NOVEL APPROACHES TO IDENTIFY T CELL-RECOGNIZED TUMOR ANTIGENS AND TO REDIRECT T CELLS FOR ADOPTIVE IMMUNOTHERAPY

by

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DEDICATION

This work is dedicated to:

My Mother, Hualian Liu
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Tumor antigens (Ags) and adoptive immunotherapy are two important topics in tumor immunology. Traditional methods for identifying T cell-recognized tumor antigens and adoptive therapy using antigen-specific T cells are laborious and difficult. Rapid developments in molecular biology and immunology have allowed us to design novel strategies to achieve these two goals more efficiently.

A novel strategy, SING (Signal transduction molecule-mediated, NFAT-controlled, GFP expression), for cloning T-cell recognized tumor Ags, was designed using Ag-specific T cells. The SING system is an artificial Ag presentation system, in which a mouse T cell line BW5147 has been manipulated to respond to stimulation by Ag-specific TCR (the resultant BW5147 cells are named BS cells). Either Ag peptide-pulsed or Ag-expressing BS cells could become transiently fluorescent (GFP+) and puromycin resistant after TCR engagement. In combination with retrovirally mediated functional genomics, the SING strategy should allow us to isolate antigen-expressing (GFP+) cells directly and retrieve sequences coding for tumor antigens by PCR amplification of genomic DNA from GFP+ BS cells.

To investigate whether three-domain single chain T cell receptors (3D-scTCR) are able to redirect T cells to recognize tumor cells, multiple scTCR constructs were constructed and retrovirally transduced into T cells. The effects of CD8, CD28 and the complete CD3 complex on scTCR-induced T cell activation were also determined. Compared with full-length TCR (fTCR)-modified T cells and native CTLs, scTCR-
modified T cells had high thresholds for response to Ag stimulation. After adoptive transfer of TCR (either scTCR or flTCR)-modified T cells into tumor-bearing mice, the \textit{in vivo} tumor growth was controlled to some extent, although most TCR-modified T cell recipient mice didn’t show significant signs of anti-tumor effects. This result suggests the possible application of scTCR- as well as flTCR-modified T cells for adoptive immunotherapy.

Finally, to accomplish the above goals, we systematically investigated the optimal conditions for transduction of murine primary T cells as well as T cell lines. Our results showed that successful infection of murine primary T cells required a combination of high titer (>10^7 CFU/ml) of ecotropic retroviral vectors and proper timing of infection (within 24 hours after mitogen stimulation).
CHAPTER 1
INTRODUCTION

1.1 Literature review

Part I: Retroviral Vectors as Important Tools in Research of the Immune System

Retroviral vectors have been widely used in both basic studies and clinical trials. These vectors are easy to manipulate and provide stable, long-term gene expression because of their ability to integrate into the genome (1). There are two major types of retroviral vectors. The first type are the murine leukemia virus (MuLV)-based vectors. The second type is a lentiviral vector derived from human immunodeficiency virus (HIV). The former type can only transduce dividing cells because this type of virus do not have a mechanism to traverse the nuclear envelope, whereas the latter one can transduce both dividing and non-dividing cells (1-4). In most applications, the retroviral vector should be replication-defective to insure safety. This feature can be accomplished by replacement of most or all of the coding regions of a retrovirus (\textit{gag, pol} and \textit{env}) with the gene(s) or sequence elements of interests, so that the vector by itself is incapable of making the proteins required for additional rounds of replication (1-6). Viral proteins needed for the initial infection can be provided in \textit{trans} by a retroviral packaging cell. Packaging cell lines are engineered to express the necessary retroviral genes required to generate an infectious retroviral particle (typically \textit{gag, pol} and \textit{env}) (5,6). Generally, these viral genes are introduced independently so that the chance that
the *gag*, *pol* and *env* genes will recombine with the retroviral vector to recreate a replication-competent virus can be significantly reduced or eliminated (7).

**Retroviral Receptors, Tropism and Pseudotyping**

The host range of a retrovirus is determined by its envelope glycoprotein. There are at least 6 types of receptors for MuLV-based vectors (3). The best characterized and most widely used are the Env proteins of the ecotropic (can only infect mouse and rat cells) and amphotropic (can infect many mammalian cells) MuLVs (8,9). Ecotropic retroviruses can only infect mouse or rat cells. It is possible to change the host range of retroviral vectors by generating viral particles that express an Env protein from a virus with a different host range (pseudotyping). The most commonly used Env is the envelope glycoprotein from vesicular stomatitis virus glycoprotein G (VSV-G) (6,10). It provides two major advantages over autologous retrovirus Env proteins. First, VSV-G imparts a very broad host range on the viral particles because it binds to phospholipid components that are expressed ubiquitously, even on insect and other nonmammalian cells (10). Second, the VSV-G envelope protein is very stable. It can withstand very strong sheering forces due to ultracentrifugation without significant loss of activity (2,10). Therefore, VSV-G-pseudotyped retroviruses can be purified by ultracentrifugation to titers that are 100–1000-fold greater than those of viruses that express autologous Env proteins (10). However, the VSV-G protein is cytotoxic. Therefore, VSV-G pseudotyped vectors are generally produced by transient transfection (2,10).
Design of Retroviral Vectors

Efficient gene transduction and integration depend on the inclusion in the retroviral vector of a number of cis-acting viral elements. These include the long terminal repeats (LTRs) at both ends of the integrated provirus. The LTRs are composed of three discrete regions; U3, R and U5 (3). Most of the elements required to transcribe the provirus (promoters and enhancers) are located in U3. The R and U5 regions are required for reverse transcription. The LTRs also contain short sequences, called attachment sites (att), which mediate integration of the retroviral DNA into the host genome. A specific packaging signal, v, located between 5' LTR and gag coding sequence, allows the retroviral genomic RNA to be introduced into a viral particle (1-5).

In most cases, expression of genes of interest is controlled by the retroviral 5' LTR. Changes can be made in the enhancer/promoter of the LTR to provide efficient virus production or tissue-specific expression (3,6,11,12). Replacement of the LTR U3 region with an early promoter/enhancer from the cytomegalovirus (CMV) (a potent viral promoter) has led to a more than 10-fold increase in virus production (13). Selectable markers, such as neomycin or hygromycin resistant genes, are generally included to facilitate selection of vector-transduced cells (3). Two general strategies have been used to design retroviral vectors that express two or more proteins: (1) use of the promoter in the LTR and internal promoters to drive transcription of different cDNAs, and (2) use of an internal ribosomal entry site (IRES) to allow translation of multiple coding regions from a single mRNA. Vectors containing internal promoters (placed between the LTRs) have been widely used to express multiple genes (2,3,14). It has been reported, in
vectors with internal promoters, that selection for the expression of one gene resulted in reduced expression from the other gene also present in the vector due to promoter interference (2.6). However, this effect is highly dependent on vector construction and, in some cases, is insignificant. To overcome possible interference, several groups have attempted to eliminate the promoter/enhancer activity of the LTR by deletion of the U3 region of the 3’ LTR. These types of vectors are known as self-inactivating vectors (SIN vectors) (15,16). After one round of vector replication, these changes are copied into both the 5’ and the 3’ LTRs producing an inactive provirus. However, any promoter(s) internal to the LTRs in such vectors will still be active. By introducing tissue-specific or inducible promoters into these vectors it is possible to get highly specific gene expression in target cells. Drawbacks of using SIN vectors include a lower viral titer in comparison with vectors having intact LTRs (17). However, introduction of the EBNA/OriP from Epstein-Bar virus (EBV) into SIN vectors allows episomal amplification of transfected DNA in packaging cells resulting in dramatic increases in virus production (18).

Virus Production Techniques

Vector production can be achieved by transient transfection of retroviral constructs into packaging cells (7,19). Transient transfection avoids the longer time required to generate stable vector-producing cell lines and is used if the vector or retroviral packaging components are toxic to cells (e.g., VSV-G) (3,7). However, virus titers generated in this way are low (~10⁴ CFU/ml) when 3T3-based packaging cells are used (3,5-7). Highly transfectable cells, such as the human embryonic kidney cell line, 293 or
its derivative 293T, are generally utilized in this case. To obtain a definitive source of retroviral vectors, stable virus-producing cells needs to be established (6).

Packaging cells are resistant to infection by vectors produced by the same cell line because the endogenously produced Env blocks the cell surface receptors required for retroviral entry (3,5). Thus, the vector copy number in a vector-producing packaging cell line remains relatively constant, although it does increase slowly. However, if vector-producing packaging cells are cocultivated with a packaging cell line that expresses an env gene which does not interfere with the env gene in the first helper cell line, the vector can "ping-pong" between cells, resulting in multiple reinfection events (20). An increase in the number of copies of the genome of the vector per cell results in an increase in the titer of produced vectors (21, 22). Although vector titer can be increased by using this “ping-pong” amplification procedure, there is also a greater chance that the vector will rearrange and that helper virus will be produced during the amplification process (22). The copy number of proviruses that integrate into a cell can also affect gene-expression levels. Replication-defective retroviruses do not show viral interference (3,5,6). As a consequence, multiple infections with retroviral vectors can lead to the integration of more than one copy of the virus in the genome, all of which express the gene of interest.
Retrovirally Mediated Functional Cloning

A preferable method for functional identification of gene targets whose expression are involved in physiological events is to screen candidate gene-containing libraries (23-25). Since it generally takes several weeks for screening processes to be finished, stable expression of transgenes is critical (23). This requirement renders the retroviral vector-based gene transfer method the best choice. Intrinsic properties of retroviruses, such as efficient gene transfer efficiency and ability to integrate, make them useful tools for drug discovery.

