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Review

What's new in the field of cancer vaccines?

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Abstract. The observation that in some cases tumors undergo spontaneous regression concomitantly with autoimmune manifestations has been interpreted as an indication of the involvement of the immune system in tumor rejection. This raised the conceptual possibility that the immune system could be used against the tumor. However, since tumor cells are poorly immunogenic by themselves, early attempts to develop immune-based approaches for cancer therapy saw the use of tumor cells transduced with genes coding for cytokines or costimulatory molecules to enhance in vivo immunity. The identification of cytotoxic T lymphocyte (CTL)-defined tumor-associated antigens has allowed the development of new strategies for cancer immunotherapy. Novel adjuvants have been identified, and different modes of antigen delivery were devised which aim at inducing efficient CTL responses in patients. This review will discuss some of what is currently considered as relevant aspects of anti-tumor immunization.

Key words. Tumor; vaccination; antigen; targeting; adjuvant; evasion.

Introduction

Over the past 2 decades, the field of cancer immunotherapy has exploded. Countless labs have devised countless techniques and approaches which aim at turning the patient's immune system against the tumor. The choice of antigen, adjuvant and mode of administration was found to be crucial. Moreover, in humans immune evasion occurs, which may partly explain why many tumor vaccination protocols have been validated in animal models but few have been successful in human clinical trials. An integrated approach to cancer immunotherapy is therefore necessary, which capitalizes on our everexpanding understanding of what turns the immune system on and off in vivo.

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Tumor antigens

Specific tumor immunotherapy requires molecularly defined Tumor-Associated Antigens (TAAs)

Specific immunotherapy relies on the recognition of antigens by specific immune effector cells. In the case of tumors, evidence suggests that in mice as well as in humans, specific protection is conferred mainly by T cells, both of the CD4 and of the CD8 types. It is the recognition at the tumor cell surface of tumor-specific antigen-derived peptides presented by major histocompatibility molecules (MHC) molecules which permits their selective destruction by T cells. However, specific T cells must first be primed by the recognition of the peptides presented by non-tumor antigen-presenting cells, mainly by dendtritic cells (DCs).

Although some approaches aim at using tumors themselves as the primary source of TAAs in vivo, it may be argued that the amount of specific TAAs presented by tumor cells is insufficient to prime or to boost a robust T cell response, but sufficient for recognition and killing by cyto-

toxic T lymphocytes (CTLs). Therefore, during the last 10 years, in a search for antigens to immunize patients, several hundred human candidate TAAs were identified. Many of those TAAs have been validated at the epitope level in a variety of tumors expressing different MHC haplotypes. Several recent reviews have been published which describe in detail the different TAAs and their potential use for tumor immunotherapy [1–5]. The purpose of this section is therefore (i) to give a general overview of the types of TAAs which have been identified and which could possibly be used for tumor immunotherapy, (ii) to illustrate through examples different approaches that can be used to identify TAAs, (iii) to evaluate criteria for selecting TAAs, and (iv) to examine how to optimize the translation from the identification of TAAs to the design of immunogen.

An updated list of all the T-cell-defined epitopes encoded by human tumor antigens that have been published has been assembled by G. Parmiani and colleagues and is accessible at the Istituto Nazionale Tumori website (www.istitutotumori.mi.it).

Overview of the identified human TAAs

Functionally, two types of TAAs may be distinguished: those which are self, and those which are nonself. Self-antigens raise two major issues: tolerance and autoimmunity. Self-antigens have been recognized by immune cells during development but have led to tolerance either through deletion (for antigens recognized with high avidity), or through anergy (for antigens recognized more loosely). Raising a protective immune response against such antigens necessitates first breaking the tolerance. An effective antitumor immune response is expected to eliminate all the cells which efficiently present peptides against which effector T cells have been raised. In the case of self-antigen, the risk of autoimmune destruction of normal tissues has been documented in animal models, and suggested by melanoma patients who have developed vitiligo. It follows that self-antigens should be considered as appropriate targets if they are expressed only by 'dispensable' normal tissues such as prostate or ovary, but not by 'vital' tissues, like the lung.

Non-self TAAs can be either of exogenous (i.e. viral) or endogenous origin: endogenous non-self TAAs are produced as a result of mutation of genes in tumors, or by expression of unmutated proteins which have not been recognized as self because they were never presented efficiently to the immune cells before (i.e. antigen expressed only during fetal life). Non-self TAAs obviate the drawbacks of both tolerance and autoimmunity, and they have been shown to generate more easily high-avidity T cell responses in mouse models.

Currently, human TAAs whose recognition at the tumor cell surface by specific T cells has been established can be grouped according to the nature and origin of the protein, and to the mode of presentation to the T cells, into the following categories.

Exogenous viral antigens expressed by virus-induced human cancers

Viruses are known to be involved in the transformation of many cell types which then express viral antigens recognised by specific T cells. These include antigens encoded by Epstein-Barr virus (EBV) (LMP-1, LMP-2a, LMP-2b, EBNA2, EBNA 3) and expressed by some B cell lymphomas and nasopharyngiomas; human T lymphotropic virus (HTLV)-1 (gag, envelope, tax) expressed by T cell leukemias; hepatitis B virus (HBV) (HbcAb-core, HbsAg-surface, polymerase) or hepatitis C virus (HCV): (core, envelope 1 and 2, NS2, NS3, NS4, NS5) in virus-induced hepatomas; and human papilloma virus (HPV) (E6, E7) in cervical cancers.

Neoantigens as TAAs; Class I HLA-restricted tumor-specific antigens

These antigens result from point mutations in normal genes whose molecular changes often accompany neoplastic transformation or progression. They are either restricted to individual tumors or shared (i.e. generated by tumor-specific alternative splicing). Examples include AFP, β-catenin, caspase-8/m, CDK-4/m, ELF2 M, GnT-V, G250, and HSP70. Fusion proteins that result from chromosomal translocation can also give rise to TAAs that are weakly immunogenic (in leukemia patients). These include both human leucocyte antigen (HLA) class I-restricted epitopes (bcr-abl, ETV6/AML) and HLA class II-restricted epitopes (bcr-abl, Dek-cain, LDLR/FUT, Pml/RARa, p190 minor bcr-abl, TEL/AML1).

