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Gene Modification Strategies to Induce Tumor Immunity

Review

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The immune system provides an attractive option for use in cancer therapy. Our increasing understanding of the molecular events important in the generation of an effective immune response presents us with the opportunity to manipulate key genes to boost the immune response against cancer. Genetic modification is being employed to enhance a range of immune processes including antigen presentation, activation of specific T cells, and localization of immune effectors to tumors. In this review, we describe how many diverse cell types, including dendritic cells, T cells, and tumor cells, are being modified with a variety of genes, including those encoding antigens, cytokines, and chemokines, in order to enhance tumor immunity.

Introduction

The immune system is a promising tool for use in the therapy of cancer due to the range of effector mechanisms possessed by a diversity of immune cell types and its ability to exert effects with exquisite specificity. A variety of tumor-associated antigens (TAA) have been identified that singly, or in combination, can be used to discriminate between normal and malignant tissue. Successes with immunotherapeutic approaches, such as vaccines, cytokine administration, and adoptive cell transfer, have demonstrated that the immune system can be manipulated to produce dramatic antitumor responses against some malignancies, and these successes are driving intense interest in the field (Rosenberg, 2001). However, complete durable responses are rare, and immunotherapies frequently fail to prevent tumor growth (Rosenberg et al., 2004).

In asking ourselves why success with current immunotherapies is so limited, it is instructive to consider the known requirements for an effective immune response to infectious disease. The immune response is a multistep process, requiring antigen presentation, activation and expansion of specific T cells, and localization of immune effectors to the site of challenge (Figure 1). This process involves a progression of molecular interactions between various cell types resulting from the coordinated expression of specific genes. Immune processes can therefore be viewed as a developing genetic program, where initiation of the response begins a genetic cascade whose individual constituents are essential contributors to the outcome of immunity. In cancer, however, the immune process can fail at several crucial stages. The identification of key failure points in this genetic program provides us with intervention strategies to correct deficiencies in the response against cancer. The following provides a review of genetic intervention strategies to overcome existing limitations of immune defenses at five crucial stages of the adaptive immune response (Figure 1) and discusses how these might be integrated to develop improved therapies for cancer.

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Stage 1: Initiation of Antitumor Immune Responses

The initiation of an immune response is a complex series of events involving danger signals, secretion of cytokines and inflammatory mediators, and the participation of antigen-presenting cells (APCs) that take up antigen, mature, and migrate to lymph nodes where they present antigen to T cells (see stage 1, Figure 1). Conceivably, tumors may not be considered as a threat to self per se, and any of these required steps might be deficient or suboptimal. A range of genetic strategies seeks to enhance one or more of these requirements in order to achieve an effective immune response against tumors.

Some of the earliest genetic vaccine studies in mice utilized a number of alternate vector forms including plasmid DNA or recombinant viruses that encoded model antigens such as β-galactosidase. Vaccination with these vectors could result in tumor growth inhibition in mice, and the therapeutic effect could be enhanced by providing cytokines such as IL-2 either exogenously or incorporated into the same vector (Irvine et al., 1996). Although encouraging, these first successes were limited to inhibition of small tumors expressing foreign antigens. In attempts to broaden the application and enhance the antitumor effect of genetic vaccines, investigators have modified enriched populations of dendritic cells in vitro using various vectors, which are then used as cellular vaccines. Ex vivo modification of both human and mouse DCs with genes encoding tumor antigens, including self-antigens, has been shown to effectively stimulate T cell responses in vitro and in various murine models, with induction of long-term immunity against tumors expressing the corresponding antigens. However, effectiveness was generally restricted to early stage disease or protection against tumor challenge (Ribas et al., 2002).

An alternative to DNA-encoded antigens is the use of RNA for the modification of DCs. Early studies utilizing mRNA encoding model tumor antigens in DC vaccine strategies served as proof of principle that antigen-specific T cells could be generated and protection conferred against tumors in this way. This work quickly led to the use of tumor-derived polyA RNA to induce immunity against poorly immunogenic tumors (Boczkowski et al., 1996). An advantage of this type of approach is that tumor RNA or cDNA may provide an unlimited source of potential antigens without the need for prior antigen identification or characterization. However, this latter approach may be limited to highly expressed anti-

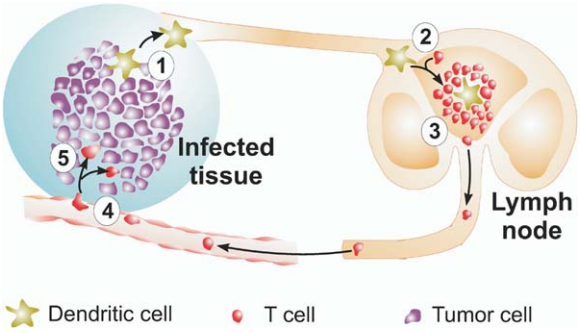

Figure 1. Five Key Stages in a Successful Immune Response

This schematic represents cells of an infected tissue (purple), together with a lymph node draining this tissue (yellow) and blood vessels supplying the tissue (pink). Initiation of the immune response is represented as stage 1, where an antigen-loaded dendritic cell (DC, in green) is shown migrating to the regional lymph node after acquiring antigen and receiving "danger" signals, indicating a potential threat to the body. It then interacts with a T cell (red) that specifically recognizes the antigen presented by the DC. This is shown as stage 2. Stage 3 signifies clonal expansion and differentiation of the relevant T cell populations within the regional lymph node, leading to stage 4, where a proportion of these cells migrates and localizes at the affected body site. Stage 5 denotes the exertion of effector functions by T cells within the affected tissue. This may constitute direct cytolytic effects and/or the production of cytokines, followed by recruitment and activation of further leukocytes. If all these steps proceed successfully, the resulting immune amplification will lead to clearance of the infection and resolution of the potential threat.

gens, and the low stability of RNA may reduce the application of these strategies.

Replicon-based RNA vaccines offer an interesting alternative to normal RNA that takes advantage of the replicase of alphaviruses that can generate large amounts of RNA. Replication of RNA can lead to very high levels of gene expression. Replicon vectors can be produced in several forms including RNA and DNA that can be delivered in various ways including gene gun or as virus particles. Replicon-based vaccines have been demonstrated to induce antitumor immune responses and impact on tumor growth in mice (Gilboa and Vieweg, 2004).

Based on the above results utilizing antigen-engineered DCs in vitro and in mouse studies, vaccination with autologous gene-modified DCs has proceeded to phase I and II clinical trials achieving some immunological responses in patients with renal (Su et al., 2003), colon (Morse et al., 2003) or other cancers (Pecher et al., 2002), with evidence of tumor-specific T cell expansion. Additionally, there was no evidence of toxicity or autoimmunity in these trials, demonstrating the safety of this therapeutic approach. However, high rates of disease progression were observed, requiring most patients to undergo alternate therapies. Clearly, the complex nature of an immune response is not easily replicated by simply providing DCs with antigen. Many other factors are important including the maturation state of DCs, whereby increases in major histocompatibility complex (MHC) and costimulatory molecule expression render DCs better able to stimulate T cells.

One tactic to trigger local DCs to mature, to secrete inflammatory mediators, and to present tumor antigen to lymphocytes is to introduce CD40 ligand (CD40L), a potent dendritic cell activation molecule, into the tumor microenvironment by gene transfer. Adenoviral transduction of CD40L into tumor cells has been demonstrated to promote the maturation of dendritic cells in vitro, as measured by an increase in the number of $CD83^+$ and MHC class II^+ DCs and increased secretion of IL-12. Intratumoral injection of this CD40L-expressing vector into tumor-bearing mice induced a 200-fold increase in IL-12 mRNA and a corresponding reduction in the DC-inhibitory cytokine IL-10 in the tumor area of mice (Buelens et al., 1997). This therapy was capable of causing regression of small tumors in mice and inhibiting the progression of larger tumors (Loskog et al., 2004). These findings are supported by several other in vitro and in vivo studies (Kikuchi and Crystal, 1999), as well as in a clinical trial of leukemia patients (Wierda et al., 2000).

Another factor important in stimulation and maturation of innate immune cells is the presence of cytokines in the tumor micromilieu, which can affect innate immune cell activity. One of the most extensively studied cytokine gene-modification approaches has been to modify tumor cells to express GM-CSF, which has been shown in multiple murine models to significantly enhance antitumor responses through improved tumor antigen presentation by recruited dendritic cells and macrophages (Dranoff et al., 1993; Levitsky et al., 1996). This immunization strategy has also been tested in clinical trials of patients with renal cell carcinoma (Simons et al., 1997), metastatic melanoma (Soiffer et al., 1998), and pancreatic cancer (Jaffee et al., 2001), with the majority of patients biopsied demonstrating extensive inflammatory infiltrate within the tumors, often in conjunction with increased tumor-specific T lymphocyte activity and, in some cases, tumor regression.

Flt3 ligand is another cytokine important in the development of DCs, and genetically modified tumor cells secreting Flt3 liter have reduced tumorigenicity in mice. However, direct comparison of GM-CSF- and Flt3-L-modified tumor cells has revealed the superior activity of GM-CSF, perhaps due to the induction of higher levels of B7.1 and CD1d on DCs (Mach et al., 2000).

