Reengineering dendritic cell-based anti-cancer vaccines

Summary: Despite initial enthusiasm, dendritic cell (DC)-based anti-cancer vaccines have yet to live up to their promise as one of the best hopes for generating effective anti-tumor immunity. One of the principal reasons for the generally disappointing results achieved thus far could be that the full potential of DCs has not been effectively exploited. Here, we argue that dramatic improvements in vaccine efficacy will probably require a careful re-evaluation of current vaccine design. The formulation of new strategies must take into account the natural history of DCs, particularly their role in helping the immune system deal with infection. Equally critical is the emerging importance of soluble factors, notably interleukin-12, in modulating the quality of immune responses. Vaccines should also be designed to recruit helper T cells and antibody-producing B cells rather than simply cytotoxic T lymphocytes. Finally, the judicious selection of tumor, target antigen, and disease stage best suited for treatment should serve as the foundation of trial designs. Our discussion addresses a recent clinical vaccine trial to treat early breast cancer, where many elements of this new strategy were put into practice.

Keywords: dendritic cell, vaccine, cancer

Introduction

The last decade has witnessed steady reductions in the death rates for many types of cancer. These reductions are largely due to improvements in early detection (1), advanced surgical techniques, refinements in the administration of radiation therapies (2), and the discovery of new, molecular-targeted chemotherapeutic agents (3). However, countless instances occur either where tumors are not amenable to any existing therapy or they respond initially only to recur in forms resistant to front-line therapies (4), leaving limited treatment options.

The development of novel treatment modalities will greatly benefit cancer patients. One such modality is immunotherapy, which posits that the immune system can be enlisted in the fight against cancer. There has existed for some time compelling evidence that cellular and molecular agents of the immune system are capable of attacking tumors, and experimental immunotherapeutic interventions have sought to take advantage of each of them (5). Although most immunotherapy trials have yielded somewhat disappointing
results, there are some outstanding examples of success, such as the recent T-cell adoptive therapy trials conducted at the Surgery Branch of the National Cancer Institute. These treatments have proven that immunotherapy can induce pronounced tumor regressions that are associated with prolonged survival for advanced melanoma (6, 7). At least for relatively advanced melanoma, such outcomes are currently superior to any other therapeutic modality available. However, this type of therapy involves the cultivation of huge numbers of patient lymphocytes, which requires uncommon technical expertise and specialized facilities. Therefore, less labor intensive forms of immunotherapy, such as vaccine modalities, are desirable for more widespread implementation. Unfortunately, vaccine strategies have underperformed these more labor-intensive adoptive immunotherapy approaches. Breakthroughs in our understanding of tumor immunology are clearly needed to advance vaccine-based immunotherapy to this next level.

One substantial hope for the development of cancer vaccines came with the development of methods to culture human and mouse dendritic cells (DCs) (8, 9). This discovery was greeted with enthusiasm because DCs were considered the most efficient known cells for the presentation of antigen to T cells. It was therefore supposed (based on some early work with murine models) that it might be relatively easy to pulse tumor antigens onto DCs and use these cells to successfully vaccinate against tumors (10). The primary source for human DC precursors was the blood and bone marrow, but the first methods produced only immature DCs. Later, ways were found to mature these cells, which usually involved a second-step culture with additional cytokines (11). Both immature and mature cells have been tested in clinical trials to treat various malignancies. Whereas occasional clinically relevant responses were observed, the overall results have been something of a disappointment. There are two opposing conclusions that can be drawn from this experience. The first is that DC-based modalities do not represent a viable pathway for the development of successful anti-cancer vaccines. The second is that DCs could still comprise an important tool for advancing immunotherapy, but efforts have been compromised by a failure to utilize their full potential. In the latter case, some careful investments in the form of new and carefully considered approaches may yield sizable returns in improved therapeutic outcomes. This review examines some of here-tofore largely neglected components in DC-based vaccine design and poses certain problems that must be overcome to increase our chances of formulating successful vaccine strategies. Finally, we propose ways in which these components can be synthesized into a coherent strategy for developing anti-cancer vaccines.

Immunosurveillance to immunoediting: defining a central problem for cancer vaccines?

When cancer came to be systematically studied in animal models using transplantable tumors, it was realized quickly that the failure of tumors to establish themselves in genetically unrelated animals was an immunological phenomenon. Further, it was demonstrated that malignancies induced by methylcholanthrene (MCA) in inbred mouse strains often expressed apparently novel antigens that could be recognized by the syngeneic host (12, 13). The question then arose that if tumors can be antigenic, why shouldn’t the immune system be capable of handling them, either naturally or as a result of vaccination? Burnet (14) and Thomas posed that lymphocytes did in fact naturally respond to cancer and constantly patrolled the body in search of newly transformed cells. When found, these nascent tumors would be quickly eliminated. This theory, called ‘Immunosurveillance’ drew considerable attention and produced a number of interesting and apparently testable predictions. Among these was ‘conditions associated with depression of the (T-cell-dependent) immune system whether genetic, induced by drugs, or of other origin should increase the likelihood of cancer’ (14). After all, if lymphocytes were really curtailing the emergence of malignancies through their constant surveillance, then the compromise of these cells in experimental animals through drugs or genetic mutation ought to result in a greater susceptibility to cancer. When this important tenet of immunosurveillance theory was put to the test, however, little evidence supporting it could be unearthed (15–17). Nonetheless, it should be noted in retrospect that the experiments seeming to refute immunosurveillance relied on nude mice. Nudes, which were the best genetically immuno-deficient mice available, are now known to be insufficiently immunocompromised to properly test the immunosurveillance hypothesis. Unfortunately, these studies using the best information and technology available at the time, prematurely laid to rest the notion that the immune system played a role in naturally controlling the appearance of malignancies.

Studies two decades later revived this notion, however, when animal models became available with more profound and molecularly defined defects in immunity. For example, mice deficient in interferon-γ (IFN-γ), elements of the IFN-γ signaling pathway (18, 19), or the T-cell cytotoxic protein perforin (19) were far more susceptible to MCA-induced tumors. The IFN-γ-deficient animals even displayed a greater
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For example, failure to present antigen in the context of class I processing and presenting function has been well documented. The presentation of antigenic targets. Loss of such antigen-carrying tumor cells, due to a fortuitous mutation, resist outright destruction, then the second phase of dynamic equilibrium ensues. Here, constant pressure is applied to the tumor in the attempt to contain it. However, due to the genetic instability of tumor cells, further mutations arise and accumulate that allow the malignant cells to become progressively more resistant to immune assault. In the third phase, escape, enough changes have occurred in the tumor cells so that they can circumvent and survive everything the immune system can deliver, and the cells thus continue to grow and spread, leading to the eventual death of the host.

The immunoediting hypothesis dictates that whereas the immune system may perhaps kill all tumor cells outright during the elimination phase, it could in other cases only attenuate the development of a malignancy, essentially sculpting the tumor into an ever more aggressive and/or immuno-evasive phenotype. It is these tumors that eventually break through containment by the immune system to ravage the host.

One of the best-understood mechanisms by which tumors change to avoid immune-mediated destruction is to minimize the presentation of antigenic targets. Loss of such antigen-processing and presenting function has been well documented. For example, failure to present antigen in the context of class I major histocompatibility complex (MHC) has been traced to point mutations and deletions that affect the expression of functional β2-microglobulin as well as individual human leukocyte antigen (HLA) molecules (22, 23). Likewise, defects in the actual antigen-processing machinery including transporter associated with antigen processing-1 (TAP-1), TAP-2, and latent membrane protein 2 (LMP-2), and LMP-7 have been reported (24–26).

A phenomenon that might be considered a form of induced immunoediting has been observed and reported in some experimental immunotherapy settings. For example, a recent study induced the inflammatory killing of normal melanocytes by repeated injection of a DNA construct encoding a suicide gene transcriptionally targeted to melanocytes (27). The mice receiving an optimized form of this treatment could reject established B16 melanomas, presumably due to shared antigens between melanocytes and melanoma. However, mice receiving a suboptimal therapy schedule did not completely reject their melanomas. Instead, the tumor broke through therapy expressing a completely amelanotic phenotype and lost expression of the tumor-associated antigen and the melanin biosynthesis pathway enzyme tyrosinase (as well as tyrosinase-related protein 2). Likewise, in clinical trials were melanoma patients were vaccinated with HLA-A2.1-restricted peptides, including those representing epitopes from tumor-associated antigens MART-1, MAGE-3, gp100, and NY-ESO-1, the appearance of antigen-loss variants were also reported (28–30).

