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Cancer immunotherapy with mRNA-transfected dendritic cells

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Summary: Bone marrow-derived dendritic cells (DCs) are the most potent antigen-presenting cells capable of activating naïve T cells. Loading DCs *ex vivo* with tumor antigens can stimulate potent antitumor immunity in tumor-bearing mice. This review describes the use of mRNA-encoded tumor antigens as a form of antigen loaded onto DCs, including our early experience from clinical trials in urological cancers. Transfection of DCs with mRNA is simple and effective. Comparative studies suggest that mRNA transfection is superior to other antigen-loading techniques in generating immunopotent DCs. The ability to amplify RNA from microscopic amounts of tumor tissue extends the use of DC vaccination to virtually every cancer patient. The striking observation from two phase I clinical trials, in patients with prostate cancer immunized with prostate-specific antigen mRNA-transfected DCs and patients with renal cancer immunized with autologous tumor RNA-transfected DCs, was that the majority of patients exhibited a vaccine-induced T-cell response. Suggestive evidence of clinically related responses was seen in both the trials. Immunization with mRNA-transfected DCs is a promising strategy to stimulate potent antitumor immunity and could serve as a foundation for developing effective treatments for cancer.

Introduction

In the first series of reports describing the use of mRNA-transfected dendritic cells (DCs), Boczkowski *et al.* (1) have shown that lipid-mediated transfection of murine bone marrow-derived DCs with chicken ovalbumin (OVA) mRNA was capable of stimulating *in vitro* OVA-specific cytotoxic T-lymphocyte (CTL) responses. CTL responses could be stimulated with total RNA or poly-A^+ but not poly-A^- fractions isolated from OVA-expressing cells or with RNA transcribed *in vitro* (IVT) from an OVA cDNA template. Mice vaccinated with DCs transfected with RNA from OVA-expressing EL-4 tumor cells were protected against a challenge with the tumor cells, and in the B16 melanoma model, a reduction in lung metastasis was seen in mice treated with B16 tumor RNA-transfected DCs. In a subsequent study, the use of mRNA-transfected DCs was extended to brain tumor models, showing that immunization of mice with B16 melanoma or SMA560 astrocytoma RNA-transfected DCs stimulated CTL responses and induced protective immunity against an

intracranial tumor challenge (2). Primary CTL responses also could be stimulated *in vitro* using human monocyte-derived DCs transfected with IVT mRNA corresponding to carcinoembryonic antigen (CEA), green fluorescent protein (GFP), or human papilloma virus (HPV) E6 protein (3).

Despite the fact that mRNA transfection was successfully used for ectopic gene expression in mammalian cells (4), the initial reports describing the immunostimulatory capacity of mRNA-transfected DCs were met with healthy skepticism, reflecting to some extent a myth that RNA is extremely labile and could not withstand the transfection protocols. The use of mRNA-transfected DCs has now been validated and extended in many laboratories, and some of these studies will be mentioned below.

Defined antigens and tumor-derived antigenic mixtures

mRNA encoding defined tumor antigens

Pulsing DCs with class I-restricted epitopes encoding 8 to 9 amino acid long peptides require a simple incubation of peptide with DCs and has been a method choice for loading DCs with antigens. To stimulate a potent immune response and reduce the risk of immune escape, multiple class I- and class II-restricted epitopes have to be identified for each haplotype, which needs to be predetermined for each patient. Identification of human leukocyte antigen (HLA)-restricted peptides, especially class II-restricted peptides, is labor intensive, and for the foreseeable future, many patients will not be eligible for peptide-based therapies. Loading DCs with whole antigen in the form of protein, cDNA, or mRNA will obviate many of those limitations; whole antigens encode multiple class I and class II epitopes that will correspond to any of the 4–6 distinct alleles present in most cancer patients. Prior determination of the patient's HLA makeup therefore will not be necessary.

Transfection of DCs with mRNA encoding defined tumor antigens is simple, reproducible, and effective. mRNA corresponding to gene products whose sequence is known can be rapidly generated *in vitro* using appropriate primers and reverse transcriptase-polymerase chain reaction (RT-PCR) coupled to transcription reactions (1, 5). Manufacture of mRNA for clinical use of this *in vitro* generated non-cell-derived product can be performed in a cost effective and defined manner, thus streamlining and simplifying the regulatory approval process. This contrasts with the complexities and limitations of using protein antigens or viral vectors, which limit their availability for investigational studies and restrict their clinical evaluation to a select few. DCs transfected with mRNA encoding a broad range of antigenic targets has been used to stimulate T-cell responses *in vitro* and tumor immunity in mice. CTL responses were stimulated *in vitro* from the peripheral blood mononuclear cells (PBMCs) of healthy volunteers or cancer patients against foreign antigens, often of viral origin, such as influenza virus matrix protein, HPV E6 and E7 proteins, human immunodeficiency virus (HIV) gag protein, Epstein–Barr virus (EBV) LMP2A product, and GFP (3, 6–9), as well as normal gene products of restricted expression patterns that could serve as tumor antigens, such as prostate-specific antigen (PSA) (10), telomerase reverse transcriptase (TERT) (11), survivin (12), oncofetal antigen (OFA) (13), and CEA (3). The ability to stimulate T-cell responses against normal gene products is conceivably a reflection of the limited nature of tolerance to self-antigens and the potency of mRNA-transfected DCs to activate the remaining lower avidity T cells. Reflecting the benefits of immunizing with whole antigen, CEA-, PSA-, or TERT-specific CTL responses could be stimulated *in vitro* from the PBMCs of healthy volunteers or cancer patients from over 100 individuals of undetermined haplotype, mostly without fail (3, 5, 10, 11, 14, and unpublished data). Stimulation of $CD4^+$ T-cell responses by mRNA-transfected DCs was also reported against CEA (3), TERT (14), EBV LMP2A (9), or HIV gag (8). CTL responses and tumor immunity could be also engendered in mice immunized against OVA (1), Muc-1 (15), survivin (12), OFA (13), and TERT (11). Recently, we have shown that CTL responses and tumor immunity can be also induced by immunization against angiogenesis-associated products, such as vascular endothelial growth factor (VEGF), VEGF receptor-2, or Tie-2, and that combined immunotherapy against angiogenic targets and tumor-expressed antigens exerts a synergistic antitumor effect (16).