The process of inflammation is central to creating a sense of danger that aids recruitment of leukocytes and initiates efficient antigen presentation. Cytokine gene transfer into tumors has been employed to address this issue. For example, tumors secreting the proinflammatory cytokine IL-1β have been demonstrated to have reduced tumorigenicity and to have effect as a vaccine against established tumors (Bjorkdahl et al., 2000). Adenovirus-mediated gene transfer of the IL-1 family member IL-1H4 into established murine sarcoma has also succeeded in inhibiting tumor growth (Gao et al., 2003b). The importance of T cells in antitumor activity was also demonstrated in these studies. However, the genetic provision of IL-1β is not always associated with tumor inhibition. Indeed, depending on the tumor model, IL-1β can increase inflammation and angiogenesis, leading to enhanced tumor growth (Saijo et al., 2002).

Other proinflammatory cytokines have also been

used in attempts to enhance antitumor responses. Genes encoding IL-12 or IL-18 have been demonstrated to promote tumor inhibition (Nishioka et al., 1999; Tatsumi et al., 2002). Utilization of these cytokines has the added benefit of promoting a Th1-type immune response that is generally more effective than a Th2 response at inhibiting tumor growth. A phase I trial involving injection of IL-12-transduced fibroblast cells into tumor sites of late-stage cancer patients has also been completed, with tumor necrosis and transient but clear reductions in tumor size observed in approximately half of patients (Kang et al., 2001).

An important consideration in the design of tumor gene-modification strategies is the mode of vector delivery. Intratumoral injection of vectors can result in safe, effective, local production of immunomodulators (Sung et al., 2002) but is impractical in many clinical situations. Targeted vectors, on the other hand, may provide tumor-specific delivery of genes with viral or non-viral vectors (Wickham, 2003). Enhanced vector targeting to tumors can be mediated through incorporation of appropriate ligands or antibodies into viral coats.

As these strategies to stimulate host antitumor immunity developed, researchers investigated engineering cells with more than one immunostimulatory gene in further attempts to induce more robust antitumor responses. For example, intratumoral injection of DCs engineered to secrete both IL-12 and IL-18—two cytokines known to act synergistically to drive Th1-type (cellular) immune responses—was found to promote tumor regression in murine sarcoma models through activation of $CD4^+$ and $CD8^+$ T cells that secreted high levels of IFN-γ. These IL-12/IL-18 cytokine gene-engineered DCs expressed higher levels of MHC and costimulatory molecules than nontransduced DCs or those engineered with either cytokine alone (Tatsumi et al., 2003). Additionally, murine studies have investigated the efficacy of supplying other cytokine genes such as TNF-α, IL-7, and IL-12, either alone or in combination with other genes, such as costimulatory or TAA genes. These studies in mice have boasted marked reduction in tumor burden, with extensive lymphocyte infiltration into the tumors, as well as enhanced survival (Chen et al., 2002; Narvaiza et al., 2000; Sharma et al., 2003; Tatsumi et al., 2003).

Transduction of more than one gene has proven superior to a single gene by targeting multiple facets of the initiation of an immune response. These results support the potential of combined gene transfer approaches to enhance multiple DC effector functions and, consequently, accelerate immune-mediated rejection of tumors. Furthermore, the localized expression of these genes more closely resembles the physiological setting and lessens toxicity concerns associated with systemic administration of DC maturation agents, such as cytokines or microbial products.

The issue of DC persistence has also received attention in efforts to generate more effective vaccines. Investigators have gene-modified mouse hematopoietic stem cells with a model tumor antigen and have demonstrated transgene expression in DCs in recipients of gene-modified stem cells (Cui et al., 2003). These gene-modified stem cells, in combination with antigen-specific T cells, soluble Flt3 ligand, and anti-CD40, were demonstrated to inhibit tumor growth. In addition, adoptive transfer of gene-modified stem cells was more effective than transfer of gene-modified DCs. Prolonged production of stem cell-derived antigen-expressing DCs with improved lymphoid localizing ability may be potential reasons for antitumor activity superior to that of transferred differentiated DCs. Although technically more challenging at present than manipulating DCs, this stem cell approach could find application in the clinic after optimization and further validation.

Other methods to increase the survival of DCs include modification with genes encoding antiapoptotic molecules. Cotransfer of genes encoding a model tumor antigen and either of several apoptosis inhibitors was demonstrated to prolong the survival of DCs and enhance the generation of antigen-specific T cells in vivo (Kim et al., 2003). Using a gene gun to deliver DNA directly into mouse skin, the authors observed improved protection from tumors in mice receiving DNA encoding both antigen and antiapoptotic molecules. Investigations are also underway to improve DC lifespan by reducing their sensitivity to tumor-derived inhibition. Transduction of DCs with antiapoptotic molecules including FLIP, XIAP/hILP, dominant-negative procaspase-9, and HSP70 can confer resistance to melanoma-induced apoptosis (Balkir et al., 2004).

Some limitations in the use of transduced DCs exist, however, such as the availability and generation of large numbers of these cells and the determination of optimal DC preparation and delivery route. Addressing the issue of response initiation can have dramatic effects by itself in some mouse tumor models, but attempts to extend these studies to the clinic have met with limited success. Clearly, there are limitations to supplying antigen or inflammatory signals alone. Perhaps paramount in these limitations is that this approach relies on stimulating the existing immune repertoire of the patient, which may often be deficient or suppressed due to tolerance induction by the tumor. Even with the best antigen presentation, an adaptive immune response cannot proceed if specific T cells are lacking.

Stage 2: The Requirement for Specific T Cells

The second crucial requirement in the generation of an effective immune response involves the existence of antigen-specific T cells with which DCs can interact (Figure 1, stage 2). Although tumor-reactive T cells have been detected in some cancer patients, these are restricted to a limited proportion of malignancies. In the majority of cases patients have become tolerant to their tumors, and tumor-specific T cells have either been deleted, are unresponsive to tumors, react only with non-physiological targets such as peptide-pulsed APC, or are not present in large enough numbers to eliminate tumors.

This high level of tolerance in some patients may pose a significant barrier to therapeutic vaccination. One avenue to increase the number of T cells that can respond to tumors is to genetically engineer T cells of a cancer patient to provide the T cells with anti-tumor specificity. One way of achieving this is by gene-modi-

fying T cells in vitro with retroviral vectors encoding TCR α and β chains of desired specificity. Adoptive transfer of these gene-modified T cells has been found to confer antitumor reactivity in vitro and in animal models (Chamoto et al., 2004; Clay et al., 1999; Stanislawski et al., 2001).

Another interesting strategy to provide tumor-reactive T cells for prolonged periods involves introduction of genes encoding TCR α and β chains into hematopoietic precursor cells. T cells derived from precursor cells in vivo were demonstrated to become activated in vivo after immunization and to proliferate and secrete cytokines in response to antigen in vitro (Yang et al., 2002). Tumor-reactive T cells generated in this manner may have a more suitable in vivo phenotype than those generated in vitro, which may lead to better trafficking and activity in vivo. In addition, their continued production from precursors may provide a more prolonged assault on tumors and perhaps protection from relapse. Suitable TCR may be derived from rare responding patients or from transgenic mice immunized with tumor antigen (Stanislawski et al., 2001). However, there are drawbacks to the clinical application of this approach, including the large number of tumor antigen-specific TCR genes that would need to be generated to cater for HLA types of every cancer patient. In addition, TCRs predominantly recognize only protein antigens, excluding a multitude of carbohydrate and glycolipid tumor antigens.

An alternative to using TCR genes to redirect T cells is to use the specificity of antibodies against TAA. An extensive selection of TAAs has been identified, and a range of specific monoclonal antibodies is available, which have higher affinities and broader recognition than TCRs. These can be engineered to behave as TCRs by constructing genes encoding chimeric antigen receptors (CARs). CARs link antigen recognition with T cell activation via fusion of the antigen binding domains of a monoclonal antibody with the intracellular signaling domains of molecules of the TCR complex in a single integral membrane protein. Genetic engineering to produce CAR-modified T cells provides an attractive means of generating large numbers of tumor-specific effector cells for adoptive immunotherapy.

Mouse monoclonal antibodies specific for a wide range of tumor antigens are readily produced, and T cells expressing any of a diverse range of CARs recognizing different tumor antigens can now potentially be used to treat multiple types of malignancies (Sadelain et al., 2003). This approach can be used to endow tumor specificity on both $CD4^+$ and $CD8^+$ T cells, and efficiently bypasses some tumor escape mechanisms, such as MHC downregulation, by recognizing tumor antigens in a non-MHC-restricted manner. Additionally, a single gene construct encoding a particular CAR can be used for multiple patients whose tumors bear common antigens. T cell modification with these genes has resulted in highly specific antigen recognition, cytokine secretion, and killing in vitro, and also in retarded tumor growth in intraperitoneal and subcutaneous murine models (Sadelain et al., 2003). In addition to these promising studies, success with genetically modified CTL has led to initiation of phase I clinical trials for relapsed B cell lymphoma (Wang et al., 2004), neuroblastoma (Rossig et al., 2002), and ovarian cancer (Parker et al., 2000).

