [79,80], emulsification in incomplete Freund’s adjuvant together with CpG-ODNs [81], covalently linked to cholerysteryl pullulan [82,83], injection into epidermal sites preconditioned by topical administration of imiquimod [84], or in conjunction with GM-CSF and BCG [85].

### 4.3. Vaccines based on long synthetic peptides

While full-length proteins provide the substrates for processing and presentation of natural antigens to T cells, a clear bias towards induction of MHC-II restricted CD4 T cell responses exists in animal models and humans. This is due to the fact that proteins are generally delivered to cells via the endocytic compartment. Coupling to membrane traversing peptides or to toxin subunits may increase direct protein translocation from the extracellular milieu to the cytoplasmic compartment and enable priming of MHC-I restricted specific CD8 T cells. Another approach to obtain immunogens capable of priming both CD4 and CD8 T cell responses is the use of long synthetic peptides (LSPs), usually 30 amino acid long. This length would preclude direct binding to MHC-I on the cell surface and ensure peptide processing and presentation by professional APCs [86,87].

One such peptide that is in advanced clinical testing is a 25-mer MUC-1 derived peptide, which recapitulates the sequence of the extracellular core repeat peptide of the glycoprotein mucin-1, expressed on epithelial cells and overexpressed and/or aberrantly glycosylated in various types of carcinomas. The peptide is formulated in liposomes together with monophosphoryl lipid A and additional lipids. Vaccination is done at weekly intervals for 8 weeks and preceded by a single low dose of cyclophosphamide. A randomized phase II trial in patients with advanced non-small cell lung cancer provided encouraging clinical results as measured by extended median overall survival [88]. Two large multicenter phase III trials, one in patients with inoperable stage III NSCLC after chemoradiation, the other in hormone receptor positive, locally advanced breast cancer, are underway. In an early phase clinical trial, a 14-mer peptide containing both MHC class-I and class-II restricted T cell epitopes recognizing NY-ESO-1 expressing tumor cells was emulsified with IFA to immunize ovarian carcinoma patients. Both CD4 and CD8 T cell responses were reported that could be detected for at least a year in the majority of patients [89].

Single long peptides, however, may not entirely target the tumor antigen of interest as proteins would do. The strategy to equate LSPs to full-length recombinant proteins is to prepare groups of long and partially overlapping synthetic peptides covering either the entire protein sequence or a region of it. Sets of overlapping 30-mers have been designed and tested in early phase clinical trials. In one, women with chronic HPV-16 infection and cervical or vulvar malignancies have been immunized with pools of HPV-16 E6 and E7 LSPs emulsified in IFA. Broad specific T cell responses were recorded in the majority of vaccinated patients [90]. In another trial, 15 out of 20 patients with pre-malignant vulvar lesions had clinical responses and 9 showed complete and durable regression of lesions [91]. A set of ten overlappings LSPs derived from the p53 has also been tried in patients with ovarian carcinoma [92] and with colorectal carcinoma [93]. In both trials, the induction of specific CD4 T cell responses in the majority of patients,
that could be detectable 6 months after the last injection, was documented.

5. Measuring T cell responses

5.1. Epitope specific T cell responses

The techniques to reveal antigen-specific T cells are based on (i) TCR mediated binding to peptide–MHC (pMHC), or (ii) detection of cytokines produced after 4–6 h triggering with antigen [94–97]. For the former, the invention of fluorescent pMHC multimers [94] (formerly called tetramers) has opened a wide array of opportunities, i.e. to quantify T cells, to characterize their function, and to molecularly dissect cellular features down to the single cell level, as discussed in more detail below. A major disadvantage of pMHC multimers is that one can only identify T cells specific for (precisely) known epitopes. Wider spectra of antigen specificities are covered by assays based on cytokine detection. Short-term (4–6 h) triggering of T cells reveals previously primed but not naïve T cells that can be detected either by flow cytometry (upon intracellular cytokine staining) or by Elispot assay [95,98]. However, these techniques only detect those T cells with functional properties corresponding to the reagents used (e.g. anti-IFNγ, anti-TNFα, or anti-interleukin antibodies). Inhibitory T cells (e.g. Treg) or T cells producing other factors (e.g. inhibitory cytokines) are rarely detected. Inhibitory cells are more frequent among CD4 T cells. Unfortunately, most CD4 T cells can still not be detected by currently available pMHC multimers. Improved multimers are urgently needed for comprehensive analysis of activatory and inhibitory T cell functions, and differentiation (polarization) of CD4 T cell responses.