The findings that support the existence of the natural process of immunoediting coupled with the phenomenon of antigen-loss variants resulting from immunotherapy (which we consider a form of induced immunoediting) reveal a central and challenging problem for the development of cancer vaccines: all immune-based therapies (including vaccination) against cancer depend upon the ability to generate immunity to defined targets (usually protein) that must be produced and presented in some manner by the tumor cell targets. It has been demonstrated in some cases that tumors expressing the defined target can be totally eradicated by vaccine therapy, thereby proving possible that essential elements of tolerance and negative regulation can be overcome. However, if tumor cells can simply escape powerful and potentially tumor-clearing immunity by shutting down either the production of the target protein or the machinery that displays these targets to T cells, what legitimate prospect exists that anti-cancer vaccines can ever achieve consistent success? Any cancer vaccine approach must eventually confront this problem, and it is one that we shall return to in this discussion. Nonetheless, it remains essential to examine how best to consistently generate, through vaccination, the strongest possible anti-tumor immunity.

Natural history and function of DCs

Why build vaccine strategies around DCs when there are so many other, more ‘traditional’ vaccine modalities that have
proven successful for a wide spectrum of infectious diseases? First, all conventional vaccines against infections are designed from the start to be protective not therapeutic. Thus, effective immunity is generated before infection, not after disease is already established. Indeed, most highly successful vaccines against microbes are usually not effective if administered post-infection. Cancer vaccines, as they are now generally conceived, are intended to induce immunity capable of rejecting malignancies after they are established and perhaps even spread extensively. An exception is the recently approved vaccines to prevent cervical cancer (although this vaccine is really protective against papilloma viruses) (31), and although a time may come when many anti-cancer vaccine are successfully developed that operate in a purely protective setting, the bulk of efforts are now therapeutic in nature. Because therapeutic immunity is more difficult to generate, it is desirable to develop even more powerful modalities of vaccination to overcome the difficulty of treating established disease. Since DCs are the most powerful antigen-presenting cells (APCs) known and are chiefly responsible for the primary sensitization of naive T cells to antigen, it is logical to try to harness these cells to achieve the strongest immunity possible.

All vaccination is essentially the creation of an artifact; a purposeful manipulation of the immune system designed to achieve resistance against a microbe or cancer. DC-based vaccines, however, differ from conventional vaccines in that they utilize a living component of the immune system to actuate the immunization. These living components carry with them a pre-programmed life cycle and a range of constitutive and inducible capabilities that cannot be exploited fully if we do not recognize their existence or the signals that trigger their expression. So, to efficiently utilize DCs in vaccination schemes, we must first understand their natural history and function.

DCs are considered the most efficient cells for presenting antigen to T cells. In the traditional view of DC natural history (32), they arise from proliferating progenitors found in the bone marrow and enter the blood stream. The non-proliferating precursor cells then gain access to virtually all peripheral tissues, particularly those underlying anatomical barriers such as the skin and mucous membranes. At this point they exist as immature DCs. Here, they take on a sentinel function, awaiting signals that will trigger their maturation, usually those indicative of infection or inflammatory tissue damage. During this period of sessile immaturity, they are highly active in the uptake and processing of protein antigens found in their vicinity (33), even though they are yet relatively poor at antigen presentation. Upon receipt of maturation signals, the DCs undergo a specialized developmental and migration program, where they decrease their uptake of antigen (33) and upregulate their expression of certain chemokine receptors meant to guide their migration (34). They then retrace a basement membrane and enter draining lymphatic vessels for the journey to the regional lymph nodes. As they mature, the DCs also upregulate the expression of certain products necessary to supply T lymphocytes with the three general signals that will determine their activation status and general fate. First, MHC molecule expression rises to increase the density of MHC:peptide complexes (signal 1) (35). Second, costimulatory molecules such as CD80 and CD86 become upregulated (36). Finally, so-called ‘signal 3’ factors, such as interleukin-12 (IL-12), type-1 IFNs, or surface molecules such as OX40L may be expressed, depending on precisely how the DCs were activated (37, 38).

Although the preceding account of the DC life cycle represents the classical view, the body of evidence clearly shows that what are commonly referred to as ‘DCs’ actually represents an extremely diverse continuum of cells, each with distinct precursors, separate functions, and unique natural histories. The differential characteristics of DC subsets have been recently reviewed (39). Briefly, the first division among DCs is the distinction between plasmacytoid and so-called myeloid or ‘conventional’ DCs. The plasmacytoid DCs are identical to the natural type-1 IFN producing cells (40) that secrete these factors in response to contact with viral and (perhaps other microbial) infections. The conventional DCs can then be further subdivided first into the lymphoid organ-resident DC subsets and second into the migratory DCs. The lymphoid organ-resident DCs do not appear to transit through peripheral tissues and thus may not acquire peripheral antigens before taking up station in the lymphoid tissues. The migratory DCs, which include Langerhans cells, interstitial (or dermal) DCs, and the monocyte-derived DCs appear to more closely correspond to the classical notion of DCs in their behavior and function. Because our discussion centers on the use of DCs in immunotherapeutic strategies against cancer, when we speak of DCs we refer almost exclusively to the migratory type of conventional DC. Although culture systems have been reported for generating large numbers of Langerhans-type DCs (41), because of the easy availability of peripheral blood monocytes and their relatively convenient conversion into DCs, this other type has most often served as the substrate for vaccine DC production, despite some controversy regarding what contribution monocytes actually make to the DC pool in vivo. There have also been published reports comparing the capacities of various types of migratory DCs, suggesting that

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some properties are best exemplified by one population and others by a different one. Such head-to-head comparisons can be especially prone to experimental bias, but evidence is accumulating that the functional capacity of migratory DCs may depend at least as much on the precise activation and maturation signals they receive as the precise subpopulation from which they are derived.

**DC activation: exploiting the potential of Toll-like receptor signaling pathways**

Most DC culture regimens that have been commonly employed in clinical trials have activated DCs through the use of individual cytokines associated with inflammation (42) or inflammatory cytokine cocktails (43). These treatments result in DCs that appear to meet most common phenotypic, morphological, and functional criteria of maturity and activation. Indeed, it is not surprising that DCs should mature and activate in response to such agents as tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2), and IL-1β, since these factors are produced in abundance during infection. However, many of these same products are also produced in instances of aseptic tissue damage or trauma (44). It stands to reason that the type and intensity of immune system involvement in instances of aseptic tissue damage would be very different from that required in the case of infection. In fact, one could argue that in the former case, the immune response should be attenuated as expeditiously as possible to avoid further tissue damage from excessive inflammation or the induction of chronic autoimmunity. Only in the case of microbial invasion should the full force of the immune response and the attendant risk of autoimmunity be brought into play and even then only so long as it takes to clear the infection. The most commonly used methods for activating DCs might be suboptimal in eliciting the type of powerful immune responses desired to reject tumors, and they actually may favor the induction of negative regulation, since the DCs are not given signals indicative of actual infection. To circumvent this difficulty and to develop better methods of DC activation, we must identify and understand both the signals and the sensory systems by which DCs perceive threats posed by infection.

Traditionally, immunologists have divided the immune system into two components: innate and adaptive. The innate immune system is the more primitive of the two and includes phagocytic, NK, and professional APCs such as macrophages and DCs. The adaptive immune system includes cells with randomly generated and clonotypically distributed antigen receptors, such as T and B lymphocytes. The recognition and successful response to infection requires the productive cooperation between both the innate and adaptive immune systems. In the past decade, Charles Janeway et al. (45) advanced a prescient and influential synthesis that described the nature of innate immunity and its relationship to adaptive immunity. It was predicted that cellular components of the innate immune system required their own receptors to facilitate the discrimination of self-tissues from infectious non-self with the goal of eliminating infection with as little damage to self as possible. Such a task is equally necessary for primitive animals without adaptive immune systems and for vertebrates including mammals. Given the vast number of possible microbial threats, how can innate immune systems, possessing a finite number of available receptors, be capable of recognizing all potential pathogens?