Despite the recognition and killing of tumors achieved by CARs against a range of malignancies, success has been limited to murine models of early disease or disseminated tumors. Treatment of established, solid tumors poses a greater challenge, most likely due to failure of T cells to expand, localize at tumor sites, and maintain their function in vivo, issues that are addressed in the following text.

Stage 3: Expansion and Differentiation of Tumor-Specific Lymphocytes

After successful interaction with APCs, activated T cells undergo clonal expansion and differentiation into effector and memory cell populations. The majority of these activated T cells will then leave the lymphoid tissue and enter the circulation (see Figure 1, stage 3). T cell proliferation and expansion to high numbers represents a key determinant of in vivo efficacy.

Although earlier designs of CAR genes could endow activated T cells with effector function, they were not able to induce proliferation in response to antigen. To overcome this limitation, more recent designs of CAR combine antigen recognition with simultaneous costimulation by incorporation of signaling domains from the well-characterized costimulatory molecule CD28 (Maher et al., 2002) and, more recently, other costimulatory molecules such as OX-40 (CD134) and 4-1BB (CD137) into the CAR (Finney et al., 2004). This results in superior signaling capacity, when compared with TCR domains alone, with increased capacity to secrete cytokines and to induce T cell proliferation in vitro.

Importantly, this approach can also achieve inhibition of tumor growth and metastases in vivo (Haynes et al., 2002b). Complete tumor regression was reported when chimeric receptor-modified T lymphocytes, recognizing the human breast cancer-associated antigen erbB2, were injected directly into the subcutaneous erbB2-expressing tumors of mice (Altenschmidt et al., 1997). Tumor growth retardation and prolonged survival has been demonstrated by Pinthus et al. in a similar model, where mice were treated with CAR-modified T cells recognizing erbB2 (Pinthus et al., 2003). Inhibition of tumor growth has also been achieved for tumors expressing erbB2 and carcinoembryonic antigen (CEA), in a variety of tumor types, in both subcutaneous and intraperitoneal murine models after systemic injection of gene-modified T cells (Haynes et al., 2002a, 2002b).

Given the importance of costimulation in the optimal activation and expansion of T cells, genetic modification of tumors with costimulatory molecules such as B7-1 (CD80) and B7-2 (CD86) has been investigated in murine models. These studies have indicated that transfection of CD80 and CD86 into tumor cells and subsequent inoculation into mice can induce T cell activation and cytokine secretion, promote tumor regression, and protect mice against subsequent challenge with parental tumors (Townsend and Allison, 1993).

Costimulatory molecules combined with antigen can also be supplied in recombinant viral vectors and immunization with these can lead to prolonged survival of tumor-bearing mice (Chamberlain et al., 1996). Interest-

ingly, in this and other studies, CD80 was demonstrated to possess greater immunostimulatory capacity than CD86. Further enhancement of antitumor activity has also been observed in mice receiving vaccination with viral vectors encoding multiple costimulatory molecules or costimulatory molecules combined with cytokine genes (Carroll et al., 1998).

Encouraged by these and other studies, a clinical trial of metastatic melanoma patients was initiated in which a vaccinia vector encoding multiple tumor epitopes, together with CD80 and CD86 costimulatory genes, was administered to patients in combination with soluble melanoma peptides and systemic GM-CSF. Specific CTL precursor frequencies were shown to briefly increase after injection (Zajac et al., 2003), and regression of individual metastases was reported in 3 of 18 patients, with no major clinical toxicity reported. Further optimization of combination strategies such as this may lead to enhanced tumor regression.

Additional costimulatory molecules, such as TNF-family members OX40 ligand and 4-1BB ligand, have also been reported to have antitumor effects in mice when introduced into tumor cells by viral vector. Engagement of these molecules upon T cell activation has been shown to increase $CD4^+$ and $CD8^+$ T cell proliferation and cytokine production and long-term persistence. Strong CTL responses leading to significant suppression of tumor growth and survival advantages were demonstrated in mice treated intratumorally with viral vectors containing these genes (Shuford et al., 1997; Andarini et al., 2004).

Antitumor effects were further enhanced when two costimulatory molecules were coexpressed in a poorly immunogenic mouse sarcoma model. Subcutaneous injection of 4-1BB ligand/CD80-transfected tumors enhanced the induction of effector CTL and tumor rejection, although neither 4-1BBL nor CD80 single transfectants were effective in this model (Melero et al., 1998). In a similar study, ligands for 4-1BB and CD28 were transfected into artificial antigen-presenting cells and found to induce activation, rapid expansion, and increased survival of antigen-specific $CD8^+$ T cells in vitro (Maus et al., 2002). These studies suggest a synergistic effect between these costimulatory pathways.

In addition to the modification of T cells and tumors, DCs can be modified to express both tumor antigens and costimulatory molecules leading to antitumor effector and memory CTL responses. The use of DC costimulatory molecules RANK (receptor activator of NF-κB) and CD40 and/or their T cell-expressed ligands RANKL and CD40L were investigated by immunization of mice with DCs expressing a combination of these molecules (Wiethe et al., 2003). Significantly elevated numbers of antigen-specific, IFN-γ-secreting effector and memory T cells were induced in immunized mice in comparison to nonimmunized mice or mice receiving DCs transduced with a control vector. Augmentation of CTL responses was correlated with upregulation of CD80 and CD86 expression in DCs transduced with costimulatory molecules, suggesting enhanced antitumor T cell activation and survival may also be achieved with this type of immunization (Wiethe et al., 2003). Another useful combination is the dual use of cytokine and costimulatory gene transfer, which has been found to synergistically augment tumor regression in mouse models in comparison to tumors transduced with single genes (Putzer et al., 1997). These results have important implications for improved tumor antigen-expressing vaccines and again emphasize the improved success of targeting multiple aspects of the immune response to achieve antitumor immunity.

Genes encoding cytokines can also be used to enhance T cell expansion and survival. Toward this aim, genes encoding the T cell growth factors IL-2 or IL-7 have been utilized to transduce tumor cells, thereby providing a constitutive source of cytokine at the tumor site. Growth inhibition of tumors has been observed after expression of IL-2 or IL-7 (Bowman et al., 1998; Hock et al., 1993).

At present, no immune strategies can enable the immune system to respond against cancer to the same degree as it responds against infectious agents. Common infectious agents such as viruses or bacteria are known to be potent immunogens and to stimulate high-level proliferation of antigen-specific effector and memory cell populations in vivo. The immune response against a virus can lead to enormous expansion of virus-specific T cells, achieving over 25% of total circulating T cells and long-lasting immunity, providing protection against subsequent challenges. This is in stark contrast to the poor immunostimulatory capacity of tumors, where T cell expansion, even against the more immunogenic malignancies such as melanoma, rarely exceeds 4% and is more often less than 1%.

In continued efforts to improve the activation, expansion and persistence of specific T cells in tumor immunotherapy, researchers have sought to combine the potential for high-level activation and expansion, exhibited in response to powerful immunogens, with the ability to react to tumors by creating T cells with two specificities. In this approach, T cell populations possessing a highly immunogenic endogenous specificity, such as viral or bacterial specificity, are genetically modified to express CARs with antitumor reactivity. This dual-specific application of gene therapy aims to efficiently stimulate T cell activation and expansion through the endogenous TCR, via immunization with a potent immunogen, while antitumor reactivity could be carried out by the genetically engineered antitumor receptor.

CAR genes have been used to generate dual-specific cells from T cells with endogenous TCR specificity for EBV (Rossig et al., 2002), CMV (Heemskerk et al., 2004), or allogeneic antigen (Kershaw et al., 2002b). Dual-specific T cells have been demonstrated to respond through both endogenous TCR and CAR in vitro and to proliferate and inhibit tumors in vivo after adoptive transfer and immunization (Kershaw et al., 2002b).

The therapeutic application of these T cells would involve their transfer into cancer patients followed by immunization with the powerful immunogen. This approach simultaneously circumvents potential impasses at several crucial immune response steps by assigning responsibility for T cell activation and expansion to a highly responsive yet separate receptor from that encoding tumor reactivity.

Therefore genetic modification strategies can be used to initiate and amplify an immune response to tumors, but this may prove fruitless if T cells are unable

to effectively home to and infiltrate tumors. Enhancing T cell migration to tumors is therefore an important goal of immunotherapists.

Stage 4: Migration and Recruitment of Immune Cells

The secretion of chemokines by cells within challenged tissues normally creates a chemoattractant gradient that leads to recruitment of activated T cells to the site (Figure 1, stage 4). A range of innate immune system cells including macrophages, neutrophils, dendritic cells, and NK cells may also be attracted to the site, amplifying the immune response, and participating in the resolution of the threat.

However, tumor tissue may not produce appropriate chemokines, and the lack of efficient trafficking of specific T cells to tumor locations is one factor that may restrict robust antitumor responses. Indeed, induction of antitumor immune responses has been shown to correlate with T cell infiltration at the tumor site, and transfer of tumor-reactive T cells has been shown to be more effective at eliminating tumors when administered within the immediate proximity of tumors in selected animal models (Pinthus et al., 2003). If localization of leukocytes can be reproducibly enhanced to a range of advanced physiologically relevant tumors, better tumor control may be achieved.