To construct retrovirally mediated libraries, several approaches have been used to create dominant genetic effectors (using cDNAs, antisense RNAs, or mini-genes) (23, 26-28). Introduction of these libraries of genetic effectors into cells using retroviral vectors yields a population of individually ‘mutagenized’ cells (23). Those cells with a new phenotypic response to biological or disease-specific stimuli owing to expression of a specific genetic effector can then be selected and isolated. By defining the interactions of these effectors with other proteins in the cell, potential targets for the development of therapeutic agents can be identified. High gene transfer efficiency allows for construction of very complex libraries ($10^8$ complexity and greater) (23, 26, 29). The power of retroviral vectors as delivery systems, coupled to functional techniques for studying the regulation of signal transduction pathways in cells, has led to identification of novel functions for a variety of cell-surface antigens, receptors and signalling proteins (30-35).
Instead of full-length cDNA libraries, fragmented cDNA libraries (36) and oligo-based libraries have been also used for drug discovery (37-39). To study antigen presentation, Tolstrup et al. (40) designed a partially randomized peptide library in a test system using a well-characterized MHC I antigen and expressed the library in a mouse antigen-presenting B-cell line. On the basis of a functional T-cell activation assay, a known antigen peptide was successfully isolated. Mutagenesis of a cDNA encoding a known pathway regulator by error-prone PCR (41), random fragmentation (42) or DNA shuffling (43,44) can be used to rapidly map functionally important domains of the molecule. For example, Kessels et al. (31) expressed a retroviral library of T-cell receptors (TCRs) mutagenized in the complementarity determining region 3 (CDR3) in a TCR-negative T-cell line to identify TCR variants with altered binding affinities for specific antigen complexes.

The ability to screen large libraries effectively and to readily isolate genetic effector elements of interest is the key to this technology (23,25). Retrovirally mediated functional genomics can be applied universally if selection methods are employed successfully. Most early studies used this technology to isolate oncogenes because the oncogene-transduced, non-transformed cells exhibited a growth advantage that could easily be identified (23,29). Other screening approaches have utilized retroviral full-length or fragmented cDNA libraries to isolate genes encoding dominant effectors of cellular phenotypes (23-25). Recent advances include uses of inducible expression systems or GFP-based reporter gene systems (45,46). Stable genetic effector expression allows for multiple rounds of selection. Once cells displaying the desired phenotype are
isolated, the genetic effector DNA is typically recovered by PCR amplification and its
sequence then determined (47). In some cases, application of inducible retroviral vectors
is preferred for the functional cloning of molecules which are involved in cell activation
and signal transduction (45). Examples include assays measuring antigen-receptor-
stimulated immune cell activation, cytokine-dependent responses, and increased tumor
cell sensitivity to chemotherapeutics (23,30,39,45). Inclusion of specific regulatory cis-
elements followed by reporter genes in SIN vector backbones will only allow gene
expression when specific pathways are activated (23,48).

Applications of Retroviral Vectors in Immunological Research

Immune cells, such as T, B and NK cells, macrophages (Mφ) and dendritic cells (DC)
play central roles in the immune system. Characterization of these cells via gene transfer
has been a powerful tool to dissect the function of transgenes (2). Many MuLV-based
vectors have been developed that can infect primary murine B and T lymphocytes and
express genes at a stable, high level in these cells (49-51). However, optimal infection
of lymphocytes only occurs when they are efficiently activated. Activation is usually
achieved by stimulating the antigen receptor and/or providing growth factors (49).
Lymphocyte progenitor cells, such as immature thymocytes and B cells, as well as
hematopoietic stem cells (HSCs), can also be infected with retroviruses (52-54) The
infected progenitor cells can then be used to study the role of genes in the development
and function of immune cells, either in culture (e.g., in fetal thymic organ cultures) or in
vivo, by using the cells to reconstitute the immune system of lethally irradiated mice
(52,53). A significant limitation of gene expression by retroviruses in HSCs is that
many vectors are silenced in these cells and, therefore, the transferred genes are not
expressed in the progeny of the infected cells (55). Retroviruses can also be used to
introduce genes into murine DCs and Mφ (56-59). The optimal time for infection is
during the first several days of culture, when these cells are undergoing maximal
proliferative expansion (56-58).

In some cases, the MuLV LTR does not remain active in embryonic stem cells or
HSCs, the precursors of all immune cells (60). Silencing of retrovirus expression may
be a result of transcriptional repression mediated by factors present in these cells, as
well as methylation of GC-rich retroviral sequences within the LTR (60,61). By altering
the enhancer region and other cis-acting elements in MuLV-based vectors, it is possible
to generate retroviruses, such as the murine stem cell virus (MSCV) (11), that are less
susceptible to these silencing mechanisms. These viruses show sustained expression in
HSCs and in their progeny, including immune cells, and have proven to be very useful
tools to study the murine immune system (50-53). Retroviral vectors are also able to
express genes under the control of heterologous promoters (3). These promoters can be
strong constitutive promoters, such as the cytomegalovirus (CMV) promoter, or tissue-
specific promoters.

The efficiency of gene expression from a retroviral vector is dependent on the site in
the genome where the virus integrates (i.e., positional effects) (3,6,23). Positional
effects can lead to a wide range of gene-expression levels. In an attempt to reduce the
impact of positional effects on retrovirus expression, a number of groups have
incorporated insulator or scaffold attachment region sequences into their vectors (62-
These sequences appear to provide a boundary between active and inactive chromosome domains and to increase the likelihood that retroviruses are expressed in stem cells.

In summary, retroviral vectors have become popular tools to study gene function in the murine immune system (Table 1, ref. 2). The vectors are easy to manipulate and provide stable, long-term gene expression. Current retroviral vectors do have limitations that affect their usefulness in certain applications. However, recent advances suggest a number of ways in which these vectors might be improved to extend their utility in immunological research.
Table 1 Examples of different uses of retroviral vectors to study immune cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Application of method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow stem cells</td>
<td>Express genes in all hematopoietic lineages <em>in vivo</em> following infection of stem cells <em>in vitro</em></td>
</tr>
<tr>
<td>Thymocytes</td>
<td>Express genes in thymocytes to study development</td>
</tr>
<tr>
<td>Mature T cells</td>
<td>Express genes in activated CD4+ or CD8+ T cells to study activation, function and cell death</td>
</tr>
<tr>
<td>Mature T cells (ex vivo)</td>
<td>Express genes in T cells that are primed <em>in vivo</em></td>
</tr>
<tr>
<td>Mature T cells (regulated gene activity)</td>
<td>Regulate activity of retrovirally expressed genes using ER system</td>
</tr>
<tr>
<td>Mature T cells (adoptive transfer)</td>
<td>Express genes in activated antigen-specific T cells <em>in vitro</em> and then study function <em>in vivo</em></td>
</tr>
<tr>
<td>Pro-B cells</td>
<td>Express genes in pro-B cells to study development</td>
</tr>
<tr>
<td>Mature B cells</td>
<td>Express genes in activated B cells to study activation, function and cell death</td>
</tr>
<tr>
<td>Bone-marrow-derived DCs</td>
<td>Express genes in bone-marrow-derived DCs to study T cell activation, DC function and cell death</td>
</tr>
</tbody>
</table>

ER, estrogen-receptor ligand-binding domain.

Adapted from Ref. 2
Part II: Genetic Manipulation of T cells for Adoptive Immunotherapy

TCR gene transfer

Adoptive transfer of T cells genetically modified with immunoreceptors, such as T cell receptor (TCR) and single chain antibody (T-body), has emerged as a promising approach to redirect specific immunity towards tumors and viral infections (65-70). This strategy avoids the limitation of low frequency of antigen specific T cells, allowing for facilitated expansion of antigen-specific T cells to therapeutic doses (70,71).

In the majority of TCR-based studies (71-75), primary T lymphocytes have been retargeted with a tumor or virus specificity by transfer of full-length TCR (fITCR) genes. These redirected T cells were shown to respond specifically toward target cells expressing both the corresponding Ag and the correct MHC molecule. The cytolytic efficiencies of the TCR-transduced T lymphocytes were in the same range as those of the parental CTL clones (74-76). Most importantly, the peptide fine specificities of the transduced TCRs were identical with those of the parental CTLs (77).

In a pioneering study by Kessels and his colleagues (74), they analyzed the in vivo feasibility of TCR gene transfer with respect to antigen-driven expansion and homing of TCR-transduced T cells. They found that TCR-transduced cells promoted tumor eradication in both immune competent and immunodeficient settings. Tumor clearance was accompanied by a prominent expansion of antigen-specific CD8$^+$ T cells and long-term survival of these mice was 100%. Upon rechallenge of the TCR-modified T cell recipient mice with antigen-expressing tumor cells after 80 days of tumor-free status, a
rapid increase in the frequency of these cells was noted and no expansion of tumor growth was observed. In addition, TCR-modified T cells showed a similar homing pattern as regular CTL.

Despite the significant success of ftTCR gene transfer in bestowing specificity and functionality, its efficiency could be compromised by several factors. First, incorrect chain pairing between endogenous and transgenic TCR molecules plus possible instability of exogenous TCR α chains might result in suboptimal expression of the desired TCR heterodimers (76, 78). Second, incorrect α and β chain pairings might alter TCR antigen recognition specificity leading to autoimmunity (74) and third, the CD3ζ chain, which is required for surface expression of the TCR complex (79-81), has been reported to be downregulated in cancer patient T cells (82), thus potentially limiting the functional efficiency of full-length transgenic TCRs.

Another concern about the efficiency of genetically modified T cells is that Moloney-based vectors are sometimes sensitive to promoter silencing in vivo (83). Therefore, the passively transferred, TCR-modified T cells might not sustain long-term memory due to the loss of expression of the transgenic TCR. Elicitation of endogenous immune system activation against tumors by massive tumor antigen release caused by exogenous Ag-specific T cells might alleviate this disadvantage. In addition, this problem can be solved by several strategies. For example, development of silencing-insensitive retroviral vectors and serial infusion of previously frozen TCR-transduced T cells (i.e., T cell “banking”).
Applications of T cell-based chimeric receptors in adoptive immunotherapy

In addition to flTCR, many T cell-based chimeric receptors can also be used for adoptive immunotherapy. In a technique called the “T-body” approach, an antibody variable region in chimeric receptor form is grafted onto a cytotoxic T (or other) effector cell (84). As such, this methodology overcomes the limitations imposed by MHC-restriction in the recognition of antigen by T cells, while taking advantage of the efficient mechanisms by which T cells can extravasate, penetrate and destroy tumor tissue (84). Moreover, the T-body takes advantage of the growing repertoire of antibodies specific for tumor-associated antigens. Such antibodies preferentially recognize antigens overexpressed on malignant tissue, which are usually shared between tumors of common histology (84, 85).