Recently, pMHC multimer based assays have been refined with the aim to simultaneously analyze T cells with many different antigen specificities. These innovative assays use 10 or more multimers, each labeled with different color combinations [99,100]. They take advantage of novel fluorochromes and quantum dots, and multi-parameter flow cytometry. The approach can be further exploited by combining with phenotypic and functional analysis, enabling more comprehensive quantitative and qualitative assessment of a spectrum of antigen-specific T cells. The large amount of data provided with multiparameter assays may also be analyzed and interpreted by specialized statistical tools, including new unsupervised analyses comparable to approaches developed for gene microarray studies.

A major challenge is to achieve and maintain high assay quality, which becomes increasingly important given the complexity and high precision requirements of sophisticated assays. Assay development generally has at least two phases, i.e. optimization and validation. Careful optimization may lead to useful results. Subsequently, the optimized assays require validation, which consists of the assessment of (i) precision (reproducibility, coefficient of variation), of (ii) accuracy or comparison with reference methods, and of (iii) robustness, i.e. the study of variations introduced by, e.g. different (batches of) reagents, different centers, etc. In case validation is not satisfactory, one needs to go back to further optimize the assay.

5.1.1. T cell receptors, T cell clonal composition and dynamics

The use of peptide MHC-I multimers allows direct identification and, when combined with other markers, phenotyping and functional characterization of single antigen-specific T cells. The current limit of detection of specific cell populations using MHC-I multimers is approximately 0.01% of antigen-specific T cells. Less frequent populations cannot be detected ex vivo. Analyses of T cells directly ex vivo enable immediate quantification of T cell numbers. Moreover, direct ex vivo analysis of phenotype and function allows concluding on T cell function in vivo. Finally, gene expression analysis can be done with T cells sorted directly ex vivo with pMHC multimers. With this approach, T cell responses can be dissected in great detail [101–105].

Studies with in vitro cultured cells are less informative, and they are often over-interpreted since in vitro alterations limit the relevance of results. Nevertheless, in vitro cultured cells usually enable to distinguish presence from absence of T cell responses. Quantitative assessment of antigen-specific T cell numbers, however, are only possible when calculations are based on so-called “limiting dilution” cultures [106], which is rarely done. Analysis of limiting dilution cultures may be performed with multimers or by a functional assay.

What are the most relevant T cell parameters, i.e. the ones that correlate best with protective immunity? Protective T cell responses are characterized by strong initial clonal bursts arising from a relatively small number of highly specific naïve precursor T cells. Indeed, analysis of T cell receptor (TCR) repertoires of protective virus specific CD8 T cells revealed that up to very high frequencies of epitope specific T cells are composed of small numbers (1 to about 20 or 50) of co-dominant clonotypes. Lymphocytes from a human being bear about 10^7 to 10^10 different TCRs, yet only an extremely small fraction of them participate in a given immune response. This extraordinary high selectivity results in immune responses dominated by progeny of only those naïve T cells with the best TCRs. Indeed, protection from disease depends on T cells with high capacity of antigen recognition, and on relatively high frequency of naïve precursor T cells with such properties [107–116].

Based on more traditional methods, the TCR repertoire is characterized by the analysis of large numbers of in vitro cultured T cell clones. Various innovative techniques made it possible to characterize TCR repertoires without the need of cell cultures [101,117]. With a strategy of analyzing large numbers of 5-cell aliquots of human virus- and tumor-specific CD8 T cells sorted directly ex vivo [104], Rufer et al. have shown that in vitro cultured T cell clones represent the in vivo existing TCR repertoire accurately, provided that slower growing clones are equally considered (manuscript in preparation). Novel refinements of cell sorting and molecular techniques progressively enable the analysis of single T cells directly ex vivo, which will bring more insight in the clonal distribution and kinetics of human T cell responses. Outstanding questions are e.g. whether virus- and tumor-specific T cells differ in their gene expression patterns, kinetics, and/or with regard to clonal burst size and number of co-dominant clonotypes. TCRs specific for tumor antigens are often of low to intermediate affinity to pMHC, likely due to negative selection and self-tolerance mechanisms. Some high affinity self/tumor-antigen-specific TCRs can be found, but they are relatively rare, and thus exist at best at low precursor frequencies. A wealth of current research is focused on the identification of optimal TCRs against human tumor antigens, and on technical improvements to insert TCRs into human T cells, enabling TCR gene therapy [118,119]. Moreover, TCRs are being optimized to increase affinity, reaching high functional potency and hopefully immune protection similar to pathogen specific T cells.