Functionally non-self TAAs; The HLA Class I-restricted cancer/testis antigens

These genes are expressed in histologically different human tumors, in spermatocytes/spermatogonia of testis (no expression of MHC I by testis cells) and occasionally in placenta and ovary. Four MAGE gene major subfamilies have been identified and located on chromosome X: the MAGE A, B and C genes are all retrogenes which either encode tumor-specific transplantation antigens, or are pseudogenes; the four members of the MAGE D genes are expressed in normal adult tissues. Interestingly, several mouse MAGE A and B orthologues are also expressed in testis and tumors only. Recently, SSX-2 has been identified as another cancer/testis antigen.

Class I tumor-specific HLA-restricted differentiation antigens that are restricted to specific normal and cancer tissues

These antigens are shared between tumors and the normal tissues from which the tumor arose. Most of these tissue-

restricted TAAs have been described in melanomas and in normal melanocytes, and are involved in the biosynthesis of melanin. Thirty-nine epitopes derived from eight antigens have been described: MART-1/Melan-A, MC1R, Gp100, PSA, PSM, tyrosinase, TRP-1 and TRP-2.

Class I HLA-restricted widely expressed antigens that are over expressed by cancer cells

These genes are expressed in many normal tissues and in histologically different types of tumors, with no preferential expression in some types of cancer. Alterations of transcription or of translation underlies the generation of T-cell-defined epitopes: overexpression in tumors, use of alternative open-reading frame (ORF) carcinoembryonic antigen (CEA), reverse transcription (RU2), and so on. These include ART-4, CAMEL, CEA, Cyp-B, HER-2/neu, HTERT, iCE, MUC1, MUC2, PRAME, P15, RU1, RU2, SART-1, SART-3, WT1, and the anti apoptotic protein surviving. Loss or downregulation of some of these antigens compromises the growth potential of tumor cells, therefore limiting the possibility of generating escape mutants [6].

Strategies for identifying TAAs

Although several strategies have been designed to identify TAAs, they can be separated into two categories, depending on the starting material: (i) the approaches which start from the T cell response of the patients and which thereby guarantee that the TAAs which will be identified are by definition T-defined antigens; (ii) the strategies based on what the tumor cells express can start from information regarding the messenger RNAs (mRNAs) or the proteins, or from the peptides that are presented by MHC molecules. These approaches provide only putative TAAs, which have to be validated for recognition at tumor cell surface by human CTL.

Increasing understanding of the rules that govern the efficiency of presentation of protein-derived peptides by MHC class I and II molecules has been gained recently. These involve (i) the role of subcellular protein localization, (ii) sensitivity to ubiquitination, (iii) specificity of proteosomal (for class I) and lysosomal (for class II) cleavage, (iv) of transporter-associated protein (TAP) transporter and (v) of N-terminal aminopeptidase, (vi) participation of each amino side chain to the binding avidity within the MHC grooves and (vii) to the recognition by the T cell receptor (TCR). Special algorithms have been designed for predicting T cell epitopes from protein sequence.

Confirmation and determination of the affinity of peptide-MHC fixation can be performed with an enzyme-linked immunosorbent assay (ELISA)-based large scale assay. Alternatively, the presentation by tumor cells of peptides derived from candidate TAAs can be confirmed directly by analyzing the peptides eluted from tumor MHC molecules.

In all cases, definitive TAA validation relies on generation of peptide-specific T cells and the demonstration that they can be activated by tumor cells. Generation of specific CTL has been facilitated by immunizing HLA transgenic mice. However, the final demonstration that the human T cell repertoire has not been deleted for epitope specificity requires the generation of specific human T cell clones.

Specific T-cell-based identification of TAAs

Using CTL derived from peripheral blood of melanoma patients, human TAA were initially characterized by screening tumor-derived complementary DNA (cDNA) libraries for genes which would convert transfected cells into targets for these tumor-specific CTLs. T-cell-epitope specificity was next determined using a series of antigen-derived synthetic peptides. Most of the cancer/testis TAAs have been identified following the CTL-based approach, including members of the MAGE, BAGE, GAGE and DAM families. The main advantage of the T-cell-based strategy is that it specifically and directly identifies antigenic peptides that are naturally processed by tumor cells and recognized by T cells. Furthermore, this strategy has been the most efficient at isolating the HLA class I-restricted cancer/testis antigens which are shared by many tumors. However, this method is best suited for tumors that are relatively easy to grow in culture, such as melanomas.

Antibody-based identification of TAAs

Serological analysis of recombinant cDNA expression libraries of human tumors with autologous serum (SEREX) has been used to isolate several putative human tumor antigens. Interestingly, many of the TAAs identified by SEREX had previously been known as T-cell-defined TAAs. Up to now, 1549 public sequences have been compiled and are accessible through the SEREX homepage (http://www-ludwig.unil.ch/SEREX/), which is run by the Ludwig Institute for Cancer Research.

Among the T-confirmed TAAs are the cancer/testis antigens NY-ESO-1 and cTAGE-1 expressed by cutaneous T cell lymphomas. Putative TAAs include SOX1, SOX3 and SOX21 (expressed during early development and in small cell lung cancer), hsp105 in pancreatic ductal and colon adenocarcinoma and HOM-MEL-40 (melanoma).

