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The transduction of dendritic cells for the immunotherapy of cancer

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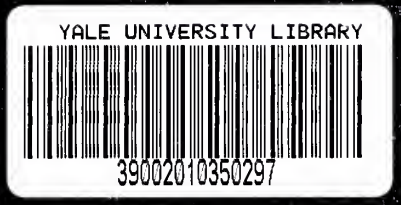

THE TRANSDUCTION OF DENDRITIC CELLS FOR THE IMMUNOTHERAPY OF CANCER

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The Transduction of Dendritic Cells for the Immunotherapy of Cancer

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THE TRANSDUCTION OF DENDRITIC CELLS FOR THE IMMUNOTHERAPY OF CANCER. J. Mark Sloan, Michael Kershaw, Chris Touloukian, Rejean Lapointe, Paul Robbins, Nicholas Restifo and Patrick Hwu. Surgery Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD. (Sponsored by Ira Mellman, Department of Cell Biology, Yale University School of Medicine, New Haven, CT.)

The unique antigen presenting capabilities of dendritic cells make them an attractive means with which to initiate an anti-tumor immune response. Using dendritic cells transduced with tumor antigens for immunotherapy has several theoretical advantages over peptide-pulsed or tumor lysate-pulsed dendritic cells. Since both $CD4^+$ and $CD8^+$ T cells have been shown to play important roles in anti-tumor immunity, one potential advantage is the possibility that transduced dendritic cells are capable of presenting multiple and undefined epitopes on both class I and class II MHC molecules. To test this theory, we inserted the human tumor antigen gp100, which has known class I and class II epitopes, into dendritic cells derived from the bone marrow of mice transgenic for HLA-DRβ1*0401. The human gp100 gene was inserted using either adenovirus or a VSV-G pseudotyped retrovirus. A murine $CD8^+$ T cell clone recognizing an H-$2D^b$ restricted epitope for gp100 and a murine $CD4^+$ T cell line recognizing an HLA-DRβ1*0401 restricted gp100 epitope were then used in a cytokine release assay to test for reactivity against the adenovirally or retrovirally transduced dendritic cells. Both the $CD8^+$ and $CD4^+$ gp100 specific T cell lines were able to recognize the gp100 transduced dendritic cells significantly more than GFP transduced controls, lending support to the use of gene-modified dendritic cells as a means to simultaneously initiate a $CD4^+$ and $CD8^+$ anti-tumor response. Additionally, VSV-G pseudotyped retroviral vectors were investigated as a means of inserting multiple genes into dendritic cells. This ability, combined with the ability to immunize mice against genes expressed by transduced dendritic cells, is explored as a potential means of antigen discovery and direct immunization against tumor.

PREFACE & ACKNOWLEDGEMENTS

These experiments were performed at the Surgery Branch of the National Cancer Institute in Bethesda, MD. I received financial support from the Howard Hughes Medical Institute through the HHMI-NIH scholars (cloister) program. All data presented here represents my work except as noted. This work was made possible through the generosity of the Surgery Branch and its employees, who shared their reagents, resources and advice. In particular, the gp100 reactive T cells were developed by other members of the branch. The pCLNCgp100 (Non-SIN) plasmid and pCLNCGFP (SIN) plasmid, along with all the tumor cell and TIL lines, developed elsewhere in the branch. Sequencing and FACS machines were generally run by Surgery Branch technicians. All adenoviral vectors were gifts from Genzyme. The remainder of the reagents described were developed by me in close collaboration with those in my lab.

I am especially appreciative of the excellent mentoring and guidance of Drs. Patrick Hwu and Michael Kershaw. I am indebted to the many other scientists and physicians at the Surgery Branch who contributed intellectually and physically to these projects: John Toso, Rejean Lapointe, Matt Lublin, My Do, Chris Touloukian, Nicholas Restifo, Kari Irvine, Willem Overwijk, Paul Robbins, Yong Li, Jim Yang, Donna Perry-Lally, Dave Jones, Arnold Mixon, Shawn Farid and Dr Steven Rosenberg.

Introduction

Cancer Immunotherapy

Evidence that the immune system can recognize and attack tumors in humans and murine models is abundant and compelling. For instance, administration of the cytokine interleukin-2 (IL-2) to patients with metastatic melanoma can induce tumor regression in 15 to 20% of patients, almost half of whom experience a complete response[1]. Of the complete responders, 80% remain in lasting remission, and are apparently cured of their disease[2]. IL-2 has no primary effect on the tumor; its action is mediated by the cytokine's ability to initiate a cellular immune response against antigens presented on the surface of the melanoma cells.

Despite a decade of intensive effort, characterizing these cellular immune responses has been difficult. These difficulties reflect the complex nature of the afferent anti-tumor immune response, and its elaborate interplay with T cell tolerance and autoimmunity. A successful anti-tumor response requires that a quiescent, tumor-reactive T lymphocyte recognize a tumor antigen, attain the state of activation required to proliferate, than traffic to and exert a cytolytic effect on an autologous tumor. Strategies used to produce this response can be divided into either active or passive approaches. Direct immunization against tumor antigens is known as active immunotherapy, while passive (adoptive) immunotherapy refers to the transfer of T cells with pre-existing reactivity to tumor antigens[3].

The majority of cancer immunotherapy work has focused on the identification and molecular characterization of melanoma tumor antigens. Antigens are identified using anti-tumor T lymphocytes to screen cDNA libraries derived from cancer cells. These

anti-tumor T lymphocytes are either grown from resected tumors to yield tumor infiltrating lymphocytes (TIL), or generated through repetitive in vitro stimulation of peripheral blood mononuclear cells (PBMC) with tumor cells in the presence of IL-2. Tumor antigens can be classified into four categories:

1) Mutated antigens unique to both patient and individual tumor
2) normal, non-mutated self-proteins presented on the surface of differentiated cells
3) embryonic or quiescent genes reactivated and expressed after carcinogenesis
4) antigens resulting from aberrant splicing or post translational protein modification prior to antigen processing

Of these antigen categories, the second has received the most scrutiny. The majority of melanoma antigens identified to date are proteins expressed in normal melanocytes, and are present in the majority of malignant melanomas.

Discouragingly, little success has been made towards generalizing from work on melanoma to more common cancers, such as breast, ovarian and colon cancer. A major stumbling block has been the lack of defined T cell antigens in these tumors, which have been resistant to the aforementioned antigen discovery techniques[4]. It is of considerable interest, therefore, to develop methods that would enable the identification and characterization of antigens in common tumors.

Dendritic Cells and Cancer Immunotherapy

Dendritic cells (DCs) play a pivotal role in the initiation of immunity[5]. Because of their unique repertoire of cell surface molecules, antigen processing machinery, and tissue distribution, dendritic cells serve as the principal activators of quiescent T cells (figure 1). These antigen presenting capabilities make dendritic cells an attractive means with which to initiate an anti-tumor immune response. Considerable effort has been

directed towards immunizing patients and murine models using dendritic cells loaded with tumor antigens, and many groups have documented their ability to induce protective and therapeutic anti-tumor immunity[6 7 8].

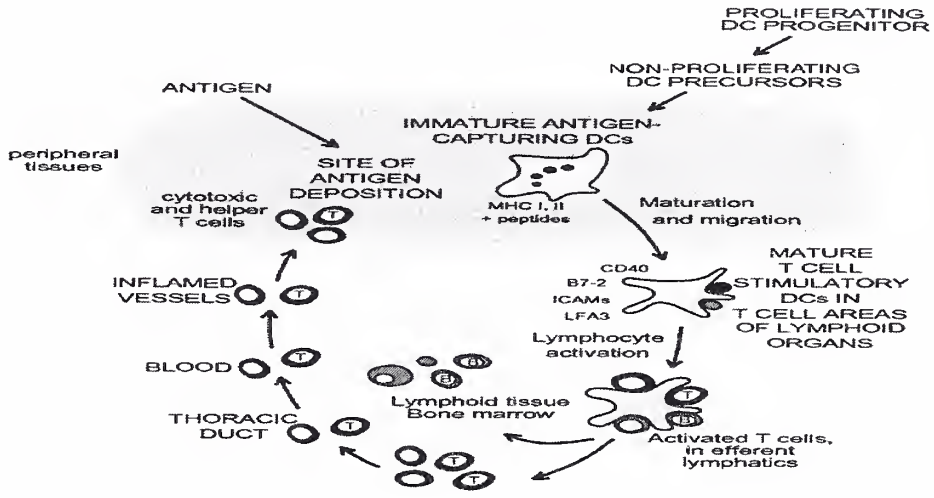

Figure 1 Dendritic cells and the immune response. Dendritic cells capture and process potential antigens, then present them to T cells in the appropriate co-stimulatory context. Figure from Banchereau, 1998.

Most studies endeavor to immunize patients with DCs that have been loaded with tumor antigens ex vivo. Common methods of antigen loading DCs include pulsing them with defined peptide epitopes or with tumor lysates[9]. These methods will primarily result in antigen presentation on MHC class I and class II molecules, respectively. However, both $CD8^+$ and $CD4^+$ T cells appear to play crucial roles in robust anti-tumor responses[10 11]. It is therefore advantageous to find a method of loading and administering dendritic cells which results in the simultaneous presentation of class I and class II tumor antigen epitopes. Since certain endogenous antigens have been shown to be efficiently presented on MHC class II molecules[12 13], the use of gene-modified DCs may be a way to present a tumor antigen on both MHC class I and class II molecules.

Gene-Modified Dendritic Cells in Cancer Immunotherapy

Groups have been able to insert genes into both human[14] and murine[15] dendritic cells using retroviral vectors[16], adenoviral vectors[17], or direct transfection of genetic material[18]. These studies demonstrate the ability of gene-modified dendritic cells to induce protective and therapeutic anti-tumor immunity in mice. Most of this work was done with highly immunogenic foreign model antigens. For example, mice can readily be immunized using dendritic cells transduced with the beta galactosidase gene; these animals are then protected from intravenous challenge with the highly immunogenic, beta galactosidase expressing tumor CT26.Cl25[19]. As with other methods of active and passive immunotherapy, treatment of autologous tumors using transduced DCs is much more difficult than treating tumors containing foreign antigens. The paucity of experiments demonstrating treatment of entirely autologous tumors supports this assertion. Those that do are often plagued by technical oversights, such as challenging mice with tumors cultured in fetal calf serum after those animals have been vaccinated using dendritic cells grown in calf serum. In this situation, the protection conferred against tumor challenge is related to anti-calf serum immunity, and has little to do with antigen specific anti-tumor immunity initiated by the dendritic cells.

Several groups have observed that immunizations with transduced dendritic cells require $CD4^+$ T cell help for optimal anti-tumor effects[20]. However, existing evidence for anti-tumor $CD4^+$ T cell activation by gene-modified dendritic cells is indirect. Using the OVA model antigen, a group has demonstrated that $CD4^+$ T cell depletion abolishes tumor protection induced by transduced DCs[21]. It is unclear whether this reflects presentation of antigen from transduced DC directly to $CD4^+$ T cells, or results from cross

presentation of antigen secreted from the transduced cell which is then processed by a native APC as an exogenous antigen. Direct evidence for the presentation of tumor antigen to $CD4^+$ T cells by transduced dendritic cells is lacking.