mRNA amplified from tumor cells

Vaccination with defined and well-characterized tumor antigens shared among many cancer patients is clearly the method of choice. However, in many instances in which the optimal antigenic targets expressed in the tumor cells are not known, the alternative choice is to vaccinate with autologous tumor-derived antigenic mixtures. Animal studies have shown that despite the small proportion of relevant tumor antigens in the mixture, this approach is remarkably effective in stimulating antitumor immunity (17–19). DCs loaded with autologous tumor-derived antigenic mixtures in the form of tumor lysates (20), peptides (21), intact or dying tumor cells (22), as well as total or poly-A^+ RNA fractions isolated from tumor cells (1, 2, 11, 23, 24) were also effective. The problem is that for most cancer patients it will not be possible to obtain sufficient

tumor tissue to generate the amount of antigens needed for an effective and sustained immunization protocol. The RNA approach offers a practical solution. The mRNA content of tumor cells can be amplified by simple and straightforward PCR-based protocols from microscopic amounts of tumor tissue, providing a virtually inexhaustible amount of antigen without the need to identify the potent tumor antigens in each patient.

We have initially shown that murine DCs transfected with mRNA amplified from the B16 melanoma cell lines stimulated potent CTL responses in mice and induced the regression of metastasis in tumor-bearing mice (5). Demonstrating the clinical utility of this approach, tumor RNA could be isolated by microdissection techniques from frozen section of tumor tissue obtained from CEA-positive colorectal cancer patients, amplified, and following transfection into HLA-matched DCs, stimulate a CEA-specific CTL response *in vitro*. In a second study, Heiser *et al.* (25) have shown that human monocyte-derived DCs generated from patients with prostate cancer and transfected with RNA amplified from microdissected frozen sections or from needle biopsies of the patient's tumor stimulated a polyclonal antitumor response which recognized the patient's tumor targets as well as PSA- and TERT-expressing targets. In a third study, Grunebach *et al.* (26) amplified RNA from a GFP-expressing renal cancer cell line. Human monocyte-derived DCs transfected with amplified RNA exhibited GFP expression and stimulated a CTL response that was comparable to that of DCs transfected with non-amplified mRNA.

Methods of transfecting DCs with mRNA

Lipid-mediated transfection, electroporation, or passive transfection

In the first study using mRNA-transfected DCs to stimulate immune responses in mice, RNA was transfected into murine DCs in the presence of a cationic lipid (DOTAP) (1). Lipid-mediated transfection was successfully used by us (2, 3, 5, 7, 11, 14) and other researchers (8, 9, 15, 23, 24, 26, 27) to load human or murine DCs with mRNA-encoded antigens. Lipids, however, are often toxic to DCs requiring careful optimization of the transfection protocol, invariably limiting the amount of RNA the DCs can be exposed to. Lipids are readily available for investigative purposes, but for clinical applications, the choices are quite limited. Originally planned as a negative control, we were surprised to find that incubation of immature human monocyte-derived DCs with CEA mRNA in medium alone, called now passive transfection, was sufficient to sensitize the DCs to stimulate a CEA-specific CTL response which was comparable if not slightly superior to that stimulated with DCs transfected in the presence of lipid (3). Passive transfection was successfully used by us in subsequent preclinical studies (10, 11, 25, 28) as well as in the two clinical trials described below (29, 30). More recently, a highly efficient method to introduce mRNA into DCs was described using electroporation (31, 32). Electroporation does not require additional reagents and is compatible with clinical use. DCs subjected to electroporation become fragile, and special care is needed to recover the shocked cells. Electroporation is rapidly becoming the method of choice to introduce mRNA into DCs and has been successfully used by an increasing number of investigators in the field (6, 13, 16, 26, 33–36).

'More is not necessarily better' or is it actually worse?

How do electroporation, lipid-mediated transfection, and passive transfection compare? When transfection efficiency is determined by measuring the expression of a mRNA-encoded reporter transgene such as GFP (26, 31, 32) or by presentation of a class I-restricted epitope to a CTL clone (33), electroporation was found to be superior to lipid-mediated transfection, whereas passive transfection was ineffective. However, when a functional endpoint was used to monitor the efficiency of mRNA transfection into DCs, for example induction of CTL responses, surprisingly all transfection methods yield a comparable CTL response (3, 26, and unpublished data). The emerging data therefore point to a remarkable discrepancy between the amount of antigen expressed in the DCs or processed for class I presentation and the stimulatory capacity of the DCs. Low to undetectable levels of antigen can stimulate effective CTL responses, and increasing the amount of antigen in the DCs does not appear to improve their immunostimulatory capacity. The likely explanation of these observations is that T cells can be activated in response to very low levels of major histocompatibility complex (MHC) epitopes presented on the surface of DCs, and these data suggest that optimizing transfection of DCs by measuring transgene expression is not time well spent.