In making chimeric immunoreceptors, the extracellular antigen recognition portions are generally fused to intracellular signalling domains (65-69). This design allows the immunoreceptor-grafted immune cells (e.g., T cells, macrophages and neutrophils) to bind antigens by an antibody or TCR-derived domain and induce cellular activation by intracellular signalling domains. Intracellular signalling domains are derived from the cytoplasmic parts of membrane-bound receptors to induce cellular activation, e.g., the FcεRI receptor γ-chain, the CD3ζ chain, CD28 and p56Lck (65-70).

Due to the critical roles of these signal transduction domains in effecting the functional activity of chimeric receptor-modified T cells, it's necessary to optimize the signalling modules. Engagement of Ig-binding FcR on myeloid/NK cells is a critical event in initiating host immune defense against virus-infected and malignant cells. The
signalling events downstream of the FcR involves rapid phosphorylation of conserved 18-aa Ig tyrosine activation motifs (ITAM), and the subsequent activation of Src family, ZAP-70, and Syk kinases (91). However, the FcεRI-γ-bearing receptor was less efficient than a chimeric receptor bearing CD3ζ chain (66). According to the dual signal model of T cell activation, a costimulatory signal in addition to signalling though the TCR/CD3 complex is required for efficient activation of primary T cells, resulting in cellular proliferation, cytokine secretion, CTL-mediated target cell lysis, and prevention of activation-induced anergy (92). CD28 plays a key role in the costimulation of naive T cells through its interaction with members of the B7 family of molecules (B7-1, CD80, and B7-2, CD86) (93). The short intracellular domain of CD28 serves to initiate a signal transduction cascade, which is distinct from the primary signal delivered by the TCR (92). On binding of the extracellular domain of CD28 to B7, the intracellular domain of CD28 becomes phosphorylated at a tyrosine residue in a motif that conforms to the consensus amino acid sequence, YMNM (94). Phosphorylated CD28 then binds to SH2 domains-containing molecules such as phosphatidylinositol 3'-kinase (PI-3 kinase), inducible T cell kinase (ITK) and the adaptor molecule Grb-2, leading to downstream signalling (94). Costimulation lowers the amount of Ag required to achieve full cellular activation (95). Moreover, CD28 costimulation in addition to IL-2 secretion synergistically prevents activation-induced T cell death by up-regulation of the anti-apoptotic proteins bcl-xL and bcl-2, respectively (96). Thus, it's conceivable that engineered T cells receiving only one signal by ligation of the tumor antigen might be predicted to be incompletely activated. Since expression of B7 molecules is generally
restricted to professional APCs (92), costimulation of primary T cells via the CD28 pathway by direct interaction with tumor cells is less possible. Integration of the CD28 signalling domains to the CD3ζ chain in the same immunoreceptor allows the co-delivery of both primary and costimulatory signals.

When cocultured with target cells, T cells grafted with immunoreceptors harboring both CD28 and CD3ζ signalling domains produce 20-fold higher levels of IFN-γ and GM-CSF than those armed with chimeric receptors with CD3ζ signalling domain only (97). The induction of these cytokines was at least equivalent to that observed in the same T cells incubated with anti-CD3/anti-CD28 mAbs. Not only in vitro, the in vivo function of genetically modified T cells with chimeric domains containing both the CD28 and TCR ζ signalling moieties fused in a single receptor is greatly enhanced (97). The superior antitumor efficacy of T cells armed with CD28-CD3ζ containing chimera in vivo was dependent on the antigen-specific secretion of IFN-γ.

A Src-family kinase p56Lck constitutively associates with coreceptor CD4 or CD8 (98). It has been shown to play an important role in initiating and propagating TCR signals by regulating the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 (99). Geiger et al found that chimeric receptors with an integrated Lck led to a significant increase in T cell activation (18). The enhancement effect may be because the integrated Lck in chimeric receptors promotes ZAP-70 phosphorylation, and thereby facilitates early signalling events. Besides CD3ζ, CD28 and Lck, tyrosine kinases (e.g. Syk and ZAP70) and another Src family kinase p595m also play important
roles in T cell activation (98). It will be of great interest to explore the potential application of these kinases in making T-cell based chimeric receptors.

One promising solution to the potential problems of using flTCR mentioned above is the use of three-domain single chain TCRs (3D-scTCR), in which the TCR Vα and Vβ chains are joined by a flexible linker with signal transduction domains fused at the carboxyl termini as signal transducers and amplifiers (69,78,86). Although many efforts have been made to construct and characterize scTCRs, most studies have focused on E.coli and yeast expression systems (87-90). Knowledge of the immunological consequences of engineering T cells with scTCR constructs remains limited. Therefore, it will be very interesting to investigate the relationship between structure and scTCR function and compare scTCRs with flTCRs in in vitro and in vivo functional activities.
Part III: Strategies for Identifying Tumor Antigens

Tumor antigens

The identification of tumor antigens (Ags) which are able to elicit an immune response in cancer patients has opened the way for specific immunotherapy of cancer (100, 101). Multiple cancer Ags, either uniquely expressed or over-expressed on cancers have been identified, and in many cases the immunodominant peptides presented on both class I and class II MHC (major histocompatibility complex) molecules have been determined. To date, such tumor Ags have been classified into five categories (102): (a) cancer testis Ags, such as MAGE, BAGE, GAGE, and more recently, NY-ESO-1; (b) differentiation Ags, such as gp100, Melan-A/Mart-1, and tyrosinase; (c) mutated Ags, such as cdk4, β-catenin, and cdc27; (d) overexpressed and/or ubiquitous Ags, such as PRAME, and p53, and (e) viral Ags, such as those from human papillomavirus (HPV) and Epstein Bar virus (EBV). Very few tumor-specific Ags have been identified in epithelial tumors, the best known of which are CEA, PSA, Her-2/Neu, and MUC1.

However, there are still several important questions that need to be answered concerning the choice of Ags to be used for immunotherapy (103). Are all Ags equally important as targets of immunotherapeutic attack, or do some antigens have unique qualities of immune stimulation that would facilitate their successful application to active immunization? How can one choose which Ags to use for active immunization from the large number of available cancer Ags? In order to solve these problems, it is
preferable to identify tumor Ags though functional approaches; namely, identification of Ags based on induction of anti-tumor responses.

**Strategies for cloning tumor Ags**

Four major strategies have been utilized for identifying tumor Ags. These methods include transfection of recombinant tumor cDNA libraries and HLA molecules into target cells ("genetic approach") (104, 105); elution of peptides from the binding cleft of tumor HLA molecules ("peptide-elution approach") (106, 107); serological analysis of recombinant tumor cDNA expression libraries (SEREX approach) (108); and deduction of peptide sequences from known oncogenes or tumor-associated proteins using known HLA-anchor motifs ("reverse immunology approach") (109, 110).

**T cell screening of cDNA libraries ("genetic approach")**

The classic way of identifying T cell-recognized tumor Ags was developed by Boon et al (111). The first step in this strategy is to establish T cell clones against tumor cell lines. Subsequently, tumor antigen expression libraries are made. Finally, these expression libraries are screened using tumor-specific T cells and antigens are identified by the ability to stimulate activation of T cells. However, the drawbacks of this method are significant. Since the avidity of the TCR on CTL for MHC/peptide is low, the sensitivity to detect tumor Ag-expressing cells based on T cell activation is limited. Therefore, to detect tumor Ag-expressing cells, the number of recombinant plasmids in a pool derived from cDNA libraries and used to stimulate the T cells is generally less than 100. Thus, to screen a library of $1 \times 10^5$ recombinant plasmids, a minimum of 1,000 recombinant plasmid pools must be screened. Assays detecting cytokines such as IFN-γ,
GM-CSF and TNF-α released in the culture supernatants are generally used. The purification of plasmids from a large number of bacterial pools, their transfection, and the subsequent T cell assays for these cytokines are therefore considerable and expensive tasks. Screening a tumor cell-derived retroviral cDNA library has been successfully utilized to isolate tumor Ags by Wang and co-workers (34). Autologous fibroblasts were transduced by tumor-derived cDNAs via retroviral vectors to display antigenic peptides to T cells. The advantage of this methodology is that there is no need to know the MHC restriction elements. The copy number in fibroblasts (1~10 copies/cell) after retroviral transduction is much lower than that in DNA-transfected COS cells (~1000 copies/cell) used in the traditional genetic approach for identifying T cell recognized tumor Ags. Therefore, the sensitivity of this methodology is lower than that of the traditional method.

Peptide elution

Ags recognized by T cells are expressed as peptides bound to MHC molecules. Direct isolation of tumor peptides by acid elution from tumor cells or Ag presenting cells (APCs), and subsequent sequencing of amino acids from MHC-associated peptides combined with (or without) mass spectrometry seems to be a straightforward method. However, peptide elution is technically demanding and it is practically impossible to purify a fraction containing only a single peptide even after repeated high performance liquid chromatography (112). Therefore, it has proven extremely difficult for the most part to resolve a reactive peptide sequence from the many contaminating peptide signals.
Serological methods (SEREX)

The development of a general method to analyze the humoral immune response in cancer patients has provided a powerful new tool to detect the immune response to cancer. This method, known as SEREX (serological analysis of recombinant cDNA expression libraries) (108), combines molecular cloning of autologous cancer cells with patient serum containing tumor-specific antibodies. However, because CTLs represent a major arm of the immune response against cancer, the elicitation of a specific CTL response against tumor Ags is one of the main aims of current immunotherapy trials. Therefore, it is crucial to define CD8⁺ T cell epitopes in SEREX-defined Ags. However, despite the large number of SEREX-defined antigens, only a few CD8⁺ T cell epitopes have been characterized thus far in SEREX proteins (other than NY-ESO-1, 113).

Reverse immunology

Unlike the previous strategies, a totally different approach, the 'reverse immunology' approach, takes full advantage of cancer gene expression profiles and bioinformatics (109,110,114,115). This strategy starts with the deduction of peptide-MHC epitopes from genes with broad overexpression in cancer, products of which have known crucial roles in the growth and development of tumors. The immunological methods employed are relatively straightforward. The potential of this approach derives from the understanding of cancer genetics made possible by the Human Genome Project and the exponentially growing repertoire of tools and technologies to study - in parallel - thousands of genes as potential therapeutic targets (including immunological targets). However, the tumor Ag candidates are chosen based on their role in cancer biology,
rather than the analysis of the immune responses of cancer patients to these gene products. Thus, these candidates might not be good tumor Ags in terms of elicitation of immune responses (114,115).