Potential Advantages of Immunization with Transduced Dendritic Cells Over Peptide Pulsed Dendritic Cells

By introducing a gene encoding an antigen into dendritic cells, one allows the antigen presenting cell (APC) to perform its own antigen processing and presentation. In addition to the possibility that gene-modified dendritic cells can present transgene-encoded class II epitopes to $CD4^+$ T cells, this method has several other potential advantages over peptide-pulsed DCs. All possible epitopes within the antigen would be available for processing and presentation, obviating the need to laboriously define and synthesize precise peptide epitopes. The epitopes in gene-modified DCs would be subjected to normal post-translational processing prior to presentation, thereby acquiring modifications that could influence epitope recognition. Finally, since the antigen continues to be expressed over time, transduced cells may sustain epitope presentation longer than their peptide pulsed counterparts.

The Transduction of Dendritic Cells

The methods currently available for the gene-modification of dendritic cells are: transfection with DNA or RNA, adenoviral transduction and retroviral transduction. Transfection and other non-viral methods of gene transfer into dendritic cells is difficult, with approximately 2% gene transfer efficiency in our experience, though others report higher numbers[22]. Transgene expression in a transfected cell tends to be short-lived, especially if RNA is used as the transfected material.

Adenovirus can insert genes into DCs with efficiencies approaching 100%. It has the additional advantage of being able to transduce non-dividing cell populations. The adenovirally-inserted transgene resides episomally within the target cell. As a result, transgene expression will be diluted and lost if the target cell proliferates after transduction. Adenoviral vector construction is technically challenging; the cost and labor involved in creating large numbers of adenoviral vectors is nearly prohibitive.

Retroviral vectors offer a unique set of advantages and disadvantages. Although dendritic cell transduction with retroviral vectors is more challenging than with adenoviral vectors, retroviral vectors are also capable of attaining high levels of gene transfer into DCs. Retroviruses can only transduce dividing cells, but do so stably, so that the progeny of transduced cells also contain the transgene. Since a mature dendritic cell does not proliferate, one must retrovirally transduce dividing hematopoietic progenitor cells, then differentiate them in vitro into dendritic cells. In comparison with adenoviral vectors, it is relatively easy to engineer a retroviral vector containing the desired transgene.

VSV-G Pseudotyped Retroviral Vectors

Several limitations of traditional retroviral vectors derived are circumvented through the use of Moloney murine leukemia virus (MMLV) based retroviral vectors pseudotyped with vesicular stomatitis virus G (VSV-G) glycoprotein. A retroviral pseudotype is created when the genome of one virus is encapsidated by the envelope protein of a second virus (Figure 2). In this situation, the host range is determined by the viral envelope protein. The VSV-G envelope protein has several properties that make it attractive for use as a retroviral pseudotype. In contrast to other viral envelopes, which

depend on specific protein receptors for viral entry and fusion, the VSV envelope proteins use phosphatidylinositol, a ubiquitous phospholipid, as their receptor[23], thus enabling the pseudotyped virus to fuse directly with the target cell membrane. This remarkable property gives VSV-G pseudotyped vectors a virtually unlimited target cell range.

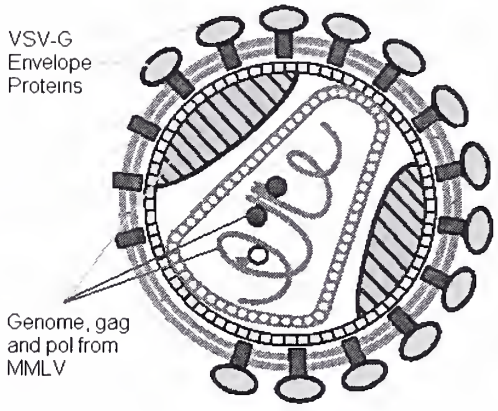

Figure 2 Retroviral Pseudotypes. VSV-G pseudotyped retroviruses have use the genome, gag and pol of the MMLV, but have a VSV envelope, making a virus an unlimited host cell range. [Figure adapted from Heaphy, 1997[24]]

The stability of the VSV envelope provides several additional advantages. While normal amphotropic/ecotropic retroviral envelopes cannot withstand the force of ultracentrifugation, VSV pseudotypes can be concentrated to extremely high titers[25]. High viral titers are crucial for obtaining maximal gene transfer efficiency, especially in difficult to transduce cell populations, such as dendritic cells. Traditional retroviral envelopes are thermolabile, and must be used immediately after collection. The VSV envelope, in contrast, has the stability needed to withstand a freeze thaw cycle. Although this is largely a matter of convenience, it allows for the generation and storage of large amounts of standardized pseudotyped virus.

Unlike adenoviral vectors and some retroviral systems, the VSV-pseudotyped system expresses no viral proteins in the target cell. After viral entry and reverse

transcription the transgene is stably integrated into the target cell genome. The highly immunogenic VSV-G envelope protein is lost as the target cell proliferates. Since a tumor antigen will be overlooked by the immune system in favor of an immunodominant antigen, it is important to keep the antigen presenting cell free of foreign, potentially immunodominant antigens. This is a theoretical contraindication for using adenoviral or certain retroviral vectors, which co-express viral proteins in the host cell, for immune-mediated gene therapy.

The Processing of Endogenous Antigens on the Class II Pathway

MHC class I and MHC class II molecules primarily associate with peptides from different cellular compartments. Peptides bound to MHC class I molecules come predominantly from proteins degraded in the cytoplasm, while peptides bound to MHC class II molecules are mostly derived from exogenous or intravesicular sources (figure 3)[26]. In this manner, they are able to present the antigen to the subset of T cells best equipped to destroy a pathogen residing in a given compartment. A more complicated picture of antigen processing is emerging, in which antigen presenting cells, and dendritic cells in particular, are able to sample proteins from either compartment for presentation on class I and class II molecules[27] [28].

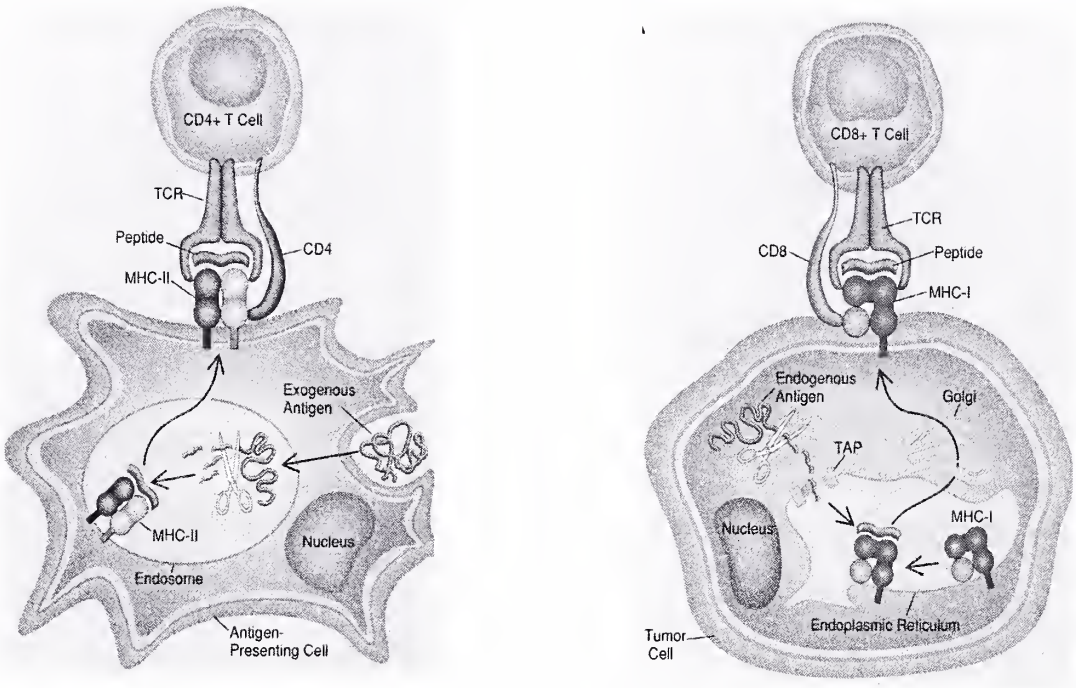

Figure 3 Endogenous and Exogenous antigen presentation pathways. The MHC class II and class I molecules primarily associate with exogenous or cytoplasmic pathogens, respectively. Despite this, antigen presenting cells may be able to sample endogenously expressed proteins on MHC class II molecules. [Adapted from Rosenberg, 1996]

The maturity of dendritic cells is known to have a substantial impact on their MHC II expression[29]. Upon activation, immature dendritic cells experience a reduction in endocytic activity and simultaneously transport intracellular peptide-MHC II complexes to the cell surface. Because of this dramatic reorganization, the activation state of the dendritic cell could have considerable impact on the MHC class II presentation of endogenous antigens. In vivo, bacterial products (LPS), inflammation markers (TNFα) or T cell interaction via CD40L are thought to be responsible for DC activation. For bone marrow derived dendritic cells, any physical manipulation appears to mature the DCs. The cell surface markers displayed on the dendritic cells used in this experiment, even without biochemical manipulation, are those of mature dendritic cells. The degree to

which further activation can be induced by addition of membrane-bound CD40L, in the form of contact with 3T3 cells expressing CD40L is unknown.

The gp100 Antigen and the Melanosome Transport Signal

Several requirements must be fulfilled in order to definitively demonstrate the simultaneous presentation of transgene-encoded epitopes to $CD4^+$ and $CD8^+$ T cells. The antigen used must contain well-defined class I and class II epitopes. Clonal $CD4^+$ and $CD8^+$ T cell populations specific for these epitopes and amenable to in vitro work must exist. Ideally, the antigen used should be a human tumor antigen in order to have immediate clinical relevance.

The nonmutated melanocyte differentiation antigen, gp100, fulfills these criteria. In humans, gp100 is expressed by normal melanocytes and the majority of malignant melanomas[30]. A close murine homologue for gp100 (also known as pmel) has been described[31]. The gp100 class I epitope is restricted by $H\text{-}2D^b$ and located at amino acids 25-33. Human and mouse gp100 differ by 3 amino acids at these positions (figure 4), but either can be recognized by mouse $CD8^+$ T cells specific for this epitope[32]. Human gp100 was used for these experiments. The class II epitope is restricted by HLA-DRß1*0401 and located at amino acids 44-59[33]. Therefore, a dendritic cell derived from a C57BL/6 mouse transgenic for human HLA-DRß1*0401 and expressing human gp100 should be able to present both the class I (25-33) and class II (44-59) gp100 epitopes.

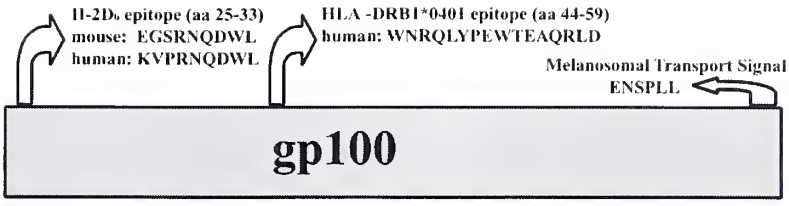

Figure 4 The gp100 tumor antigen. Both human and murine gp100 have well-defined MHC class I and class II epitopes, and a melanosome transport signal.