SEREX takes advantage of the humoral immune response that cancer patients often develop against the tumor and only requires a patient's serum and tumor cDNA library as starting material. Several previously T-cell-defined TAAs have been identified through the SEREX approach. Reciprocally, some TAAs that were first found through the SEREX approach were secondarily confirmed as T-defined TAAs. However, most of the genes identified by SEREX remain candidate TAAs, as they have not yet been

shown to lead to the recognition of tumor cells by specific T cells. Furthermore, information regarding the tumor specificity must be confirmed (and is still lacking) for the vast majority of those genes.

Identification of TAAs by the reverse genomic approach

Identification of TAAs by reverse genomics involves a comparative analysis of mRNA expression by cancer versus normal tissues. Methods include cDNA library subtraction, representational differential analysis (RDA) or serial analysis of gene expression (SAGE). MAGE-B5, MAGE-B6, MAGE-C2, MAGE-C3, SAGE, HAGE, CT10 and CTp11 are all cancer-testis antigens that were first identified by RDA.

Recently, comparison of expressed sequence tag (EST) cDNA libraries derived from cancer versus normal tissues through bioinformatics or genomic microarray analysis has opened novel opportunities for identifying genes specifically expressed by tumor cells. The reverse genomic approach takes advantage of the huge amounts of information contained in public and proprietary databases for selection of tumor specificity to predict peptides which could be used as immunogens. Both the quality and size of the sequence database are critical, as they set the level of tumor specificity. Predicted proteins for which cancer-restricted patterns of expression are confirmed (e.g. through microarray or Taq-man analysis) can be considered as putative TAAs. Computational algorithms for protein sequence analysis allow the prediction of candidate TAA peptides.

Identification of TAAs from the analysis of proteins expressed by tumors

Starting from the identification of the proteins that are specifically expressed or overexpressed by tumor cells, it is possible to test whether those proteins could be the source of peptides allowing the destruction of the tumor by specific T cells. Proteins are classically resolved by two-dimensional (2D) gel electrophoresis. Single spots are automatically picked from the gel and enzymatically digested, and the peptides are analyzed by mass spectrometry. Masses are compared with databases for sequence identification. Sequences of peptides of interest can be confirmed by tandem mass spectrometry. Through a proteomic approach, β-tubulin isoforms have been identified as potential tumor antigens that are recognized by a neuroblastoma patient's antibodies [7].

In contrast to the reverse genomic approach, proteomics starts with direct quantitative and qualitative information about the proteins present in tumor cells. However, accuracy of the data relies on the amount and purity of the cells analyzed. While this may be less of a problem for cell lines, purity is more critical for fresh tumors.

Use of proteomics for TAA identification requires comparison of proteins expressed in cancer cells versus normal tissues. The quality of such subtraction is highly dependent on technical considerations, including: (i) the relative difficulty of purifying some proteins (i.e. membrane-bound proteins) (ii) the resolution capability of the preparative gels (which often excludes proteins with extreme immunoprecipitation), (iii) the reproducibility and sensitivity of the procedures, (iv) the absence of contamination and (v) well-defined criteria for data comparison.

Identification of proteins from peptide mass analysis relies on the size and quality of the databases to which these can be matched. Protein identification can sometimes be hampered by the superposition of several proteins on the same 2D gel spot. Also, the limited amounts of peptide available do not always allow the confirmation of the sequence.

Again, proteins specifically expressed by tumor cells remain putative TAAs as long as their immunogenicity and antigenicity have not been confirmed (see above).

Identification of TAAs by analysing peptides eluted from tumors

In order to be recognized by T cells, TAAs have to be converted by tumor cells into peptides presented at their surface in the context of MHC molecules. In this protocol, MHC molecules are immunopurified from tumor cells, and the acid-eluted peptides are fractionated [e.g. by high-performance liquid chromatography (HPLC)]. Pools of fractionated peptides are next analyzed by mass spectrometry. The masses are then compared with those of peptides predicted to be generated from proteins expressed by the tumor and to be able to bind to MHC molecules. Any mass match can be experimentally confirmed by coelution experiments. Alternatively, collision-activated dissociation (CAD) mass spectra databases can be assembled with peptides eluted from the MHC molecules of cancer cells. This allows direct verification whether any protein expressed by this tumor can give rise to peptides presented by the MHC molecules.

Until now, only a few novel TAAs have been identified through this approach: a peptide derived from a mutated elongation factor 2 has been characterized after elution from squamous cell lung carcinoma [8]. The same strategy also led to identification of a natural HLA-A31-restricted CTL epitope presented by human gastric carcinoma [9].

Compared with other tumor-based approaches, analysis of MHC-bound peptides provides information that is more directly relevant for tumor T cell recognition. This strategy does not rely on the uncertain assumption that gene transcription, protein expression and peptide generation are quantitatively linked. Furthermore, posttranslationally modified peptides (e.g. phosphorylated, glyco-

sylated or cysteinylated peptides) or peptides differentially processed by the tumor can be characterized after elution. Therefore, antigens identified through this method are most likely to satisfy the criteria of immunogenicity and antigenicity, although final proof depends on recognition of tumor by peptide-specific T cells.

Limitations of this method include (i) its limited sensitivity, which allows identification of about only 70% of the peptides presented by a given HLA haplotype and (ii) correlation between the repertoire of peptides that are presented by tumor cells versus the repertoire of peptides that can be eluted from MHC molecules. In particular, the absence of several well-characterized tumor antigen-derived epitopes among the peptides eluted from tumors raises the possibility that peptides bound to MHC molecules with high affinity may not be efficiently recovered by elution, and (3) the number of cells needed for the analysis ($>10^{10}$), which can be a limitation for fresh tumors.

Optimization of TAAs for tumor immunotherapy

While natural TAA-derived T cell epitopes represent potential targets for tumor immunotherapy, they may still be suboptimal immunogens. Peptides which differ from naturally processed proteins may (i) bind to a broader spectrum of MHC molecules without changing TCR recognition, (ii) increase the affinity for MHC binding, which correlates usually with increased immunogenicity and (iii) be recognized with higher avidity by specific TCRs. Moreover, technology is available to produce polypeptides with multiple TA peptides to significantly enhance the probability of covering different types of cancer (i.e. with multiple TAAs) or multiple MHC molecules, to limit the consequences of tumor escape variants, and to associate both MHC class I and class II epitopes in the same vaccine.