Enhancing the transfection efficiency of mRNA into DCs may be in fact counter-productive and may yield diminished protective immunity in the patient. This outcome is predicted because T cells exposed to a high antigenic dose will exhibit enhanced propensity to undergo activation-induced cell death, which will preferentially affect the higher avidity T cells in the population and lead over time to the selection and persistence of lower avidity T cells. This result was seen *in vitro* (37, 38) as

well as *in vivo* (39–42). We would therefore argue that one should strive to reduce (above a certain minimum that can be easily achieved experimentally, including passive transfection), rather than enhance, the efficiency of loading DCs with mRNA-encoded antigens.

Mechanism of RNA uptake by DCs: is timing everything?

RNA uptake by DCs can follow several routes, depending on the transfection protocol, and is conceivably facilitated by the efficient and unique antigen-capture mechanisms characteristic of DCs (43). Macropinocytosis, a non-receptor-mediated, high-throughput process that captures media and solutes, may be the primary conduit for RNA uptake via passive or lipid-mediated transfection. Alternatively, electroporation could provide direct access for the RNA to the cytoplasm through temporary damage to the outer cellular membrane and may be independent of uptake mechanisms operating in the DCs.

If RNA uptake is mediated via DC-specific antigen-capture pathways, and because by-and-large such pathways and/or the processing of the captured antigen are downregulated during maturation, transfection of immature DCs with mRNA would be more effective. Two studies have shown that *in vitro* stimulation of CTLs by DCs transfected with mRNA is optimal, if immature DCs are first transfected and then matured, using either tumor necrosis factor-α (TNF-α) or soluble CD40 ligand as maturation agents, compared to transfection of mature DCs (3, 44). This issue has not been satisfactorily resolved and may not apply in every case; timing and sequence of mRNA transfection could depend also on the type of DCs and specific experimental conditions used. Importantly, the ability to stimulate $CD4^+$ T cells also should be considered when determining the optimal timing of mRNA transfection.

How efficient is loading DCs with mRNA-encoded antigens?

Several methods have been used to load DCs with antigen: pulsing with peptides, incubating with protein, transfecting with plasmids, or transducing with viral vectors. We have observed that mRNA-transfected DCs were equally or more potent than peptide-pulsed DCs in stimulating CTL responses *in vitro* (1, 3, 7). Strobel *et al.* (6) have shown that electroporation of mRNA into DCs was more effective than cDNA plasmids. A thorough comparative analysis of different loading techniques was carried out by Weissman *et al.* using the HIV p55 gag product as a model antigen: incubation with whole protein, pulsing with a peptide mixture spanning the protein, transducing with a vaccinia vector, and lipid-mediated transfection with mRNA. Remarkably, in this study, mRNA-transfected DCs were the most effective antigen-presenting cells (APCs) capable of stimulating $CD8^+$ as well as $CD4^+$ T-cell responses *in vitro*. Thus, observations from several laboratories suggest that mRNA transfection is an effective, if not superior, method to generate immunostimulatory DCs.

It is not clear why mRNA transfection, when functional endpoints are used to determine the outcome, is so efficient, especially because antigen loading of DCs with peptides or viral vectors is much more efficient when measured by standard techniques. Several factors could contribute to the efficiency of mRNA transfection. One important factor could be that mRNA provides a supply of antigen to generate antigenic peptides over an extended period of time. This may be particularly important in the setting of *in vivo* immunization, because considerable time will elapse between exposure of the DCs to antigen in peripheral tissues and encounter of cognate T cells at the draining lymph nodes, estimated to take between 6 and 48 h. To prevent loss of MHC–peptide complexes, DC maturation is accompanied by what appears to be a partial, though definitely not complete, reduction in the turnover of MHC class I and class II–peptide complexes on the cell surface (45, 46). A continuous supply of antigen translated from the transfected mRNA could ensure that a sufficient density of MHC–peptide will be maintained until the DC has encountered its cognate T cell. While the same can be argued for DCs transfected with cDNA expression constructs, for reasons that are not altogether clear, transfection of DCs with cDNA plasmids using non-viral methods have been so far ineffective (31). Viral vectors, such as adenovirus- or poxvirus-based vectors, result in very efficient transfection and high levels of transgene expression in DCs, yet they also negatively impact DC function (47–49). In addition, immunodomination by viral products could further suppress an immune response against the transgene in an unpredictable manner (50, 51). Another reason why mRNA transfection is so effective is that it can induce or contribute to the DC maturation process that was seen in some (8, 29, 52) but not other (9, 15, 31, 33) instances. Whether and to what extent mRNA induces DC maturation may depend on the RNA used, method of transfection, state of the DCs, as well as the parameters measured.

The Achilles' heel of the mRNA approach – induction of $CD4^+$ T-cell immunity

Induction of potent $CD8^+$ CTL responses has been the main goal in developing immunotherapeutic strategies for cancer,

yet emerging evidence points also to the pivotal role of $CD4^+$ T cells in tumor immunity (53, 54). $CD4^+$ T cells provide important functions for the expansion and persistence of $CD8^+$ CTLs (55), stimulate the innate arm of the immune system at the tumor site, and inhibit local angiogenesis (56). The importance of the $CD4^+$ T-cell response in tumor immunity was highlighted in murine studies showing that $CD4^+$ T cells can eradicate tumor in the absence of $CD8^+$ T cells (57–59) or constitute the dominant effector arm in the antitumor response (60). An optimal antitumor immune response therefore will require the concomitant activation of both the $CD8^+$ and the $CD4^+$ T-cell arms of the immune response. Endogenously expressed antigens, such as antigens expressed in DCs from the transfected mRNA, will be channeled preferentially into the class I processing pathway and activate $CD8^+$ but not $CD4^+$ T cells. Thus, immunization with mRNA-transfected DCs will be deficient in stimulating the $CD4^+$ T-cell arm of the immune response.