Like other melanosome membrane glycoproteins, gp100 contains a melanosome transport signal (MTS)[34]. This transport signal has been shown to traffic antigens to the endocytic pathway for class II presentation[35]. The MTS would be expected to enhance the class II presentation of endogenously expressed gp100 protein. Other groups use lysosomal-associated membrane protein (LAMP)[36] or invariant chain (Ii)[37] targeting signals in an effort to increase class II presentation of endogenous proteins. The relative efficiency of these targeting signals for increasing class II presentation of endogenous antigens is unknown.

The Retroviral Transduction of Dendritic Cells with Pools of Genes

Many of the experiments in this project depend on the ability to simultaneously transduce cells with more than one gene. The efficiency of DNA or RNA co-transfection into DCs becomes vanishingly small when working with multiple transgenes. Likewise, using adenovirus for multiple gene insertions is impractical, given the aforementioned difficulties in vector construction and that toxic levels of adenovirus would be required for multiple transductions. Multiple gene insertions using a retrovirus are only allowed in certain situations. In nature, only one retrovirus can infect a cell before others are excluded. This is secondary to the sequestration of required binding and fusion proteins after entry of the initial virus. This mechanism can be circumvented by using replication-incompetent retroviruses. VSV-G pseudotyped virions are ideally suited to insert

multiple genes into a single cell, since they do not require surface proteins on the target cell for infection; therefore, their entry is not limited by receptor saturation. The ease of retroviral vector construction in the VSV system aids in the development of retroviral libraries. By simultaneously infecting cells with supernatant containing a mixture of virions, each with a unique transgene, one can create cells expressing extremely large numbers of inserted genes.

The behavior of individual cells containing multiple transgenes, or multiple copies of a single transgene, all of which are driven by highly active viral promoters, has not been studied in great detail. In this situation, competition for ribosomes and other cellular machinery and resources certainly plays a role in limiting the expression of individual transgenes. For instance, if 1000 copies of gene A (driven by a CMV promoter) were inserted into a single cell, large amounts of protein A would be produced, perhaps to the exclusion of the cell's normal housekeeping functions. The situation is less clear when 1000 unique copies of different transgenes are inserted into a cell. Even after each has successfully integrated into the host genome, these transgenes would compete with each other for transcription and translation. At some point, a single gene in an increasingly large pool of transgenes would be expressed at either undetectable levels or not at all.

The complexity increases when one applies this situation to an antigen presenting cell, and asks which antigens in a dendritic cell saturated with multiple transgenes will be presented to T cells. It is the function of an APC to present foreign antigens at a sufficient level even when sampling from amongst much more numerous native proteins. It does so through a combination of stochastic efficiency and intrinsic preference of the

immunoproteasome, TAP protein, and other components of the antigen processing machinery for proteins that appear foreign[38]. It is thus intuitively favorable to presume that a DC transduced with a pool of genes will be able to present most potential antigens encoded in that pool, but will do so only haphazardly if the pool is so large that individual transgenes are not all expressed.

Transduced Dendritic Cells for Antigen Discovery

Beyond the potential importance of active immunotherapy by vaccination with gene-modified dendritic cells, there are several possible in vitro applications of transduced dendritic cells. These applications could make gene-modified dendritic cells important tools for antigen discovery and for gaining insight into the anti-tumor immune response.

It is a strong possibility that some antigens are better than others, although we know little about *what it is* that makes a good tumor rejection antigen. Many variables could affect the ability of an antigen to initiate and sustain an anti-tumor response. These variables include the antigen category (i.e. whether it is a mutated antigen or a normal differentiation antigen), the number of epitopes on an antigen, as well as their type, dominance and affinity for the associated MHC molecules. Also important is the level of antigen expression and its heterogeneity within the tumor, as well as the susceptibility of a particular antigen to loss during immunoselection. If the antigen is a normal differentiation antigen, its expression level and pattern in normal tissues will impact the available T cell repertoire and tolerance. Finally, the presentation context and co-stimulatory setting of the antigen is crucial to promoting an afferent immune response.

As described above, antigen discovery has previously been limited to seeking the epitopes recognized by tumor infiltrating lymphocytes (TIL) or PBMC from mixed tumor-lymphocyte cultures. These methods are a convenient means to define an antigen, although they may only be finding a particular subset of antigens. In fact, this subset contains perhaps the worst antigens to characterize, as tumors generally continue to grow despite the presence of endogenous or adoptively transferred TIL specific for these antigens. A more comprehensive approach would be one that uses tumor rejection itself as the means for antigen identification.

The ability to efficiently transduce dendritic cells with pools of genes, combined with the ability to successfully immunize an animal using transduced dendritic cells makes such an approach possible. By immunizing mice with dendritic cells transduced with pools of genes derived from a tumor cDNA library, and then challenging with the corresponding tumor, one could identify pools of genes that confer tumor protection. These pools must contain one or more rejection antigens. Individual antigens could then be identified through repeated rounds of sub-pooling and tumor challenge (figure 5).

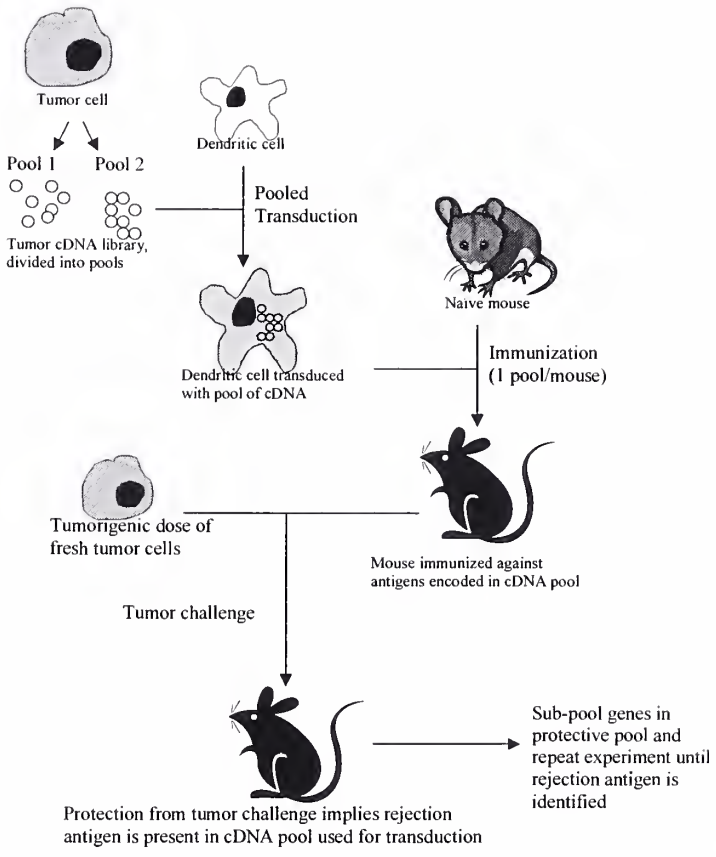

Figure 5 A method of antigen identification based on tumor protection. Dendritic cells are transduced with pools of genes derived from a tumor cDNA library. Mice are immunized with the transduced cells, and then challenged with tumor. In the cases where mice are protected from tumor challenge, there must be a tumor rejection antigen present in the pool used to transduce the DCs. The particular antigen responsible for protection can be identified through repeated rounds of sub-pooling.

This pooling method of antigen discovery has several potential advantages over conventional antigen finding methods. Since the most protective antigens would presumably immunize even in very large pools (with corresponding low levels of expression within the APC), the best tumor rejection antigens may be found first. The antigens found using this approach may provide insight into the nature of a rejection antigen. For example, if all the antigens discovered were mutated proteins, it may speak to the inability of animals to mount an equally efficient response against non-mutated differentiation antigens. This would represent a major paradigm shift in current thought on tumor antigens, as it was always hoped that the continued growth of tumors in the

presence of TIL known to recognize non-mutated antigens reflected the inadequate activation state or numerical advantage of the TIL, rather than anything intrinsically wrong with differentiation antigens themselves.

Since a retroviral library can be made from any tissue type, this approach may also be a means to discover antigens for more common, less immunogenic tumors. This would be an extremely valuable asset, as it would allow antigen-based immunotherapy to move away from melanoma to more important cancers. In addition, work using appropriate animal models may lead directly to clinically relevant antigens because murine tumor antigens often have human homologues,. This point is illustrated by the murine melanoma antigens mgp100, mtrp1 and mtrp2, which all have homologous human antigens[39].

Potential Uses of Dendritic Cells Transduced with Tumor cDNA Libraries for Direct Immunization Against Tumor

The ability to actively immunize animals and patients with dendritic cells expressing pools of transgenes has many potential applications. It is possible that one could directly immunize against a tumor using dendritic cells that have been transduced *en masse* with an entire tumor cDNA library. This would represent the ultimate in customized immunotherapy, where potential rejection antigens come directly from the cDNA library of the specific tumor to be treated. This approach would be a means to take advantage of mutated, tumor and patient specific antigens, as well as shared differentiation antigens. The method has the added advantage of being applicable to any tumor type, regardless of its tissue of origin.

The possibility for success hinges on the dendritic cell's ability to process and present individual antigens even when they are expressed in the presence of more numerous non-antigenic proteins. Obviously, the more antigens present within the tumor, the greater the chance of presenting a given antigen at levels visible to the immune system. Although the amount of potential antigens in a tumor is unknown, the substantial genetic instability, rapid mutation rate and genetic dysregulation in neoplastic cells could produce multiple potential antigens.

MATERIALS AND METHODS

VSV-G Pseudotyped Retroviral Vectors and Retroviral Transduction of DCs

The procedure for creating VSV-G pseudotyped virus and infecting target cells is schematized in Figure 6. Because the VSV-G envelope is toxic to virus-producing cells, it is not possible to engineer a suitable stable packaging cell line. Therefore, pseudotyped virus must be created by co-transfecting the transgene encoding retroviral plasmid and the VSV-G encoding plasmid into cells that constitutively express the gag and pol proteins. The 293GP cell line is easily transfectable and was developed for this purpose.