Specific example of TAA usage: NY-ESO-1

The NY-ESO-1 cancer testis antigen (CTA) has been abundantly analyzed and will serve to illustate several recent aspects of the research pertaining to TAA usage [10]. NY-ESO-1, a member of the cancer/testis family of antigens, is expressed in normal testis germ cells (particularly spermatogonia) and in a range of human tumor types: 80% of synovial sarcomas, 50% of esophageal carcinomas, 20–30% of lung cancers (mostly the small-cell lung cancers), bladder cancers and melanoma (with statistically significant correlation between the expression of NY-ESO-1 in melanoma and advanced stages of disease). However, NY-ESO-1 is not expressed in colon and renal cancer. Although NY-ESO-1 protein and mRNA expression showed a good correlation in a large panel of lung cancers, there appears to be great variability in NY-ESO-1 expression in individual tumors, ranging from an infrequent homogeneous pattern of staining to highly heterogeneous antigen expression [11].

The NY-ESO-1 gene is located on chromosome Xq28. Two closely related genes were subsequently identified on chromosome X: (i) LAGE-1 (ESO2), which shares similar biological features and >80% protein sequence identity with ESO1, and (ii) ESO3, whose mRNA is ubiquitously expressed in somatic tissues and which has lower (<50%) amino acid homology with ESO1.

NY-ESO-1 is regarded as one of the most immunogenic antigens ever isolated, eliciting both antibody and cell-mediated immune responses in 50% of patients with NY-ESO-1-expressing neoplasms. Both NY-ESO-1 MHC I- and MHC II-restricted epitopes have been characterized in detail. The HLA-A2-restricted epitope, NY-ESO-1 peptide 159–167 (L9L), is strongly recognized by CD8+ T cells as a result of peptide vaccination of cancer patients. However, L9L-specific CD8+ T cells fail to recognize tumor cells naturally expressing NY-ESO-1. Processing of L9L can be rescued after interferon (IFN-γ) treatment of tumor cells or by using dendritic cells pulsed with NY-ESO-1 protein/antibody immune complexes [12]. A dual specificity within peptide S11L has been described, with S9C (peptides 157–165) as the natural antigenic tumor epitope, and L9L (159–167) as a cryptic epitope with dominant immunogenicity upon vaccination that diverts the immune response from tumor recognition. Moreover, modification of S9C peptide at the carboxy terminus enhances HLA-A2.1 binding affinity and stability in solution (165V) and stimulates in vitro CTL, which recognize peptide-pulsed target cells and HLA-A2.1+ NY-ESO-1+ tumor cells, suggesting that this peptide may be clinically valuable for the treatment of patients with NY-ESO-1+ tumors. The NY-ESO-1 119–143 peptide that can be presented in the context of multiple HLA-DR alleles is also capable of inducing specific CD4+ T cells in vitro from peripheral blood lymphocytes of normal donors and patients with melanoma who express these HLA-DR alleles. Furthermore, peptide 157–170 contains an HLA-DP4-restricted helper T cell epitope as well as an HLA-A2-restricted cytotoxic epitope recognized by CD4+ and CD8+ T-cell clones that could be efficiently generated from the peripheral blood of multiple melanoma patients [13]. Dual-specific peptides containing both cytotoxic T cell and helper T cell epitopes may represent an attractive strategy of NY-ESO-1 vaccine design. These observations emphasize the importance of analyzing the fine specificity of vaccine-induced T cell responses in patients as a basis for constructing effective cancer vaccines.

Although renal carcinoma and malignant mesotheliomas cells do not express NY-ESO-1, DNA hypomethylating agent 5-aza-2′-deoxycytidine (DAC) induces persistent de novo expression of several CTAs, including NY-ESO-1, suggesting that systemic administration DAC may repre-

sent a promising strategy to enhance the constitutively poor immunogenic potential cancer cells [14]. Furthermore, the histone deacetylase inhibitor depsipeptide FR901228 (DP) has been shown to enhance NY-ESO-1 induction mediated by DAC in cell lines established primarily from thoracic cancers. After sequential DAC-DP treatment, HLA-A*0201 cancer cells were not only recognized by an HLA-A*0201 CTL specific for NY-ESO-1 but also induced to apoptosis through as yet undefined mechanisms. Thus, sequential DAC-DP treatment may be a novel strategy to augment antitumor immunity in cancer patients.

Criteria that may govern the selection of TAAs for tumor immunotherapy

In summary, novel cancer vaccine TAAs should have following characteristics:

- they should have high immunogenicity
- they should preferably be non-self, to avoid the problems of tolerance and autoimmune destruction of normal tissues
- they should not be presented efficiently by vital non-cancer cells: e.g. hepatitis C viral antigen might not be the best candidate, as nontransformed hepatocytes express these antigens and could become the target of effective CTL
- they should be presented by as many types of tumors or as many individual tumors of a selected type as possible to offer broad application
- they should include both MHC class I and class II epitopes
- they should contain multiple TAAs to broaden the population coverage and to prevent immune escape of tumor variants
- they should preferentially correspond to proteins involved in tumor transformation, to prevent immune counterselection
- they should be homogeneously expressed in a given tumor.

Adjuvants

The term ‘adjuvant’ is derived from the Latin ‘adjuvare’, which means to help. If adjuvants have been closely linked to the succesful history of antiinfectious vaccines, they will certainly also be a key component of efficient preventive or curative antigen-specific antitumor therapies. Adjuvants can help vaccines in a quantitative way, that is by enhancing the overall response to a given amount of antigen, but also have a qualitative impact on the immune response. From this point of view, there are probably different requirements for the adjuvants designed for antiinfectious versus anticancer vaccines. The type of immune system sought in cancer is preferentially a strong MHC class I-restricted, Th1-type response, while protection against extracellular pathogens is usually better in case of a Th2 response.