5.2. Functional characterization of vaccine elicited T cell responses: effector versus memory T cells

T cell function is highly dynamic, particularly during acute disease phases. After priming or boosting, the first few days are characterized by extraordinary strong T cell proliferation, which is often followed by a steep decline of immune responses. Such acute T cell responses are better understood as opposed to T cells in chronic disease and cancer, which is perhaps one reason why it
is frequently argued that effector T cell responses are short-living. Indeed, in case of pathogen elimination, a small number of memory T cells persist, and they are thought to proliferate only at low rates but are long-living. In contrast, in malignant disease or chronic inflammation, numbers of T cells are larger, and the majorities of them are constantly active and proliferate at relatively high rates.

Some studies have shown decreased immunocompetence with advanced age, associated with cellular T cell senescence and progressive loss of proliferative capacity [120–126]. Yet, prevention and particularly treatment of intracellular infections or cancer requires continued T cell proliferation, emphasizing the need to determine if and how T cells are capable for this task at medium- and long-term. Novel data indicate that clonotypic effector T cell populations can indeed persist over many years in cancer patients, and that these T cells may be frequently and successfully induced, boosted and maintained at high numbers and high effector activity by repetitive vaccination [117,127]. Even in absence of antigen, CD8 T cell clonotypes may not only persist as memory but also as effector cells [128–130].

5.3. “Global” monitoring

In malignant diseases, the number of T cell antigens is usually large, with potentially hundreds of unknown epitopes. Although difficult to achieve, it is necessary to assess the complete spectrum of anti-tumor antigen-specific T cell responses. Measuring cytokine production allows the detection of such T cells, provided that they are triggered with the relevant antigens. Autologous tumor cells are the most comprehensive source of antigens, but they are often not available. Alternatively, whole tumor antigenic proteins, and/or reagents that counteract tumor mediated immune suppression. Abbreviations of cytokines and enzymes: TGF, transforming growth factor; PGE2, prostaglandin E2.

Fig. 1. Left: Active immunotherapy is being developed by optimizing antigens, adjuvants and delivery systems. Furthermore, optimal dose, route and schedule of vaccine administration are identified, and prime/boost strategies are evaluated (not shown). Right: Cancers are associated with local and systemic immunosuppressive mechanisms. Novel immune therapies need to overcome these obstacles, by the induction of highly competent systemic immune responses, and/or by local targeting of reagents that counteract tumor mediated immune suppression. Abbreviations of receptors: CTLA-4, cytotoxic T lymphocyte antigen-4; PD1, programmed cell death-1; BTLA, B and T lymphocyte attenuator; LAG-3, lymphocyte activation gene 3. Abbreviations of cell populations: T reg, regulatory T cell; NKT, natural-killer T cell; MDSC, myeloid derived suppressor cell; TAM (M2), tumor associated macrophage (type 2); DC, dendritic cell. Abbreviations of cytokines and enzymes: TGF, transforming growth factor; IL, interleukin; IDO, indoleamine 2,3-dioxygenase; NOS, nitric oxide synthase; Cox-2, cyclooxygenase 2; VEGF, vascular endothelial growth factor; PGE2, prostaglandin E2.

6. Immune modulation

Type 1 immune responses can eliminate a variety of infections, and have the potential to eliminate tumors. Interestingly, a history of multiple infections and vaccinations against various microbes is associated with reduced incidence of malignant disease, and better prognosis for patients with cancer [134]. However, type 1 immune responses are often inhibited by several biological mechanisms (Fig. 1). Immune cells and inflammatory cells (other than Th1 and CD8 T cells) frequently support tumor growth, and they may do so in conjunction with suppression of type 1 immune responses [135]. While several responsible mechanisms are well characterized in animal models, they require further studies, particularly in humans. It is important to identify the mechanisms of immune inhibition that are dominant in individual patients and pathologies, and thus require specific therapeutic interventions.