Expression of specific chemokines at the tumor site is one approach that may enable specific T cells bearing the relevant ligands to be better directed toward tumors. Genetic modification of tumors to express chemokines such as lymphotactin (XCL1) has been shown to attract $CD4^+$ and $CD8^+$ antigen-specific T cells in vitro and to eradicate well-established subcutaneous tumors in vivo when used in combination with adoptive T cell transfer (Huang et al., 2002a). This was in comparison to mice treated with either the chemokine gene or T cells alone. Similar reports of T cell infiltration and tumor regression have been observed in other mouse studies where tumor cells were modified with chemokine genes, including CCL27 (Gao et al., 2003a), macrophage-derived chemokine gene (CCL22) (Guo et al., 2002), IFN-γ-inducible protein 10 (IP-10, CXCL10) (Huang et al., 2002b), and macrophage inflammatory protein 3α (MIP-3α, CCL20) (Fushimi et al., 2000). Combinations of chemokine genes have also been demonstrated to stimulate proliferation of $CD8^+$ T cells and to chemoattract T cells more efficiently than either chemokine alone. In a mouse model, intratumoral injection of both XCL1 and CXCL10 chemokine genes, together with adoptive T cell transfer, not only significantly enhanced T cell infiltration of tumors when compared to either gene alone, but the majority of treated mice were tumor-free (Huang and Xiang, 2004).

Another molecule of interest is the TNF superfamily ligand LIGHT, which regulates T cell immune responses by signaling through the herpesvirus entry mediator (HVEM) and the lymphotoxin-β receptor. Gene transfer of LIGHT into tumor cells has been shown to induce massive infiltration of T cells that correlated with increased expression of chemokines and adhesion molecules and eradication of established tumors (Yu et al., 2004).

As an alternative to tumor cell modification, DCs modified to secrete T cell-attracting chemokines may also find application in tumor immunotherapy. Intratumoral injection of DCs expressing CCL21/SLC has been demonstrated to enhance the antitumor response in mice when compared to treatments involving fibroblasts expressing CCL21 or DCs lacking CCL21 (Yang et al., 2004). Increased recruitment of T cells was associated with the antitumor response. It was thought that transferred DCs persisting at the injection site attracted T cells that could then be stimulated in situ within tumors and exert subsequent effector function locally.

As further examples of how combinations of strategies can be used to enhance the antitumor response, genes encoding lymphocyte-attracting chemokines can be successfully combined with cytokine genes. Intratumoral coinjection of two adenoviruses, one encoding the chemokine CXCL10 and another encoding IL-12, resulted in marked antitumor synergy. This generated a powerful tumor-specific T cell response with both $CD4^+$ and $CD8^+$ T cells present in the infiltrate of regressing tumors (Narvaiza et al., 2000). Similarly, coinjection of adenovirus vectors coding for CXC chemokines and IL-12 into murine adenocarcinoma or fibrosarcoma resulted in considerable tumor regression and increased survival of injected mice. However, T cell infiltration was not specifically demonstrated in this case (Palmer et al., 2001). In another study, T cell activation and infiltration into tumors in association with a type 1 immune response was demonstrated upon coinjection of both IP-10 and IL-18 genes at the same tumor nodule, causing complete regression of established tumors in 8 of 10 mice, compared with either gene alone (Liu et al., 2002).

An alternate approach to modifying cells at the tumor site is to engineer T cells with receptors for chemokines expressed on tumors. Growth-regulated oncogene-α (Gro-α, CXCL1) was demonstrated to be secreted by several human tumor cell lines, but T cells did not express the appropriate chemokine receptor, CXCR2 (Kershaw et al., 2002a). In subsequent experiments, T cells were retrovirally transduced with a vector encoding CXCR2, and a chemotactic response was demonstrated in vitro toward tumor-derived CXCL1 (Kershaw et al., 2002a). Redirecting T cell migration toward tumor-secreted chemokines may enhance effector cell localization at the tumor site.

The use of chemokine and cytokine genes to both activate and attract tumor-specific T cells may be further enhanced by the inclusion of genes encoding adhesion molecules, such as the intercellular adhesion molecule-1 (ICAM-1), which has been shown to facilitate cellular migration into inflammatory sites and cytolytic interaction with tumors (Sartor et al., 1995) Aiding these types of interactions between lymphocytes and tumors may result in more efficient tumor targeting and thus prove beneficial to achieving tumor eradication.

Although localization of immune cells to tumors can be genetically augmented, simply getting to the site is not sufficient to eradicate tumors. Immune cells must be able to exert robust effector and auxiliary functions within tumors, and enhanced action might be expected if these functions can be maintained for prolonged periods of time.

Stage 5: Persistence and Enhancing Antitumor Functions

Approaches to enhance persistence of T cells may involve genetic modification of tumors with costimulatory molecules such as CD80 and CD86, which deliver survival signals to T cells (see "Stage 3" text above). Direct modification of T cells with receptors such as CD28, CD134, and CD137, mentioned previously in Stage 3 can also enhance T cell persistence. Increased T cell survival through the use of costimulatory genes likely results from inhibition of apoptosis, but direct manipulation of genes important in apoptosis of T cells is another method of inhibiting the early demise of specific T cells. Indeed, genes encoding overexpression of the antiapoptotic molecules Bcl-2 or Bcl-X(L) have been demonstrated to enhance the survival of T cells (Eaton et al., 2002; Lin and Wang, 2002).

Along with the importance of long-term persistence of tumor-specific T cells is the ability of these cells to execute potent antitumor effects. Potential mechanisms to enhance the cytolytic activity of lymphocytes include gene transfer into tumor of ligands for apoptosis-inducing cytokines such as lymphotoxin β (Browning et al., 1996) and tumor necrosis factor (TNF) (Gnant et al., 1999). Tumor regression and growth inhibition has been demonstrated upon subsequent systemic administration of the relevant T cell-derived cytokines. It is hoped that by genetically increasing the susceptibility of tumors with receptors to apoptosis-inducing cytokines they may become more sensitive to tumor-specific T cells or doses of these cytotoxic agents.

Other approaches utilizing members of the TNF superfamily include TNF-related apoptosis-inducing ligand (TRAIL), which is thought to be nontoxic to normal cells while killing a broad range of tumor cells. Gene transfer of TRAIL into tumor cells has induced apoptosis and blocked growth of TRAIL receptor-expressing human tumor cell lines derived from colorectal, lung, prostate, and liver cancer in vitro, and local administration of viral vectors expressing the TRAIL gene has been demonstrated to be useful in treating established tumors in animals (Jacob et al., 2004; Mohr et al., 2004). However, expression of the TRAIL gene is likely to require regulation by a tumor-specific promoter to prevent potential liver toxicity demonstrated in some experiments (Armeanu et al., 2003; Lin et al., 2002).

Many of these attempts to enhance tumor susceptibility to immune eradication are reliant on intratumoral gene delivery, which can induce a strong and specific immune response in murine studies. However, this approach would likely be more limited in humans to easily accessible tumors. In addition, as with many approaches advocating genetic modification of tumors in situ, success may be dependent on improvements in vector technology that enable modification of the majority of tumor cells. An alternative to this is to transfer genes enabling increased cytotoxic functions into T cells. This was shown to be possible in experiments where tumor-reactive lymphocytes were transduced with the gene for TNF in an attempt to deliver high concentrations of TNF to the tumor site without dose-limiting systemic toxicity (Hwu et al., 1993). However, TNF production was low and not durable, and future therapeutic applications of this may depend on the development of vectors enabling higher production of this cytokine in an inducible manner.

Another important factor to consider is the secretion of immunomodulatory cytokines by tumor cells and surrounding stromal cells. This can have dramatic effects on the persistence of tumor-reactive T cells and the maintenance of their activity within tumors. For example, a T cell inhibitory cytokine, transforming growth factor-β (TGF-β), has been demonstrated to be secreted by a variety of tumors, resulting in inhibition of antitumor immunity. Strategies aimed at circumventing the effects of TGF-β include genetic modification of tumor-reactive T cells with a dominant-negative TGF-β receptor. T cells modified in this manner were found to possess enhanced antitumor function in vitro when compared to unmodified T cells (Bollard et al., 2002). This tumor-mediated immunosuppression may in part explain previous failures of adoptively transferred T cells to mediate antitumor effects. Bestowing resistance to these effects on T cells by gene transfer may prove an important strategy to allow T cells to better defend themselves in their confrontation with tumors.

Technical and Safety Issues and Concluding Remarks

In this review, we have covered a wide range of genetic modification strategies that are aimed at inducing an immune response against tumors. However, it is important to consider these genetic strategies in relation to nongenetic approaches to immunotherapy where the major areas of investigation include vaccines, monoclonal antibodies, cytokines and adoptive cell transfer.