It was proposed that the innate immune system should have evolved pattern recognition receptors that would probably not recognize proteins unique to specific pathogens (as do the antigenic receptors of T and B lymphocytes of the adaptive immune system). Instead, they would recognize generalized molecular patterns common to broad classes of potential pathogens. These patterns were termed pathogen-associated molecular patterns (PAMPs). The biomolecules representing these PAMPs would be so crucially linked to microbial physiology that they would be unlikely to vary or be eliminated by selective immune pressure. Finally, such structures would be virtually absent from host tissues so that a discriminatory capacity could be attained and preserved. Several candidate patterns were proposed at the time this hypothesis was enunciated, including lipopolysaccharide (LPS) and lipoteichoic acid (from the cell walls of Gram-negative and Gram-positive bacteria, respectively), Mannans (yeast), double-stranded RNA (some viruses), and unmethylated CpG DNA motifs (all bacteria).

The hypothesis of Janeway et al. was in many ways validated with the discovery of several types of pattern recognition receptors, including NOD (nucleotide-binding oligomerization domain) (46), retinoic acid inducible gene 1 (RIG1) (47) and PKR (double-stranded RNA – dependent protein kinase) (48). The archetypical and perhaps most functionally diverse family of pattern recognition receptors are, however, the Toll-like receptors (TLRs) (49). This family of receptors was discovered by a somewhat circuitous route that began with the study of invertebrate genetics and development. Investigators seeking to determine the role of two genes, named Toll and 18-wheeler, thought to be important in Drosophila developmental biology, created transgenic fruitflies with targeted disruption of these two genes. Surprisingly, 18-wheeler-
Toll-KO animals were found to be highly susceptible to fungal (50) and bacterial (51) infection, respectively. Investigators quickly realized that these genes were somehow connected to some fundamental mechanism of resistance in the innate immune system, and a search quickly ensued to discover homologs in mammals (52). Subsequent molecular cloning efforts revealed that mammals possessed at least 10–11 separate genes with strong homology to the original Drosophila Toll. These TLRs quickly became the focus of intense study, and individual receptors were connected fairly quickly with their respective ligands. Thus, TLR2 was found to be involved in the recognition of bacterial lipopeptide and lipoteichoic acid, TLR3 was activated by double-stranded RNA, TLR4 was the much searched-for LPS receptor, TLR5 was activated by bacterial flagellin, TLRs 7 and 8 recognized single-stranded RNA, and TLR9 responded to unmethylated CpG motifs of bacterial DNA (49).

There is great diversity in TLR signaling, however, providing numerous opportunities to fine-tune responses to given microbial threats. These intricacies of TLR signaling have been extensively reviewed in detail elsewhere; however, a brief discussion is helpful for understanding how TLR signaling might be exploited for DC-based vaccines. First, it should be kept in mind that TLRs have particular distribution patterns among various cell types, meaning that B cells, macrophages, plasmacytoid DCs, and conventional DCs do not respond to identical sets of TLR ligands, owing to these differential TLR distributions. For example, plasmacytoid DCs in humans express TLR7 and TLR9, while myeloid DCs express TLR8 but not the other two (53). Sometimes such distributions vary even between species and should be kept particularly in mind when comparing studies done in mice with humans (54). Second, various TLRs inhabit distinct subcellular compartments. For example, the TLRs primarily responsible for recognizing nucleic acids tend to be intracellular (although this is a trend, not an absolute finding), which probably facilitate recognition of intracellular pathogens. TLR4 and TLR2, in contrast, can be found at the cell surface. Third, TLRs exist as dimers, and some are capable of heterodimerization, which can affect ligand specificity and serves to leverage the number of recognized ligands without requiring additional receptors (55). For example, if TLR2 heterodimerizes with TLR1, triacylated peptides are recognized, but if TLR2 instead dimerizes with TLR6, recognition of diacylated peptides results.

Perhaps the distinctions between different TLRs that have the most profound functional consequences are the differences in signal transduction pathways activated by the different TLRs. These, in turn, are regulated by the distinct adapter proteins associated with each receptor. Adapter proteins are required for signal transduction through the TLRs and assemble on the cytoplasmic domain of the TLRs upon ligand binding. There are four principal known adapter proteins involved in positive signaling [a fifth, SARM (sterile-α and Armadillo motif-containing protein) appears to have a negative regulatory function] that share a common conserved structural feature known as the Toll-IL1 receptor (TIR) domain. These adapter proteins are MyD88 (myeloid differentiation factor 88), TRIF (TIR-domain-containing adapter-inducing IFN-β), MAL (MyD88-adaptor-like protein), and TRAM (translocating chain-associating membrane protein). Although new complexities are constantly being identified, the TLRs are commonly divided into those that are MyD88 dependent and those that are TRIF dependent. TLR5, TLR7, TLR8, and TLR9 require the MyD88 adapter protein. Heterodimers of TLR1/2 and TLR2/6 are also MyD88 dependent, but for signaling they also need MAL as a bridge protein to recruit MyD88. TLR3, in contrast, is TRIF dependent. TLR4 exhibits elements of pathway duality, with a MyD88/MAL-dependent branch and a TRIF/TRAM-dependent branch. The reason that the various adapter proteins are so crucial for signaling through individual TLRs is not merely that they transmit the activation signals. Rather, do the different adapters and adapter combinations activate distinct downstream cascades that differentially affect the expression of various genes? This idea means that PAMPs indicative of certain types of microbes have the potential to activate tightly defined gene expression programs, which may in turn be adaptations for most efficiently dealing with particular classes of pathogens. Some of these expression programs may also be highly beneficial for generating anti-cancer responses.

One potentially compelling observation is that when two TLR agonists are used to stimulate DCs in such a way as to activate both MyD88- and TRIF-dependent pathways simultaneously, a phenotype emerges that is not seen when either TLR agonist alone is used (56). This phenotype includes greatly enhanced expression of IL-12, a cytokine that appears intimately associated not only with infection control but also resistance to cancer.

**Uniquely balanced impacts of IL-12 for anti-tumor immunity**

IL-12 is a cytokine that was first detected in the supernatants of stimulated Epstein–Barr virus-infected human B cells and was initially characterized as a factor important for the stimulation of NK cells and the maturation of cytotoxic T lymphocytes.
(CTLs) (57). IL-12 was subsequently identified as a major factor for polarizing T-helper (Th) cells toward the Th1 phenotype (58), which was defined as a producer of high-level IFN-γ and relatively low amounts of IL-4 and IL-5 (59). This phenotype of T cell was found to be critical for controlling certain types of infection, particularly intracellular protozoan pathogens (60, 61). IL-12 is a heterodimeric cytokine (57) composed of a p40 and a p35 subunit. It is the archetypical member of a small family of cytokines (62) that includes IL-23 and IL-27. IL-12 and IL-23 share the common p40 chain, but IL-23 possesses the unique p19 subunit. Although IL-12 can be secreted either as the p70 heterodimer or as free or homodimeric p40 subunit, only the heterodimer seems to possess the biological activities ascribed to IL-12. Homodimers may serve an inhibitory function (63). The IL-12 subunits appear to be independently regulated and require the activity of multiple nuclear factors acting in concert, including IFN response factor (IRF) as well as nuclear factor (NF)–κB family members, which have been linked to TLR signaling. Although some details remain incompletely clarified, the complexity of nuclear factors required for IL-12 transcriptional control and the diversity of TLR signaling through differentially distributed adapter proteins and subsequent signaling pathways seem to largely account for the observed requirements of multiple TLR engagement for maximized IL-12 production.

IL-12 possesses a number of powerful anti-cancer activities, but not all of them are immunologically related, nor do they always act directly. For example, IL-12 plays a role as an antiangiogenic agent (64) that can strongly inhibit the formation of neovasculature. As stated above, one of the chief functions of IL-12 appears to be the polarization of T cells toward the IFN-γ-secreting Th1 type. IFN-γ in turn has been shown critical for tumor rejection in a number of models (18, 65). IL-12 also supports the activation of NK cells (66) that can have powerful effects against tumor cells, particularly those with attenuated MHC expression.