Endogenously expressed antigens can be redirected into the endocytic compartment by appending a leader sequence to the amino end and a lysosomal sorting signal to the carboxyl end of the endogenously expressed antigen (61). DCs transfected with mRNA modified in this manner exhibited a modest enhancement in their capacity to stimulate $CD4^+$ T-cell responses *in vitro* (3, 14). Access to the endocytic compartment where class II-restricted peptides can be generated is, however, not sufficient. MHC class II loading of endogenously derived peptides is inefficient because of the presence of the invariant chain, which forms complexes with the nascent MHC class II molecules. This finding is supported by the observations that presentation of endogenous peptides is often favored in cells expressing class II molecules in the absence of invariant chain (62–64). This roadblock can be overcome, as we have recently shown, by transiently inhibiting invariant chain expression using antisense oligonucleotides co-delivered with the mRNA. In this study, partial inhibition of invariant chain expression led to enhanced class II presentation and enhanced potency of antitumor immunity in mice immunized with mRNA-transfected DCs (36). The effects seen so far in the animal studies were modest, and it remains to be seen whether this approach can be translated to human clinically relevant settings.

Clinical trials with mRNA-transfected DCs

The efficacy of RNA-transfected DC vaccination in stimulating T-cell immunity and therapeutic antitumor responses in murine models established a scientific rationale for human studies. In 1999, we initiated a series of clinical trials to explore the use of mRNA-transfected DCs in patients with renal and prostate cancer. These two tumor systems were chosen because conventional therapies have failed to improve survival, and access to well-defined patient populations for clinical trials can be secured. Furthermore, prostate and renal cancers are distinctly different with regard to immunogenicity, biologic aggressiveness, and availability of serum markers; hence studies performed in both systems should provide complementary information as to vaccine safety as well as to biologic and clinical response to therapy. The primary endpoints of these phase I clinical trials were to determine the safety of administering mRNA-transfected DCs to cancer patients. Secondary end points were induction of T-cell immunity and clinical responses.

In these clinical trials, immature monocyte-derived DCs were passively transfected with mRNA and injected into patients. Yet, recent studies have not only underscored the importance of DC maturation to enhance the immune potency of the administered DCs, but also raised the concern that vaccination with immature or suboptimally matured DCs could induce immune suppression (65–69). Exposure of immature DCs to RNA can provide a maturation stimulus and in our system, passive transfection of the monocyte-derived immature DCs with mRNA led to increased CD83 expression on the mRNA-transfected DCs (29). In view of the immunopotency of the mRNA-transfected DCs seen in these clinical trials (see below), it appears that the RNA-exposed immature DCs underwent a partial maturation *in vitro* and remained responsive to additional maturation stimuli encountered *in vivo*.

Clinical trials were preceded by extensive validation studies. A key parameter was to determine the capacity of clinically processed mRNA-transfected DCs to stimulate specific T-cell responses *in vitro* from the PBMCs of patients. Presentation of antigen to T-cell clones, which merely demonstrates the successful loading of DCs with antigen (or for that matter any MHC-expressing cell), is not informative in this instance, because it fails to measure the critical feature of immunopotent APCs, namely the ability to activate and expand antigen-specific T cells. Such preclinical validation studies provide critical information on the immunopotency of DCs, the mRNA and the transfection protocol, and the ability of the patients to mount an immune response. They also provide an opportunity to fine-tune and optimize various aspect of the procedure and should be a mandatory step prior to initiation of the clinical trials.

A phase I clinical trial with PSA mRNA-transfected DCs in patients with advanced prostate cancer

In the first clinical trial, DCs transfected with mRNA encoding the PSA were used to immunize patients with hormone-refractory,

metastatic prostate cancer. As illustrated in *Fig. 1*, preclinical studies demonstrated that DCs generated from the PBMCs of prostate cancer patients and transfected with PSA mRNA were capable of stimulating PSA-specific T-cell responses *in vitro*. Moreover, PSA mRNA-transfected DCs generated from male or female healthy volunteers or from cancer patients were equally effective in stimulating PSA-specific CTLs, suggesting that neither natural tolerance to PSA nor tumor-mediated T-cell anergy will represent major barriers for CTL generation against this self-antigen (10). Interestingly, no cross-reactivity was seen in the CTL assays between PSA and kallikrein, a highly abundant serum protein, despite a considerable (60–80%) homology between the two proteins at the amino acid level. The ability to stimulate PSA-specific CTL responses without fail from over 50 individuals without regard to their HLA composition also illustrates the advantages of vaccinating with whole antigen compared to HLA-restricted peptides.

Based on the preclinical studies, a total of 16 patients with hormone-refractory, metastatic prostate cancer were vaccinated with escalating doses of PSA mRNA-transfected DCs at biweekly intervals. These cell doses were chosen based on the maximal number of cells that could be generated from a PBMC batch collected during a single leukapheresis. A traditional, three-tiered dose escalation design was chosen to provide