Applications of NFAT-Based Inducible Gene Expression Systems in Measuring T cell Activation

NFAT signalling Pathway

The nuclear factor of activated T cells (NFAT) plays a critical role in the coordinate induction of expression of many genes (116,117). These genes include cytokines, such as interleukin (IL)-2, IL-3, IL-4, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, and tumour necrosis factor (TNF)-α, and cell-surface receptors, such as CD40L, CTLA-4, and FasL (116). Activation of T cells results in the rapid calcineurin-dependent translocation of NFAT transcription factors from the cytoplasm to the nucleus. This translocation process coupled to the subsequent active maintenance of NFAT in the nuclear compartment is critical for the induction of expression of several genes encoding cytokines and membrane proteins that modulate immune responses (116-118). There are four major members of the NFAT family: NFAT1, NFATc, NFATx and NFAT3 (116,117). Members of the NFAT family have in common the following characteristics: (1) they are related by sequence similarity; (2) all four members cooperate with AP-1 polypeptides and bind the distal NFAT site in the IL-2 promoter in vitro; and (3) when over-expressed in appropriate cell lines, all can induce transcription from NFAT reporter constructs upon stimulation. Three major domains have been identified in the NFAT family members (116). They are: the Rel-
similarity domain (RSD), which is responsible for the DNA-binding activity and interaction with AP-1 polypeptides; the NFAT-homology region (NH), which regulates the intracellular localization; and the transcriptional-activation domains (TADs). Besides the positive regulator calcineurin, NFAT pathway can also be negatively regulated. One such negative regulator is a kinase called GSK-3 (117). It is constitutively active in resting cells, and its activity is reduced during T-cell activation (119). GSK-3 as an NFAT kinase significantly reduced nuclear import of NFATc and increased nuclear export (117,119).

**NFAT-Based Inducible Gene Expression System**

NFAT-based inducible gene expression systems have been applied for many purposes involving T cell activation, such as imaging the T cell-APC interaction, isolation of Ag-specific T cells and identification of T cell-recognized tumor Ags (18, 120-123). Reporter gene constructs (GFP, LacZ and luciferase) driven by multiple copies of NFAT-binding sites are routinely used as output signals to test the activation of the NFAT pathway downstream of the T-cell receptor (TCR) (120,122,123).

Underhill *et al* (123) used Ag-specific T cells, which were modified with a NFAT-responsive promoter-controlled GFP expression vector, to visualize productive Ag presentation at the single T cell level. The advantages of this method include rapid readout (within 2 hours) and easy analysis by video microscopy. Hooijberg *et al* (18) applied a similar strategy to isolate viable, Ag-specific, human T cells from a heterogeneous pool of T cells. Primary T cells were transduced with a retroviral vector containing NFAT-controlled GFP expression elements. Stimulation of the transduced T
cells with cognate Ag resulted in GFP expression. This NFAT-GFP reporter system allows for an easier and more rapid isolation of Ag-specific T-cell clones than the frequently used method of repeated in vitro stimulations of polyclonal T cells, followed by random cloning of the responding T cells by limiting dilution. Compared with the tetramer technology, no prior knowledge of the MHC restriction element and the antigenic peptide is required for isolation of Ag-specific T cells.

Large quantities of tumor-specific CTLs are required to allow for multiple rounds of screening in identification of T cell-recognized tumor Ags (124). But this approach is often hampered by the limited amount of CTLs available. This issue can be circumvented by transferring the TCR of a tumor-specific T cell to an immortalized T-cell line. In work done by Aarnoudse et al (122), Ag-specific TCR was reconstituted in a TCR-negative Jurkat T-cell line (JRT3), which was equipped with a NFAT-luciferase reporter construct to allow measurement of TCR-mediated activation. When pools of the cDNA library from which a tumor Ag CAMEL was originally cloned were transfected in COS-1 cells and screened with CAMEL-specific TCR-reconstituted JRT3 cells, identical cDNA pools were found that were positive with these cells.

The major advantage of the NFAT-based inducible system is the high signal/noise ratio (120,121). Without stimulation, the weak strength of the basal IL-2 promoter plus the seldom leaky NFAT nuclear translocation leads to “nearly zero” background. After stimulation, NFAT-IL-2 promoter drives high gene expression. In contrast, NF-κB and AP-1-based inducible systems give lower signal/noise ratio (125).
Taken together, the NFAT-based inducible system currently is one of the most faithful systems to measure T cell activation. Combining chimeric receptors with T cell signalling domains with the NFAT inducible system may allow for the detection of many types of ligand-receptor binding, including the TCR and MHC-peptide interaction. Therefore, a NFAT inducible system can be useful for identifying T cell-recognized Ags based on the interactions between an Ag-specific TCR and its MHC-peptide complex.
1.2 SPECIFIC AIMS

Part I: Optimization of protocols for efficient transduction of murine primary T cells.

1) Establishment of an efficient protocol for retroviral transduction of murine primary T cells.

2) Exploration of various factors that affect transduction efficiency, including virus titer, times of exposure, timing of infection, low-speed centrifugation and use of fibronectin fragments.

Part II: Transgenic TCR expression: comparison of single chain with full-length receptor constructs for T cell function.

1) Determination of the effects of TM region and signal transduction domains on surface expression and intracellular signalling.

2) Determination of the effects of CD8, CD28 and the complete CD3 complex on scTCR-induced T cell activation.

3) Comparative analysis of flTCR and scTCR in functional efficiency.

4) Preliminary investigation of Ag-binding property using H-2K^b-Ig dimers.

5) Exploration of the effects of TCR-modified T cells on in vivo tumor growth.


1) Establishment of an indicator system (SING) for Ag presentation.

2) Functional test of the BS clones for responses to TCR engagement.
EFFICIENT TRANSDUCTION OF MURINE PRIMARY T CELLS REQUIRES A COMBINATION OF HIGH VIRAL TITER, PREFERRED TROPISM AND PROPER TIMING OF TRANSDUCTION

2.1 OVERVIEW

T cells play a central role in the immune system. Genetic modification of T cells has become an important tool for both basic research and clinical immunotherapy (49). Currently, retroviral transduction is the predominant method used to deliver genes into T cells (49). However, most T-cell infection protocols have been developed for human T cells whereas systematic investigations of the optimal conditions for transduction of murine primary T cells are limited and the efficiency of transduction of murine primary T cells is very low. There are several obstacles in retrovirus transduction, such as short viral half-life (126), low virus motion rate (127), limited viral receptor expression on target cells (128), inhibitory factors (129) and interferons (130). To overcome these hurdles, various strategies have been utilized. First, T cells have been transduced using viruses with high or extremely high titers. High-titer viruses can be obtained by repeated rounds of cross-infection ("ping-pong" infection) and concentration (20, 131). Second, low speed centrifugation combined with fibronectin during infection can increase transduction efficiency by up to several fold (132). Third, prior to transduction, T cells can be activated using optimal stimuli (133). Fourth, T cells can be transduced at
low temperatures (*i.e.*, at $32^\circ$C instead of $37^\circ$C) (134). Although these strategies have been shown to be effective in retroviral transduction of human primary T cells, limited efforts have been made to characterize the obstacles in infection of murine primary T cells.

In this study, ecotropic and 10A1-pseudotyped retroviral vectors were compared for their efficiency in infecting murine primary T cell cells, as well as T cell lines. Various factors that affect transduction efficiency were also explored, including virus titer, times of exposure, timing of infection, low-speed centrifugation and use of fibronectin fragment.

Our results showed that up to 80% of murine primary T cells could be infected after a single exposure. Successful infection required a combination of high virus titer ($>10^7$ CFU/ml), proper timing of infection (within 24 hours after mitogen stimulation) and preferred tropism (ecotropic vectors). These optimization results may help to establish a standard protocol for infection of murine primary T cells and provide some insight into the obstacles to retroviral infection of T cells.
2.2 METHODS AND MATERIALS

Cell lines and reagents

GP-293 packaging cells were purchased from Clontech (Palo Alto, CA). The packaging cell lines PT67 and GP+E-86, mouse T cell lymphoma BW5147 (TCR αβ), EL-4 (thymoma), mouse fibroblast NIH/3T3 and human T cell leukemia cell line Jurkat cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Jurkat cells were cultured in RPMI-1640 (GIBCO-BRL Life Technology, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, Utah), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37°C in 5% CO₂. The other cell lines were cultured in Dulbecco's modified Eagle medium with a high glucose concentration (4.5 g/liter) plus 10% FBS. Recombinant human fibronectin fragment CH-296 (TaKaRa, Japan) was obtained from Pan Vera Co. (Madison, WI). Coating of CH-296 was performed according to the manufacturer's instruction. Briefly, 8 μg CH-296 (40 μg/ml diluted in PBS) was coated onto each well of non-tissue culture treated 48-well plates (Falcon, NJ) at room temperature for two hours. Coated wells were then blocked with 2% bovine serum albumin (BSA) for 30 minutes and washed once with PBS before use.

Retroviral construct and production of retroviral supernatants

A retroviral construct (LXSN-EGFP) encoding enhanced green fluorescent protein (EGFP) was constructed by inserting the EGFP gene from pEGFP-1 (Clontech, CA)
into a retroviral vector LXSN (a gift from Dr. Dusty A. Miller, ref. 135) under the transcriptional control of the 5' LTR.

Eighteen hours before transfection, 5 \( \times 10^6 \) GP-293 cells were plated on a 100mm culture dish. Transfection of retroviral constructs into GP-293 cells was performed using LipofectAMINE 2000 according to the manufacturer's instruction. Briefly, 10 \( \mu \)g VSV-G expressing plasmid pVSV-G (Clontech, Palo Alto, CA) plus 10 \( \mu \)g LXSN-EGFP were mixed with 60 \( \mu \)l LipofectAMINE 2000 in 3.5 ml OPTI-MEM (Gibco-BRL) were added directly to plates. Four hours later, media were replaced with 10 ml fresh DMEM-10 media (containing 10% FBS). After twenty hours, media were changed again with 7 ml DMEM-10. Viral supernatants were collected 48 and 72 h post transfection, filtered through 0.45 \( \mu \)m filters and frozen at -80°C. Viral titers were estimated by transduction of NIH/3T3 cells with serial 10-fold dilutions of virus-containing supernatant and titer was determined by counting colonies after 10 days of G418 selection (0.6 mg/ml).