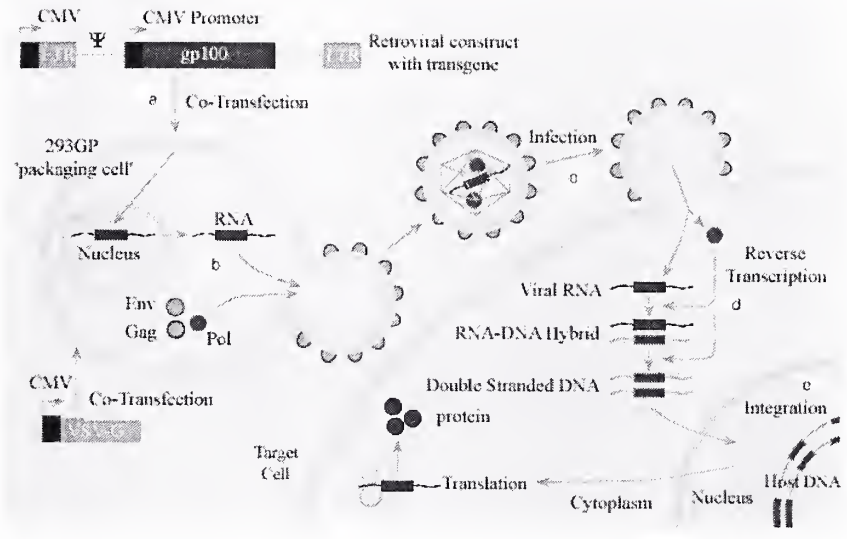

Figure 6 Creation of VSV-G pseudotyped retrovirus. VSV-G pseudotyped retrovirus is created by co-transfection of retroviral plasmid and a VSV-G encoding plasmid into a 293 cell constitutively expressing gag and pol proteins. The pseudotyped virions are then capable of infecting a target cell. Once infected, the retrovirus undergoes reverse transcription and integration into the host cell genome. [Figure adapted from Verma, 1998[40]]

A number of retroviral vectors were tested for these projects. The most effective vectors were those that contained a CMV promoter driving transcription of the provirus within the packaging cell (data not shown). The pCLNCx construct contains the early region of the CMV enhancer-promoter fused to the MMLV LTR in the 5' U3 region[41]. Once integrated, the 5' LTR is responsible for promoting the neomycin resistance gene. Another CMV promoter is located downstream of the neo gene and drives transcription of the transgene.

The original plasmid was modified to create a more functional multiple cloning site (figure 7). Also, a self-inactivating (SIN) form of this plasmid was created by removing the portion of the U3 region containing the NFκB transcription factor binding site (figure 8, 9). Since the U3 region is copied to the 5' end of the provirus during reverse transcription, this excision greatly reduces neo expression. By removing the

powerful promoting elements from the 5'LTR, the SIN plasmid is thought to eliminate promoter interference between the LTR and CMV promoters. The SIN and NON-SIN versions of this plasmid behaved comparably for dendritic cell transduction, and were used interchangeably except where noted.

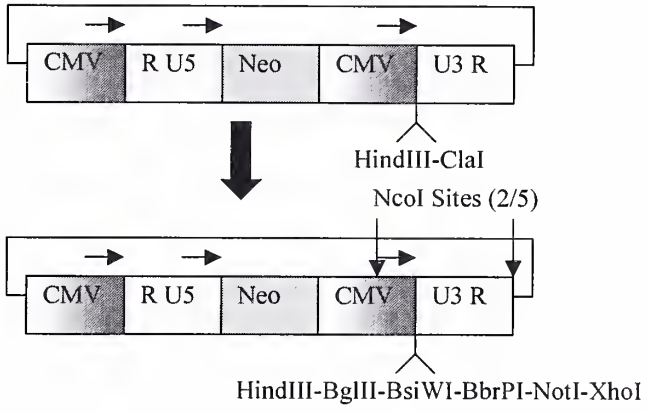

Figure 7 pCLNC plasmid and its modifications. pCLNC was modified to contain a more useful multiple cloning site. This was accomplished by ligating annealed 5' phosphorylated primers into HindIII-ClaI cut pCLNC. 2/5 NcoI sites present in pCLNCX are shown to aid in interpretation of figure 9.

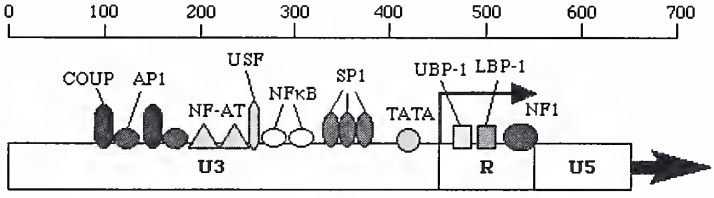

Figure 8 The U3 region contains retroviral transcription factor binding sites. The U3 region lies at the 3' end of the provirus, but is copied to the 5'end during reverse transcription, forming the 5' LTR. It contains the transcription factor binding sites responsible for promoting neo in the pCLNCx plasmid. Several of these sites are removed in the SIN plasmid, which contains a several hundred bp deletion from the U3 region. [Adapted from Heaphy, 1997]

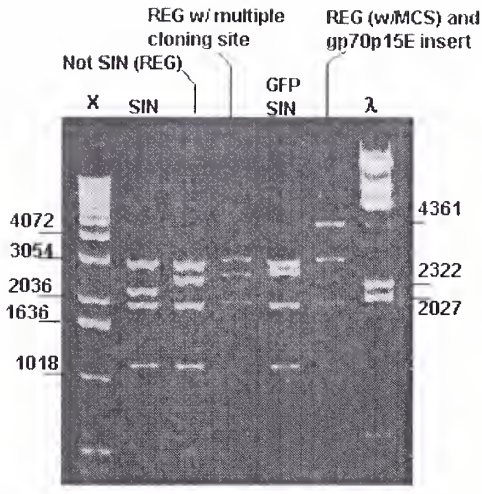

Figure 9 NcoI restriction digests of pCLNC plasmids. NcoI cuts pCLNCx in 5 places, but does not cut GFP or gp70p15E. The SIN plasmid contains a several hundred bp deletion from the U3 region. The NcoI fragment containing the U3 region also contains the multiple cloning site and any insert. Therefore, the size of this band indicates whether the plasmid is SIN or non-SIN, and the size of inserted gene. The lanes are as follows: pCLNCx (SIN Lane 1, Non-SIN Lane 2, Non-SIN w/ multiple cloning site Lane 3), pCLNCGFP (SIN) Lane 4, and pCLNCgp70p15E (Non-SIN) Lane 5. GFP is 800bp, gp70p15E is 2009bp.

To manufacture pseudotyped virus, the pCLNC plasmid and a plasmid encoding the VSV-G envelope protein were co-transfected into semi-confluent 293GP cells using commercially available lipofectamine reagents (GIBCO). Serial supernatant harvests of the virus were performed on days 2,3,4 and 5 post-transfection, passed through a 0.45 μm filter (Millipore) and frozen. Supernatants were thawed on the day of transduction and concentrated in an ultracentrifuge (50,000g, 1.5 hrs, 4°C). The viral pellet was gently resuspended to achieve appropriately concentrated virus.

One day after bone marrow harvest, concentrated supernatant containing VSV pseudotyped retrovirus (described above) was added to the dendritic cells along with 8 ug/ml polybrene (Sigma) and 10mM HEPES (Biofluids). Transduction was performed by spinoculation on a table-top centrifuge for 1 hour (2800 RPM, 32°C).

Adenoviral Vectors and Adenoviral Transduction of DCs

The recombinant adenoviral vectors Ad.GFP and Ad.hgp100 were a gift from Bruce Roberts (Genzyme) and are described in detail elsewhere[42]. These Ad vectors were derived from Ad serotype 2, and a CMV promoter driving expression of the transgene was inserted into the deleted E1 region. The adenovirus was thawed on the day of transduction.

Recombinant adenovirus was added at an MOI of 500 to the dendritic cells after replating on day 6. The transduction was carried out for 24 hours at 37°C, 5% CO_2.

Animals

Murine class II-deficient, DR4-IE transgenic mice express chimeric class II molecules consisting of the antigen binding domains from HLA-DRA and and HLA-DRß1*0401 molecules and the remaining domains from murine IE^d-α2 and IE^d-β2 chains[43] (figure 10). This allows these animals to present HLA-DRß1*0401 epitopes while preserving the species-specific interaction between the murine $CD4^+$ molecule and the β2 domain of the murine MHC Class II molecule. These animals were obtained from Taconic and maintained in accordance with institutional standards. C57BL/6n ($H\text{-}2^b$) mice were obtained from the Frederick Research Center.

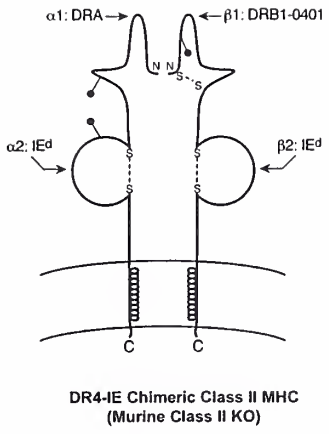

Figure 10 DR4-IE transgenic mice. These mice are murine class II knockouts; they have chimeric class II molecules consisting of the antigen binding domains from HLA-DRA and HLA-DRβ1*0401 molecules, and the remaining domains from murine IE^d-α2 and IE^d-β2 chains. Figure adapted from Ito, 1996.

Tumors and Cell Lines

The spontaneous murine melanoma B16 expresses the known tumor antigens gp100, MART-1, tyrosinase, Trp-1 and Trp-2. MC38 is a moderately immunogenic colon adenocarcinoma that was generated by administration of oral 1,2-dimethhydrazine in C57BL/6 mice. MC38 is known to present an antigen to MC38 TIL derived from the endogenous retrovirus envelope protein, p15E [44]. The melanoma cell line 526 expresses gp100 at high levels, and is frequently used as a positive control for gp100 recognition assays. 3T3-CD40L and 3T3-SAMEN are 3T3 cells retrovirally transduced with murine CD40L and SAMEN, respectively. SAMEN is an irrelevant retroviral protein, and is used in these experiments as a negative control for 3T3-CD40L mediated dendritic cell activation. All tumor lines were maintained in RPMI medium with 10% heat-inactivated fetal bovine serum, 0.03% L-glutamine, 100 μg/ml of streptomycin, 100 μg/ml of penicillin and 50 μg/ml of gentamicin sulfate (NIH media center).

Dendritic Cell Harvest and Differentiation

By harvesting mouse bone marrow, depleting the marrow of all ells except stem cells, and then differentiating those cells in the presence of GM-CSF and IL-4, dendritic cells are produced[45]. These cells can be transduced prior to proliferation with a retrovirus, or after proliferation with an adenovirus (figure 11)

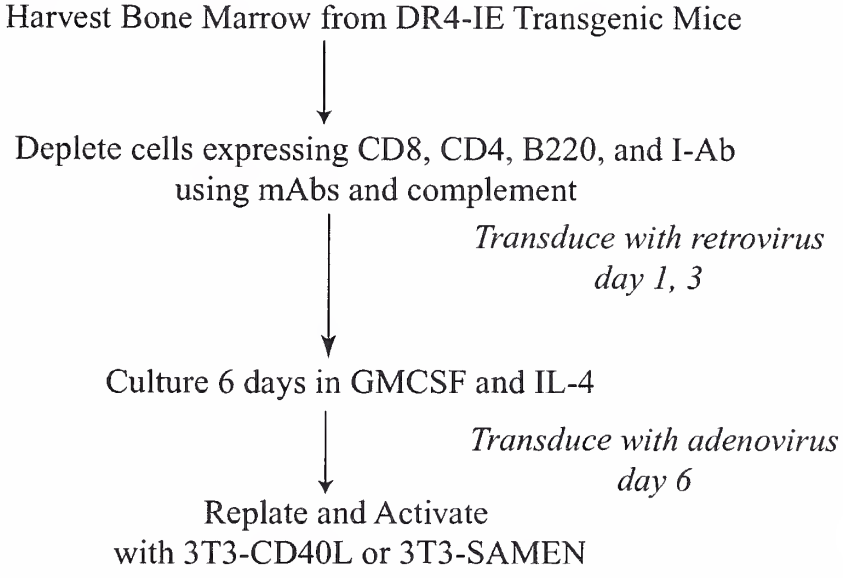

Figure 11 Dendritic cell manufacture and transduction. Dendritic cells are made by harvesting the bone marrow of mice and depleting it of all except stem cells. Growing the stem cells in GMCSF and IL-4 differentiates them into dendritic cells. They can be transduced with a retrovirus prior to proliferation or transduced with adenovirus afterwords.