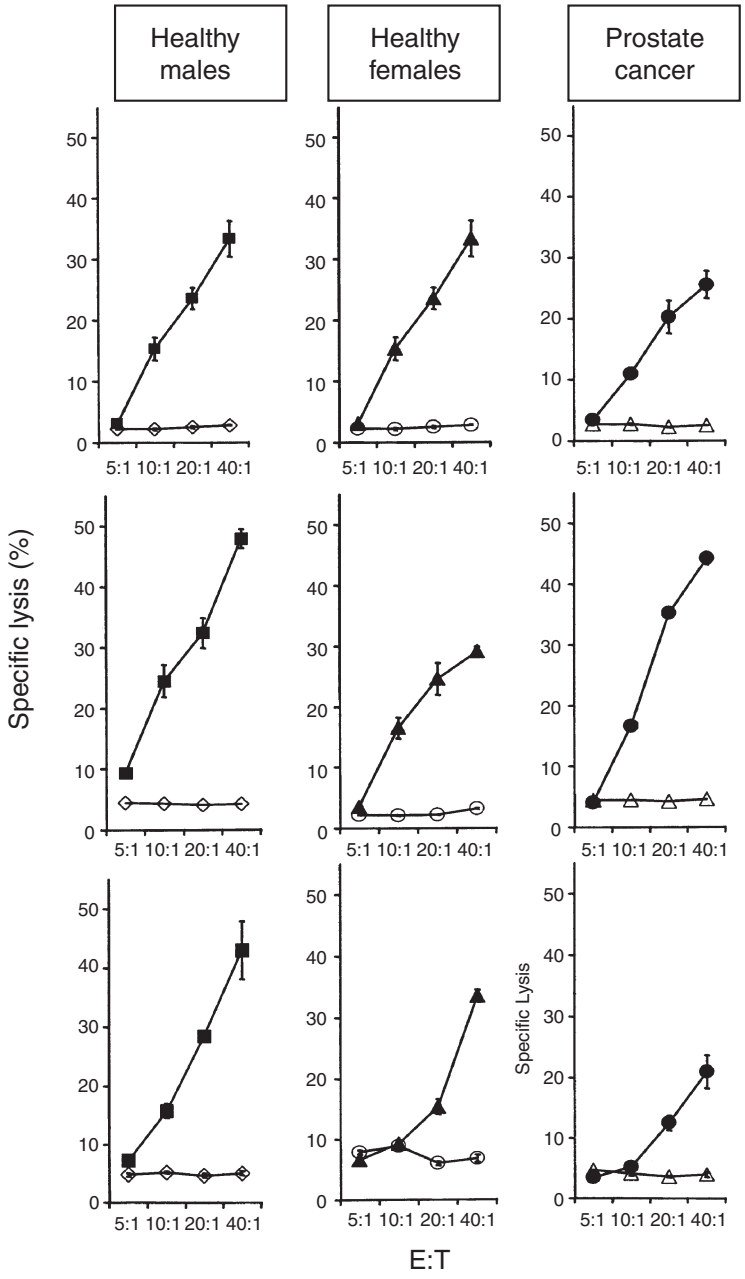

***Fig. 1.* Prostate-specific antigen (PSA) mRNA-transfected dendritic cells (DCs) stimulate PSA-specific cytotoxic T-lymphocyte (CTL) responses *in vitro*.** DCs were generated from 14 randomly selected healthy volunteers and cancer patients of undetermined human leukocyte antigen haplotype (six healthy females, four healthy males, and four prostate cancer patients). DCs passively transfected with PSA mRNA were used to stimulate CTL responses *in vitro* from autologous peripheral blood mononuclear cells. Cytotoxicity was determined by a standard ^{51}Cr release assay using PSA (closed symbols) or green fluorescent protein (open symbols) mRNA-transfected DCs as targets. Three representative results are shown from each group. Reproduced with permission from Heiser *et al.* (10). E:T, effector:target ratio.

information on vaccine safety and toxicity, induction of PSA-specific T-cell immunity, and impact on tumor burden. The vaccine was well tolerated; only minimal toxicities were observed that were limited to grade I skin reactions and/or flu-like symptoms. There was no clinical evidence of prostatitis or other vaccine-mediated autoimmune manifestations. Remarkably, as shown in *Fig.* 2, all patients who completed the full treatment course developed T-cell immunity against PSA, as measured by enzyme-linked immunospot (ELISPOT) analysis and standard cytotoxicity assays (29).

Interestingly, in six of seven evaluable subjects (patients which did not undergo subsequent treatments that could impact on the PSA levels), vaccination with PSA RNA-transfected DCs was associated with a small but statistically significant decrease in the log PSA slope (PSA velocity); three of the patients that provided sufficient material for additional analysis exhibited a transient molecular clearance of circulating tumor cells (29). Although such measurements remain, at this point, unvalidated surrogates for clinical benefit, the identification of biologic and clinically related markers provide further guidance to improve vaccine potency and identify responding patients in future vaccine trials. Clearly, the impact of this immunization strategy on tumor burden was minimal and unlikely to translate into clinical benefit, not the least because in this group of patients only a fraction of tumor cells express PSA (70). Yet, the fact that an immune response and a modest effect on tumor burden were consistently seen in the majority of the treated patients suggest that this is a promising platform and future improvements may be beneficial.

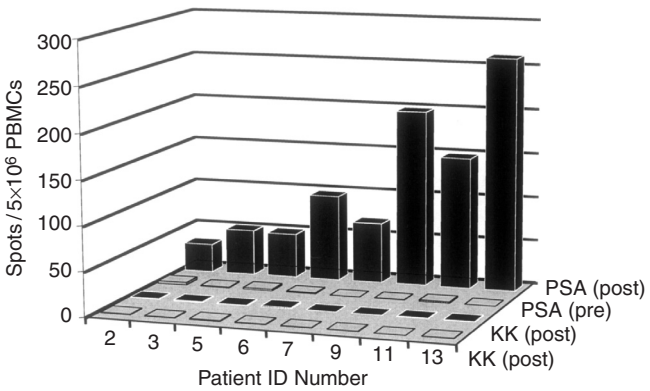

***Fig.* 2. Induction of prostate-specific antigen (PSA)-specific T-cell responses in prostate cancer patients immunized with PSA-specific mRNA-transfected dendritic cells (DCs).** Peripheral blood mononuclear cells (PBMCs) obtained before (pre) and after (post) three cycles of vaccination with PSA mRNA-transfected DCs were incubated with PSA or kallikrein (KK) protein. Interferon-γ-secreting T cells were detected by enzyme-linked immunospot (ELISPOT) analysis. Reproduced with permission from Heiser *et al.* (29).