Nonetheless, some of the most important anti-cancer activities of IL-12 involve its direct effects on T cells. Some recent studies, for example, have demonstrated IL-12’s role as a central regulator of CD8+ T-cell function. In murine studies using the OT-1 mouse strain, which is transgenic for a T-cell receptor recognizing an immunodominant H-2Kb-restricted epitope from ovalbumin (OVA), the presence of IL-12 during naïve T-cell sensitization appeared to determine whether the resulting CTL would attain full activation versus a functionally deficient state resembling tolerance (67). In these studies, artificial APCs in the form of microbeads coated with MHC-peptide complexes (at defined peptide densities) illustrated that at high peptide concentrations, naïve CD8+ T-cell proliferation was independent of IL-12. At low concentrations, however, IL-12 was required to expand the antigen-specific T cells. In vivo, high antigen concentrations promoted early expansion of OT-1 T cells comparable to peptide plus IL-12, but by later time points, recovery of OT-1 cells was limiting unless exogenous IL-12 was supplied. This finding suggested that IL-12 enhances T-cell survival in vivo. Of further interest, IL-12 was required at both high and low peptide concentrations for the attainment of effector function, as defined by the capacity to lyse OVA-expressing target cells. These results showed that without IL-12, the sensitized T cells would be fewer and functionally incapacitated, despite their initial clonal expansion. This finding regarding function was also confirmed in vivo, with lytic CD8+ T cells arising only when mice were vaccinated with peptide plus IL-12 adjuvant. It has also been reported that in vivo administration of IL-12 leads to the development of a functional memory population (68).

Somewhat surprisingly, the capacity of CTL to secrete IFN-γ was not dependent upon the presence of IL-12, but it did approximately double the proportion of cells that became specific IFN-γ secretors (67).

Other studies from the same laboratory gathered evidence that the enhanced levels of proliferation may be linked to the capacity of IL-12 to upregulate and prolong the expression of CD25 (the IL-2 receptor α chain), which is an important growth factor receptor for CD8+ T cells (69). Enhanced survival promoted by IL-12 may be in part due to the upregulated expression of the transcription factor Bcl-3, which opposes cell death (70). From a functional standpoint, however, the most interesting finding was the role played by granule proteins (71). Again, using the transgenic mouse model, it was shown that OVA-specific CD8+ T cells sensitized in the presence or absence of IL-12 each exhibited comparable capacities to mobilize intracellular calcium and degranulate upon contact with appropriate specific targets. The levels of perforin, a protein critical for the transfer of cytotoxic granule contents from the T cell to target, also appeared at comparable levels. However, the granule-associated serine protease, granzyme B, a caspase activator and initiator of apoptosis, was strongly expressed in T cells sensitized in the presence of IL-12 but poorly expressed in the absence of the cytokine. The levels of granzyme expression appeared to correlate with cytotoxic activity. These experiments were capped with a fascinating study where OT-1 transgenic T cells were adoptively transferred into mice and then challenged with an OVA-expressing tumor cell. It was demonstrated that transferred OT-1 T cells proliferated (indicating that they were antigenically stimulated...
by the presence of tumor) and infiltrated the tumor. This approach, however, did not affect tumor growth. In contrast, when recombinant IL-12 was supplied, tumor growth kinetics were significantly decelerated, an activity that was dependent on IL-12 receptor expression on the transferred T cells (72). It is also interesting to note that in one of the clinical vaccine trials that reported the appearance of antigen-loss variants, vaccination was accomplished using class I-restricted melanoma antigen peptides with an IL-12 adjuvant (73).

In our own studies with human cells, we observed that healthy donor CTLs could be sensitized in vitro against HLA-A2.1-restricted synthetic peptide epitopes based on the sequence of tumor-associated antigens such as MART-1, gp100, and HER-2/neu. In vitro sensitizations were performed using monocyte-derived DCs that were specially cultured and activated by various techniques to become DCs that were comparably mature as judged by common surface immunophenotype, but differed in their capacity to secrete high levels of different soluble factors, including IL-12 (74, 75). We found that both IL-12-secreting and non-secreting DCs could comparably sensitize T cells for recognition of high concentrations of peptide antigen as presented by the HLA-A2.1-expressing ‘professional target cell’ line T2. However, direct recognition of cultured, HLA-A2.1-expressing ‘professional target cell’ line T2. However, direct recognition of cultured, HLA-A2.1 melanoma and breast cancer lines could only be achieved if the sensitizing DCs secreted IL-12 or if recombinant IL-12 was added to sensitizing co-cultures where the DCs themselves did not produce the cytokine. It was further discovered that when T2 cells were pulsed with successively lower concentrations of peptide antigen, T cells sensitized in the presence of IL-12 were 10–100-fold more sensitive that those sensitized without IL-12. This increased antigen sensitivity or high functional avidity correlated with the enhanced expression of CD8β relative to CD8α. Other investigators have demonstrated that CD8 heterodimers composed of the CD8α and CD8β chains are capable of forming a more efficient T-cell receptor signaling apparatus that CD8α homodimers (CD8β heterodimers are not known to form), contributing to the enhanced functional avidity (76). Considering these powerful anti-tumor properties possessed by IL-12, tumor vaccination strategies might consistently benefit from incorporation or endogenous induction of this cytokine.

**CD4⁺ T cells: take all the help you can get**

Cancer immunotherapy has witnessed an historical bias toward MHC class I-restricted CD8⁺ CTLs rather than class II-restricted CD4⁺ Th cells. There are probably several reasons for this bias. First, it is conceptually more satisfying to envision CTLs, best known for mediating the direct killing of virally infected cells, to be attacking and killing tumor cells. Second, solid tumors rarely express HLA class II molecules but often express class I, making tumor cells obvious targets for direct attack by CTLs. Finally, early studies in mice appeared to show a greater dependency on CTLs for tumor rejection, and the relative ease in which CD8⁺ T cells could be grown from human tumors cemented the reputation of CTLs as the primary effectors for anti-tumor immunity. However, studies have steadily accumulated indicating that Th cells are not only important for anti-tumor activity but also can sometimes mediate tumor rejection without any apparent participation by CD8⁺ CTLs (77–80).

It is now appreciated that Th cells can even possess the capacity to directly kill tumor cells, at least in some special circumstances. Induction of apoptosis has been shown to be a means by which Th cells kill lymphoma and other types of malignant cells. There are at least two mechanisms. One mechanism has been shown to be operative against Burkitt’s lymphomas. Here, Th cells specific for the Epstein–Barr virus antigen EBNA-1 are capable of killing lymphomas through a cell contact-dependent process relying on Fas/Fas ligand interactions (81). A second apoptosis-inducing pathway utilized by Th cells against melanoma has been described that involves the TNF-related apoptosis-inducing ligand (TRAIL) (82).

Th cells also appear to support the activation of CTLs. There are at least three mechanisms by which it may be accomplished. In the classic mechanism, Th cells participate in the autocrine/paracrine secretion of the T-cell growth factor IL-2, which in most circumstances CD8⁺ CTLs require but cannot produce themselves. Such help could in theory be supplied without direct cell–cell contact. The second mechanism relies on a three-way contact-dependent interaction between a Th cell, an APC, and a CTL (83, 84). Here, an APC presenting cognate antigen to a Th cell activates the Th cell and induces upregulation of CD40L. This in turn signals the APC through CD40. This interaction somehow primes or ‘licenses’ the APC to appropriately activate the CD8⁺ CTL, although the exact mechanism by which this occurs is not completely clear. The Th cell can substitute, however, for agonal CD40L antibody. Another interesting study, however, has suggested that CD8⁺ T cells could themselves express CD40 and might be stimulated directly by Th through their surface CD40L (85). Finally, a soluble factor of the TNF family has been identified called TRANCE (TNF-related activation-induced cytokine), that appears to be critical for a CD40 ligand-independent mechanism of T-cell help (86).
Apart from CTL priming, Th cells promote the long-term survival of CTLs and their memory function in models dealing with cancer, infectious diseases, and allograft rejection (87–89).

Among Th cells, the polarized Th1 type can contribute to tumor immunity in additional ways via their maximized production of IFN-γ (which is favored in the presence of IL-12). IFN-γ can have a variety of effects on tumors that are both direct and indirect. For example, IFN-γ has been shown to inhibit the proliferation of malignant cells via a signal transducer and activator of transcription-1 (STAT-1)-dependent transcriptional activation of genes that inhibit cell cycle progression and work through cyclin-dependent kinases (90, 91). Also through STAT-1, IFN-γ can upregulate the expression of a variety of genes that promote apoptosis, including caspase-1, Fas, and Fas ligand (92–94). IFN-γ also has been shown to possess indirect anti-angiogenic properties through the induction of IFN-inducible protein-10 (IP-10) (95–97). Finally, IFN-γ can induce the upregulation of MHC molecules and the machinery of antigen processing and presentation, making tumor cells more vulnerable to immune-mediated attack (98).