Inhibitory mechanisms can be classified in T cell intrinsic and extrinsic pathways. With regard to the former, the co-inhibitory receptors CTLA-4 and PD-1 are co-responsible for the suppression of human effector T cell responses to infectious diseases and cancer [136,137]. Therapeutic blockade of these two pathways is in promising clinical development. Lympocytes can express additional inhibitory receptors, such as Killer-Inhibitory-Receptors and C-Lectin-type-receptors [138]—both of these classes, however, are expressed by only small subsets of T cells [138,139]. T cell inhibition can also be mediated via LAG-3 [140], 2B4, CD160 and further pathways, and these receptors may co-regulate T cell inhibition [141].

Further inhibition is mediated by a more recently described co-inhibitory receptor, the so-called B and T lymphocyte attenuator (BTLA; CD272). It is an immunoglobulin-like molecule belonging to the CD28:B7 family, and is expressed by the majority of lymphocytes [137,142–144]. In animal models, BTLA has been shown to be involved in peripheral T cell tolerance induction [145], and in early control of tissue damage and of anti-bacterial immunity [146]. In humans, BTLA expression may be altered by specific immunotherapies with allergens, as shown for allergic rhinitis [147]. We have found that BTLA is downregulated during human CD8 T cell differentiation to effector cells, which was however not the case for tumor antigen-specific T cells, that persistently expressed BTLA despite effector cell differentiation in unvaccinated melanoma patients [148]. In contrast, when CpG-ODNs were used as adjuvant for vaccination, tumor-specific T cells downregulated BTLA, developed strong effector functions and became independent of BTLA mediated inhibition. The ligand of BTLA is HVEM, a TNFR superfamily member [142,143], and is expressed by the majority of melanoma metastases [148]. Functionally, BTLA–HVEM interactions inhibited T cell proliferation and cytokine secretion (Fig. 2). Thus, the T cell intrinsic pathway via BTLA inhibits human tumor-specific CD8 T cells, and vaccination with CpG-ODNs as adjuvant can partially overcome this blockade.

A relatively large number of T cell extrinsic inhibitory mechanisms have been reported, which are briefly mentioned here (Fig. 1), and are reviewed e.g. in the papers cited below. Regulatory T cells (“Treg”; CD4+ CD25+ FoxP3+ cells) have been shown to downregulate T cell responses in cancer and allograft rejection [149–151]. We could combine MHC-II/Melan-A peptide multimers with FoxP3 labeling in flow cytometry to track antigen-specific regulatory T cells in the course of vaccination with Melan-A peptide
and adjuvants, mainly IFA plus CpG-ODNs. Interestingly, while the relative levels of Melan-A specific Tregs were elevated before vaccination, those cells were markedly reduced after vaccination and replaced by Th1 type specific CD4 T cells [152]. Regulatory functions have also been identified in NKT cells, which include CD4+ and CD4− subsets. CD4+ NKT cells show a functional Th2 profile and are enriched in livers of patients with hepatocellular and colorectal cancer, suggesting that NKT cells failed to mount Th1 polarized responses [153].

Increasing attention is given to so-called myeloid derived suppressor cells (MDSC), which comprise a heterogenous family of immature hematopoietic cells with immune suppressive functions (reviewed in [154,155]). Granulocytes, immature DCs, or tumor-associated macrophages (TAM) exert a variety of activities that can promote tumor growth, neo-angiogenesis and T1 immune inhibition [154–160]. These functions may be mediated by enzymes such as IDO, arginase, nitric oxide synthase, or Cox-2 [161–164], and soluble factors e.g. TGFβ, IL-10 or IL-13 [165–168]. Many of these mechanisms are promoted by inflammatory processes. Central players such as members of the IL-1 family [169] may play important roles, and require further characterization to fully elucidate their effects on tumor growth and immune defense.

CTLA-4 may also act in a T cell extrinsic fashion [170]. It has been proposed that CTLA-4 signals to DC via their CD80/CD86 receptors, resulting in Foxo3 activation and production of the inhibitory enzyme IDO [171–173]. This may be preferentially triggered by Treg cells, which express CTLA-4 constitutively, in contrast to the remaining T cell populations. Further studies are needed to determine whether CTLA-4 mediated T cell extrinsic pathways are more important than the more frequently cited T cell intrinsic inhibition mediated by CTLA-4. Given the extraordinary powerful effects of CTLA-4 blockade, it is surprising that research in this area has not evolved more rapidly.