The VSV-G pseudotyped viruses (titers > 5\( \times 10^5 \) colony-forming units, CFU/ml) were used to transduce both the ecotropic packaging cell GP+E-86 and the 10A1 envelope-pseudotyped dual tropic packaging cell PT67. Approximately 1\( \times 10^5 \) GP+E-86 and PT67 cells were transduced overnight with 1 ml of virus supernatants in each well of 6-well plates in the presence of polybrene (8 \( \mu \)g/ml). After three rounds of transduction, both GP+E-86 and PT67 cells were selected in G418 (1 mg/ml) for 7 days. The viral supernatants from both packaging cells were used to cross-infect each other three times.
Through this process, the virus titer from pooled packaging cells generally ranged from 1 to $5 \times 10^6$ CFU/ml.

**Concentration of retroviruses by Polyethylene glycol (PEG)**

Concentration of retrovirus stocks was performed according to the modified Aboud's method (136). Briefly, 24 h before collecting the retrovirus from nearly confluent virus producing cells (GP+E-86/LXSN-GFP or PT67/ LXSN-GFP), 6~8 ml of DMEM-5 media (containing 5% FBS) was added to each 100mm culture dish. Ice-cold NaCl (5 M) and PEG 6000 (36%) were slowly added to the filtered viral supernatants to the final concentration of 0.4 M NaCl and 8.5% PEG. After the mixture was stirred at $4^\circ$C for 30 minutes, virus supernatants were centrifuged at 5000x_g (Sorval RC-5B, SS-34 rotor, ~6500 rpm), $4^\circ$C for 10 minutes. Virus pellets were resuspended in 1~5% of original volume of RPMI-10. Virus concentrates were aliquoted in 0.5 ml per tube (FACS tubes, Falcon, cat#: 2054), and stored at -80^\circ C.

**Transduction of primary T cells**

Murine primary T cells were obtained from spleens of C57BL/6 (B6) and BALB/C mice. Mononuclear cells were isolated using Lympholyte M (CEDARLANE, Canada). Cells were cultured at a density of 2x10^6/ml in RPMI-10 containing concanavalin A (ConA, 2.5 µg/ml) or anti-CD3 mAb (1 µg/ml, Pharmingen, San Diego, CA) plus anti-CD28 mAb (0.1 µg/ml, Caltag, Burlingame, CA) in 6-well plates (4 ml per well). At various times, 50 µl (6x10^6/ml) of T cells were mixed with 250 µl of retroviral supernatant in the presence of 25 U/ml of recombinant human IL-2 (Genzyme,
Cambridge, MA) and 4 µg/ml of polybrene (Sigma, St. Louis, MO.) in CH-296 precoated or non-coated 48-well plates. Cells in plates were placed in microplate carriers and centrifuged at 1200×g (Beckman Allegra 6R, 2500 rpm) for 1 hour at 30°C, incubated at 37°C for an additional 6 h unless indicated otherwise and then the media was changed with regular RPMI-10 containing 25 U/ml IL-2. The T cell lines, Jurkat and BW5147, were infected without prior stimulation. All samples were subjected to flow cytometry analysis after either 2 or 5 days.

**Flow cytometry**

Cells were analyzed using an Epics XL flow cytometer (Beckman Coulter, Inc., Miami, FL). GFP expression was analyzed 5 days (unless indicated) after vector exposure. For two-color flow cytometric analysis of primary T cells expressing CD3 and GFP, cells were incubated with either the PE-labeled anti-CD3 antibody or an irrelevant isotype control (Pharmingen) for 30 min at 4°C. The cells were washed twice, and fixed in PBS containing 2% paraformaldehyde.

**Statistical analysis**

The data were analyzed to determine any significant differences among the experimental groups using Student’s *t* test. A value of *p*≤0.05 was considered significant. The correlation between the virus titer and infection efficiency was evaluated using the curve-fitting (Logarithmic) function of EXCEL2000 software (Microsoft, Redmond, CA).
2.3 RESULTS

10A1-pseudotyped retroviral vectors are efficient in transduction of murine T cell lines but not murine primary T cells

The murine T cell line BW5147 and human T cell line Jurkat were used to analyze the transduction efficiency of 10A1-pseudotyped retroviral vectors. The results showed that ~60% of BW5147 cells could be transduced (at a multiplicity of infection of 5, MOI=5) after a single infection by 10A1-pseudotyped vectors, whereas the transduction efficiency in Jurkat cells was low (~25% at MOI=5) (Fig. 1B, E). However, after G418 (0.8 mg/ml) selection for a week, the majority of both cells became GFP-positive (Fig. 1C, F). Similar results were observed using other T cell lines, such as EL-4 (Fig. 1I-J) and a T cell hybridoma, B3Z (Fig. 1G-H). In an attempt to investigate the relationship between virus titers and transduction rates, BW5147 cells were exposed to increasing MOI's of viral vectors. As shown in Figure 2A, there was a positive correlation between virus titer and infection efficiency. More than 90% of BW5147 cells could be infected when the virus titer was as high as $5\times10^6$ CFU/ml (MOI=25). However, the infection efficiency in murine primary T cells was quite low. Five days post transduction, the percentage of GFP$^+$ T cells was less than 3% even when extremely high viral titer supernatants ($>10^8$ CFU/ml, MOI>500) were used (Figure 2B). In this case, no correlation between virus titer and transduction efficiency was observed.
Figure 1. GFP expression in T cell lines. BW5147 (A-C), Jurkat (D-F), B3Z (G-H) and EL-4 (I-J) cells were either mock infected (A, D, G, I) or transduced with 10A1-pseudotyped LXSN-GFP vectors (B, E, H, J) described in Methods and Materials. Some transduced T cells were subjected to G418 (0.8 mg/ml) selection for a week (C, F). GFP expression was determined by flow cytometry.
Figure 2. Correlation between virus titer (10A1-pseudotype) and T cell transduction efficiency. Both BW5147 (A) and murine primary T cells (B) were transduced with various titers of 10A1-pseudotyped retroviral vectors. GFP expression was determined five days after transduction. R: Correlation coefficient.
Ecotropic retroviral vectors are efficient in transduction of murine primary T cells

In contrast to dual tropic retroviral vectors, ecotropic vectors were very efficient in the transduction of murine primary T cells. Figure 3A shows the infection results from 3 independent experiments. Concanavalin (ConA)-activated BALB/C and B6 spleen cells were used. Both B6 and BALB/c mice spleen cells were efficiently transduced. The percentage of GFP^+ cells determined 5 days post infection however, was lower than that determined 2 days post infection. To make sure that gene expression at day 5 was due to stable integration, the culture time post-infection was extended to 10 days. The values obtained at day 5 and day 10 were similar, indicating stable gene expression (Table 2). When selection with G418 was performed at day 5, by day 10 most T cells became GFP^+. In a representative experiment, the transduction efficiency in murine primary T cells was close to 80% after only a single infection using ecotropic vectors (Figure 3C).
<table>
<thead>
<tr>
<th>Time(^a)</th>
<th>% of GFP+ T cells (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 5</td>
<td>18.7±11.9</td>
</tr>
<tr>
<td>Day 10</td>
<td>19.0±12.1</td>
</tr>
<tr>
<td>Day 10 (+G418)(^b)</td>
<td>76.3±8.5</td>
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The percentages of GFP positive T cells were determined by flow cytometry. The value was presented as mean ± SD of three independent experiments.

\(^a\)-Days after virus exposure.
\(^b\)-Infected T cells were selected with G418 (0.4 mg/ml) beginning at day 5.
High virus titers are necessary for ecotropic vectors to transduce primary T cells

In order to assess the relationship between virus titer and infection efficiency using ecotropic retroviruses, virus stocks with different titers were used to infect primary splenic T cells. Similar to 10A1-pseudotyped retroviral vector transduction of T cell lines, the efficiency was closely correlated with the titer of ecotropic vectors. Virus stocks with a titer of $10^7$ CFU/ml (MOI=10) could infect approximately 30% of primary T cells. When virus titers were increased to $10^9$ CFU/ml (MOI=100), more than 60% of primary T cells could be stably transduced (Figure 3B).

Timing of exposure of activated T cells to retroviruses is critical for efficient transduction

Unlike T cell lines, which can grow continually without stimulation, primary T cells must be activated before transduction. Therefore the activation status may have significant effects on transduction efficiency. In most of our experiments, T cells were transduced 24 h post-stimulation. Infection was also tried at 48 h and 72 h after ConA activation. However, transduction efficiency decreased dramatically when activation lasted more than 48 h before infection. In this case, repeated infection at 24-hour intervals was also not helpful (Figure 4).
Figure 3. Retroviral transduction of murine primary T cells using ecotropic vectors. ConA-stimulated murine spleen cells from Balb/C and B6 strains were infected with ecotropic retroviral vectors (A). Results from three independent experiments are shown. GFP and CD3 expression were determined using two-color flow cytometry described in Methods and Materials at either two (shaded bar) or five (blank bar) days post stimulation. Correlation between virus titer (ecotropic vectors) and transduction efficiency in primary T cells were also plotted (B). A representative transduction result was shown in Figure 3C. R: correlation coefficient.
Figure 4. Effects of timing of exposure to ecotropic vectors and repeated infection on the T cell transduction efficiency. T cells were infected at 24, 48 and 72 h after ConA activation. For double and triple transductions, cells were infected at 24-hour intervals beginning at 24 h post stimulation. The results were presented as mean ± SD of 3 independent experiments.*: $p<0.05$
Centrifugation, but not CH-296 fragments and the time of exposure to virus can enhance transduction efficiency

As shown in Table 3, low-speed centrifugation (at 1200\(\times\)g for 1 h) had a significant enhancement effect upon retrovirus infection, ranging from 30-80%. No enhancement was observed when the time of exposure to virus was extended to 8 h or more. It has been reported that fibronectin fragments could significantly enhance retrovirus-mediated gene transduction into mammalian cells. However, in our protocol, the recombinant fibronectin fragment CH-296 was not helpful.
TABLE 3: Effects of fibronectin, time of exposure and centrifugation on retroviral transduction of murine primary T cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>CH-296</th>
<th>Time of exposure (h)</th>
<th>Centrifugation</th>
<th>Relative efficiency</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>4</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>3</td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>56±12*</td>
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<tr>
<td>4</td>
<td>-</td>
<td>8</td>
<td>+</td>
<td>92±9</td>
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<tr>
<td>5</td>
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<tr>
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<td>-</td>
<td>8</td>
<td>-</td>
<td>56±8*</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>16</td>
<td>-</td>
<td>59±5*</td>
</tr>
</tbody>
</table>

Coating of CH-296 and centrifugation were performed according to Materials and Methods. After indicated times of virus exposure, cells were washed and cultured in regular DMEM-10 containing 25 U/ml IL-2. GFP expression was determined 5 days after transduction by flow cytometry and normalized to relative values. The results represent mean ± SD of two independent experiments. The p values were determined between group 1 (standard condition) and other experimental groups using Student's t test. *: p<0.05.
2.4 DISCUSSION

The 10A1 retrovirus is a dual tropic murine leukemia virus (MuLV) as 10A1 envelope-pseudotyped viruses have the ability to infect cells via two receptors: the amphotropic receptor (Ram1 or Pit2) and the GALV receptor (Pit1) (142, 143). 10A1-pseudotyped retroviral vectors have been shown to efficiently transduce human primary T cells (126,144). However, limited efforts have been made to explore the possibility of infecting murine primary T cells using dual tropic vectors.