When considering antiinfectious vaccines, there is a shift towards the use of synthetic, molecularly defined – and therefore safer – antigen moieties that makes the use of adjuvants even more necessary. Yet, the molecular basis of the biological properties of many adjuvants was still unknown, and strong adjuvants such as complete Freund’s adjuvant (CFA) often caused undesirable side effects. On the other hand, second-generation approved adjuvants such as the water-in-oil emulsion Montanide, currently used in cancer vaccine trials [15], may have a much lower risk of secondary effects but also decreased immunogenicity. We shall see that some of the adjuvant signals are starting to be deciphered and that the modern design of vaccines will probably combine the right set of molecularly defined antigens together with molecularly and biologically customized adjuvants.

While large-scale cancer vaccines will probably fit into this definition, there is currently a great enthusiasm in using DCs as a cellular therapy for cancer. This strategy relies upon the capacity of DCs to capture and present exogenous antigens in both the MHC class I or MHC class II pathways to naive T cells, but also upon their key role in shaping the immune response [16]. Indeed, dendritic cells have been called ‘nature’s adjuvant’, and this property is probably related to the fact that DCs are equipped to sense proinflammatory signals derived from pathogens, which are similar to those of vaccine adjuvants [16, 17]. Thus, the numerous experimental models and now clinical trials using DCs [18] represent an interesting opportunity to select adjuvants based on the intrinsic properties of such or such DC vaccine. For example, there exist several DC subsets in humans which are equipped with different sets of receptors for pathogen-derived molecular signals [19]. Thus, the relative efficiency of different DC subsets in cancer vaccines may guide the selection of an adjuvant that would trigger a particular set of receptors expressed by one DC subset.

Therefore, as we will see in the following paragraphs, the required properties for an efficient adjuvant for cancer vaccines is tightly related to its impact on DC biology. Moreover, the paradigm of vaccination with DCs suggests that these different properties should be gathered into one formulation in order to trigger an optimal immune response.

Adjuvants which promote DC recruitment

DCs form a network of sentinels throughout most tissues, yet they are mostly abundant at the interface with the external milieu. For example, Langerhans-type cells colonize the epithelium of the skin, lung and intestine, while

dermal-type DCs are found in connective tissues [16]. Blood represents a critical barrier because it could allow for the uncontrolled spreading of microbes through the body. It contains different subsets of immature DCs and in particular the plasmacytoid subset of DC precursor, which is the only cell type able to produce large quantities of type I interferon in response to certain viral infections [19]. The other DCs are usually referred as myeloid DCs, although it is increasingly obvious that they also represent multiple functional subsets.

Immature DCs arise from blood precursors under the influence of several growth factors. The injection of such factors could increase the number of DCs either throughout the body or at the site of tumor antigen delivery. Depending upon the specificity of the growth factor, one subset of DCs could be preferentially targeted. For example, Fms-like tyrosine kinase receptor 3 ligand (Flt3-L) induced an increase of all DC subsets in humans and mice, including the plasmacytoid DCs [20, 21]. In contrast, granulocyte-macrophage colony-stimulating factor (GM-CSF) injection seemed to preferentially induce myeloid DCs while granulocyte colony-stimulating factor (G-CSF) induced preferentially plasmacytoid DCs [22]. Recombinant GM-CSF has been used for quite a long time as an adjuvant of cancer vaccines [23–25], although it is not proven that this property was only related to the recruitment of DCs. GM-CSF is also an adjuvant for viral-based cancer vaccines [26], and formulations allowing for its slow release are probably more efficient [27]. Flt3-L has been used through a systemic injection to increase the overall DC number, followed by HER-2/neu peptide vaccination [28] or followed by an ex vivo step of DC purification and loading with an altered tumor antigen [29]. But results are too preliminary to conclude the adjuvant potency of Flt3-L. It will certainly be extremely relevant in the future to define the potential role of the different DC subsets in antitumor response in order to select the most suitable DC growth factor [22].

Another strategy developed to recruit DCs at the site of antigen delivery is to use chemokines, which are small proteins controlling the navigation of leukocytes. While chemokines represent a large family of over 30 proteins, we and others have shown that subsets of DCs respond to a restricted set of chemokines [30], suggesting that these chemokines could be used to attract the desired subset. Indeed, many known adjuvants are probably inducing a variety of so-called inflammatory chemokines, which are mainly those active on immature DCs. The use of a single recombinant chemokine in vivo, however, may be insufficient, since it is possible that DCs require a multiple-step process involving different chemokines in order to reach tissues [31].

For both growth and recruiting factors for DCs, an attractive strategy is to include them by gene transfer into the tumor cells used as vaccine. This approach is at the frontier of the vast domain of cancer gene therapy, but must still be considered as vaccines since the antigenic material is of exogenous origin, even though in some instances it derives from the patient himself. The best-known example of such vaccines is modification of an allogeneic tumor vaccine with GM-CSF, which gave preliminary encouraging results in the clinic [32]. There are also many reports that the introduction of a chemokine gene into a tumor cell line induces strong DC recruitment and antitumor immunity, but so far most of this work has been done looking at the transfer of modified live tumor cells, therefore suggesting the use of chemokines in gene therapy rather than as adjuvants for cancer vaccines [33].