A phase I clinical trial with autologous tumor mRNA-transfected DCs in patients with advanced renal cell cancer

PSA is a prototype tumor-associated antigen, and it was used to demonstrate the safety, feasibility, and biological activity of mRNA-transfected DC-based vaccination. A PSA-targeted vaccine fails to take into account that only a fraction of tumor cells in the metastatic patient express antigen (70) and that tumors could express more relevant, largely unidentified, tumor antigens (71). As discussed above, the limitation of vaccinating against single antigens can be addressed by using autologous tumor-derived antigenic mixtures, and this approach was explored in the second clinical trial. In this trial, patients with metastatic renal cell cancer (RCC) were treated with the patient's own tumor RNA (non-amplified)-transfected DCs. In preclinical studies, we first validated the ability of the tumor RNA-transfected DCs to stimulate *in vitro* a polyclonal tumor-specific CTL responses. How were we able to measure immune responses against a polyclonal, largely patient-specific, unidentified set of tumor antigens? The mRNA technology provided a useful tool.

mRNA-transfected DCs as surrogate targets for measuring T-cell responses

The induction of antitumor T-cell immunity in patients is currently monitored by measuring T-cell responses stimulated against shared antigens known to be expressed in the tumor cells. Because shared tumor antigens are often immunosubdominant in nature and their contribution to the overall antitumor response is likely to vary from patient to patient (71), measuring immune responses against such antigens may not be representative of the overall antitumor response. An accurate measure of an antitumor response has to account for all tumor-specific T cells, in particular T cells corresponding to immunodominant antigens. Because the composition and contribution of such antigen is not known and will vary from patient to patient, an accurate measure of tumor immunity would require the use of the patients' own tumor cells as targets. The problem is that from most patients it is not possible to obtain sufficient tumor tissue of appropriate purity needed for such assays, and therefore a reliable assessment of tumor immunity in most cancer patients is lacking. Tumor

RNA-transfected autologous DCs used as surrogate targets for tumor cells in T-cell assays constitutes a simple and broadly applicable, if not perfect, solution (of note, other autologous cell types, provided they express MHC and are transfectable with RNA, such as fibroblasts or B cells, will be also appropriate). Because tumor cells often downregulate MHC class I expression and/or secrete immune suppressive factors, T cells recognized by the mRNA-transfected DCs may not recognize the real targets, the tumor cells. Thus, use of mRNA-transfected autologous targets will provide a reliable and accurate measure of a tumor-specific T-cell response but cannot determine whether the measured immune response will recognize the actual targets, the tumor cells. Nevertheless, in a limited number of cases that tumor RNA-transfected DCs and parental tumor cells were compared, both targets exhibited comparable sensitivity to the effector T cells (11, 26, 34, 35). As discussed below, use of tumor RNA-transfected DCs as surrogate targets in T-cell assays was instrumental in monitoring the induction of T-cell immunity in renal cancer patients.

Immunological monitoring of RCC-specific T-cell responses

In preclinical studies, we have shown that granulocyte–macrophage colony-stimulating factor- and interleukin (IL)-4-cultured, non-matured, monocyte-derived DCs passively transfected with autologous (non-amplified) tumor RNA from RCC patients were capable of stimulating tumor-specific T-cell responses *in vitro* (28). The tumor specificity of the CTL responses stimulated *in vitro* was shown by the fact that the CTLs lysed DC targets transfected with RCC RNA but not DC targets transfected with RNA isolated from PBMCs or from normal renal epithelia. The selective stimulation of tumor-specific, but not normal renal epithelial specific, CTL responses suggest that risk of autoimmunity will be diminished, despite the fact that patients will be immunized with unfractionated renal tumor-derived antigenic mixtures.

Based on these findings, a phase I clinical trial was carried out in which 10 patients with metastatic RCC were immunized with autologous RCC RNA-transfected DCs, and the induction of RCC-specific T cells in the treated patients was monitored by direct ELISPOT analysis using RNA-transfected DCs as targets (30). There was no evidence of treatment-associated adverse effects. Furthermore, no clinical syndromes indicating autoimmune pathology, such as vasculitis, thyroiditis, cardiomyopathy, or vaccine-induced anti-DNA or antimitochondrial antibodies, were observed in the study patients. As shown in *Fig.* 3, five of six patients analyzed exhibited a tumor-specific T-cell response after immunization. Using ELISPOT analysis, T-cell responses were detected in the PBMCs of vaccinated patients, when incubated with RCC RNA-transfected DCs but not with PBMCs or normal renal epithelial RNA-transfected DCs used as surrogate targets for tumor cells and normal epithelial cells, respectively. The immune response was polyclonal in nature, because it was also directed against TERT, OFA, and G250 [an RCC-specific antigen (72)], determined by incubating the patients' PBMCs with DCs transfected with the corresponding mRNAs.

In summary, it is quite remarkable that despite the advanced state of diseases in the first two clinical trials described above, the majority of patients responded immunologically to vaccination with mRNA-transfected DCs. This response is also seen in a third clinical trial currently underway in which patients with prostate cancer are vaccinated with TERT mRNA-transfected DCs. Unlike the first two trials, in this trial, DCs are electroporated with RNA (31, 32) and matured with cytokines (73, 74) before administration. Despite those modifications, the magnitude of the TERT-specific immune responses seen in the vaccinated patients does not appear to be strikingly different from what was seen in the first clinical trial (where patients were immunized with immature DCs passively transfected with PSA mRNA). It is possible, though unlikely, that the weakness of TERT compared to that of PSA as tumor antigen has offset the benefits of DC maturation and RNA electroporation. The possibility that the electroporation or DC maturation protocols are detrimental has to be considered as well.