Obstacles to retroviral infection can be classified into two major groups: inefficient viral entry and internal viral instability (145, 146). It's generally thought that the level of viral receptors is a key factor controlling viral entry. Several groups have found that the retroviral transduction efficiency with a particular pseudotype correlates with the relative abundance of its receptor (128). The inefficiency of transduction of murine primary T cells using 10A1-pseudotyped viruses suggests a limited expression of the viral receptors pit1 and pit2 on murine primary T cells. In contrast, ecotropic vectors are more efficient at transducing murine primary T cells, probably due to ubiquitous expression of ecotropic receptors on murine cells (147). However, a pattern of universal receptor expression does not guarantee the sensitivity of mouse cells to ecotropic vectors. A critical factor, which affects the binding of the receptors to ecotropic viruses, is production of endogenous retroviruses (148, 149). In the mouse genome, especially inbred mice, there are multiple copies of endogenous retroviral sequences (150). Although in most cases these viruses are defective, some elements can be expressed in either a constitutive or inducible manner (149). Endogenous Env gene expression has
been studied intensively. A mouse melanoma Ag H52 isolated from the C57BL/6-derived melanoma cell line B16 is an envelope protein of endogenous ecotropic MLV of AKV-type (151). Northern blot analysis showed that this transcript was also detected in the EL-4 T lymphoma line but not in normal C57BL/6 tissues. It has been reported that mitogen-stimulated murine spleen cells express endogenous retrovirus envelope proteins of both xenotropic and ecotropic origins, whereas non-activated mouse T lymphocytes did not (152). Therefore it’s conceivable that there is only a narrow window of time after T cell activation that permits the efficient transduction of mouse T cells by ecotropic vectors without significant interference by endogenous ecotropic envelope proteins. Our results demonstrated that the optimal time for transduction of murine T cells was within 24 hours of stimulation, supporting the hypothesis. Another important mechanism for interference of viral infection is the production of interferons (130). This mechanism may be more significant in the transduction of primary T cells as activated T cells release large amounts of interferon-γ (153, 154). Unlike endogenous ecotropic envelope proteins that prevent external virus entry, interferons induce an antiviral state, in which viral replication is arrested (155).

For some applications, it’s necessary to concentrate virus stocks to increase titers. In our experiments, PEG precipitation was chosen as a standard procedure for virus concentration because no ultracentrifugation is required, which can lead to significant loss of infectivity (10).

The effects of repeated infection on the transduction efficiency of T cells were also tested. A triple infection could increase transduction efficiency of T cell lines by
30~100%. However, triple infection of murine primary T cells at 24-hour intervals was no more advantageous than a single infection. That result may be due to the inefficiency of transduction at 48 hours post-stimulation. However, it's possible that multiple rounds of infection could enhance the transduction efficiency when infections are performed at shorter intervals (i.e., 6~8 h).

Currently, most transduction protocols require the use of an expensive reagent, recombinant fibronectin (CH-296), for efficient transduction of primary T cells (49,126,132,133). The possible mechanisms for the enhancement effects on infection may be attributed to the colocalization of retroviral particles and target cells on the molecules of fibronectin (156). Our experiments did not show any advantageous effects of CH-296. However, murine fibroblast NIH 3T3-based packaging cells have been shown to produce and secret fibronectin (157). Therefore, viral particles from these packaging cells are more likely to be in the form of virus-fibronectin complexes. This notion is supported by the observations that anti-fibronectin antibody-coated magnetic beads and a fibronectin-binding reagent (Pansorbin) could significantly increase transduction efficiency (158,159). Based on this concept, viral particles may co-precipitate with fibronectin by PEG precipitation. After resuspension, the virus-fibronectin complexes may readily access target cells to overcome the low motion rate of viral particles (i.e., “Brownian movement”) (127). This reasoning can also explain why low-speed centrifugation is effective in promoting viral transduction. Although several reports (126, 160) have shown that extended exposure to viruses could increase infection efficiency, our results did not show this approach to be advantageous.
Increased toxicity either caused by the concentrated virus stocks, polybrene or both may compromise the positive effects of extended exposure. Furthermore, facilitated access of viral particles to target cells using the described protocols together with the short half-life of the retroviruses may render extended exposure unnecessary or non-beneficial.

Compared with other protocols for transduction of primary T cells, our protocol has several advantages. First, a combination of cross-infection between packaging cells of different pseudotypes with virus concentration allows rapid production of extremely high titers of viral vectors (~10^8 CFU/ml) in less than three weeks. This procedure avoids the laborious work in screening “high virus producers” which generally takes more than one month. Second, no requirement for the use of expensive reagents (i.e., CH-296, anti-CD3 and anti-CD28 mAb) makes our protocol more applicable. Third, the transduction protocol is comparatively simple. Multiple rounds of infection and coculture of T cells with virus producing cells are not necessary. Thus, our protocol is more useful for efficient genetic modification of murine primary T cells in terms of time, costs and simplicity.
CHAPTER 3

TRANSGENIC TCR EXPRESSION: COMPARISON OF SINGLE CHAIN WITH FULL-LENGTH RECEPTOR CONSTRUCTS FOR T CELL FUNCTION-PART I: IN VITRO STUDIES

3.1 OVERVIEW

Genetic modification of T lymphocytes with T cell receptor (TCR) genes provides a novel tool for adoptive immunotherapy. However, the efficiency of full-length TCR (fTTCR)-modified T cells could be limited by many factors; such as incorrect pairing between exogenous and endogenous TCR chains, instability of transgenic TCR molecules and downregulation of the CD3 complex. To overcome these hurdles, one promising strategy is to use three-domain single chain TCRs (3D-scTCR), in which TCR Vα and Vβ chains are joined by a linker with signal transduction domains fused at the carboxyl termini as signal transducers and amplifiers (69, 86, 98).

In this study, multiple scTCR constructs were analyzed to demonstrate the effects of 3D-scTCR design on surface expression and intracellular signalling in T cells. scTCR constructs were introduced into a mouse T cell line (BW5147) as well as primary splenic T cells via retroviral transduction. scTCRs could be efficiently expressed on both types of T cells. Surface expression of scTCRs was affected by the origin of the transmembrane (TM) region and placement of signalling domains. scTCR-modified T cells were functional as shown by cytokine (IL-2 and IFN-γ) release in response to Ag
stimulation and cytolytic activity against specific target cells. Integration of CD3ζ and CD28 with or without p56Lck in the same 3D-scTCR allowed stronger signalling than constructs with CD3ζ alone. The influence of other molecules, such as CD8, CD28 and the complete CD3 complex on scTCR-induced T cell activation was also investigated. Compared with flTCR-modified T cells and native CTLs, scTCR-modified T cells require higher thresholds of Ag stimulation (~10^{-8} M peptide) to be functional as compared to flTCR-modified T cells (<10^{-10} M peptide), suggesting that factors other than high surface expression and the presence of multiple signalling domains also play critical roles in scTCR function.

Despite the low affinity of scTCR-modified T cells in response to Ag stimulation at physiological levels, our data demonstrate the feasibility of redirecting T cells with 3D-scTCRs to respond specifically against Ag-expressing cells and provide some insight into further improvements in development needed to generate highly efficient scTCRs for in vivo applications.
3.2 METHOD AND MATERIALS

Cell lines

GP-293 packaging cells were purchased from Clontech (Palo Alto, CA). The packaging cell line GP+E-86, mouse T cell lymphoma BW5147, EL-4 (thymoma), OVA-expressing EL-4 clone EG7 (137) and mouse fibroblast NIH/3T3 were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). All cell lines were cultured at 37°C in Dulbecco's modified Eagle medium with a high glucose concentration (4.5 g/liter) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, Utah), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