Vaccines can take one of several forms, including tumor-associated proteins or peptides either alone, with adjuvant, or pulsed onto dendritic cells. The most promising results with vaccines have been observed against melanoma and viral-induced malignancies, which have demonstrated that the immune system can be manipulated to produce dramatic antitumor responses against some malignancies (Rosenberg, 2001). However, although precursor frequencies of tumor-specific T cells can be increased after vaccination, complete durable responses are rare, and vaccines frequently fail to prevent tumor growth (Rosenberg et al., 2004). A large field of study focuses on loading dendritic cells (DCs) with tumor antigens in an effort to better stimulate endogenous T cell responses. Tumor antigen peptide- or protein-pulsed DCs can be effective at raising antitumor responses in mice, and multiple clinical trials have evaluated the safety and efficacy of cancer vaccines based on tumor antigen-pulsed DCs (Engleman, 2003). However, this approach has disadvantages associated with the short duration of antigen-MHC complexes and the requirement for matching defined peptides with MHC haplotypes. In contrast, modification of DCs with genes encoding tumor antigen can result in prolonged presentation of multiple epitopes by various class I and class II MHCs, with the ability to activate antigen-specific T cells. Nevertheless, vaccines are appealing from the point of view of ease of administration, and future advances in our understanding of immune activation and maintenance may lead to more effective vaccination methods.

Table 1. Summary of Genetic Modification Strategies to Enhance Tumor Immunity

Immune Stage	Gene	Cell	Reference	Comments	Clinical Trial
Initiation	Antigen	DCs	(Ribas et al., 2002)	Induction of tumor-specific T cells and enhanced survival of tumor-bearing mice	yes
	CD40L	DCs	(Kikuchi et al., 2000)	Regression of day 8 s.c. murine tumors, survival advantage, and protection against subsequent challenge	no
	CD40L	Tumor	(Kikuchi and Crystal, 1999)	Sustained regression of established s.c. tumors and protection against subsequent challenge in mice	yes
	GM-CSF	Tumor	(Dranoff et al., 1993; Levitsky et al., 1996)	Enhanced survival of tumor-bearing mice	yes
	Flt3-L	Tumor	(Mach et al., 2000)	Prevention of tumor growth in mice	no
	IL-1β	Tumor	(Bjorkdahl et al., 2000; Saijo et al., 2002)	Differential effects in mice depending on use of active or inactive forms	no
	IL-12	DCs	(Nishioka et al., 1999)	Regression of established (day 7) weakly immunogenic tumors in mice	yes
	IL-18	DCs	(Tatsumi et al., 2002)	Inhibition of day 7 s.c. tumors in mice	no
	IL-12 + IL-18	DCs	(Tatsumi et al., 2003)	Regression of day 7 s.c. tumors and increased survival	no
	Antigen	Stem cells	(Cui et al., 2003)	Decreased tumour growth and increased survival in treated mice	no
	Antiapoptotic molecules	DCs	(Kim et al., 2003)	Increased DC survival in vitro and enhanced T cell generation and tumor inhibition in mice	no
Specific T cells	TCR	T cells	(Chamoto et al., 2004)	Eradication of small A20-OVA tumors in mice	yes
	TCR	Progenitors	(Yang et al., 2002)	Prolonged production of specific T cells	no
	CARs	T cells	(Sadelain et al., 2003)	Regression of early or deseminated tumors in mice	yes
Expansion	CARs + CD28	T cells	(Finney et al., 2004; Haynes et al., 2002b)	T cell proliferation in vitro and eradication of 5 day lung metastases in mice	no
	CD80, CD86	Tumor	(Li et al., 1994; Townsend and Allison, 1993)	Eradication 8 day s.c. tumors	yes
	OX40, 4-1BB	Tumor	(Andarini et al., 2004)	Tumor inhibition and increased tumor-specific T cells	no
	RANK/RANK-L	DCs	(Wiethe et al., 2003)	Enhanced T cell response in mice	no
	IL-2, IL-7, IL-4	Tumor	(Bowman et al., 1998; Hock et al., 1993; Levitsky et al., 1996)	Leukocyte infiltration, reduced tumorigenicity, enhanced survival in mice	yes
	CAR	Dual-specific T cells	(Kershaw et al., 2002b)	Tumor inhibition and T cell expansion in mice	yes
Leukocyte recruitment	Chemokine	Tumor	(Gao et al., 2003a)	Lymphocyte infiltrate and reduced tumorigenicity	no
	LIGHT	Tumor	(Yu et al., 2004)	Massive leukocyte infiltrate and eradication of established allogeneic tumors	no
	Chemokine	DCs	(Yang et al., 2004)	Eradication of 5-day s.c. tumors in 60% of mice	no
	Chemokine receptor	T cells	(Kershaw et al., 2002a)	T cell migration in vitro	no
	ICAM-1	Tumor	(Sartor et al., 1995)	Reduced tumorigenicity	no
Persistent and maintaining activity	Bcl-2	T cells	(Lin and Wang, 2002)	Enhanced survival of T cells in vitro	no
	TNF-R	Tumor	(Gnant et al., 1999)	Tumor inhibiton	no
	TRAIL	Tumor	(Lin et al., 2002)	Tumor inhibition	no
	TNF	T cells	(Hwu et al., 1993)	Constitutive expression by T cells in vitro	no
	Dominant – ve TGFβ-R	T cells	(Bollard et al., 2002)	T cell resistance to TGF-β-mediated inhibition in vitro	no

Strategies are grouped according to the most relevant stage of the immune response involved. A diverse range of genes and target cell types is employed to effectively stimulate an antitumor response. An appraisal of the relative capacity of each approach to impact on tumor immunity can be obtained from the Comments column. References are representative of each area.

Monoclonal antibodies, either alone or conjugated to drugs or toxins, are gaining acceptance in the clinic and can be useful against lymphoma or breast cancer (von Mehren et al., 2003). Humanized antibody can be produced in large amounts and easily administered to patients. However, issues such as low-level penetration of tumor, poor ability to recruit appropriate effector function, and toxicity have resulted in limited application and low response rates for most malignancies.

Similarly, cytokines including interferons and IL-2 can

significantly impact on some cancers, but current clinical effectiveness is largely restricted to melanoma, renal cell carcinoma, and some hematologic malignancies, where low-to-moderate response rates have been observed (Smyth et al., 2004). Although large amounts of recombinant cytokines can be produced and easily administered, severe toxicity is often observed with this type of therapy that activates immune mechanisms in a largely nonspecific manner.

Adoptive immunotherapy, involving the ex vivo generation of large numbers of activated tumor-reactive lymphocytes, can have dramatic effects on established tumors particularly when used in combination with IL-2 (Rosenberg, 2001). However, the approach is cumbersome at present and again responses are limited to only a proportion of patients with melanoma.

Thus, the above nongenetic approaches can have advantages in certain situations, and some remarkable responses have been documented. However, the majority of malignancies do not respond to current immunotherapies, and issues regarding toxicity are concerning. Clearly, there is a need for more precise therapies that can enhance specific immune elements against a broader range of cancers.

Elucidation of the many molecular and genetic events underlying each of the key steps involved in a successful immune response has led to the genetic manipulation of many immune targets in attempts to reinforce crucial components of the anti-tumor response. Here, we have outlined many strategies utilizing genetic modification in attempts to boost the immune system against cancer that are summarized in Table 1. An estimate of the relative effectiveness of each strategy can be gained from comparison of the "best achieved to date" comments for each approach. Some strategies have only progressed as far as demonstrating function in vitro or tumor inhibition in simple mouse models, whereas others have achieved tumor eradication in mice and have progressed to clinical trial. However, in the clinic, responses are infrequent and rarely durable, and are limited to a minority of patients with one of only a limited range of malignancies. The studies detailed in this review suggest a need to combine several approaches to enhance multiple aspects of the immune response in order to generate robust anti-tumor responses. For example, optimal therapy may involve transfer of DCs transduced with genes encoding antigen, in combination with supplying autologous T cells modified to respond to antigen, along with a vector for expression of appropriate chemokine in tumor tissue.

At present, technological limitations render genetic modification of DCs or T cells laborious and expensive. However, improved approaches may lead to effective in vivo gene transfer that enables stable gene expression. Similarly, genetic modification of tumor is limited at present. Better targeted, more stable transgene expression in the majority of tumor cells may enhance many of the approaches described above. Future improvements in vector design and specific targeting of tumors may make this a reality.

A number of vector systems are available for investigators to choose from that have a variety of advantages and disadvantages (Kay et al., 2001). Nonviral vectors can be used to deliver large gene "payloads" into cells although their transfection efficiency is currently limited. Viral vectors include retrovirus that can produce stable gene expression in cells and their progeny over prolonged time periods due to genomic integration, but active cell division is required for integration and the amount of exogenous DNA is limited to approximately 8000 bp. Lentiviral vectors can be used to stably express genes in nondividing cells but, again, genes are of limited size, and there are some misgivings about these vectors due to their derivation from HIV. Vaccinia, adenovirus and others can incorporate larger amounts of DNA, but expression is transient and their application in vivo may be limited by preexisting immunity.

Safety issues are also of concern with cutting edge technology such as that embodied in the approaches described in this review. Of particular concern is the safety of viral vectors used for in vivo gene modification. Recent incidents involving toxicity after gene transfer through adenovirus highlight the need for a detailed appraisal of risk for each area of application (Somia and Verma, 2000). Also of concern is potential malignant transformation of immune system cells following modification with vectors that can integrate into the genome potentially disrupting oncogene regulation. Recent examples of this in children receiving retrovirally gene-modified stem cells to treat immune deficiency highlight this concern (Hacein-Bey-Abina et al., 2003). However, potential safety concerns need to be weighed against potential benefit, and greater risk may be justified in patients with otherwise terminal disease.