Th cells can therefore contribute to anti-tumor activity through direct tumor killing of tumors, by supporting both the activation and long-term maintenance of CD8+ T cells, and through the production of cytokines. But let us not neglect the initial function that earned Th the moniker of ‘helper’, that is their capacity to support the production and class-switching of antibodies by B cells.

The case for antibodies

Once thought to have only a limited potential for controlling tumors (with the possible exception of some lymphomas), appreciation of antibodies’ anti-tumor potential now appears to be undergoing a reevaluation, with several immunoglobulin-based drugs having proved useful when supplied as passive immunotherapy against solid tumors. Chief among these are humanized antibodies targeting members of the epidermal growth factor receptor (EGFR) family, including cetuximab (anti-EGFR) and trastuzumab (anti-HER-2/neu). There are several mechanisms by which these antibodies may act, which are not mutually exclusive. Evidence has been presented that these antibodies, when bound to their target, can interfere with or alter transmembrane signaling, can induce antibody-dependent cellular cytotoxicity, or can participate in complement-mediated cytotoxicity (99, 100). These drugs, by themselves or in conjunction with other therapies, have significantly impacted the treatment of several tumors. The question arises, however, as to whether it is possible to generate, in vivo through a vaccination procedure, an antibody response that would be at least as effective if not more effective than the antibody-based drug. For example, it has been demonstrated that pairing trastuzumab with two other monoclonal antibodies that recognize different epitopes on the HER-2/neu extracellular domain appeared to have more potent antitumor activity than trastuzumab alone (100). Because a vaccine-induced antibody response would almost certainly be highly polyclonal, and would have the advantage of being self-renewing either upon continued presence of antigen or artificial boosting, there may be considerable advantages to such a strategy.

An approach very similar to the one described above has been attempted with considerable success in an animal model. In a fascinating series of studies (65, 101, 102), Forni et al. examined a mouse strain transgenic for rat HER-2/neu overexpression that spontaneously develops adenocarcinomas of the breast. They observed that vaccination with HER-2/neu in conjunction with IL-12 adjuvant could prevent mice from developing breast tumors. An extensive and detailed investigation showed that CTLs were not necessary for tumor control in this model. Th cells clearly played an important role but were required only at early time points, coinciding with vaccination, but not later during tumor protection. This observation suggested an early helper role rather than a later effector role for the Th cells. However, antibodies proved both necessary and sufficient to provide protection. It also appeared that antibody effectiveness was subtype dependent, with IgG2a being most effective and its production to some degree dependent upon IFN-γ. Presumably, a strong Th1 steer provided by IL-12 enhances IFN-γ secretion, which in turn promotes class switching to the IgG2a subtype.

Although identified targets for effective antibody-mediated immunity against tumors are far less numerous than those identified for T cells, the available data are highly encouraging and support the strategy that anti-cancer vaccines should also be designed with antibody production in mind. Indeed, the discovery of additional tumor-associated antigens that can serve as antibody targets would be a welcome development.

The opportunity of early disease settings

Most cancer immunotherapy trials have focused on locally advanced or metastatic disease, for several reasons. Experimental therapies by their very nature are usually called upon only after current standards of care have been attempted and failed. For cancer, the disease is usually fairly advanced before patients...
opt for experimental therapies. There are several arguments one could make, however, as to why this patient population might be exactly the wrong one on which to test novel immunotherapies. First, it is asking much of the immune system, even under the best of circumstances, to eliminate large quantities of malignant tissues such as are found with advanced, bulky disease. Second, patients with advancing cancer typically display certain immune dysfunctions induced by the tumors themselves (103). In addition, it is possible that some harsh chemotherapies can induce lingering pathologies to the immune system that may hamper responsiveness to therapy (104). Finally, patients with advanced cancer may be in generally declining health and thus may mount poor responses.

Many experimental vaccine therapies may therefore be most meaningfully evaluated in earlier disease settings. This way, vaccine strategies that are shown to be effective in patients without these other potentially confounding issues can then be confidently tested and adapted to those with more advanced disease. It is possible, however, that the best vaccines we can possibly make will be effective only in early disease settings. Such a finding should by no means be considered disastrous for the future of cancer vaccines, because continuing technological advances will allow cancer to be detected (or even predicted) at earlier and earlier stages. Therefore, in the future, it can be expected that there will be a growing opportunity and need to treat less advanced stages of disease.

Putting it all together: constructing a DC-based vaccine

Many of the disappointing results historically achieved by DC-based anti-cancer vaccines stem from a strategic rather than a conceptual deficiency. There is nothing wrong with employing DCs as the basis of cancer vaccines; indeed, DCs are perhaps the ideal platform for this task. The trouble instead lies in the manner in which they have been employed. We adhere to a working principle that DC vaccination strategies should be designed around the natural behaviors and functions of DCs, and investigators should avoid, where possible, trying to engineer or otherwise force the DCs or other immune system components to perform tasks they were not innately programmed to undertake. It is therefore our goal to consider everything that is currently known about DC biology as well as cancer immunology, many components of which had been previously tested clinically (albeit individually or in incomplete combinations), and formulate a fully integrated strategy that should stand the greatest chance of successfully demonstrating the potential of DC-based vaccines. In the remainder of this review, we put into practice, in a sense, the components detailed in the first half, formulating an integrated vaccine strategy (Fig. 1) and describing a recent clinical trial that exemplifies many of these concepts.

Choosing a culture and activation regimen

The first studies demonstrating that human DCs could be derived from peripheral blood monocytes described a culture system that required cultivation of monocytic DC precursors for over a week with the cytokines granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (8). After this initial culture, the immature DCs could be activated and matured with additional cytokines (11, 43). These techniques were rightly regarded as unprecedented advances in our ability to study and use DCs clinically. However, it was difficult to envision an anatomical location or biological situation where monocytes would be exposed in vivo to these cytokines for a week or more. In fact, if monocytes are to play any role in mobilizing immune responses against infection, they must be capable of transforming from monocytes into fully activated DCs fairly quickly, probably on the order of no more than a day or two. We therefore sought to independently develop culture and activation systems that would more closely mimic the natural pace of DC activation.

We first established a rapid system whereby freshly isolated CD14+ peripheral blood monocytes could be made to adopt the phenotype and function of fully mature DCs in 24–48 h using pharmacological agents that mobilized intracellular calcium (105). These DCs expressed all of the surface markers associated with mature DCs and also possessed DC morphology and the capacity to sensitize healthy donor CTLs to synthetic tumor antigens. Whereas calcium mobilization alone probably did not represent a truly physiological activation pathway for DCs, these studies served as a proof of principal that DCs could be derived rapidly from monocytes. It also established a convenient in vitro system whereby DCs could be used to sensitize healthy donor CTLs to tumor antigens in a single round of in vitro sensitization. This advance was important, since it allowed us to develop and test new and different DC activation regimens and compare them for their capacity in sensitized CTLs to foster various desirable properties including favorable cytokine secretion profiles and the capacity to recognize and kill tumor cells.

Follow-up studies, however, revealed that similar rapid conversion of monocytes into DCs could also be achieved by more physiological stimuli, including bacterial LPS, cytokines, and viral infection (106, 107), particularly if human serum was omitted from culture medium (106). Rapid DC
conversion in serum-free media presented several advantages. First, by adhering to physiologically meaningful activation timeframes, the DCs generated were likely to possess better functional capacities. Second, from a practical standpoint, it is safer (less chance of introducing contamination), less expensive (no need to continually replenish cytokines), and more convenient to generate potential vaccine DCs over the course of 2 days rather than a week or more of culture. Finally, by eliminating the need for serum, an undesirable variable is removed, and the medium becomes more defined and consistent (a plus for both basic and clinical research). Alternate rapid activation strategies have also been demonstrated by other laboratories (108).