Plasmid construction

All scTCR constructs were made in a cassette fashion (see Figure 5). Briefly, TCR portions and signal transduction domains were constructed separately, ligated and introduced into LXSN (a gift from Dr. Dusty A. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) at the EcoRI site. The fragments Va2-Ja23 (nucleotides 1-420, primers #1 and #2) and Vp5.2-Djp2.3-Cp (nucleotides 88-804, Cp stops just before the cysteine, primers #7 and #8) were amplified by PCR using the plasmid TCR70 (ref. 137, a gift of Dr. Larry Pease, Mayo Clinic at Rochester) as a template. The sequences of PCR primers are shown in Table 4. The plasmid TCR70 contains the OVA-specific, full-length TCR genes coding for both α and β chains. The α and β chain PCR fragments were joined with a universal (Gly4Ser)3 linker (G4S)3 (138). In the
constructs denoted scTCR-Cys-ζ, scTCR-Cβ-ζ and scTCR-Cβ-28-ζ-Lck, either the
cysteine residue (nucleotides 805-807, primers #7 and #9) or the Cβ region extending to
the transmembrane domain (nucleotides 805-930, primers #7 and #10) was included.
The mature CD3ζ chain (nucleotides 64-492, primers #14 and #16) and CD3ζ
cytoplasmic tail sequences (nucleotides 154-492, primers #15 and #16) were derived
from the full-length mouse CD3ζ chain cDNA (obtained from Dr. Allan M. Weissman,
NIH, Bethesda, Maryland) by PCR. In scTCR-28-ζ and scTCR-28-ζ-Lck, the DNA
sequence encoding amino acids 117-199 of mature mouse CD28 including the hinge
region, transmembrane domain and cytoplasmic tail was amplified by PCR (primers #19
and #23 or #24) from a plasmid containing the full-length CD28 (kindly provided by Dr.
James P. Allison, University of California at Berkeley, CA). In scTCR-ζ-28, scTCR-ζ-
28-Lck and scTCR-Cβ-28-3ζ-Lck, only the CD28 cytoplasmic tail sequence
(nucleotides 535-654, primers #20 and #24 or #21 and #23) was used. The CD28-CD3ζ-
Lck fragment in scTCR-Cβ-CD28-CD3ζ-Lck was generated by PCR (primers #20 and
#25) using H-2Kb-28-3ζ-Lck (Ref. 70, kindly provided by Dr. Terrence L. Geiger, at St.
Jude Children's Research Hospital, Memphis, TN), whereas in scTCR-28-ζ-Lck, the
CD28 cytoplasmic tail was replaced by CD28117-199 as mentioned above. The Lck region
(amino acids 33-509) containing SH2, SH3 and kinase domains in scTCR-3 was excised
from the plasmid NT18 (63228, ATCC, Manassas, VA). The construct scTCR-B7-ζ
contains the sequences coding for the hinge region, transmembrane and cytoplasmic
domains of mouse B7.1 (amino acids 207-269). The B7-1 fragment was obtained by
RT-PCR (primers #27 and #29) using mRNA from LPS-stimulated C57BL/6 spleen cells. The full-length mouse CD8α cDNA (kindly provided by Dr. Dan R. Littman, New York University, NY), CD28 and B7.1 genes were inserted into retroviral vectors LXSN, LNCX2 (Clontech, Palo Alto, CA) and pFB-neo (Stratagene, La Jolla, California), respectively. The full-length TCR α and β chain were generated by PCR (TCR α chain: primers #3 and #4; TCR β chain: primers #5 and #6) using the plasmid TCR70 as a template and then inserted into the Xhol /SalI sites of the SAMEN vector (a gift of Dr. Michael Nishimura, University of Chicago, IL). Full-length CD3δ and ζ chain cDNAs were amplified (CD3δ chain: primers #30 and #31; CD3ζ chain: primers #13 and #16) using the plasmid pCD-mCD3δ (a gift of Dr. Cox Terhorst, Harvard Medical School, Cambridge, MA) and pGEM-CD3ζ, respectively. Construction of a mouse CD3δ and ζ chain co-expressing vector, SAMEN-CD3ζδ, was performed similar to that of SAMEN-TCRβα. The sequences of all PCR amplified fragments were confirmed by sequencing (Appendix D).

Retrovirus production and transduction

Eighteen hours before transfection, GP-293 cells were plated in 6-well plates at a density of 8x10^5 cells per well in 2 ml of DMEM-10. In order to increase the adhesion of GP-293 cells, plates were precoated with 0.01% poly-L-lysine (P6282, Sigma) accord to the manufacturer’s instructions. Transfection of retroviral constructs into GP-293 cells was performed using GenePORTOR2 (Gene Therapy System, San Diego, CA). Briefly, 1μg VSV-G expressing plasmid pVSV-G (Clontech, Palo Alto, CA) plus 1 μg
retroviral constructs were complexed with 10 μl GenePORTOR2 and added directly to plates. After twenty hours, 1 ml fresh DMEM-10 was added. Viral supernatants were collected 48 and 72 h post-transfection and filtered (0.45μm). The VSV-G pseudotyped viruses were used to transduce the ecotropic packaging cell GP+E-86 in the presence of polybrene (8 μg/ml). After three rounds of transduction, GP+E-86 cells were selected in G418 (1 mg/ml) for 7 days. Through this process the virus titer from pooled GP+E-86 cells generally was over 1×10^6 CFU/ml. Concentration of retroviruses by polyethylene glycol (PEG) was performed according to a method described previously (139) except that virus supernatants were centrifuged at 2000×g (Beckman GPR, ~3200 rpm), 4°C for 15 minutes. Virus concentrates were aliquoted at 0.5-1 ml per tube, and stored at -80°C. Viral stocks with high titer (>10^7 CFU/ml) unless indicated, were used for transduction of T cells. BW5147 cells were transduced three times (once a day on 3 consecutive days), whereas primary T cells from spleens of C57BL/6 (B6) mice were infected only once 18-24 h after concanavalin A (ConA, 2.5 μg/ml) stimulation based on our previous protocol (139). Three days after infection, transduced primary T cells (0.5-1 ×10^6/ml) were selected in RPMI-10 media containing G418 (600 μg/ml) plus 25 U/ml rHuIL-2 for an additional 3 days. Viable cells were isolated using Lympholyte-M (Cedarlane Laboratories, Hornby, Canada) and expanded for 2 days without G418 before functional analyses.

**Cytokine production by TCR-modified T cells**

For IL-2 production, TCR-modified BW5147 cells (5×10^5) were cocultured with an equal number of EL-4 cells in the presence of either OVA peptide (10^{-10}~10^{-4} M) or a
control peptide (Trp2, SVYDFFVWL) in 96-well, round bottom plates (200μl/well). To test the ability of TCR-modified BW5147 cells to respond to Ag stimulation at physiological levels, OVA-stably transfected EG7 cells (137) instead of OVA-loaded EL-4 cells were used. For IFN-γ release, coculture of TCR-transduced primary T cells (1×10^5) with OVA peptide-pulsed EL-4 cells (1×10^5) were set up in the same way. Before incubation at 37°C, plates were centrifuged at 450xg for 5 min to initiate close cell contact. Twenty-four hours later, supernatants were collected. IL-2 and IFN-γ were assayed by ELISA using murine IL-2 and IFN-γ ELISA kits (BD Pharmingen, San Diego, CA), respectively. Mock-transduced T cells were used as negative controls and T cells from OT1 mice {OVA257-264 specific TCR transgenic mice, strain name: C57BL/6-Tg(TcraTcrb)1100Mjb, Jackson Laboratory, Bar Harbor, Maine} served as positive controls.

**Dimer staining and Flow cytometry**

H-2Kb:Ig dimer (Pharmingen) staining was performed according to the manufacturer’s protocol. Briefly, either the OVA peptide or the Trp2 control peptide was mixed with an H-2Kb:Ig dimer at a ratio of 160:1 (0.7 μg peptide for 1 μg dimer) at 37°C overnight. T cells (~5×10^5), which were blocked with anti-CD16/CD32 mAb (clone 2.4G2, Pharmingen), were incubated with 2 μg peptide-loaded dimer in 50 μl of FACS buffer (PBS, 1% FBS, 0.09% NaN₃) at 4°C for 1 h. Cells were washed once with FACS buffer and resuspended in 100 μl diluted PE-labeled anti-mouse IgG1 mAb (clone A85-1, Pharmingen) and FITC-anti-CD8a mAb (clone 53-6.7, Pharmingen) at 4°C for 30 min. After two rounds of washing, cells were fixed in 2% paraformaldehyde. Detection of scTCR surface expression on GP+E86 and BW5147 cells was performed using a FITC-
anti-TCR β chain mAb H57-597 (Caltag, Burlingame, CA). Before staining, GP+E86 cells were detached from plates using 0.02% trypsin plus EDTA. Cell-surface phenotyping of transduced cells was determined by direct staining with PE-anti-CD8a (clone 53-6.7, Caltag), PE-anti-CD80 (clone 16-10A1, Caltag), PE-anti-CD28 (Clone, Caltag), FITC-anti-TCR Vβ5 (clone MR9-4, Pharmingen) and PE-anti-TCR Vα2 (clone B20.1, Pharmingen) mAbs. Cell fluorescence was monitored using an Epics XL flow cytometer (Beckman Coulter, Inc., Miami, FL). Cell sorting was performed on a Becton Dickinson FACStar flow cytometer (Franklin Lakes, NJ).

**Cytotoxicity assay**

Three days before CTL activity was assayed, TCR-modified primary T cells were restimulated with mitomycin-C treated, OVA peptide-pulsed (10^{-5} M) stimulator splenocytes in RPMI-10 containing 25 U/ml IL-2. OVA peptide loading was performed at 37°C for 2 h and washed twice before use. After restimulation, viable lymphocytes were recovered by centrifugation over Lympholyte-M and washed. EG7 and OVA peptide-pulsed EL-4 were used as target cells. Effector cells (1x10^5) and target cells (1x10^4) were added to each well of 96-well U-bottom culture plates. Plates were incubated at 37°C for 5 h, and supernatants were recovered. Specific lysis was measured with the non-radioactive CytoTox 96 kit (Promega, Madison, WI) according to the manufacturer’s instructions.

**Statistical analysis**

Statistical significance of IL-2 production among different groups was determined by an unpaired Student’s t test. A value of p≤0.05 was considered significant.
Table 4. Primers used in retroviral vector construction

<table>
<thead>
<tr>
<th>#</th>
<th>Primers</th>
<th>Sequences</th>
</tr>
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<tbody>
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<td>1</td>
<td>5’ Va2-Ja23</td>
<td>CCGAATTCGCCGCCACCATGGACAAAGATCTCTGACA</td>
</tr>
<tr>
<td>2</td>
<td>3’ Va2-Ja23</td>
<td>GCGGAAGCTCTGTTAGGGAGAAATGGAATGTTG</td>
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<td>20</td>
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<td>GCAATAGCTTGAAGCTTGCCTGGATCCTCCAA</td>
</tr>
<tr>
<td>21</td>
<td>5’ CD28 (CYP)-downstream^</td>
<td>GCCAAGCTTGAAGCTTGCCTGGATCCTCCAA</td>
</tr>
<tr>
<td>22</td>
<td>3’ CD28-TGA^</td>
<td>GCCCTAGATCTTTGCTGATGCTGATCTG</td>
</tr>
<tr>
<td>23</td>
<td>3’ CD28-non stop^</td>
<td>GCCCTAGATCTTTGCTGATGCTGATCTG</td>
</tr>
<tr>
<td>24</td>
<td>3’ CD28-CD3ζ-Lck</td>
<td>CCTCCACCAGTTGTCTGGGCTTT</td>
</tr>
<tr>
<td>25</td>
<td>5’ B7-1</td>
<td>CCGAAGCTTGGCCACCACATGGCTGCAATTGCTCAG</td>
</tr>
<tr>
<td>26</td>
<td>5’ B7-1(TM)</td>
<td>CGACGATAGCTTACGTACGTCAGGACTTACC</td>
</tr>
<tr>
<td>27</td>
<td>3’ B7-1-TAG^</td>
<td>GGAACGGCTTCTAAAGGAGACGCGCTTGTCA</td>
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<td>3’ B7-1-non stop^</td>
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</tr>
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<tr>
<td>30</td>
<td>3’ CD3δ</td>
<td>GCCCTGAGATGATTTCTTTTCTGGGCTG</td>
</tr>
</tbody>
</table>

a: Primers with stop codon.
b: Primers without stop codon.
c: Amplified CD28 cytoplasmic tails is upstream of the CD3ζ moiety.
d: Amplified CD28 cytoplasmic tails is downstream of the CD3ζ moiety.