Our knowledge of key cellular and molecular stages in immunity together with our ability to gene modify cells provides us with extraordinary opportunities to circumvent impasses in immunity to tumors. Genes may be used with greater precision and lower toxicity than systemically applied adjuvants. Technological advances and an increasing understanding of immunoregulation may lead to combined immunomodulatory gene approaches with enhanced effectiveness for the treatment of cancer patients.

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References

Altenschmidt, U., Klundt, E., and Groner, B. (1997). Adoptive transfer of in vitro-targeted, activated T lymphocytes results in total tumor regression. J. Immunol. *159*, 5509–5515.

Andarini, S., Kikuchi, T., Nukiwa, M., Pradono, P., Suzuki, T., Ohkouchi, S., Inoue, A., Maemondo, M., Ishii, N., Saijo, Y., et al. (2004).

Adenovirus vector-mediated in vivo gene transfer of OX40 ligand to tumor cells enhances antitumor immunity of tumor-bearing hosts. Cancer Res. *64*, 3281–3287.

Armeanu, S., Lauer, U.M., Smirnow, I., Schenk, M., Weiss, T.S., Gregor, M., and Bitzer, M. (2003). Adenoviral gene transfer of tumor necrosis factor-related apoptosis-inducing ligand overcomes an impaired response of hepatoma cells but causes severe apoptosis in primary human hepatocytes. Cancer Res. *63*, 2369–2372.

Balkir, L., Tourkova, I.L., Makarenkova, V.P., Shurin, G.V., Robbins, P.D., Yin, X.M., Chatta, G., and Shurin, M.R. (2004). Comparative analysis of dendritic cells transduced with different anti-apoptotic molecules: sensitivity to tumor-induced apoptosis. J. Gene Med. *6*, 537–544.

Bjorkdahl, O., Dohlsten, M., and Sjogren, H.O. (2000). Vaccination with B16 melanoma cells expressing a secreted form of interleukin-1beta induces tumor growth inhibition and an enhanced immunity against the wild-type B16 tumor. Cancer Gene Ther. *7*, 1365–1374.

Boczkowski, D., Nair, S.K., Snyder, D., and Gilboa, E. (1996). Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J. Exp. Med. *184*, 465–472.

Bollard, C.M., Rossig, C., Calonge, M.J., Huls, M.H., Wagner, H.J., Massague, J., Brenner, M.K., Heslop, H.E., and Rooney, C.M. (2002). Adapting a transforming growth factor beta-related tumor protection strategy to enhance antitumor immunity. Blood *99*, 3179–3187.

Bowman, L., Grossmann, M., Rill, D., Brown, M., Zhong, W.Y., Alexander, B., Leimig, T., Coustan-Smith, E., Campana, D., Jenkins, J., et al. (1998). IL-2 adenovector-transduced autologous tumor cells induce antitumor immune responses in patients with neuroblastoma. Blood *92*, 1941–1949.

Browning, J.L., Miatkowski, K., Sizing, I., Griffiths, D., Zafari, M., Benjamin, C.D., Meier, W., and Mackay, F. (1996). Signaling through the lymphotoxin beta receptor induces the death of some adenocarcinoma tumor lines. J. Exp. Med. *183*, 867–878.

Buelens, C., Verhasselt, V., De Groote, D., Thielemans, K., Goldman, M., and Willems, F. (1997). Human dendritic cell responses to lipopolysaccharide and CD40 ligation are differentially regulated by interleukin-10. Eur. J. Immunol. *27*, 1848–1852.

Carroll, M.W., Overwijk, W.W., Surman, D.R., Tsung, K., Moss, B., and Restifo, N.P. (1998). Construction and characterization of a triple-recombinant vaccinia virus encoding B7–1, interleukin *12*, and a model tumor antigen. J. Natl. Cancer Inst. *90*, 1881–1887.

Chamberlain, R.S., Carroll, M.W., Bronte, V., Hwu, P., Warren, S., Yang, J.C., Nishimura, M., Moss, B., Rosenberg, S.A., and Restifo, N.P. (1996). Costimulation enhances the active immunotherapy effect of recombinant anticancer vaccines. Cancer Res. *56*, 2832–2836.

Chamoto, K., Tsuji, T., Funamoto, H., Kosaka, A., Matsuzaki, J., Sato, T., Abe, H., Fujio, K., Yamamoto, K., Kitamura, T., et al. (2004). Potentiation of tumor eradication by adoptive immunotherapy with T-cell receptor gene-transduced T-helper type 1 cells. Cancer Res. *64*, 386–390.

Chen, Z., Huang, H., Chang, T., Carlsen, S., Saxena, A., Marr, R., Xing, Z., and Xiang, J. (2002). Enhanced HER-2/neu-specific antitumor immunity by cotransduction of mouse dendritic cells with two genes encoding HER-2/neu and alpha tumor necrosis factor. Cancer Gene Ther. *9*, 778–786.

Clay, T.M., Custer, M.C., Sachs, J., Hwu, P., Rosenberg, S.A., and Nishimura, M.I. (1999). Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. J. Immunol. *163*, 507–513.

Cui, Y., Kelleher, E., Straley, E., Fuchs, E., Gorski, K., Levitsky, H., Borrello, I., Civin, C.I., Schoenberger, S.P., Cheng, L., et al. (2003). Immunotherapy of established tumors using bone marrow transplantation with antigen gene–modified hematopoietic stem cells. Nat. Med. *9*, 952–958.

Dranoff, G., Jaffee, E., Lazenby, A., Golumbek, P., Levitsky, H., Brose, K., Jackson, V., Hamada, H., Pardoll, D., and Mulligan, R.C. (1993). Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc. Natl. Acad. Sci. USA *90*, 3539–3543.

Eaton, D., Gilham, D.E., O'Neill, A., and Hawkins, R.E. (2002). Retroviral transduction of human peripheral blood lymphocytes with Bcl-X(L) promotes in vitro lymphocyte survival in pro-apoptotic conditions. Gene Ther. *9*, 527–535.

Engleman, E.G. (2003). Dendritic cell-based cancer immunotherapy. Semin. Oncol. *30*, 23–29.

Finney, H.M., Akbar, A.N., and Lawson, A.D. (2004). Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. J. Immunol. *172*, 104–113.

Fushimi, T., Kojima, A., Moore, M.A., and Crystal, R.G. (2000). Macrophage inflammatory protein 3alpha transgene attracts dendritic cells to established murine tumors and suppresses tumor growth. J. Clin. Invest. *105*, 1383–1393.

Gao, J.Q., Tsuda, Y., Katayama, K., Nakayama, T., Hatanaka, Y., Tani, Y., Mizuguchi, H., Hayakawa, T., Yoshie, O., Tsutsumi, Y., et al. (2003a). Antitumor effect by interleukin-11 receptor alpha-locus chemokine/CCL27, introduced into tumor cells through a recombinant adenovirus vector. Cancer Res. *63*, 4420–4425.

Gao, W., Kumar, S., Lotze, M.T., Hanning, C., Robbins, P.D., and Gambotto, A. (2003b). Innate immunity mediated by the cytokine IL-1 homologue 4 (IL-1H4/IL-1F7) induces IL-12-dependent adaptive and profound antitumor immunity. J. Immunol. *170*, 107–113.

Gilboa, E., and Vieweg, J. (2004). Cancer immunotherapy with mRNA-transfected dendritic cells. Immunol. Rev. *199*, 251–263.

Gnant, M.F., Berger, A.C., Huang, J., Puhlmann, M., Wu, P.C., Merino, M.J., Bartlett, D.L., Alexander, H.R., Jr., and Libutti, S.K. (1999). Sensitization of tumor necrosis factor alpha-resistant human melanoma by tumor-specific in vivo transfer of the gene encoding endothelial monocyte-activating polypeptide II using recombinant vaccinia virus. Cancer Res. *59*, 4668–4674.

Guo, J., Wang, B., Zhang, M., Chen, T., Yu, Y., Regulier, E., Homann, H.E., Qin, Z., Ju, D.W., and Cao, X. (2002). Macrophage-derived chemokine gene transfer results in tumor regression in murine lung carcinoma model through efficient induction of antitumor immunity. Gene Ther. *9*, 793–803.

Hacein-Bey-Abina, S., von Kalle, C., Schmidt, M., Le Deist, F., Wulffraat, N., McIntyre, E., Radford, I., Villeval, J.L., Fraser, C.C., Cavazzana-Calvo, M., and Fischer, A. (2003). A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N. Engl. J. Med. *348*, 255–256.

Haynes, N.M., Trapani, J.A., Teng, M.W., Jackson, J.T., Cerruti, L., Jane, S.M., Kershaw, M.H., Smyth, M.J., and Darcy, P.K. (2002a). Rejection of syngeneic colon carcinoma by CTLs expressing single-chain antibody receptors codelivering CD28 costimulation. J. Immunol. *169*, 5780–5786.