Adjuvants which promote MHC class I delivery

As mentioned above, one main goal of cancer vaccines is to promote the development of a strong MHC class I-restricted CTL response. If special antigen-delivery vehicles, and in particular viral vectors, can efficiently promote CTL (see next section), certain adjuvant formulations seem to also favor CTL induction. In most cases, their effect is to bypass or reduce the degradation of exogenously acquired proteins in lysosomal compartments. Certain proteins such as listeriolysin from *Listeria monocytogenes* can allow exogenous proteins to be delivered into the cytosol by forming pores in the cell membrane rather than by a receptor-based mechanism [34]. This property is rather similar to that of several oil or lipid-based formulations which contain detergents allowing for direct cytosolic delivery, such as immunostimulating complexes (ISCOMs), QS21 and AF [35]. These compounds, however, have no specificity and therefore cannot direct the antigen into an antigen-presenting cell (APC). Similar targeting of exogenous antigens to the MHC class I pathway might also be achieved with liposomes, which are initially internalized in the endosomal pathway but could deliver their antigen load in the cytosol [36]. The use of liposomes could provide some selectivity since they are preferentially engulfed by macrophages, but their optimal composition for cancer vaccines is still being debated [35].

Intracellular trafficking of certain toxins may make them useful in targeting proteins or peptides to the cytosol. The B fragment of the Shiga toxin from *Shigella Dysenteriae* could present human or mouse tumor-associated antigens in the context of MHC class I molecules in vitro [37] and induced CTL in vivo [35]. Similarly, the adenylate cyclase toxin CyaA could deliver CTL epitopes directly into the cytosol [38] and induce a protective antitumor response when fused to the model tumor antigen ovalbumin [39]. The fact that the B fragment of Shiga toxin binds to the glycolipid Gb3 [35] and that *Bordetella* CyaA binds to CD11b [40], both molecules being highly expressed by

DCs, might explain a good adjuvant effect through cell specificity.

More recently, the outer membrane protein A from *Klebesiella pneumoniae* (kpOmpA) was also shown to be able to deliver exogenous ovalbumin into the cytosolic MHC class I pathway and subsequently induce antitumor immunity against ovalbumin-expressing tumors [41]. An interesting feature of kpOmpA is that it binds to the toll-like receptor 2 (TLR-2), providing a strong activation signal in addition to its antigenic package to APCs, and especially immature DCs [41]. It seems that CpG immunostimulatory sequences could have similar dual-adjuvant properties, since they are also able to facilitate the presentation of exogenous protein via the MHC class I pathway [42] and to activate cells expressing the TLR-9 receptor, including DCs [43].

Aduvants which promote dendritic cell activation and Th responses

In infectious diseases, traditional vaccines included inflammatory components, which are strong activators of the immune response. For example, the whole-cell diphteria-tetanus-pertussis, typhoid and cholera vaccines all contained lipopolysaccharide (LPS), and the BCG vaccine took advantage of the immunostimulatory properties of mycobacteriae [44]. One of the challenges of adjuvant design in the past decades was to break down microorganisms and isolate their adjuvant component, while trying to eliminate as much as possible undesirable side effects. For example, the adjuvant-added effect of CFA versus IFA is derived from mycobacterial cells, and it was discovered that the cell-wall skeleton fraction of mycobacteria retained the main immuological properties of CFA, with some effects probably related to the muramyldipeptide (MDP) and trehalose dimycolates [45]. Interestingly, although microorganisms show an enormous diversity, their components stimulating the immune system of mammals seem to belong to a restricted family of conserved structures such as LPS for Gram-negative bacteria, peptidoglycan (PGN), lipoteichoic acid (LTA) for Gram-negative bacteria, lipoarabinomannan (LAM) for mycobacteria, unmethylated CpG DNA and bacterial lipoproteins [46, 47]. These structures are now acknowledged as pathogen-associated molecular patterns (PAMPs) [47]. In the last decade, the molecular nature of the interaction of PAMPS with host cells was partly unveiled. In particular, the family of the TLRs encompass 10 members which are specialized in the recognition of PAMPs and trigger several activation pathaways related to Myd-88 and/or mitogen-induced protein (MAP) kinases [46, 47]. In parallel, the role of TLRs in the development of innate [47] but also adaptive [17, 48] immunity is being unraveled. Interestingly, it seems that DC interaction with PAMPs is a key event in the intiation of both types of immune responses, although it seems that TLRs preferentially drive a Th1-type response [48]. Together with the understanding of the function of particular subsets of DCs [19] and the distribution of TLRs in those subsets [49], this recent advance could lead to the development of more efficient, more specific and less toxic cancer vaccines. As an example, there is now a vast literature on the use on CpG sequences as an adjuvant of immune responses [50], in particular cancer, and TLR9 was recently described as a receptor for CpG sequences [43].

Beside PAMPs, endogenous signals can also trigger DC activation and thus be applied, in theory, to adjuvant design. These signals mainly originate from dying or stressed cells [51]. Interestingly, it seems that some of these endogenous signals, namely heat-shock proteins (HSPs), could also stimulate the immune system via the TLR-4 [52]. HSPs have recently been shown to chaperone antigenic peptides, including tumor-associated antigens, as well as to promote the maturation of DCs [53]. Thus, HSPs in recombinant form or purified from tumor extracts could be a promising adjuvant for antitumor therapy.

If PAMPs or other types of danger signals seem to be a prerequisite for an optimal adaptive immune response, several other pathways will amplify or bias the immune response, and those could be harnessed for designing anticancer vaccines. Most strategies aim at stimulating a productive cross-talk between T helper cells, CD8+ effector T cells and antigen-presenting cells, namely DCs. The addition of an exogenous immunogenic protein such as keyhole limpet hemocyanin has been known for a long time to provide help for a response to otherwise poorly immunogenic tumor-associated antigens, and this approach is being tested in the clinic [54–56]. Nowadays, the cognate and soluble molecules involved in T cell help are also being tested in experimental cancer vaccines, especially members of the tumor necrosis factor (TNF) TNF-R families such as OX-40 [57] and CD40 [58], as well as the ligand for 41-BB, CD137 [59].