Bone marrow was expelled from the long bones of C57BL/6 or DR4-IE mice. Erythrocytes were depleted in ACK lysing buffer (Biofluids). Cells expressing B220, I-Ab,d, Lyt-2.2 and $CD4^+$ were removed using hybridoma supernatant from TIB-146, TIB-229, TIB-150, TIB-207 (ATCC) followed by incubation with rabbit complement (Cedarlane). The remaining cells were plated at a concentration of $7x10^5$ cells/ml in 6 well plates, and cultured for six days in DC medium (RPMI supplemented with 5% inactivated FCS (GIBCO), 2 mM glutamine (Biofluids), 100 U/ml penicillin and 100ug/ml streptomycin (Biofluids) and $5x10^{-5}$ M 2-ME (Sigma)). Recombinant murine

GM-CSF was added to a final concentration of 20 ng/ml (Peprotech) and recombinant murine IL-4 (Peprotech) added to a final concentration of 100 ng/ml. Cytokines were replenished on days 2, 4 and 6. On day 6, non-adherent cells were collected and replated at $1x10^6$ cells/ml in a 6 well plate. Where indicated, the cells were replated onto irradiated 3T3 cells expressing CD40L or SAMEN (as a control for the CD40L activation). On day 7, the non-adherent cells were collected and washed three times in PBS before use.

Murine gp100 Specific $CD8^+$ T Cell Lines

The generation of the $CD8^+$ T cell clone specific for the H-$2D^b$ restricted epitope of gp100 ($gp100_{25-33}$) used in this experiment has been described in detail elsewhere (Overwijk, 1998). They were derived from splenocytes of mice immunized by gene gun with human gp100 DNA. This line was thereafter restimulated with irradiated syngeneic splenocytes pulsed with 1μM mouse $gp100_{25-33}$ peptide. These cells are able to recognize both human and murine $gp100_{25-33}$ epitopes presented on D^b molecules, despite a three amino acid difference between the murine and human epitope. All T cells were used between 5 and 10 days after re-stimulation.

Murine gp100 Specific $CD4^+$ T Cell Lines

The generation of the $CD4^+$ T cell line specific for the HLA-DRß1*0401 gp100 epitope used in this experiment has been described in detail elsewhere (Touloukian, 2000). They were derived from DR4-IE transgenic mice which were originally immunized with hgp100 protein. Cells were subsequently stimulated with irradiated syngeneic DR4-IE splenocytes pulsed with $hgp100_{44-59}$.

Tumor Infiltrating Lymphocyte Lines

The MC38 reactive TIL used in this project have been described elsewhere[46]. These TIL are known to recognize a class I epitope encoded on the endogenous ecotropic murine leukemia virus, p15E. The B16 TIL were isolated by Dr Michael Kershaw from mice bearing subcutaneous B16 tumors which were retrovirally transduced with the B-7.1 co-stimulatory molecule. All TIL lines were re-stimulated with irradiated tumor every week, and supplemented with IL-2 every other day.

Creation of Tumor Retroviral Library

A high quality cDNA library was made from 1987 MC38 and cloned into a pCLNCx retroviral construct by the method schematized in figure 12, using enzymatic reagents from GIBCO and custom primers.

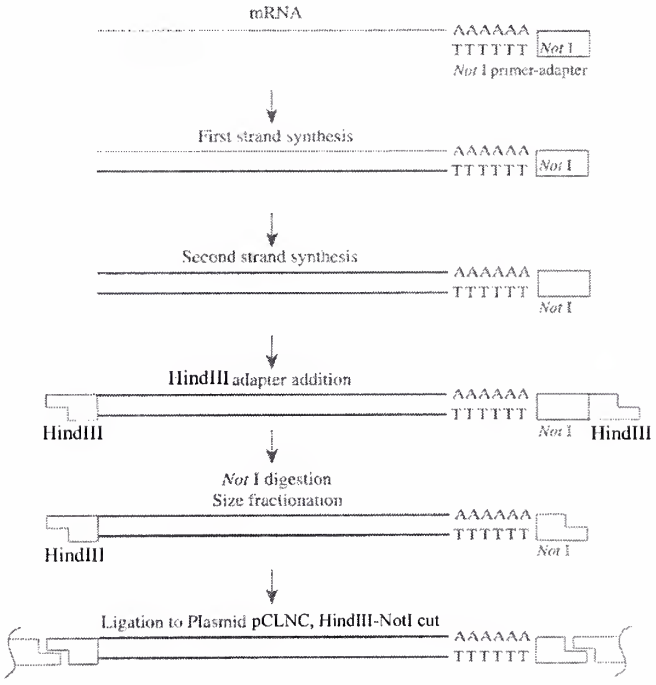

Figure 12 pCLNCMC38 library construction. pCLNCMC38 was created by first and second strand synthesis from MC38 polyA mRNA, followed by ligation into pCLNC cut with HindIII and NotI.

The pCLNC plasmid used for ligation was prepared from a HindIII-NotI digest of pCLNCGFP (Non SIN) plasmid, which was purified by agarose gel. Thus, the library

background resulting from autoligation of incompletely cut plasmid should be pCLNCGFP rather than empty pCLNC. This was done so that the self-ligation background could provide a useful parameter via fluorescence (by FACS or examination under UV scope) to assess the efficiency of gene expression during pooled transduction. Post-ligation colony counts are given in figure 13. This library is abbreviated as pCLNCMC38.

Elution fraction	10 uL	100uL
I-1	66	~500
I-2	160	>500
I-3	242	>500
II-1	154	>500
II-2	234	>500
II-3	252	>500
plasmid only (V)	2	80

Figure 13 pCLNCMC38 library post-ligation colony counts. The different ligation conditions and elution fractions were electroporated into bacteria to characterize their insert to background ratio. These colony counts of pCLNCMC38 show a high quality library with low (plasmid only) background. Roman numerals (I,II) indicates the elution fraction of polyA mRNA used in library synthesis. Arabic numerals (1,2,3) indicate different ligation conditions into pCLNC plasmid.

HindIII and NotI digests (liberating cDNA insert) were performed on 18 colonies chosen at random. Gel electrophoresis (figure 14) and sequencing (figure 15) of these randomly selected clones confirms that they contain a wide variety of genes, with a good range of insert sizes. A significant portion of the cDNA inserts include a start codon.

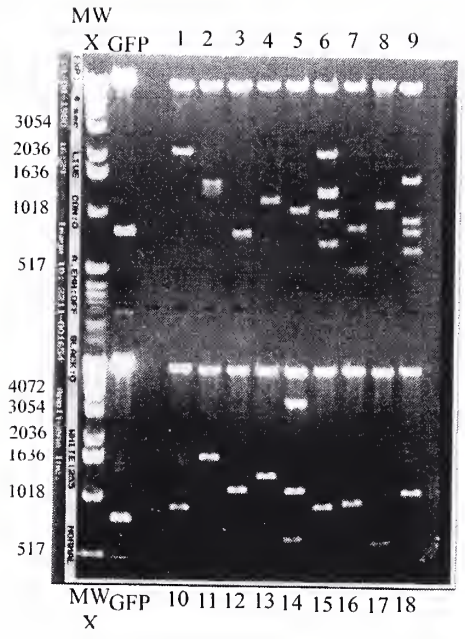

Figure 14 pCLNCMC38 library insert sizes. Gel electrophoresis of 18 randomly selected colonies from pCLNCMC38 library after restriction digest to liberate insert. This gel demonstrates a variety of cDNA insert sizes, from 800 bp to 3500^{+} bp.

Elution Group, ID	Highest Homology**	Homology	genbank sequence size	insert size by gel	Notes
I-2 1	mus musculus oxidative stress induced protein	152/162	2000 bp	2200	Includes start codon
I-2 2	No significant homology			1600	
I-2 3	rattus norvegicus mRNA for oligomycin sensitivity conferring protein (subunit c of mitochondrial H+ ATPase)	224/249		800	includes protein coding region but not start codon or mitochondrial targeting sequence
I-2 4	Bad Sequence			1400	
I-2 5	homo sapiens homeobox prox 1 mRNA	117/146		1300	
I-3 6	No significant homology			3500+	
I-3 7	No significant homology			800	
I-3 8	mus musculus MD3 mRNA (cell surface Antigen)	91/104		1100	
I-3 9	mus musculus stearyl-coenzyme A desaturase 2 (scd2)	212/215	5032	2000+	does NOT include start codon
II-2 10	mus musculus ferritin heavy chain mRNA	189/213	866	1000	Includes start codon
II-2 11	No significant homology			2000	
II-2 12	mus musculus ubiquitin-like 1 (ubl1)	207/211	1187	1200	Includes start codon
II-2 13	Bad Sequence			1400	
II-2 14	No significant homology			6000+	
II-3 15	mus musculus proteasome subunit B, type 2	32/36, 26/30		1000	
II-3 16	rattus norvegicus mRNA for ribosomal protein S3a	185/212		1000	
II-3 17	mus musculus ribosomal protein S8 (Rps8)	63/77, 31/33		700	
II-3 18	not sequenced			1200	

** No Significant Homology = BLAST score <= 50

Figure 15 pCLNCMC38 library characterization. The same 18 randomly selected colonies shown in figure 14 were sequenced for the first several hundred base pairs. These sequences were then matched for known homology by BLAST search. When known, the existence or lack of a start codon is noted.

Immunization of Mice Against MC38 Using Dendritic Cells Transduced en masse with an MC38 cDNA Library

Dendritic cells from C57BL/6 mice were transduced two times with pCLNCMC38 or pCLNCGFP. MC38 transduced dendritic cells were injected into the footpads of C57BL/6 mice. Control mice were injected with equivalent numbers of GFP transduced dendritic cells on the same schedule. Three separate immunizations of $3x10^6$ DCs were given on days 0, 13 and 15. These mice were randomized after immunization. Mice were challenged with subcutaneous bilateral tumors approximately 3 weeks after the final immunization with $2x10^5$ MC38 cells. The MC38 used in the tumor challenge was harvested from tumor-bearing mice in order to avoid anti-fetal calf serum mediated effects. Tumor growth was measured bilaterally on days 4, 7, 9, 11, 13, 18 and 21.

IFN-γ Release Assay

After the final dendritic cell harvest on day 7, $1x10^5$ viable T cells and $1x10^5$ dendritic cells were incubated in duplicate for 12 hours in 96 well plates. The murine IFN-γ concentration in the supernatant was determined by ELISA, using commercially available reagents (Endogen). Recognition was considered significant when IFN-γ secretion was greater than twice the appropriate background measurement. Significant values are bolded in tables.