Haynes, N.M., Trapani, J.A., Teng, M.W., Jackson, J.T., Cerruti, L., Jane, S.M., Kershaw, M.H., Smyth, M.J., and Darcy, P.K. (2002b). Single-chain antigen recognition receptors that costimulate potent rejection of established experimental tumors. Blood *100*, 3155–3163.

Heemskerk, M.H., Hoogeboom, M., Hagedoorn, R., Kester, M.G., Willemze, R., and Falkenburg, J.H. (2004). Reprogramming of virus-specific T cells into leukemia-reactive T cells using T cell receptor gene transfer. J. Exp. Med. *199*, 885–894.

Hock, H., Dorsch, M., Kunzendorf, U., Qin, Z., Diamantstein, T., and Blankenstein, T. (1993). Mechanisms of rejection induced by tumor cell-targeted gene transfer of interleukin *2*, interleukin *4*, interleukin *7*, tumor necrosis factor, or interferon gamma. Proc. Natl. Acad. Sci. USA *90*, 2774–2778.

Huang, H., and Xiang, J. (2004). Synergistic effect of lymphotactin and interferon gamma-inducible protein-10 transgene expression in T-cell localization and adoptive T-cell therapy of tumors. Int. J. Cancer *109*, 817–825.

Huang, H., Li, F., Gordon, J.R., and Xiang, J. (2002a). Synergistic enhancement of antitumor immunity with adoptively transferred tu-

mor-specific CD4+ and CD8+ T cells and intratumoral lymphotactin transgene expression. Cancer Res. *62*, 2043–2051.

Huang, H., Liu, Y., and Xiang, J. (2002b). Synergistic effect of adoptive T-cell therapy and intratumoral interferon gamma-inducible protein-10 transgene expression in treatment of established tumors. Cell. Immunol. *217*, 12–22.

Hwu, P., Yannelli, J., Kriegler, M., Anderson, W.F., Perez, C., Chiang, Y., Schwarz, S., Cowherd, R., Delgado, C., and Mule, J. (1993). Functional and molecular characterization of tumor-infiltrating lymphocytes transduced with tumor necrosis factor-alpha cDNA for the gene therapy of cancer in humans. J. Immunol. *150*, 4104–4115.

Irvine, K.R., Rao, J.B., Rosenberg, S.A., and Restifo, N.P. (1996). Cytokine enhancement of DNA immunization leads to effective treatment of established pulmonary metastases. J. Immunol. *156*, 238–245.

Jacob, D., Davis, J.J., Zhang, L., Zhu, H., Teraishi, F., and Fang, B. (2004). Suppression of pancreatic tumor growth in the liver by systemic administration of the TRAIL gene driven by the hTERT promoter. Cancer Gene Ther. *12*, 109–115.

Jaffee, E.M., Hruban, R.H., Biedrzycki, B., Laheru, D., Schepers, K., Sauter, P.R., Goemann, M., Coleman, J., Grochow, L., Donehower, R.C., et al. (2001). Novel allogeneic granulocyte-macrophage colony-stimulating factor-secreting tumor vaccine for pancreatic cancer: a phase I trial of safety and immune activation. J. Clin. Oncol. *19*, 145–156.

Kang, W.K., Park, C., Yoon, H.L., Kim, W.S., Yoon, S.S., Lee, M.H., Park, K., Kim, K., Jeong, H.S., Kim, J.A., et al. (2001). Interleukin 12 gene therapy of cancer by peritumoral injection of transduced autologous fibroblasts: outcome of a phase I study. Hum. Gene Ther. *12*, 671–684.

Kay, M.A., Glorioso, J.C., and Naldini, L. (2001). Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat. Med. *7*, 33–40.

Kershaw, M.H., Wang, G., Westwood, J.A., Pachynski, R.K., Tiffany, H.L., Marincola, F.M., Wang, E., Young, H.A., Murphy, P.M., and Hwu, P. (2002a). Redirecting migration of T cells to chemokine secreted from tumors by genetic modification with CXCR2. Hum. Gene Ther. *13*, 1971–1980.

Kershaw, M.H., Westwood, J.A., and Hwu, P. (2002b). Dual-specific T cells combine proliferation and antitumor activity. Nat. Biotechnol. *20*, 1221–1227.

Kikuchi, T., and Crystal, R.G. (1999). Anti-tumor immunity induced by in vivo adenovirus vector-mediated expression of CD40 ligand in tumor cells. Hum. Gene Ther. *10*, 1375–1387.

Kikuchi, T., Moore, M.A., and Crystal, R.G. (2000). Dendritic cells modified to express CD40 ligand elicit therapeutic immunity against preexisting murine tumors. Blood *96*, 91–99.

Kim, T.W., Hung, C.F., Ling, M., Juang, J., He, L., Hardwick, J.M., Kumar, S., and Wu, T.C. (2003). Enhancing DNA vaccine potency by coadministration of DNA encoding antiapoptotic proteins. J. Clin. Invest. *112*, 109–117.

Levitsky, H.I., Montgomery, J., Ahmadzadeh, M., Staveley-O'Carroll, K., Guarnieri, F., Longo, D.L., and Kwak, L.W. (1996). Immunization with granulocyte-macrophage colony-stimulating factor-transduced, but not B7–1-transduced, lymphoma cells primes idiotype-specific T cells and generates potent systemic antitumor immunity. J. Immunol. *156*, 3858–3865.

Li, Y., McGowan, P., Hellstrom, I., Hellstrom, K.E., and Chen, L. (1994). Costimulation of tumor-reactive CD4+ and CD8+ T lymphocytes by B7, a natural ligand for CD28, can be used to treat established mouse melanoma. J. Immunol. *153*, 421–428.

Lin, C.M., and Wang, F.H. (2002). Selective modification of antigen-specific CD4(+) T cells by retroviral-mediated gene transfer and in vitro sensitization with dendritic cells. Clin. Immunol. *104*, 58–66.

Lin, T., Gu, J., Zhang, L., Huang, X., Stephens, L.C., Curley, S.A., and Fang, B. (2002). Targeted expression of green fluorescent protein/tumor necrosis factor-related apoptosis-inducing ligand fusion protein from human telomerase reverse transcriptase promoter elicits antitumor activity without toxic effects on primary human hepatocytes. Cancer Res. *62*, 3620–3625.

Liu, Y., Huang, H., Saxena, A., and Xiang, J. (2002). Intratumoral coinjection of two adenoviral vectors expressing functional interleukin-18 and inducible protein-10, respectively, synergizes to facilitate regression of established tumors. Cancer Gene Ther. *9*, 533–542.

Loskog, A., Dzojic, H., Vikman, S., Ninalga, C., Essand, M., Korsgren, O., and Totterman, T.H. (2004). Adenovirus CD40 ligand gene therapy counteracts immune escape mechanisms in the tumor Microenvironment. J. Immunol. *172*, 7200–7205.

Mach, N., Gillessen, S., Wilson, S.B., Sheehan, C., Mihm, M., and Dranoff, G. (2000). Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. Cancer Res. *60*, 3239–3246.

Maher, J., Brentjens, R.J., Gunset, G., Riviere, I., and Sadelain, M. (2002). Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRzeta /CD28 receptor. Nat. Biotechnol. *20*, 70–75.

Maus, M.V., Thomas, A.K., Leonard, D.G., Allman, D., Addya, K., Schlienger, K., Riley, J.L., and June, C.H. (2002). Ex vivo expansion of polyclonal and antigen-specific cytotoxic T lymphocytes by artificial APCs expressing ligands for the T-cell receptor, CD28 and 4–1BB. Nat. Biotechnol. *20*, 143–148.

Melero, I., Bach, N., Hellstrom, K.E., Aruffo, A., Mittler, R.S., and Chen, L. (1998). Amplification of tumor immunity by gene transfer of the co-stimulatory 4–1BB ligand: synergy with the CD28 co-stimulatory pathway. Eur. J. Immunol. *28*, 1116–1121.

Mohr, A., Henderson, G., Dudus, L., Herr, I., Kuerschner, T., Debatin, K.M., Weiher, H., Fisher, K.J., and Zwacka, R.M. (2004). AAV-encoded expression of TRAIL in experimental human colorectal cancer leads to tumor regression. Gene Ther. *11*, 534–543.

Morse, M.A., Nair, S.K., Mosca, P.J., Hobeika, A.C., Clay, T.M., Deng, Y., Boczkowski, D., Proia, A., Neidzwiecki, D., Clavien, P.A., et al. (2003). Immunotherapy with autologous, human dendritic cells transfected with carcinoembryonic antigen mRNA. Cancer Invest. *21*, 341–349.

Narvaiza, I., Mazzolini, G., Barajas, M., Duarte, M., Zaratiegui, M., Qian, C., Melero, I., and Prieto, J. (2000). Intratumoral coinjection of two adenoviruses, one encoding the chemokine IFN-gamma-inducible protein-10 and another encoding IL-12, results in marked antitumoral synergy. J. Immunol. *164*, 3112–3122.

Nishioka, Y., Hirao, M., Robbins, P.D., Lotze, M.T., and Tahara, H. (1999). Induction of systemic and therapeutic antitumor immunity using intratumoral injection of dendritic cells genetically modified to express interleukin 12. Cancer Res. *59*, 4035–4041.