We also recognized the potential importance of IL-12 secretion by DCs and adapted methods for inducing this cytokine (109) to a 1 to 2 day serum-free culture (74). We later showed that DCs capable of secreting large amounts of IL-12, generated by overnight culture in GM-CSF and IL-4, followed by maturation in IFN-γ, TNF-α, and CD40L possessed capabilities that exceeded even the current ‘gold standard’ method of generating DCs, which included culture in GM-CSF and IL-4 followed by a maturation cocktail of TNF-α, IL-6, IL-1B, and PGE2 (75). For example, CTLs from healthy donors, when sensitized by any DC in the absence of IL-12, could recognize T2 target cells pulsed with high concentrations of the sensitizing peptide. They could not, however, recognize

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**Fig. 1. A summary of an integrated approach for cancer vaccination with DCs.** (A) Monocytes are converted into activated DCs via a rapid culture regimen that employs overnight culture in serum-free medium with GM-CSF and IL-4. The next day, cells are activated with IFN-γ and a TLR agonist (bacterial LPS), pulsed with HER-2/neu class I and II-restricted peptides, and harvested just prior to IL-12 secretory burst. (B) Antigen-loaded DCs are administered intranodally to assure timely arrival in force at the lymph node. (C) DCs present antigen to both class II-restricted Th cells and class I-restricted CTLs. The influence of IL-12 polarizes Th cells toward the type 1 phenotype and enhances survival and functional avidity of CTLs. B cells are recruited and class switch through T-cell help. (D) Lymphocytes traffic to distant tumor deposits and attack HER-2/neu-expressing tumor cells through HLA-restricted and HLA non-restricted mechanisms. (E) Targeted immunoediting of tumor occurs that either eliminates tumor outright or destroys those cells exhibiting the target tumor phenotype associated with greatest aggressiveness, leaving behind only tumor cells with more clinically manageable characteristics.
melanoma or breast tumor lines endogenously expressing the corresponding tumor antigen. In contrast, DCs producing their own IL-12 or non-secretors supplemented with recombinant IL-12 could sensitize CTLs that could react both to peptide-pulsed T2 as well as naturally antigen-expressing melanoma and breast cancer lines. Specific recognition of melanoma or breast cancer lines was determined by the secretion of IFN-γ or the cytolysis of the target cell. This capacity was found to stem from an IL-12-dependent enhancement of functional avidity (i.e. antigen sensitivity) that allowed CTLs sensitized in the presence of this cytokine to recognize peptide at 10–100-fold lower concentrations than CTLs sensitized in its absence (75).

Our own studies as well as the work done by others regarding the adjuvant properties of IL-12 underscored the potential critical importance of utilizing DCs that secreted large amounts of this cytokine. Logically, if the very effectors of immunity were unable to recognize and kill tumor cells unless exposed to IL-12 during sensitization, how could one envision a successful DC-based vaccine that did not incorporate cells primed to secrete generous amounts of this cytokine in a timeframe coincident with antigen presentation?

We also believed that it was crucial to activate DCs with TLR agonists, given the central role TLRs play in activating innate immunity. We therefore chose a combination of agents we had previously shown to induce high levels of IL-12 in the rapid immune response (75). These agents were INF-γ and bacterial LPS (107). Although LPS is widely regarded as anathema for human use, in fact the National Institutes of Health maintains a reference standard endotoxin that has been Food and Drug Administration-approved for parenteral administration in humans (110). We believed that by pulsing DCs with tumor antigens and activating them with a combination of agents that induced IL-12, including a TLR agonist, we could essentially disguise self-proteins as infectious non-self and set in motion an immune response of an intensity usually reserved only for dangerous infections. We therefore settled on this rapid DC production regimen consisting of overnight culture of peripheral blood monocytes in serum-free medium with IL-4 and GM-CSF followed by activation with IFN-γ and LPS. There was one important unresolved issue, however, and that was selecting the right moment to harvest DCs for use in vaccination.

**IL-12 secretion: timing is everything**

Previous studies have shown that mature DCs are superior to immature DCs for generating immune responses (111, 112). Therefore, most clinical trials now employ fully mature DCs.

However, given the demonstrated importance of IL-12 for generating anti-tumor immunity, the timing of IL-12 secretion must be taken into account when determining the optimal time point for harvesting in vitro-activated DCs and administering them to patients. To complicate these issues, IL-12 is generally secreted in a fairly narrow window of time that not only peaks but virtually ends prior to attainment of full DC maturation. In our hands, DCs activated with IFN-γ and LPS begin secretion of IL-12 around 6 h after activation with IFN-γ and LPS (Fig. 2). Production maximizes at around 8–10 h and falls precipitously afterward, with secretion virtually completed 15–24 h later. If DCs are not harvested and administered until they display maximized phenotypic maturity, their IL-12 secretory burst will have been wasted in tissue culture, and no benefit from IL-12 can necessarily be expected as a result of vaccination. If IL-12 secretion is to be exploited optimally, it stands to reason that DCs must be harvested after full maturation/activation signals have been administered in vitro (since mature DCs are more effective immunogens than immature DCs), but before full maturity is reached (an optimal harvest time may be around 6 h post-activation). There are no reported observations to suggest that DCs, having received all necessary activation signals in vitro, would not continue on to full maturation after they are administered. It should be noted that we have demonstrated that DCs activated with IFN-γ and LPS are capable of generating a second burst of IL-12 secretion in vitro many hours after the initial one, provided they are re-stimulated with recombinant soluble CD40 ligand trimer (113). We hypothesize that such DCs could secrete a second IL-12 burst in vivo should they encounter CD40L-expressing Th cells. However, this idea has not been formally demonstrated, and it is probably the better way to go.
strategy to design vaccine strategies and procedures that mainly exploit the initial burst of IL-12. The take-home lesson is that if the desirable effects of IL-12 are to maximized, vaccine production methods must be built around the DCs pre-programmed cytokine secretion schedule. This timing must be empirically determined for any given activation regimen.

Vaccine administration: location, location, location
It is part of the developmental program of migratory DCs to home, after activation, to the lymph nodes, which are the cradles of T-dependent immune responses. Therefore, DCs must somehow relocate to the lymph nodes if they are to activate adaptive immunity and serve as effective cancer vaccines. Studies have demonstrated, however, that only a variable proportion of cultured DCs can find their way to regional lymph nodes after administration to a peripheral anatomic site (114). Such findings have spurred studies to determine requirements for DC migration and homing, so that appropriate signals can be administered to DCs or to DC injection sites to facilitate their successful migratory behavior. Although the study of the biology of DC migration is a worthy endeavor, from the practical standpoint of vaccine strategy, we have observed that this problem can be side-stepped altogether by using ultrasound guidance to facilitate the direct injection of DCs into lymph nodes distal from sites of malignant disease (115). We believe this simple solution offers at least two advantages. First, it allows administration of a defined quantity of DCs directly to the site of T-cell sensitization. The second reason relates again to the kinetics of IL-12 secretion. Since DCs activated in vitro to make IL-12 will probably reach peak secretion capacity shortly after vaccine administration, it would defeat the purpose of priming for IL-12 secretion if the DCs are forced to dwell initially at peripheral locations, trying to locate afferent lymphatic channels and thus wasting the cytokine where it will do little good. By administering the DCs intranodally, peak IL-12 secretion will be synchronized with their proximity to T cells, where IL-12 can exert its full effects during antigen presentation. We therefore suggest that the intranodal route of administration, particularly when vaccinating with DCs primed to secrete IL-12, is a practical and safe way to reliably co-localize IL-12 production with the anatomical site of T-cell sensitization.

Selection of tumor antigen: the targeted immunoediting approach
Once a method to culture and activate DCs is chosen, a strategy must be devised to select a tumor antigen that will maximize the effectiveness of the vaccine. We have already detailed how the immune system naturally sculpts the phenotype of emerging tumors through a process known as immunoediting (21). During immunoediting, the immune system attacks tumors and eliminates those with phenotypes that mark them as most visible and vulnerable to destruction. Then, a period of equilibrium ensues, as surviving cells adapt to immune system attack. During the escape phase, tumor cells that have acquired properties that allow them to evade immune recognition and destruction grow, spread, and kill the host. In some cases, cancer immunotherapy can lead to an induced form of immunoediting, whereby tumor cells that express specific therapeutic targets are selectively eliminated, leaving antigen-loss variants to continue to grow and spread. These antigen loss variants may selectively eliminate the expression of individual immunotherapeutic targets, or they may suppress antigen presentation altogether.