Besides the challenges to identify the most dominant inhibitory pathways in individual pathologies, it will be important to determine which of these mechanisms act locally, and which of them reach sites distant from tumor tissue and act systemically. For novel therapeutic approaches, it will also be necessary to determine whether metastases can and need to be targeted directly for successful intervention. Alternatively, but not mutually exclusively, powerful vaccines may activate type 1 immune responses adequately such that effector cells can overcome peripheral inhibition, as shown for CpG-ODN based vaccines that can overcome BTLA mediated downregulation of T cell function [148].

7. Concluding remarks

A great deal of experience with tumor antigen based vaccination has been accumulated in patients with many cancer types. It is not easy to develop effective cancer vaccines, but recent progress in the development of a few lead candidates may give reasons for qualified optimism of identifying vaccines with therapeutic efficacy. It is becoming increasingly clear that objective tumor response criteria commonly used in the evaluation of the efficacy of cancer treatments may not be appropriate to evaluate the anti-tumor activity set in motion by vaccination. It has been often noted that responses associated with immunotherapy tend to be slow, and only become apparent after multiple immunizations, possibly even after a period of apparent tumor progression. Because tumor responses follow similar patterns in many of the patients responding to therapy with humanized anti-CTLA-4 antibody, a new set of modified tumor response criteria has been proposed [174]. Moreover, novel paradigms of clinical testing tailored to development of vaccines and biological therapy of cancer have been proposed [175].

Immune monitoring of vaccination has made significant progress since the initial trials were reported. The ability to directly identify antigen-specific T cells by the use of fluorescent MHC-antigen peptide multimers has been a breakthrough enabling T cell quantitation. In addition many functional assays have become available to characterize tumor reactive T cells. However, an important obstacle to the identification of appropriate immune surrogate(s) of effective immunity remains. Since no vaccine has shown major clinical efficacy in trials that involve small numbers of patients, it is at present difficult to conclude on what immune parameters of the specific vaccine response may be correlated with clinical tumor protection. Many researchers agree that a trend for an association between strong specific T cell responses elicited by vaccination and a favorable clinical outcome can be seen in meta-analyses of clinical trials reported. The challenge for the future is to pinpoint the attributes of the immune response that can be explored as predictive biomarkers of clinically effective vaccination. A reasonable set of those attributes include high frequency of tumor reactive T cells, ability to migrate to the tumor sites, intrinsic high avidity of antigen recognition, one which components is high TCR affinity, and well developed effector function. Another critical component is the generation of long-term memory T cells. However, our understanding of how vaccine formulation and regimen impact on formation of long lived memory T cells is still
in its infancy, and none of the available vaccines is ideal in this regard. A no less important issue concerning immune monitoring is the comparability of assay results between clinical centers and monitoring laboratories. This depends on the development of sensitive immune assays which are optimized and properly validated. Important initiatives are underway to promote specific T cell assay harmonization [176–178] as well as standard procedures to report T cell monitoring results [179]. It will be necessary to reach some harmonization as well as standard procedures to report monitoring laboratories. This depends on the development of sen-

The use of complex antigens for vaccination such as recombinant proteins and even LSPs requires the use of assays able to detect all the T cells in vivo activated by the vaccine. These include both CD4 and CD8 T cells and clonal populations recognizing epitopes in the context of all the MHC alleles expressed by the vaccinated individual. New assays heavily relying on multiparametric flow cytometry use the transient expression of T cell activation mark-

Finally, it might be that even potent vaccine formulations inducing strong effector lymphocyte populations and integrated immune responses at the tumor site are not enough to contain advanced tumors or to fully prevent tumor relapse. The apparent multiplicity of suppressive factors operating locally within tumors may require the combination of vaccination with agents that alleviate immune modulatory pathways. Lead candidates are TLR agonists, COX-2 inhibitors, IDO substrate analogues and antibodies that mediate either blockade of co-inhibitory receptors (CTLA-4, PD-1, BTLA, NKRs) or agonist activity of costimulatory molecules (41BB, ICOS). Moreover, recent work has suggested that certain chemotherapeutic agents may enhance tumor immunity. Future research will elucidate the mechanisms of possible syner-

In conclusion, recent immune response and clinical data suggest that T cells have a large potential and thus encourage the further development of T cell based immunotherapy against persistent infection and cancer.

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