Peptides

Peptides used in recognition assays were pulsed onto dendritic cells then washed four times in PBS prior to use in recognition assays. They were synthesized by Surgery Branch technicians and confirmed by mass spectroscopy. Human $gp100_{25-33}$: KVPRNQDWL, human $gp100_{44-59}$: RQLYPEWTEAQRL, $p15E_{604-611}$ (MHC Class I irrelevant control peptide): KSPWFTTL, HA (MHC Class II irrelevant control peptide).

RESULTS

Dendritic Cells Derived from Mouse Bone Marrow can be Efficiently Transduced Using Adenovirus and VSV-G Pseudotyped Retrovirus

To determine how amenable dendritic cells are to gene transfer using these methods, DCs were transduced with genes for GFP or human gp100. To determine whether the transduced cells efficiently expressed the transgene, a FACS analysis of the GFP transduced cells was performed (figure 16). Both adenovirally and retrovirally GFP transduced cells displayed cell surface markers similar to non-transduced dendritic cells. Specifically, the dendritic cells were strongly positive for CD11c, B7.1 and B7.2 expression (data not shown). For the purpose of calculating transduction efficiency, any $CD11c^+$, GFP^+ cells were considered to be GFP transduced dendritic cells.

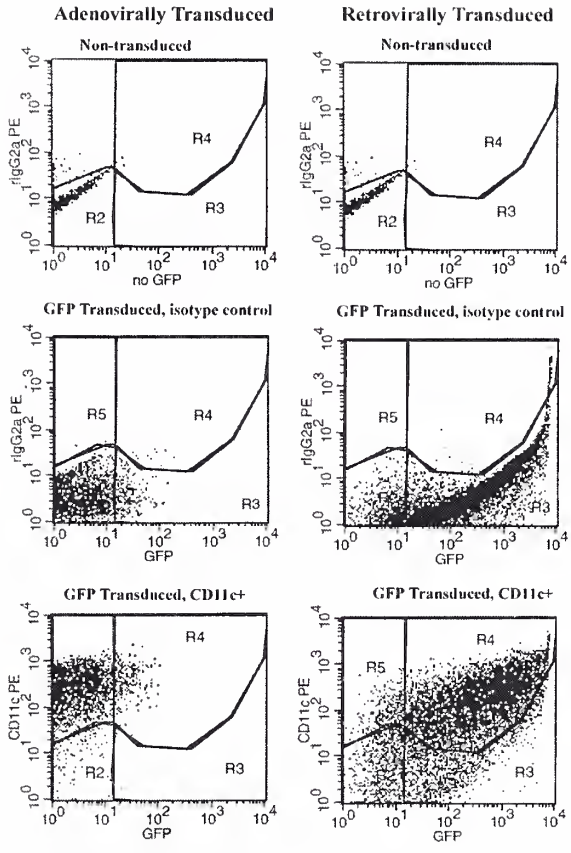

Figure 16 Adenoviral and retroviral transduction of dendritic cells with GFP. The green fluorescent protein (GFP) was inserted into dendritic cells using either a VSV-G pseudotyped retrovirus or an adenovirus. RatIgG2a is the isotype control for the antibody recognizing murine CD11c. Retrovirus was more efficient than adenovirus at transducing these dendritic cells.

For retrovirally transduced dendritic cells, the number and timing of transductions, along with the titer of the viral supernatant, determined the level of transgene expression in cells (figure 17). More frequent transductions with more concentrated supernatant resulted in higher transgene expression (23% for one transduction with unconcentrated supernatant vs. 79% for two transductions with 100x concentrated supernatant), but resulted in fewer dendritic cells per mouse. This reduction in dendritic cell yield most likely reflects direct toxicity from the VSV envelope or injury from the polybrene or spinoculation process.

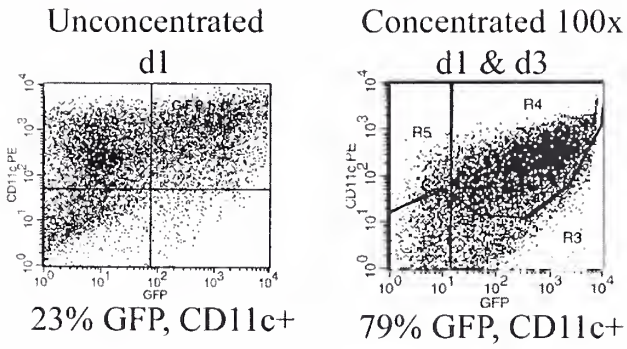

Figure 17 Transduction number and virus concentration affects transgene expression in DCs. The number of transductions and concentration of supernatant determines the level of transgene expression in retrovirally transduced dendritic cells.

Efficiency of adenoviral transduction of dendritic cells was related to the quality of the adenovirus used. In all circumstances, however, retroviral transduction resulted in higher levels of transgene expression than with adenoviral transduction, as measured by GFP fluorescence.

DR4-IE Dendritic Cells can be Transduced with Adenovirus and VSV Pseudotyped Retrovirus, and express DR4

Dendritic cells derived from the bone marrow of DR4-IE mice were efficiently transduced with genes for GFP or human gp100, using adenovirus or retrovirus (figure 18). GFP expression in retrovirally transduced cells was stronger than GFP expression in adenovirally transduced cells. In addition to the cell surface markers described above (CD11c, B7.1 and B7.2), these DCs were strongly positive for HLA-DR, indicating the presence of the chimeric MHC class II molecule on the surface of these cells.

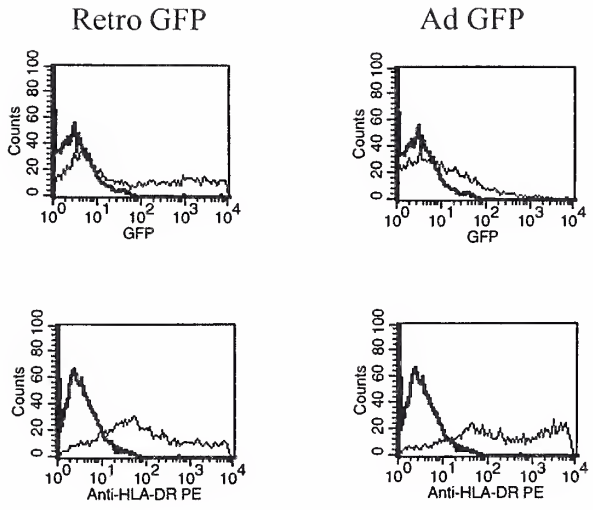

Figure 18 DR4-IE Dendritic cell transduction. DR4-IE dendritic cells transduced with GFP using a VSV-pseudotyped retrovirus or an adenovirus have good transgene expression, and are strongly positive for HLA-DR. Thick lines indicate FACS histogram of gp100 transduced dendritic cells, thin line indicates GFP transduced dendritic cells.

Dendritic Cells Can Be Transduced With Pools of Genes Using VSV Pseudotyped Retroviral Vectors

Fluorescence studies

To assess the behavior of individual transgenes transduced into dendritic cells with pools of other genes, the pCLNCGFP plasmid was diluted in empty pCLNCX plasmid prior to transfection of 293GP cells. This was done at ratios of 1:0 (i.e. 1 part pCLNCGFP to 0 parts pCLNCX), 1:10, 1:100 and 1:500. Since 293GP cells transfected with pCLNCX make a retrovirus with titers comparable to pCLNCGFP (data not shown), this provides a convenient model for assessing the behavior of individual transgenes in a pool. Assuming that pCLNCGFP represents the gene of interest, and that pCLNCX represents all other genes, the cell's fluorescence indicates the level of expression of an individual transgene in a gene pool whose size is determined by the dilution ratio. The dendritic cells in this experiment were transduced using unconcentrated supernatant, on day 1 only. Transduction efficacy could be improved by more frequent transductions or

more concentrated supernatant. The FACS analysis shows GFP expression, albeit at low levels, even at a pool size of 500 (figure 19). This indicates that transgene expression persists in large pools, but decreases rapidly with increasing pool size.

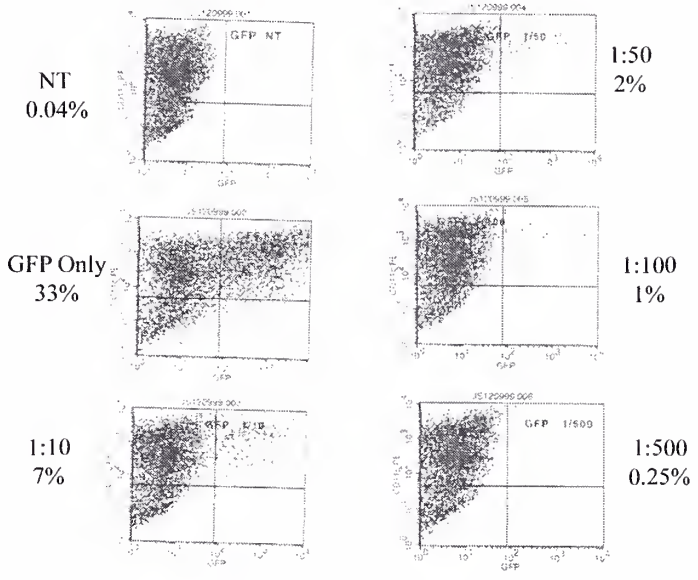

Figure 19 Dendritic cell transduction with gene pools. FACs analysis of dendritic cells transduced with pools of genes. PCLNCGFP plasmid was diluted in empty pCLNCx plasmid at the ratio given prior to transfection into 293GP cells. At a pool size of 1:100, 1% of dendritic cells are expressing an individual transgene.

Recognition Studies

Recognition studies were used to demonstrate that transduced dendritic cells can express and present individual transgenes to T cells, even when they are transduced using large pools of genes. The plasmid pCLNCgp100 was diluted in pCLNCGFP plasmid prior to transfection into 293GP cells. This was done at ratios of 1:0 (pCLNCgp100 only), 1:10, 1:100, 1:1000, and 0:1 (pCLNCGFP only). In this model, specific recognition of gp100 indicates the presentation of an individual transgene in a gene pool whose size is determined by the dilution ratio.

The unconcentrated supernatant was used to transduce the dendritic cells on day 1 only, and then $1x10^5$ mature dendritic cells were used in a IFN-γ release assay with $1x10^5$ $CD8^+$ T cells specific for the $gp100_{25-33}$ epitope. These pooled data demonstrate that gp100 transduced dendritic cells efficiently present antigen at a pools size of 100, but not a pool size of 1000 (figure 20).

Conc (mIFNg, pg/ml)	media	Non-transduced	GFP Transduced	gp100 transduced	1:10 gp100:GFP	1:100 gp100:GFP	1:1000 gp100:GFP	526
CD8 anti gp100	155	1180	1699	**6895**	**9934**	**8105**	1123	**5362**

Figure 20 Recognition of Dendritic cells transduced with gene pools. Dendritic cells transduced with supernatant derived from pCLNCgp100 diluted in pools of pCLNCGFP prior to 293GP transfection are recognized by gp100 specific $CD8^+$ T cells at a pool size of 1:100 but not 1:1000.