Therapeutic benefit of RCC patients immunized with mRNA-transfected DCs?

Tumor-related mortality in the study subjects was unexpectedly low, with only three of 10 patients dying from disease after a mean follow up of 20 months and seven of 10 experiencing stabilization of metastatic disease or attenuated tumor growth. This outcome is considered unusual for this group of patients (75), especially because no deliberate attempts of patient selection enrolled in this trial was made. It should be noted, however, that the majority of the patients underwent additional treatments subsequent to vaccination that could have influenced the course of the disease.

Despite such encouraging results derived from uncontrolled phase I trials, these observations cannot be viewed as indicative of clinical benefit. First, patients are often subjected to various treatments before and/or after vaccination, and their contribution to the outcome is not clear. Second, RCCs, as well as other

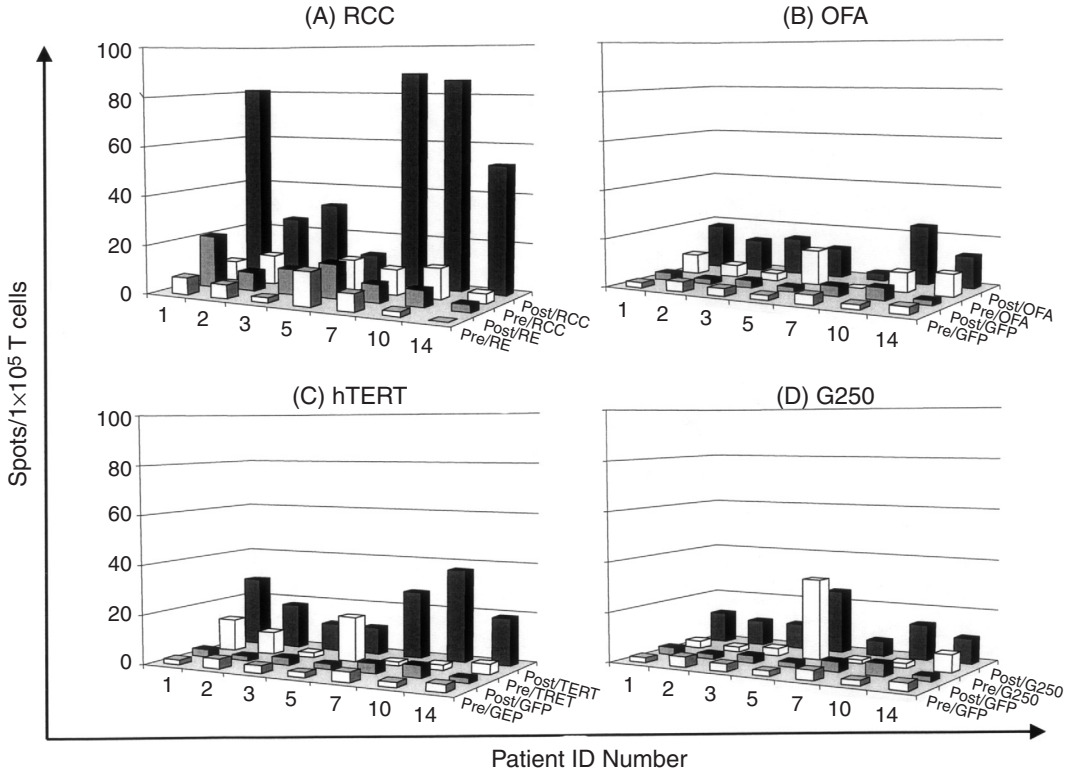

***Fig.* 3. Induction of polyclonal cytotoxic T-lymphocyte (CTL) responses in renal cancer patients immunized with autologous tumor RNA-transfected dendritic cells (DCs).** Peripheral blood mononuclear cells (PBMCs) obtained before (pre) and after (post) three cycles of vaccination with autologous renal tumor RNA-transfected DCs were analyzed by enzyme-linked immunospot (ELISPOT) for specific T-cell responses using RNA-transfected DCs as targets/stimulators. (A) DCs transfected with autologous renal epithelial RNA (RE) or tumor RNA [renal cell cancer (RCC)]. (B) DCs transfected with green fluorescent protein (GFP) or telomerase reverse transcriptase (TERT) mRNA. (C) DCs transfected with GFP or oncofetal antigen (OFA) mRNA. (D) DCs transfected with GFP or G250 mRNA. Note that with the exception of patient no. 5, all patients exhibited a specific T-cell response against tumor-derived antigenic mixtures and specific antigens expressed in the tumor, TERT, OFA, or G250, but not normal renal epithelial antigens or GFP. Reproduced with permission from Su *et al.* (30).

cancers such as melanoma, are characterized by an unpredictable clinical course in which spontaneous regressions of metastatic lesions are not uncommon. Third, genetic instability of tumor cells and the presence of heterogeneous clones within the tumor mass may explain so-called mixed clinical responses (one metastasis progresses/appears and one metastasis regresses/disappears), as others and we have often seen in the course of immunotherapy trials in patients with metastatic disease. In our experience, the demonstration of shrinkage of isolated tumor lesions, without rigorous imaging of other potential metastatic sites, is not informative and cannot be presented as evidence for therapeutic benefit. Fourth, despite best efforts, in single-arm clinical trials some measure of patient selection bias is inevitable, which may skew the conclusions in either direction. Controlled and statistically powered phase II clinical trials therefore will be required to explore the clinical efficacy of vaccination with mRNA-transfected DCs.

Perspectives and future directions

Immunotherapy with *ex vivo* generated DCs is a patient-specific form of cell therapy, founded on the premise that the benefit to the patient will outweigh the added cost and complexity associated with this form of therapy. Preclinical studies and early clinical experience suggest that vaccination with mRNA-transfected DCs is a promising approach that could serve as a foundation for an effective treatment. The clinical benefit associated with this treatment in its current form appears to be minimal at best.