Palmer, K., Hitt, M., Emtage, P.C., Gyorffy, S., and Gauldie, J. (2001). Combined CXC chemokine and interleukin-12 gene transfer enhances antitumor immunity. Gene Ther. *8*, 282–290.

Parker, L.L., Do, M.T., Westwood, J.A., Wunderlich, J.R., Dudley, M.E., Rosenberg, S.A., and Hwu, P. (2000). Expansion and characterization of T cells transduced with a chimeric receptor against ovarian cancer. Hum. Gene Ther. *11*, 2377–2387.

Pecher, G., Haring, A., Kaiser, L., and Thiel, E. (2002). Mucin gene (MUC1) transfected dendritic cells as vaccine: results of a phase I/II clinical trial. Cancer Immunol. Immunother. *51*, 669–673.

Pinthus, J.H., Waks, T., Kaufman-Francis, K., Schindler, D.G., Harmelin, A., Kanety, H., Ramon, J., and Eshhar, Z. (2003). Immunogene therapy of established prostate tumors using chimeric receptor-redirected human lymphocytes. Cancer Res. *63*, 2470–2476.

Putzer, B.M., Hitt, M., Muller, W.J., Emtage, P., Gauldie, J., and Graham, F.L. (1997). Interleukin 12 and B7–1 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression. Proc. Natl. Acad. Sci. USA *94*, 10889–10894.

Ribas, A., Butterfield, L.H., Glaspy, J.A., and Economou, J.S. (2002). Cancer immunotherapy using gene-modified dendritic cells. Curr. Gene Ther. *2*, 57–78.

Rosenberg, S.A. (2001). Progress in human tumour immunology and immunotherapy. Nature *411*, 380–384.

Rosenberg, S.A., Yang, J.C., and Restifo, N.P. (2004). Cancer immu-

notherapy: moving beyond current vaccines. Nat. Med. *10*, 909–915.

Rossig, C., Bollard, C.M., Nuchtern, J.G., Rooney, C.M., and Brenner, M.K. (2002). Epstein-Barr virus-specific human T lymphocytes expressing antitumor chimeric T-cell receptors: potential for improved immunotherapy. Blood *99*, 2009–2016.

Sadelain, M., Riviere, I., and Brentjens, R. (2003). Targeting tumours with genetically enhanced T lymphocytes. Nat. Rev. Cancer *3*, 35–45.

Saijo, Y., Tanaka, M., Miki, M., Usui, K., Suzuki, T., Maemondo, M., Hong, X., Tazawa, R., Kikuchi, T., Matsushima, K., and Nukiwa, T. (2002). Proinflammatory cytokine IL-1 beta promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: in vivo analysis of tumor-stromal interaction. J. Immunol. *169*, 469–475.

Sartor, W.M., Kyprianou, N., Fabian, D.F., and Lefor, A.T. (1995). Enhanced expression of ICAM-1 in a murine fibrosarcoma reduces tumor growth rate. J. Surg. Res. *59*, 66–74.

Sharma, S., Batra, R.K., Yang, S.C., Hillinger, S., Zhu, L., Atianzar, K., Strieter, R.M., Riedl, K., Huang, M., and Dubinett, S.M. (2003). Interleukin-7 gene-modified dendritic cells reduce pulmonary tumor burden in spontaneous murine bronchoalveolar cell carcinoma. Hum. Gene Ther. *14*, 1511–1524.

Shuford, W.W., Klussman, K., Tritchler, D.D., Loo, D.T., Chalupny, J., Siadak, A.W., Brown, T.J., Emswiler, J., Raecho, H., Larsen, C.P., et al. (1997). 4–1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. J. Exp. Med. *186*, 47–55.

Simons, J.W., Jaffee, E.M., Weber, C.E., Levitsky, H.I., Nelson, W.G., Carducci, M.A., Lazenby, A.J., Cohen, L.K., Finn, C.C., Clift, S.M., et al. (1997). Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by ex vivo granulocyte-macrophage colony-stimulating factor gene transfer. Cancer Res. *57*, 1537–1546.

Smyth, M.J., Cretney, E., Kershaw, M.H., and Hayakawa, Y. (2004). Cytokines in cancer immunity and immunotherapy. Immunol. Rev. *202*, 275–293.

Soiffer, R., Lynch, T., Mihm, M., Jung, K., Rhuda, C., Schmollinger, J.C., Hodi, F.S., Liebster, L., Lam, P., Mentzer, S., et al. (1998). Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma. Proc. Natl. Acad. Sci. USA *95*, 13141–13146.

Somia, N., and Verma, I.M. (2000). Gene therapy: trials and tribulations. Nat. Rev. Genet. *1*, 91–99.

Stanislawski, T., Voss, R.H., Lotz, C., Sadovnikova, E., Willemsen, R.A., Kuball, J., Ruppert, T., Bolhuis, R.L., Melief, C.J., Huber, C., et al. (2001). Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. Nat. Immunol. *2*, 962–970.

Su, Z., Dannull, J., Heiser, A., Yancey, D., Pruitt, S., Madden, J., Coleman, D., Niedzwiecki, D., Gilboa, E., and Vieweg, J. (2003). Immunological and clinical responses in metastatic renal cancer patients vaccinated with tumor RNA-transfected dendritic cells. Cancer Res. *63*, 2127–2133.

Sung, M.W., Chen, S.H., Thung, S.N., Zhang, D.Y., Huang, T.G., Mandeli, J.P., and Woo, S.L. (2002). Intratumoral delivery of adenovirus-mediated interleukin-12 gene in mice with metastatic cancer in the liver. Hum. Gene Ther. *13*, 731–743.

Tatsumi, T., Gambotto, A., Robbins, P.D., and Storkus, W.J. (2002). Interleukin 18 gene transfer expands the repertoire of antitumor Th1-type immunity elicited by dendritic cell-based vaccines in association with enhanced therapeutic efficacy. Cancer Res. *62*, 5853–5858.

Tatsumi, T., Huang, J., Gooding, W.E., Gambotto, A., Robbins, P.D., Vujanovic, N.L., Alber, S.M., Watkins, S.C., Okada, H., and Storkus, W.J. (2003). Intratumoral delivery of dendritic cells engineered to secrete both interleukin (IL)-12 and IL-18 effectively treats local and distant disease in association with broadly reactive Tc1-type immunity. Cancer Res. *63*, 6378–6386.

Townsend, S.E., and Allison, J.P. (1993). Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. Science *259*, 368–370.

von Mehren, M., Adams, G.P., and Weiner, L.M. (2003). Monoclonal antibody therapy for cancer. Annu. Rev. Med. *54*, 343–369.

Wang, J., Press, O.W., Lindgren, C.G., Greenberg, P., Riddell, S., Qian, X., Laugen, C., Raubitschek, A., Forman, S.J., and Jensen, M.C. (2004). Cellular immunotherapy for follicular lymphoma using genetically modified CD20-specific CD8+ cytotoxic T lymphocytes. Mol. Ther. *9*, 577–586.

Wickham, T.J. (2003). Ligand-directed targeting of genes to the site of disease. Nat. Med. *9*, 135–139.

Wierda, W.G., Cantwell, M.J., Woods, S.J., Rassenti, L.Z., Prussak, C.E., and Kipps, T.J. (2000). CD40-ligand (CD154) gene therapy for chronic lymphocytic leukemia. Blood *96*, 2917–2924.

Wiethe, C., Dittmar, K., Doan, T., Lindenmaier, W., and Tindle, R. (2003). Enhanced effector and memory CTL responses generated by incorporation of receptor activator of NF-kappa B (RANK)/RANK ligand costimulatory molecules into dendritic cell immunogens expressing a human tumor-specific antigen. J. Immunol. *171*, 4121–4130.

Yang, L., Qin, X.F., Baltimore, D., and Van Parijs, L. (2002). Generation of functional antigen-specific T cells in defined genetic backgrounds by retrovirus-mediated expression of TCR cDNAs in hematopoietic precursor cells. Proc. Natl. Acad. Sci. USA *99*, 6204–6209.

Yang, S.C., Hillinger, S., Riedl, K., Zhang, L., Zhu, L., Huang, M., Atianzar, K., Kuo, B.Y., Gardner, B., Batra, R.K., et al. (2004). Intratumoral administration of dendritic cells overexpressing CCL21 generates systemic antitumor responses and confers tumor immunity. Clin. Cancer Res. *10*, 2891–2901.

Yu, P., Lee, Y., Liu, W., Chin, R.K., Wang, J., Wang, Y., Schietinger, A., Philip, M., Schreiber, H., and Fu, Y.X. (2004). Priming of naive T cells inside tumors leads to eradication of established tumors. Nat. Immunol. *5*, 141–149.

Zajac, P., Oertli, D., Marti, W., Adamina, M., Bolli, M., Guller, U., Noppen, C., Padovan, E., Schultz-Thater, E., Heberer, M., and Spagnoli, G. (2003). Phase I/II clinical trial of a nonreplicative vaccinia virus expressing multiple HLA-A0201-restricted tumor-associated epitopes and costimulatory molecules in metastatic melanoma patients. Hum. Gene Ther. *14*, 1497–1510.