A Murine $CD8^+$ T Cell Clone Specific for $hgp100_{25-33}$ Recognizes hgp100 Transduced Dendritic Cells

To determine if the transduced DR4-IE dendritic cells were able to process and present a class I epitope encoded by the transgene, a murine $CD8^+$ T cell clone specific to the $H\text{-}2D^b$ epitope of gp100 ($hgp100_{25-33}$) was used in a cytokine release assay with gp100 transduced dendritic cells. This clone was able to specifically recognize the gp100 transduced dendritic cells above GFP transduced DC controls, for both adenovirally and retrovirally transduced cells (figure 21). Recognition of retrovirally transduced dendritic cells was greater than that of adenovirally transduced dendritic cells. Numbers given are results from a representative experiment. For controls, the $CD8^+$ T cells recognized DCs pulsed with $gp100_{25-33}$ peptide over DCs pulsed with an irrelevant MHC class I peptide ($p15E_{604-611}$). These findings indicate that transduced dendritic cells can efficiently present transgene-encoded epitopes along the endogenous pathway.

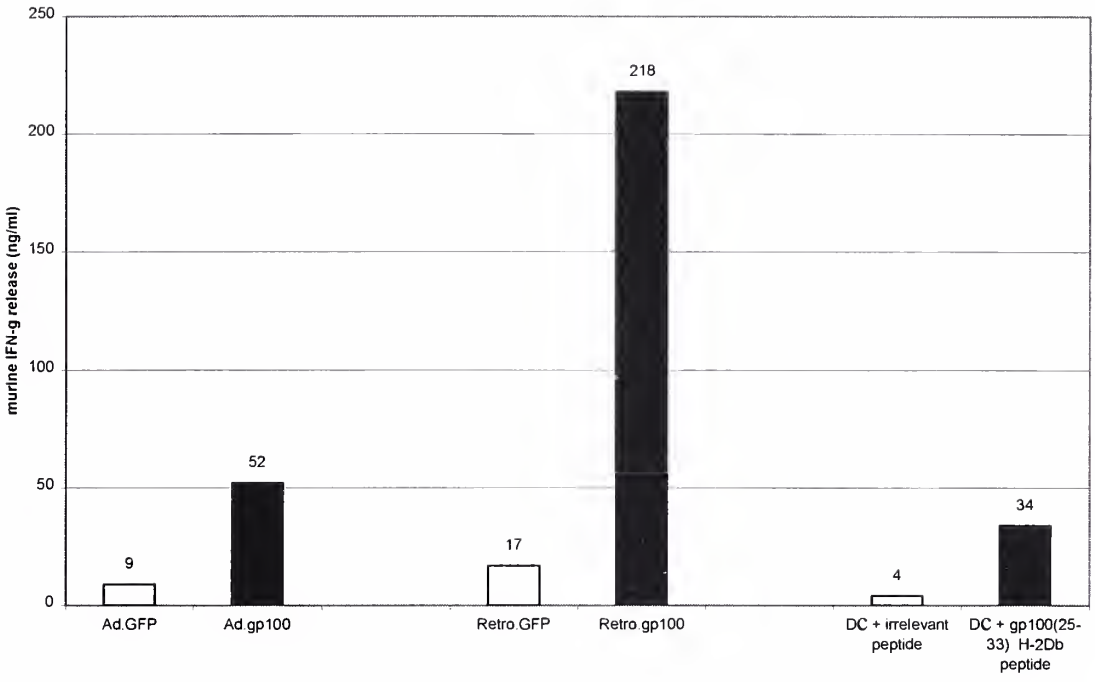

Figure 21 CD8$^+$ recognition of gp100 transduced dendritic cells. The CD8$^+$ T cell clone specific for $gp100_{25\text{-}33}$ recognized adenovirally and retrovirally gp100 transduced dendritic cells more than GFP transduced controls. DCs pulsed with $gp100_{25\text{-}33}$ peptide were recognized more than DCs pulsed with an irrelevant H-2D^b epitope.

A Murine CD4$^+$ T Cell Line Specific for $hgp100_{44\text{-}59}$ Recognizes hgp100 Transduced Dendritic Cells

DR4-IE dendritic cells identical to those used in the CD8$^+$ assay were used in a cytokine release assay with CD4$^+$ T cells recognizing the HLA-DRß1*0401 restricted $hgp100_{44\text{-}59}$ epitope. These CD4$^+$ T cells were able to specifically recognize the gp100 transduced dendritic cells above GFP transduced controls, for both adenovirally and retrovirally transduced dendritic cells (figure 22). This indicates that transduced dendritic cells were also able to process and present a class II epitope from an endogenous antigen. Again, recognition of retrovirally transduced dendritic cells was greater than that of adenovirally transduced dendritic cells. DCs pulsed with $gp100_{44\text{-}59}$ peptide were appropriately recognized over DCs pulsed with an irrelevant MHC class II peptide.

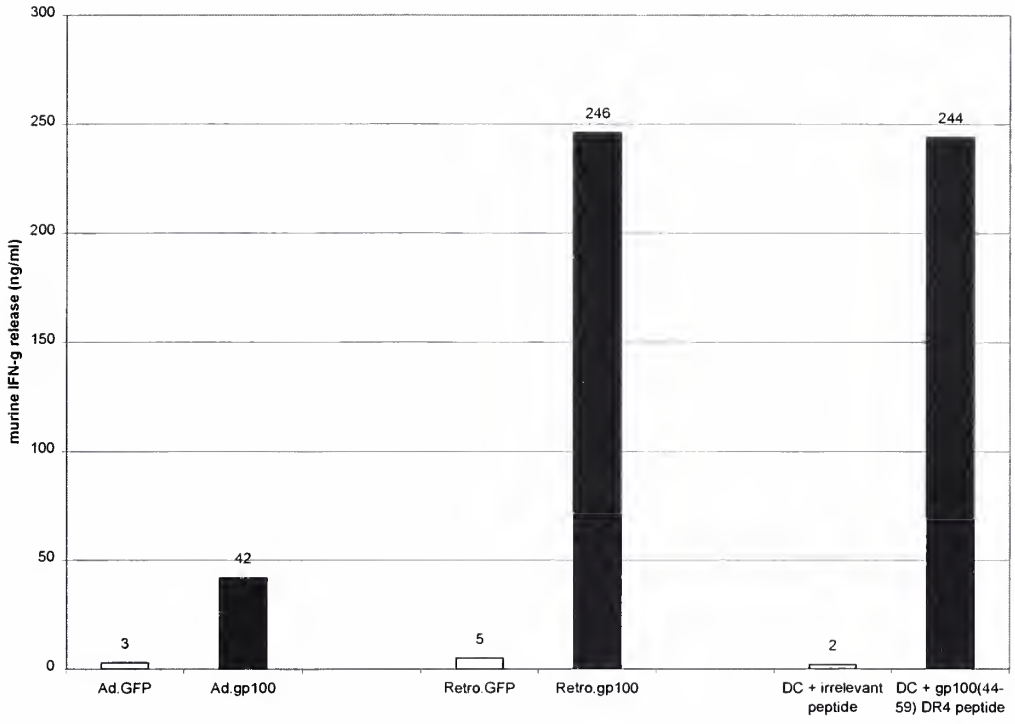

Figure 22 CD4$^+$ recognition of gp100 transduced dendritic cells. The CD4$^+$ T cell clone specific for gp100$_{44\text{-}59}$ recognized adenovirally and retrovirally transduced dendritic cells more than GFP transduced controls. DCs pulsed with gp100$_{44\text{-}59}$ peptide were recognized more than DCs pulsed with an irrelevant HLA-DRβ*0401 epitope.

CD40L Activation of Dendritic Cells Has Little Effect on Class I or Class II Presentation of Transgene

To determine whether further activation of the dendritic cells has any impact on class I expression of the transduced dendritic cells, the gp100 or GFP transduced, DR4-IE DCs were activated by replating them onto irradiated 3T3 cells expressing CD40L, or 3T3 cells expressing SAMEN. $1x10^5$ dendritic cells were then incubated overnight with $1x10^5$ gp100 specific T cells, and the cytokine concentration in the supernatant was measured.

Regardless of means of transduction or their method of activation, both CD4$^+$ and CD8$^+$ T cells were able to recognize gp100 transduced dendritic cells over their GFP

transduced counterparts (figure 23). Specific recognition of retrovirally transduced DCs was higher than adenovirally transduced DCs in all cases. In this experiment, CD40L activation seemed to have little effect on either class II or class I presentation, with the possible exception of dampening $CD4^+$ recognition of retrovirally transduced cells. This finding may indicate that dendritic cells produced using the methods described above are already maximally stimulated, and further activation may be non-productive.

CD8 Recognition	Media	Ad.GFP	Ad.gp100	Retro.GFP	Retro.gp100	Ad.GFP	Ad.gp100	Retro.GFP	Retro.gp100	DC + $gp100_{25-33}$ peptide	DC + p15E peptide
Activation		3T3-CD40L	3T3-CD40L	3T3-CD40L	3T3-CD40L	3T3-Control	3T3-Control	3T3-Control	3T3-Control		
media	3	4	5	4	3	17	15	26	16	27	7
CD8 gp100	12	6	**29**	5	**60**	12	**32**	10	**50**	**34**	4

CD4 Recognition	Media	Ad.GFP	Ad.gp100	Retro.GFP	Retro.gp100	Ad.GFP	Ad.gp100	Retro.GFP	Retro.gp100	DC + $gp100_{44-59}$ peptide	DC + HA peptide
Activation		3T3-CD40L	3T3-CD40L	3T3-CD40L	3T3-CD40L	3T3-Control	3T3-Control	3T3-Control	3T3-Control		
media	1	1	1	1	2	1	2	6	2	1	1
CD4 gp100	4	5	**14**	3	**82**	1	**11**	1	**225**	**244**	2

Figure 23 The effect of CD40L on antigen presentation in transduced dendritic cells. CD40L activation of gp100 transduced dendritic cells had no reliable discernable effect on class I or class II presentation of gp100 epitopes. In all cases, gp100 transduced DCs were recognized significantly more than GFP transduced dendritic cells.

Lack of Protection Against MC38 Using Dendritic Cells Transduced en masse With an MC38 cDNA Library

Dendritic cells were transduced with an entire MC38 cDNA library (one transduction only, using 10x concentrated supernatant) and then used in a recognition assay with MC38 TIL. The results are given in figure 24. Although MC38 TIL recognition of MC38 transduced DCs was higher than that of GFP transduced DCs, this recognition did not reach significance (twice background). This finding is not unexpected, given the scarcity of antigens recognized by 38 TIL and the very large number of plasmids in the library.

mIFNg (1:0, avg conc)	media	GFP DC	MC38 DC	B16 Tumor	MC38 Tumor
media	6	10	12	7	8
38 TIL	530	506	748	>1000	>1000
B16 TIL	275	261	243	**>1000**	**>1000**

Figure 24 Lack of Recognition of dendritic cells transduced with MC38 library. MC38 TIL do not significantly recognize dendritic cells transduced with an entire pCLNCMC38 library above GFP transduced dendritic cell controls. Both MC38 TIL and B16 TIL recognize tumor as positive controls for the T cells.

The MC38 or GFP transduced dendritic cells were injected into the foot pad of mice for a total of three immunizations over 15 days. These mice were randomized and challenged subcutaneously with a reliably tumorigenic dose of freshly isolated MC38 tumor. Results of tumor growth measurements are given in figure 25. There are no significant differences in tumor growth between mice immunized with GFP transduced dendritic cells or dendritic cells transduced with the MC38 library. This indicates that DCs transduced with an entire MC38 cDNA library were an ineffective means of immunizing against MC38 tumor.