Improving the DC-based vaccination protocol is therefore an immediate goal. Further enhancing RNA transfection efficiency may not be necessary and perhaps even counter-productive as discussed above; current protocols may already deliver saturating, if not excess, amounts of RNA into DCs. Our view is to reduce, rather than increase, RNA transfection efficiency into DCs (above a certain threshold to be determined by functional analysis: the ability to

stimulate T-cell responses, not expression of a reporter gene or presentation to T-cell clones) and pay special attention to the consequences of the transfection procedure on DC viability. A second key parameter is DC maturation. The objective is to induce the differentiation of the DCs *ex vivo* to a stage that they are optimally poised to migrate and respond to additional stimuli from the cognate T cells encountered at the lymph, notably CD40 ligand-mediated signals. The current gold standard for *ex vivo* DC maturation is incubation of the DCs with a cytokine cocktail consisting of IL-1β, IL-6, TNF-α, and prostaglandin E_2 (PGE_2) (73, 74). The evidence in support of this protocol is derived mostly from *in vitro* studies. However, recent studies argue that this maturation protocol will not be useful for *in vivo* vaccination protocols, because the presence of PGE_2, which is required for DC migration to the lymph nodes, inhibits DC responsiveness to CD40 ligand-mediated signaling encountered at the lymph node (76, 77). Of added concern is the finding that defects in CD40 signaling in DCs lead to the activation of immunosuppressive T cells (78). These observations underscore the need to identify alternative maturation protocols. One option termed *in situ* DC maturation is to eliminate altogether the need for *ex vivo* maturation by injecting the antigen-loaded immature DCs into sites that were pretreated in a manner causing an inflammatory reaction and local DC maturation (79). Finally, there are a multitude of important parameters that can influence the induction of immunity, such as the injection protocol, site of injection, dose and frequency, etc., which are often difficult to explore systematically in clinical trial settings.

Clinical trial strategy with mRNA-transfected DCs

Informative clinical trials are the bottlenecks in guiding the development of clinically useful DC-based immunotherapies. Phase I clinical trials have established the general safety of administering mRNA-transfected DCs to cancer patients, and the majority of the vaccinated patients exhibited an immunological response. Traditional clinical trial designs developed for testing cytotoxic or biologic agents are, however, ill suited for cell-based therapies such as DC vaccines. Typically, phase I trials are carried out in subjects with advanced or metastatic disease to whom escalating doses of a cytotoxic or biologic agent are administered in order to define a maximally tolerated dose. This dose is then used in subsequent phase II and phase III protocols with the goal to demonstrate clinical efficacy. There are, however, several important differences between cell-based cancer vaccines and chemotherapeutic agents. Because DC vaccines appear to be inherently safe, dose escalation will always proceed to the highest dose level feasible, yet it may not necessarily be the dose that provides the optimal immunologic or clinical response. Therefore, the dose–toxicity relationships as seen with cytotoxic drugs will not apply to DC vaccines. Importantly, DC-based therapy is a complex process influenced by multiple parameters, such as what antigens to vaccinate against, how to mature DCs, how to preserve the vaccine-induced immune response, etc. Each parameter has to be tested in human subjects to determine whether it represents an improved outcome from a biological or clinical efficacy standpoint.

Because the traditional two-arm trials are clearly too expensive and time consuming, we are now performing randomized phase II trials to evaluate multiple parameters of DC vaccination, while maintaining strict safety monitoring (80, 81). Unlike traditional two-arm trials, these trials are not comparative by nature, yet they allow for rapid screening of the most promising regimens that warrant further clinical testing (the so-called best-bet approach). The primary endpoints in these trials are immunological, T-cell responses, which can help to define the underlying biologic mechanisms for potentially encountered clinical responses and guide the further refinement of the clinical strategy. Importantly, in this trial design, the number of patients in each arm is comparable to that typically used in traditional phase I trials, therefore offering a rapid and less-expensive path to explore multiple parameters before proceeding to phase II trials with clinical endpoints. We are currently using this clinical trial strategy to test several novel concepts that may have a considerable impact on enhancing the immunologic and clinical efficacy of the RNA-transfected DCs approach: a novel *in situ* DC maturation protocol (79), the impact of removing regulatory T cells (82), and an alternative DC administration protocol. In the near future, we plan to test a method to enhance the induction $CD4^+$ T-cell responses from endogenous (mRNA-encoded) antigens (36) and CTLA-4 blockade using a CTLA-4-binding aptamer (83). A striking feature of our early clinical experience is the consistency of detecting T-cell responses in patients immunized with mRNA-transfected DCs (*Figs 2 and 3*) (29, 30), thus providing a baseline of biological activity that we seek to improve. Despite the non-comparative nature of the randomized phase II trials we are currently conducting, this information is arguably a tremendous asset that will provide increasing confidence in the decision-making process to systematically develop increasingly effective DC vaccination protocols.

On a final note, successful therapy of complex diseases, such as cancer, will require a combination approach. DC vaccination is designed to activate tumor-specific T cells, and the

effectiveness of the vaccination is reflected in the type and frequency of T cells activated. The overall potency of an anti-tumor immune response will, however, also depend on the persistence of the immune response induced by the DC vaccine. Thus, combination of DC therapy with treatments that will enhance persistence of immunity, such as 4-1BB and/or OX40 costimulation (84), CTLA-4 blockade (85), and/or removal of regulatory T cells (82), may be highly beneficial. Cancer therapy may also benefit from combining immunotherapy with other compatible modalities such as anti-angiogenic therapy, localized radiation therapy, or chemotherapy.

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