Antigen delivery

Protein antigens

To increase antitumor immune responses, tumor-derived proteins can be fused with other proteins. For instance, a better response against cervical intraepithelial neoplasia was obtained using a fusion protein consisting of HPV L2, E6 and E7 in a protein-based vaccine than with a vaccinia-based vaccine, as studied previously in a phase I trial [60]. In vitro, the fusion protein obtained by the association of a renal cell carcinoma antigen and GM-CSF induced DC maturation, and CD4 and CD8 T cell response [61]. These results suggest that fusion proteins could be used either directly or for DC transduction in DC-based vaccines.

Tumor-derived proteins can also be protected by chaperones. Among these, HSPs are overexpressed in a wide variety of cancer cells and virally transformed cells. It was demonstrated that HSPs derived from tumor cells were excellent immunogens capable of inducing a robust host immune response against the tumor [62, 63]. The success of preclinical animal studies in which HSP-peptide complexes directly purified from tumor were used led to clinical phase I/II trials of tumor-derived HSP preparations [64]. HSPs (particularly HSP 110) can also bind to and stabilize large proteins. In preclinical studies in which recombinant HSP 110 was noncovalently associated with the recombinant tumor protein antigen HER2/neu, the activation of a robust immune response was observed [65]. Among proteins used in immunotherapy, immunoglobulins have been used as tumor antigens or delivery systems. In one study, B-cell lymphoma patients were immunized with immunoglobulin expressed by their own tumors and chemically coupled to the foreign carrier protein KLH and emulsified in adjuvant [66]. Forty-nine percent of the treated patients generated a specific immune response against the idiotype of their tumor immunoglobulin (Ig). Importantly, the extent of the immune response was correlated with a favorable clinical outcome. The main limitation of this approach is the need to prepare tumor Ig protein for each patient. Antibody can also be used for the delivery of immunogenic peptides to APCs such as DCs. Recently, Lunde and colleagues described the construction of recombinant, APC-specific antibodies that have antigenic peptides integrated into their constant regions. They demonstrated that when these recombinant antibodies were internalized and degraded by APC, the T cell epitopes were efficiently loaded onto MHC molecules and presented to T cells [67, 68]. The targeting of MHC class I-restricted epitopes to APC via APC-specific antibodies could be a promising approach to elicit specific T cell responses.

DNA vaccines

Naked DNA with its simplicity, stability and inexpensiveness is another vaccination strategy being explored for immunotherapy. In addition, plasmid DNA is easily modifiable, and many genes or combinations of genes or DNA encoding epitopes can be engineered ([69] for review). Vaccination with DNA leads to uptake into cells including APC and sustains expression of the antigen. In a phase I//II trial, half of the prostate cancer patients administrated with DNA encoding PMSA antigen and the costimulatory molecule CD86 developed delayed-type hypersensibility to PMSA. Boosting with a replication deficient viral vector encoding PMSA induced immunity in all of the patients [70].

An elegant DNA-based approach consisting of recombinant chimeras composed of ligands of APC cell surface molecules fused to TAA sequences has been recently investigated. For instance, a chimeric recombinant construct encoding a MAGE-3/IgG Fc fusion protein mediated efficient presentation by MHC class I and class II [71, 72]. In other studies, the inclusion of HSP70, CTLA-4 or FLT3-L into constructs expressing TAA sequences led to a considerably better presentation than immunization with TAA sequences alone [73–76].

In addition, considerable efforts have been devoted to developing formulations that would protect DNA and improve cellular uptake. The main destination of liposome-encapsulated macromolecules is intralysosomal degradation. In this context, one approach to enhance cytosolic delivery through escape from endosomes is the use of reconstituted envelopes of influenza virus (virosomes), Sendai virus (fusogenic liposomes) or hemagglutinating virus of Japan (HVJ liposome). The acidic environment within endosomes triggers fusion of the viral envelope with the endosomal membrane, resulting in release of plasmid into the cytosol of the host cell. Preclinical studies performed with a plasmid encoding the parathyroid hormone-related peptide, a protein secreted by prostate and carcinoma cells [77], or a melanoma-associated antigen [78], demonstrated the induction of specific immune responses.

Cell-based vaccines

As discussed above, the role of DCs as potent professional APC able to prime T cell responses in vivo provides a rationale for using DC for human immunotherapy. In DC-based vaccine design, the most common approach involves loading MHC class I molecules with exogenous peptides. Early clinical evaluation of peptide-loaded DCs was performed in melanoma patients and revealed that such vaccines were safe and that they induced durable remissions in patients with metastatic melanoma [79–82]. Promising results have also been reported in patients with other tumors such as colorectal and lung carcinoma [83], hormone refractory prostate cancer [84], breast and ovarian cancer [85], and malignant glioblastoma [86]. Alternatively, the use of full-length native or recombinant protein as antigen allows the induction of responses against different epitopes restricted by several MHC alleles. An example of successful vaccination is the use of DCs loaded with idiotype Ig derived from patients with follicular lymphoma and multiple myeloma [87, 88].

Another strategy which does not require the knowledge of the patient MHC and antigenic epitopes involves gene delivery to DCs by transduction with recombinant viruses or transfection with DNA or RNA encoding tumor antigen [83, 89–91]. In the same context, DCs loading with tumor lysates have been developed [92–94]. Tumor antigens may also be delivered to DCs by fusing DCs and tumor cells. This approach has been tested and yielded signifi-

cant clinical responses in patients with renal carcinoma [95].

However, as in peptide-based vaccines, data are complicated by reports indicating that immature DCs can induce tolerance to antigen. Injection of peptide-pulsed immature DCs but not mature DCs into healthy individuals can result in antigen-specific inhibition of effector T cell functions in vivo [96]. Therefore, caution should be exercised when using DC in antitumor vaccination.

Immune evasion

Despite these considerable advances, it is becoming increasingly clear that negative signals exist that are deleterious to the generation of an effective antitumor immune response. It is largely accepted that many such signals are the products of the tumor environment.