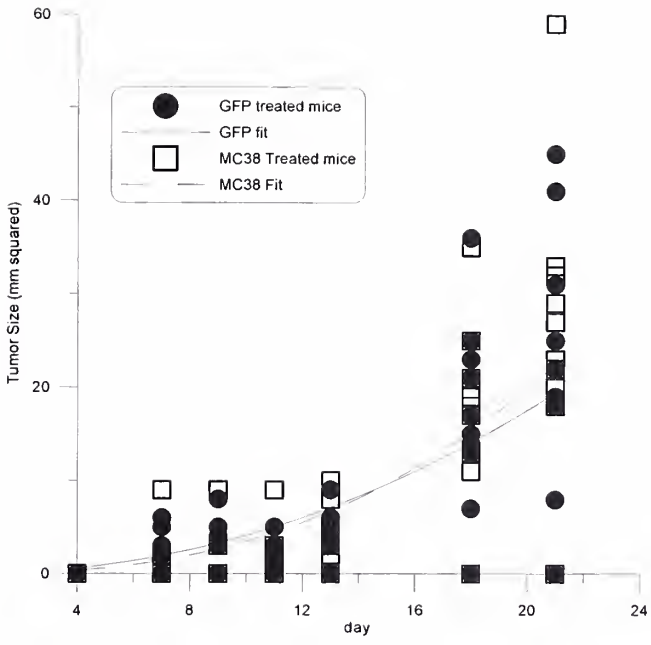

Figure 25 Lack of protection from MC38 by immunization with MC38 transduced dendritic cells. Repeated immunizations with pCLNCMC38 transduced dendritic cells failed to protect mice against subcutaneous MC38 tumor challenge beyond immunization with pCLNCGFP transduced dendritic cell controls.

Discussion

VSV-G Pseudotyped Retroviral Transduction Efficiency

The relative ease of dendritic cell transduction and high levels of transgene expression obtained with VSV-G pseudotyped retroviral vectors is evident throughout these experiments. It is crucial to interpret these transduction efficiencies with caution, since experience with these retroviral constructs suggests that viral titer depends greatly on the size and properties of the insert. For example, pCLNCx (no insert), pCLNCgp100 (2 kb insert) and pCLNCGFP (800 bp insert) all give unconcentrated titers of 10^7 colony forming units (cfu)/ml but pCLNCgp70p15E (2.2 kb) produced unconcentrated virus with a much lower titer, 10^5 cfu/ml. This is perhaps due to unusual tertiary structure in the gp70p15E RNA, or secondary to interference from the p15E gene product with viral budding from the virus-producing cell. A plasmid containing a much larger insert,

pCLNCβgal, produced an intermediate viral titer of 10^6 cfu/ml. These variations in viral titer caution against inferring the transduction efficiency of one construct using data extrapolated from the transduction efficiency of another plasmid.

Even taking into account the variability between specific retroviral plasmids, and the differences between batches of adenoviral vectors, VSV-G pseudotyped retroviral vectors consistently outperformed adenoviral vectors in both fluorescence and recognition assays of gene transfer efficiency. This suggests that other investigators may wish to use pseudotyped retroviral vectors as a convenient, efficient means of dendritic cell transduction.

MHC Class I and Class II Presentation of Tumor Antigen in Transduced Dendritic Cells

The data presented here demonstrate that adenovirally and retrovirally transduced dendritic cells are capable of presenting class I and class II epitopes of the melanocyte differentiation antigen, gp100. CD40L activation of the dendritic cells had little impact on their ability to present either epitope, perhaps reflecting the mature state of the dendritic cells prior to biochemical manipulation. These findings join a growing body of evidence suggesting that gene-modified dendritic cells can present both MHC class I and class II epitopes, and therefore may be a means to simultaneously initiate a $CD4^+$ and $CD8^+$ anti-tumor response.

As discussed in the introduction, gp100 contains a melanosome transport signal (MTS) known to traffic antigens to the endocytic pathway for class II presentation. What role, if any, the transport signal plays in the class II epitope presentation of this epitope is not known. Furthermore, the class II presentation of any given cytoplasmic antigen must

be determined individually, as it involves many factors, including the rapidity of protein turnover in the cytosol[47]. Because of these caveats, class II presentation of all endogenous antigens cannot be presumed from the finding that transduced dendritic cells are capable of presenting a gp100 derived class II epitope. Nonetheless, it would be important to compare the abilities of different targeting signals (MTS, LAMP and the Invariant chain) to augment class II presentation. This may allow investigators to generalize about the most efficient class II targeting signal, and take this into account when designing the constructs used for dendritic cell transduction.

The Potential for Using Dendritic Cells Transduced with Pools of Genes to Find New Tumor Antigens

These data demonstrate that dendritic cells can be efficiently and simultaneously transduced with large numbers of genes using VSV-G pseudotyped retrovirus. This was shown both using FACS data gathered from cells which were transduced with pCLNCGFP diluted in pCLNCX and using recognition data of cells transduced with pCLNCgp100 diluted in pCLNCGFP. The ability to transduce DCs with pools of genes, combined with the ability to protect mice from tumors after immunization with transduced dendritic cells may provide a new and important means of discovering tumor antigens.

By immunizing mice with DCs transduced with pools of genes encoding potential antigens obtained from a tumor and then challenging immunized mice with that tumor, one could identify mice that were protected from tumor challenge. The antigen responsible for that protection could then be isolated from the original gene pool by repeated rounds of sub-pooling and tumor challenge (as shown in figure 5). The

importance of this method is that it uses tumor rejection as its endpoint, and therefore discerns only potentially useful rejection antigens. It can be applied to any type of tumor, and may give an indication of which types of cancers will prove ultimately amenable to immunotherapy. The system also overcomes a major drawback in current antigen finding methods, which only identify the subset of antigens recognized by T cells that have a high precursor frequency in peripheral blood or infiltrate tumors in vivo.

The recent push to develop better murine models for human cancers will result in more tumor models amenable to immunotherapy work and antigen discovery using these techniques. For example, current breast cancer models are generally inappropriate for immunotherapy study, because they depend on tumors induced by the murine mammary tumor virus (MMTV). MMTV greatly alters the antigen profile on the tumor, and does not have a clinically relevant human counterpart. Once better models are acquired, the antigens discerned from work in mice may have homologous human antigens, as was found to be the case with melanoma.

The major disadvantage to this method of antigen finding is that it is extraordinarily labor intensive. Given the evidence that individual transgenes are expressed in > 1% of cells and capable of being effectively presented on DCs at a pool size of 100, this is an appropriate pool size for initial screening. Assuming that approximately 20,000 genes are expressed in a well-differentiated cell[1], it would require

[1] The number of genes expressed in a given cell varies greatly depending on its tissue type and condition. For example, human kidney expresses 19477 genes, 3034 of which are unique (only 30 of the unique genes are known). The best estimates come from expressed sequence tag (EST) databases, available on line at the Cancer Genome Anatomy Project (http://www.ncbi.nlm.nih.gov/CGAP/hTGI/sumtab/cgapba.cgi). As preliminary analyses of the human genome project draft arrive (International Human Genome Sequencing Consortium, Nature 409: 860-921, 2001), it appears that the EST databases have overestimated the number of genes present in any given cell.

200 pools to thoroughly screen the library. Even this would not ensure that all antigens have been screened, as more common genes would be included multiple times, while less common genes may not be included in the screening at all.

There are a number of strategies that could reduce the number of potential antigens needing to be screened using this method. Through library subtraction techniques, one could develop a pool of genes expressed in a neoplastic cell but not in a normal cell of the same tissue type from the same patient. This method would more efficiently identify potential antigens that have become activated during oncogenesis, e.g. embryonic antigens, and would obviate the need to screen extremely common genes, such as actin, that have little to no potential for tumor-selective antigenicity. Unfortunately, using the same tissue type for library subtraction would prevent the identification of normal differentiation antigens and mutated antigens, as these genes would be removed during subtraction. This process can be visualized and analyzed using bioinformatics tools, such as digital differential display[48]. Variations on this idea produce slightly different results. For example, subtracting the library of the tumor cell from a different cell type would enrich for differentiation antigens, but still eliminate mutated antigens of proteins shared by the cell types.

Dendritic Cells Transduced with Genetic Material Obtained from Tumor as a Means of Tumor Protection

In these experiments, mice were immunized with dendritic cells transduced en masse with a tumor cDNA library from MC38, and then challenged with the corresponding MC38 tumor. This immunization resulted in no protection from tumor challenge beyond control mice immunized with GFP transduced dendritic cells. Given

the data in figure 20 demonstrating the lack of specific recognition of dendritic cells transduced with gp100 in gene pools of 1000, the lack of immunization by DCs transduced with a much larger gene pool (the 38,400 plasmid library) is not entirely surprising. A more vigorous schedule of immunization with similarly transduced dendritic cells is unlikely to result in protective immunity.

An intriguing question is how less efficient strategies for gene-modifying DCs were able to generate protective immunity. For instance, a group has reported for a number of different tumor types that dendritic cells transfected with RNA obtained directly from the tumor are able to immunize mice despite transient and mediocre amounts of transgene expression[49]. The difference cannot be explained by relative over-representation of antigens in RNA, as they should be proportionately represented in a tumor cDNA library. Given the data described in these experiments, it is difficult to imagine that an antigen in a complete library of RNA (driven by weaker, physiologic promoters) will be expressed at useful levels, even transiently. It would be important to validate these approaches using pooled transfections similar to those described in this paper before proceeding to clinical trials of RNA transfected DCs. The recent, well publicized[50] work on poorly characterized tumor cell-dendritic cell hybrids[51] raises similar concerns. Identifying and cloning antigens recognized by the T cells involved in the anti-tumor response remains the gold standard for validating immunization methods. The many groups that claim success in immunizing patients with dendritic cells transfected with genetic material obtained directly from tumor have yet to meet this standard.

Conclusion

The same qualities that make dendritic cells ideal for initiating an immune response to infection make them attractive for generating an anti-tumor immune response. The simplicity of this logic has sparked manifold experiments in animals and humans using antigen loaded DCs for the immunotherapy of cancer. Data on the efficacy of tumor antigen presentation to T cells by dendritic cells is rapidly accumulating. Amongst these data, there is increasing evidence that gene-modified dendritic cells are an efficient means of presenting both class I and class II tumor antigens. The data presented in this paper strongly support this assertion.

Each APC must present antigen on its surface at levels detectable by T cells. The most efficient known method of DC transduction is described in this paper, but is still unable to raise protective immunity using DCs transduced with entire tumor libraries. These experiments describe the importance of delineating individual trasngene expression and antigen presentation in cells modified with tumor-derived genetic material.

The anti-tumor immune response has limitations that cannot be circumvented through more efficient antigen presentation. Although dendritic cells in general, and transduced dendritic cells in particular, show promise as a means of presenting tumor antigens to T cells, they will not overcome problems related to tolerance, tumor escape, T cell suppression and improper T cell trafficking and activation. While these considerable barriers are being studied, transduced dendritic cells may prove useful for antigen discovery and gaining insight into the anti-tumor immune response.

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