Earlier in our discussion we defined this situation as a central problem for immunotherapy. We did so because completing the daunting task of developing vaccines capable of destroying tumors that express particular antigens will be of no avail if tumors can simply escape destruction by ceasing antigen production or disabling their capacity to process and present antigen to T cells. We propose that this problem can be most effectively solved by a two-part strategy. The first is to select a target antigen that is either vital to the survival of the tumor or, at the very least, contributes strongly to the aggressiveness of the disease. Thus, if the tumor continues to produce the target antigen, it will be vulnerable to immune attack. If it desists in the production of the target antigen, it will either die or be forced to adopt a phenotype that presents a better prognosis and is more clinically manageable. The second part of this strategy is designed to overcome the disabling of antigen processing and presentation, which is accomplished through the selection of a target antigen that is highly vulnerable to a non-MHC-dependent modes of immune attack, such as those provided by antibody.

This suggested approach therefore rationally selects tumor vaccine candidate antigens based on their capacity to contribute directly to tumor survival or capacity to produce disease. Since selective elimination of these phenotypic qualities will lead either to overall tumor death or an induced immune-mediated sculpting of tumor phenotype that forces the emergence of variants with degraded aggressiveness and capacity to cause disease; we call this general strategy ‘targeted immunoediting’.

An excellent choice for testing this targeted immunoediting approach would be to design a vaccine based on the HER-2/
neu antigen. There is considerable evidence to support such a choice based on the requirements for targeted immunoediting. HER-2/neu (c-erb-B2) is a member of the human EGFR family and functions as a transmembrane tyrosine kinase. Although HER-2 does not signal by itself and has no unique known ligand, it forms functional signaling complexes through heterodimerization with the three other known EGFRs (HER-1, 3, and 4). The downstream targets activated by HER-2 are unusually diverse, owing to variations in signaling made possibly by the multiple heterodimers formed and variations in possible ligands. HER-2 can be overexpressed by a number of different tumor types, including but not limited to breast, ovarian (116), pancreatic (117), lung (118), and squamous cell carcinomas of the head and neck (119). However, not all tumors of these types express HER-2, but substantial percentages of each do. For example, about 20–30% of breast cancers overexpress HER-2. HER-2, and indeed, organ-specific overexpression of HER-2/neu by itself has been shown to initiate carcinogenesis in transgenic mice (120). HER-2 overexpression is also considered to be a poor prognostic indicator in established disease, because signaling through HER-2 can lead to the acquisition of a number of characteristics that increase tumor aggressiveness and survival. For example, apart from the obvious conferral of growth factor signaling, HER-2 expression appears to be associated with increases in the activity of matrix metalloproteinase-2 (MMP-2) and MMP-9 (121, 122). These enzymes are zinc-dependent proteinases capable of degrading elements of the extracellular matrix and basement membrane barriers including collagen, fibronectin, and laminin. With these barriers degraded, tumor cells can presumably migrate more easily. HER-2 overexpression is also associated with the enhanced cytoplasmic expression of CXCR4 (123, 124). CXCR4 is known to play an important role in development, particularly organogenesis, when cellular migration is an integral part of this process. In breast cancer, elevated CXCR4 expression is believed to be involved in the directional migration of breast cancer cells toward sites of metastasis and has been correlated with lymph node-positive disease (125). Also, a compelling study recently suggested a connection between HER-2 expression and the biosynthesis of estrogen. Here, HER-2 over-expression led to increases in the production of cyclooxygenase-2 (COX-2), an enzyme critical for the production of PGE2 as well as increases in the activity of the enzyme aromatase. Aromatase is responsible for the conversion of androgens into estrogen, which is an important growth factor for a majority of breast cancers. HER-2 overexpression therefore enhanced the availability of additional breast cancer growth factors (126).

Finally, evidence exists that HER-2 overexpression can be at least partly responsible for tumor resistance to front-line chemotherapies. This includes anti-hormone therapies such as tamoxifen (127), which may be accomplished through cross-talk involving estrogen and HER-2 signaling pathways. Another study indicates that HER-2 can enhance the expression of the protein p21Cip1, which inhibits taxol-induced apoptosis and protects HER-2 overexpressing tumor cells from the drug’s effects (128). In addition to these features of HER-2/neu, the now widespread and successful use of antibody-based drugs and the evidence from transgenic mice discussed previously clearly indicate that a non-MHC-based (i.e. antibody) attack can have positive therapeutic effects on HER-2/neu-expressing tumors. HER-2/neu overexpression therefore confers upon tumors the two necessary properties that we seek: a strong contribution to tumor aggressiveness and survival and a proven capacity to be effected by HLA-independent effectors of immunity.

Another advantage to selecting HER-2/neu as a cancer vaccine target antigen stems from the remarkably large body of information available regarding its immunology, particularly the identification of T-cell epitopes that can be represented in the form of synthetic peptides (129–131). Most peptide-based cancer vaccines focus on achieving CTL-based immunity against those peptides capable of binding to the HLA-A2 allele. The A2 allele is one of the most commonly expressed class I antigens, and it facilitates both the enrollment of subjects into trials to test vaccines and also maximizes the proportion of patients capable of benefiting should vaccine strategies prove successful. Perhaps more importantly, a number of HLA class II epitopes have also been identified on HER-2/neu, allowing for the simultaneous sensitization of Th cells, should these peptides also be included in a vaccine. This identification has been accomplished thanks largely to the efforts of the Disis laboratory and has required the detailed examination of the HER-2/neu sequence by algorithms predictive of potential class II epitopes followed by their subsequent painstaking testing in human vaccination trials (131). This work paid off handsomely in the discovery of a number of epitopes with somewhat promiscuous HLA-binding capacities that are collectively capable of stimulating Th immunity across a wide range of MHC types.

**Selection of disease model: ductal carcinoma in situ**

Widespread use of screening mammography has made ductal carcinoma in situ (DCIS) the most frequently diagnosed malignancy of the breast (reviewed in 132). DCIS displays...
considerable heterogeneity and may exist in a low-grade form that develops into invasive cancer slowly or not at all. High grade DCIS that displays comedonecrosis almost always progresses to invasive cancer in 5–7 years. Conventional treatments include lumpectomy, lumpectomy plus radiation, or mastectomy, and the overall prognosis for this disease tends to be excellent. However, a considerable proportion of patients (about 30%) require the most aggressive treatment (mastectomy), because the disease is extensive or because of concerns for recurrence. The question of recurrence is particularly relevant for the younger patient. Additional neoadjuvant therapies would therefore be welcome to reduce the risk of recurrence or perhaps to decrease tumor size before surgery, so that less aggressive options would be available.

DCIS presents a fruitful disease setting to test novel vaccine therapy for several reasons. First, because of the relatively long latency period between DCIS and invasive disease, there is a considerable window of time in which to test novel vaccine therapies. Second, DCIS is one of the cancers that can overexpress the HER-2/neu protein (133), which we have identified as a nearly ideal vaccine candidate model. Most of the data linking HER-2 overexpression and poor prognosis comes from studies with breast cancer. Finally, since the DCIS lesion will be removed surgically as a matter of routine, we can observe any histopathological effects vaccination has on the tumor.

The trial design

Patients presenting with HER-2-overexpressing DCIS (>2+ intensity) were recruited for this trial. Subjects were screened by magnetic resonance imaging to exclude individuals with any detectable invasive disease. After obtaining informed consent, subjects underwent a pre-vaccination leukapheresis with subsequent countercurrent centrifugal elutriation of the blood product to obtain monocyte-rich and lymphocyte-rich fractions. The monocyte-rich fractions were used as source material for DCs and the lymphocytes were cryopreserved to serve as a pre-vaccination immune baseline. Monocytes were cultured overnight in a serum-free culture medium supplemented with GM-CSF and IL-4. The next day, cells were pulsed with six 14-mer peptides shown previously to stimulate Th cells (131). After 8–12 h, IFN-γ was added, and cultures continued overnight. Six hours before harvest, LPS was added. Cells were further incubated with two HLA-A2-binding peptides for the final 2 h of culture. Cells were then harvested, washed, and, after screening and clearance for microbial and endotoxin contamination, administered directly into inguinal lymph nodes of subjects. Three additional vaccinations were likewise given at weekly intervals. Then, a post-vaccination leukapheresis and tandem elutriation was performed to collect large numbers of post-vaccination leukocytes. Finally, residual tumor was then removed by definitive surgical resection. The tumor specimen was then evaluated by immunohistochemistry for changes in HER-2/neu expression, cellular infiltrates, and total tumor size.