It was reported early on that T cells from tumor-bearing individuals are deficient in one or more aspects of their effector function. For instance, it was demontrated that tumor-infiltrating lymphocytes (TILs) from patients with solid tumors had lower proliferation frequencies and cytotoxicity than peripheral blood mononuclear cells (PBMCs) from the same subjects, and that incubation of PBMCs from normal individuals with tumor cells or tumor supernatants significantly reduced their proliferation and cytotoxicity ([70, 97, 98]; and reviewed in [99] and [100]). One frequently observed defect in T cells from cancer patients is the downregulation of the ζ chain of the TCR [101–103]. TCR ζ is a key signaling molecule involved in T cell activation. Interestingly, a correlation was established between the absence of ζ or its low level of expression and poor prognosis in patients with oral carcinoma [104] and squamous cell carcinoma of the head and neck [105].

It has been postulated that soluble factors secreted by tumor cells are largely responsible for impaired TIL function. One such factor that has been extensively studied is transforming growth factor-β (TGF-β). TGF-β exerts a wide range of effects on immune cells, including suppression of TCR- and interleukin (IL)-2-mediated T cell proliferation [106–108], of natural killer (NK) and lymphokine-activated killer activity [109], and of T cell cytotoxicity [110], the latter being probably due in part to a decrease in expression of perforin by CD8+ T cells [110]. More recently, it has been suggested that in presence of TGFβ, DCs polarize T cell responses toward a T helper (TH)-2 phenotype [111]. In the context of human cancer, a correlation was found between serum TGF-β levels and tumor progression and resistance to immunotherapy [112, 113]. While lacking in humans, a causal relationship between local TGF-β expression and tumor immune evasion was clearly established in rodent tumor models. For instance, highly immunogenic tumor cells that were made to express TGF-β usually evaded immunosurveillance, while interference with TGF-β expression in the tumor micoenvironment often led to eradication of the tumor [114]. IL-10 is another immunoregulatory cytokine that has been suggested to be implicated in the modulation of tumor-specific immune responses. IL-10 is a so-called Th-2-promoting cytokine in that it downregulates Th-1-type cytokines such and IFN-γ and TNF-α, and promotes the production of IL-3, IL-4, IL-6 and IL-10 by T cells [115]. Since IL-10 has been found to be produced both by tumor cells and TIL [116], and since antitumor immune responses have traditionally been associated with a delayed-type hypersensitivity (DTH)-type Th1 response, this has been taken as an indication that IL-10 may be implicated in the establishment of an immunosuppressive tumor milieu. However, testing of this hypothesis by providing or inhibiting IL-10 locally in tumor animal models has yeilded varying results ranging from tumor rejection to tumor growth enhancement [115], further illustrating the pleiotropic nature of this cytokine.

The detection of functional Fas ligand (CD95L) on the surface of some tumors has led to the so-called Fas counterattack hypothesis, whereby tumor-expressed FasL would interact with Fas on the surface of activated CTL, killing the latter and therefore evading a deleterious (from the tumor's point of view) immune response [117–120]. Although initially attractive, this hypothesis is now being seriously challenged [121, 122]. Not only were some groups unable to detect FasL on the surface of many tumors, but transfection of tumor cells with FasL rendered them more susceptible to rejection in vivo [123–125]. Similar observations were made in transplantation, whereby FasL-expressing islet β cells, myoblasts and other cells were more rapidly rejected than their $FasL^-$ counterparts [126–129]. Some insight into this apparent paradox could be gained from a recent study in mice using elegant genetic polymorphism analysis and adoptive transfer [130]. In this study, Kurooka and colleagues show that although some tumor cell lines do not express FasL, in vivo tumors formed by these same cells are $FasL^+$. Interestingly, The FasL detected in these tumors does not come from donor tumor cells but is of host origin, apparently from tumor-infiltrating macrophages. However, the relevance of this FasL expression is unclear, since tumor growth into mouse strains that are genetically deficient in Fas or FasL does not differ from that in wild-type mice. It is safe to say at this point that the issue of the contribution of the Fas system to antitumor immunity remains controversial.

Supposing they overcome all of the above obstacles, tumor-specific T cells often face yet another hurdle, namely their inability to find their specific ligand on tumor cells due to loss of antigen or MHC class I expression by a variety of tumors. Downregulation of MHC class I occurs frequently in human tumors, particularly in

metastatic lesions [131–134]. The observation that residual cells following immunotherapy express reduced levels of MHC class I argues for the idea that MHC class I downregulation is a contributing factor to immune evasion [135]. Antigen downregulation has also been shown to modulate CTL recognition of melanoma cells [136–140]. Antigen and/or MHC class I loss or reduction can be the result of a number of events. Tumor cells may mutate peptide antigens that associate with MHC class I [141–143]. They may develop defects in components of the antigen-processing machinery such as the proteasome subunits LMP2 and LMP7 [144]; downregulate [145–147] or mutate [148] the TAP transporter; or lose the expression of β2-microglobulin [149–151]. It was shown that some of these effects may be mediated by oncogenes such as RAS or MYC [152]. Theoretically, loss of MHC class I should make tumor cells susceptible to NK killing. There is recent evidence that many tumors express the NK-inhibitory molecule HLA-G [153–155], suggesting that these tumors can evade NK killing. However, this conclusion is contested by a number of groups who have failed to detect HLA-G on the surface of tumor cells [156–158].

Summary

As is apparent from the above, cancer immunotherapy carries great promise, but also poses a number of important challenges. Although clinical trials with the current generation of cancer vaccines have so far yielded somewhat disappointing results, the increasingly large body of knowledge being generated in the field of tumor immunology will certainly bring us closer to developing the ideal cancer vaccine, one which will incorporate the best combination of tumor antigen, adjuvant and delivery vector.

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