This study design therefore integrated several elements that had not been combined before in a single trial. First, it utilized DCs activated via a rapid serum-free culture system with a TLR ligand to simulate microbial infection. Second, the DCs were specially activated to secrete IL-12. Third, all aspects of vaccine production were carefully choreographed so that IL-12 secretion would coincide with the timing of vaccine administration. Fourth, the vaccine was administered intranodally to circumvent the need for trafficking and to deliver a defined number of IL-12-secreting DCs directly to the site of T-cell activation. Fifth, the vaccine utilized peptides that could stimulate both CD8+ CTLs and CD4+ Th cells. Sixth, a vaccine target antigen was selected based on its association with tumor aggressiveness and survival to exploit the concept of targeted immunoediting. Seventh, although this vaccine was not specifically optimized to induce antibodies, the induction of Th cells was likely to facilitate antibody production, shown to be important for HER-2/neu immunity. Finally, we chose to vaccinate in an early disease setting to avoid the problems associated with advanced disease such as overall declining health, extensive bulky disease, tumor-induced immunosuppression, and exposure to prior treatment regimens such as chemo- and radiation-therapy that might inhibit immune responsiveness.

Results from the first 13 subjects enrolled in this trial were highly encouraging (113). We observed peptide-specific sensitization rates of 85% for CD4+ Th cells and 80% for CD8+ CTLs (among the HLA-A2+ subjects). As expected, these T cells secreted relatively high levels of IFN-γ, while IL-4 and IL-5 were not detected. This finding suggests that the T cells were strongly type-1 polarized, as would be predicted when sensitization was performed with IL-12-secreting DCs (75). Historically, vaccination with tumor peptides has yielded CTLs that can recognize peptide-pulsed targets but usually fail to recognize tumor cell lines that naturally express the same antigen (134). Because we had shown previously that IL-12-secreting DCs could sensitize, in vitro, high functional avidity CTLs capable of recognizing breast and melanoma cell lines (75), we expected that the sensitized CTLs from trial subjects would likewise be capable of specifically recognizing HLA-A2+...
HER-2/neu breast cancer lines. This in fact turned out to be the case, with the capacity to directly recognize tumor cell lines observed about 80% of the time, almost the exact proportion predicted by our in vitro sensitizations with healthy donors (75). Although we have not yet formally determined the peptide sensitivities of these vaccinated subject-derived CTLs, their capacity to recognize tumor cell lines (that express relatively low densities of specific peptide:MHC complexes compared to peptide-pulsed targets) suggest the likelihood that we have induced high functional avidity CTLs through our DC vaccination regimen.

Perhaps most importantly, we observed apparent alterations in the tumor itself. First, immunohistochemical staining and subsequent comparison of pre-vaccine biopsies with post-vaccine surgical tumor specimens showed robust T-cell infiltrates in the region of the tumor. The bulk of the cells appeared to be CD4+ T cells, with relatively less CD8+ lymphocytes. Most of the cells congregated in tightly-packed 'collars' surrounding ducts that housed residual tumor cells, although T cells could also be observed directly infiltrating tumor masses. Interestingly, we also observed substantial B-cell infiltrates into the peritumoral regions. These B cells were usually the only class II-expressing cells detectable, raising the possibility that that they may be involved in antigen presentation to the CD4+ T cells. The second possibility (not a mutually exclusive one) is that the B cells were congregating to tumor sites to increase the effective concentration of secreted anti-tumor antibody. Indeed, we found some evidence for antibody directed against tumor. Immunohistochemical staining for human IgG appeared to show enhanced staining of tumor cells after vaccination. Furthermore, post-vaccine antisera appeared to have a greatly enhanced capacity to lyse HER-2-expressing tumor cell lines in a complement-dependent lytic assay (113).

Another important change observed in the tumor after vaccination was the apparent alteration in levels of HER-2/neu expression. In about half of the vaccinated subjects, HER-2/neu expression dropped precipitously, with some individuals (originally 2+ intensity) becoming virtually undetectable for HER-2/neu expression (Fig. 3). No such marked decreases were observed in unvaccinated controls. In contrast, levels of the estrogen receptor remained unchanged, demonstrating the specificity of this alteration. There are several explanations. First, the subset of tumor cells that were highest HER-2/neu expressers was destroyed outright, by either cellular immunity, humoral immunity, or both. Second, the tumor cells may still produce HER-2/neu at original levels, but endogenously produced antibody either competes for detection antibody binding or induces internalization and degradation of receptor. This trivial explanation offered by this latter possibility cannot account for all of the change, however, since fluorescence in situ hybridization (FISH) assays performed on three subjects demonstrating HER-2/neu loss showed that evidence for gene amplification also disappeared in two of them. These data seem to indicate that this vaccine approach did in fact result in the targeted immunoediting of...
HER-2/neu expression in the tumors of a large proportion of vaccinated subjects.

We found evidence that actual tumor size may have decreased for some subjects as an apparent consequence of vaccination. We determined this by first estimating total tumor volume at the time of diagnosis using multidimensional mammography. DCIS tumors themselves are often difficult to visualize this way. However, high grade DCIS deposits microcalcifications during the process of comedonecrosis, which do show up on mammography. Areas subtended by microcalcifications have been shown to be highly accurate predictors of actual tumor size (135, 136). We therefore compared for each evaluable subject the estimated tumor size prevaccination with the actual size of the tumor excised after completion of the 4-week course of vaccination and looked for any apparent decrease in volume. We found that in about half of the subjects, apparent reductions of greater than 50% were observed. In one patient, of a 19 × 50 × 44 mm estimated DCIS mass at the time of diagnosis, only a 1 mm focus of malignancy could be identified in the postvaccination surgical specimen (113). Taken together, these results suggest that this vaccination regimen has induced a robust immunity that is detectable not only by in vitro monitoring but also by dramatic changes in the tumor itself.

Concluding remarks

Because DCs are the best known APCs and since they serve as a major bridge between innate and adaptive immunity, great hope has been invested in their capacity to serve as tools for generating effective anti-tumor immunity. However, the generally disappointing results achieved thus far in most DC-based immunotherapy trials have forced a re-evaluation of how such vaccines are to be formulated. Because effective anti-tumor immunity will probably require at the very least the cooperation of CTLs, T cells, and antibody-producing B cells, vaccine strategies that are not designed to recruit each of these populations are probably minimizing their own potential for success. Furthermore, unlike conventional vaccines, DCs are living cells that adhere to various physiological programs that must be taken into account when devising vaccination protocols. All aspects of vaccine design, including DC activation regimens, routes of administration, and critical aspects of timing must be engineered around the evolutionarily programmed behavior of the DC, rather than the expectation that the DC will obligingly meet the requirements of the immunotherapist. We subscribe to the simulation of microbial infection through the use of TLR agonists as an ideal way to activate DCs, since the immune system’s primary task is the detection and elimination of pathogens. By exposing vaccine DCs to tumor antigens in the presence of TLR agonists, one essentially disguises self-proteins as infectious non-self and sets in motion an immune response of a quality and intensity usually reserved only for combating dangerous infections. The right combination of TLRs and cytokines can lead to robust IL-12 secretion, which is paramount for generating long-lasting, high functional avidity and type-1 polarized T-cell responses. The benefits of IL-12 may best be attained, however, if DCs are administered before their narrow window of IL-12 secretion is passed, which may require intranodal delivery of the vaccine. Vaccine target antigens should ideally be selected based on their relationship to tumor aggressiveness and survival, so that if the tumor is not destroyed outright, any antigen loss variants that break through immunotherapy will be less viable or will present a phenotype associated with a more favorable prognosis. Another favorable target antigen characteristic is the vulnerability to non-HLA-restricted immune attack, such as sometimes can be achieved by antibody. Finally, cancer vaccines may be more fruitfully employed in earlier rather than later disease settings, so that complications arising from heavy tumor burdens, tumor-induced immunosuppression, or immune system damage due to pretreatment with chemother- or radiation-therapy can be minimized. By combining each of these features into a unified strategy, DC-based vaccines may advance to the next higher level of effectiveness.

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