For facilitated cloning, restriction sites were inserted into primers (underline). Optimal Kozak sequences (in bold) were added to the 5' primers of full-length cDNAs and scTCR genes.
### Figure 5. Structure of retroviral constructs

Schematic diagram of scTCRs is shown in (A). Each scTCR construct was composed of the Vα and Vβ regions of the OVA TCR (TCR70) joined by a flexible linker, a membrane-proximal hinge region, and the transmembrane (TM) and cytoplasmic regions (CYP). The scTCR genes were inserted into the LXSN vector under the control of the 5' LTR. (B). SAMEN vector containing dual promoters: CMV/LTR chimeric promoter and SRα promoter, allows simultaneous expression of α and β chains of fITCR or CD3ζ and δ chains. Expression of TCR α and CD3ζ chains was driven under the control of the CMV/LTR promoter, whereas TCR β and CD3δ gene expression was controlled by the SRα promoter. CD8α and CD28 cDNAs were inserted into LXSN and pFB-neo, respectively. Their expression was driven under the control of the 5' LTR. The B7.1 gene was inserted downstream of CMV promoter in LNCX2.

<table>
<thead>
<tr>
<th>scTCR</th>
<th>Hinge</th>
<th>TM</th>
<th>CYP</th>
</tr>
</thead>
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<td>Cβ</td>
<td>CD3ζ</td>
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<td>Cβ</td>
<td>CD3ζ</td>
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<td>Cβ</td>
<td>CD3ζ</td>
</tr>
<tr>
<td>scTCR-Cys-ζ</td>
<td>Vα-Linker-Vβ</td>
<td>Cβ</td>
<td>CD3ζ</td>
</tr>
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<td>scTCR-28-ζ</td>
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<td>Cβ</td>
<td>CD28</td>
</tr>
<tr>
<td>scTCR-28-ζ-Lck</td>
<td>Vα-Linker-Vβ</td>
<td>Cβ</td>
<td>CD28</td>
</tr>
<tr>
<td>scTCR-Cβ-ζ</td>
<td>Vα-Linker-Vβ</td>
<td>Cβ</td>
<td>CD3ζ</td>
</tr>
<tr>
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<td>Vα-Linker-Vβ</td>
<td>Cβ</td>
<td>CD28</td>
</tr>
<tr>
<td>scTCR-B7-ζ</td>
<td>Vα-Linker-Vβ</td>
<td>Cβ</td>
<td>B7.1</td>
</tr>
</tbody>
</table>

**B**

- **fITCR**: CMV/LTR → TCRα → SRα → TCRβ → IRES → neo → 3'LTR
- **CD3ζδ**: CMV/LTR → CD3ζ → SRα → CD3δ → IRES → neo → 3'LTR
- **CD8α**: LTR → CD8α → SV40 → neo → LTR
- **CD28**: LTR → CD28 → IRES → neo → LTR
- **B7-1**: LTR → neo → CMV → LTR

<table>
<thead>
<tr>
<th>Construct</th>
<th>Promoter</th>
<th>TCRα/LTR</th>
<th>SRα/CD3ζ/CD3δ</th>
<th>IRES</th>
<th>neo</th>
<th>3'LTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>fITCR</td>
<td>CMV/LTR</td>
<td>TCRα</td>
<td>SRα</td>
<td>TCRβ</td>
<td>IRES</td>
<td>neo</td>
</tr>
<tr>
<td>CD3ζδ</td>
<td>CMV/LTR</td>
<td>CD3ζ</td>
<td>SRα</td>
<td>CD3δ</td>
<td>IRES</td>
<td>neo</td>
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<tr>
<td>CD8α</td>
<td>LTR</td>
<td>CD8α</td>
<td>CD3ζ</td>
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<td>neo</td>
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<tr>
<td>CD28</td>
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<td>CD28</td>
<td>IRES</td>
<td>neo</td>
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<td>B7-1</td>
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<td>neo</td>
<td>CMV</td>
<td>B7-1</td>
<td>LTR</td>
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</tbody>
</table>
3.3 RESULTS

Surface expression of single-chain and full-length TCR

To systematically investigate the effects of scTCR structure on membrane expression, Ag recognition and T cell activation, nine recombinant scTCRs were made (Fig. 5A). These scTCRs differed in TM regions derived from different origins (TCR Cβ, CD3ζ and CD28 and B7.1) and combinations of signal transduction domains (CD3ζ, CD28 and p56Lck). scTCR surface expression was first determined on the NIH-3T3 derived packaging cell, GP+E86. Since packaging cells generally contain multiple copies of retroviral constructs and copy number can increase over time (22), this approach is a sensitive platform to study chimeric receptors display. As shown in Fig. 6A, CD3ζ(TM)-, CD28(TM)- and B7.1(TM)-containing scTCRs (except scTCR-ζ-28) could be efficiently expressed on GP+E86 cells as determined using the pan-TCR antibody H57. Anti-Vα2 mAb B20 and anti-Vβ5 mAb MR9-4 gave similar results as the H57 mAb (Fig. 6D). Inclusion of a cysteine residue at the hinge region of scTCR-ζ had no significant effect on the surface expression. When the hinge and TM regions of wild-type TCR Cβ chain were kept in the scTCRs, the surface expression was relatively low. As shown in Fig. 6B, scTCRs could be expressed on the CD3ζ- and ζ chain- double negative T cell line BW5147 (140) but at low efficiency. In an attempt to investigate the relationship between virus titer and surface expression, BW5147 cells were exposed to viral vectors at increasing multiplicity of infection (MOI). Triple infection gave much more reliable results than a single infection. After triple transduction, a positive correlation between virus titer and scTCR expression was clearly shown (Fig. 6C).
Figure 6. Characterization of scTCR and flTCR expression on packaging cell GP+E86 and T cell line BW5147. (A). scTCR expression on GP+E86 cells was determined 3 weeks after triple infection by VSV-G-pseudotyped retroviral vectors. (B). BW5147 cells were transduced 3 times using concentrated ecotropic viruses (titers: 1~3 x10^7 CFU/ml) from TCR-expressing GP+E86 cells. Both scTCR and flTCR expression on BW5147 cells were measured 7 days after the last retroviral transduction using FITC-conjugated pan-TCR mAb, H57. (C). Effects of viral titer and time of transduction on scTCR surface expression were determined by exposing BW5147 cells to various titers of scTCR-1 retroviral vector either once (●) or 3 times (▲). The data are the mean ± SD of 2 independent experiments. (D). High TCR expression population (TCR*) of scTCR-ζ-transduced BW5147 cells were enriched by FACS sorting. scTCR expression was determined using pan-TCR Ab H57, anti-Vβ5 Ab MR9-4 and anti-Vα2 Ab B20.1. Isotype controls are shown in dashed lines.
Unlike the scTCRs whose expression was controlled by a single promoter, the long terminal repeat (LTR) from murine leukemia retroviruses (MuLV), simultaneous expression of flTCRs requires both the TCR α and β chains to be driven under two separate promoters (Fig. 5B). To achieve co-expression, the SAMEN vector was used. This vector has been reported to be efficient in directing gene expression of both TCR α and β chains on the same vector (71,72,76). In order to restore the expression of CD3δ and ζ genes, which are required for the surface expression of the TCR-CD3 complex (79-81, 140), an additional vector (SAMEN-CD3δζ) for co-expression of both CD3δ and ζ genes was constructed. After CD3δ, ζ and flTCR genes were co-transduced into BW5147 cells three times, more than 80% of BW5147 cells were detected positive using the H57 mAb (Fig. 6B). Similar results were observed using the B20 and MR9-4 mAbs (Fig. 6D).

**scTCR-transduced BW5147 cells produce IL-2 in response to Ag stimulation but only at high Ag levels**

Prior to functional analyses, TCR⁺ BW5147 cells were isolated from the scTCR-transduced population by FACS sorting using the H57 mAb. Approximately 80~100% of the sorted cells expressed the transgenic scTCR. The mean fluorescence intensity (MFI) of sorted cells is shown in Table 5. scTCR-transduced BW5147 cells produced a large amount of IL-2 in response to OVA257-274 peptide stimulation at higher concentrations (10⁻⁶ ~10⁻⁴ M) (see Fig. 7A). However, when the OVA257-264 peptide concentration was lowered to 10⁻⁸ M, no significant IL-2 production was observed in any of the scTCR-expressing BW5147 cells. In contrast, flTCR-reconstituted BW5147
cell could respond to OVA257-264 peptide stimulation at concentrations as low as $10^{-10}$ M. As expected, negative control TRP2 peptide-pulsed EL-4 cells did not stimulate TCR-modified BW5147 cells to produce significant amounts of IL-2. The effects of the CD8 coreceptor and the CD3 complex on scTCR-induced activation were investigated by transducing the CD8α and CD3ζ genes into scTCR-modified BW5147 cells, respectively. CD8αα expression could increase the maximal IL-2 production by 30–300%. Restoration of CD3δ and ζ expression in BW5147 cells had no significant effect on scTCR-induced IL-2 production. Integration of CD3ζ and CD28 with or without p56Lck in the same 3D-scTCR allowed a two-fold increase in maximal IL-2 production over that seen with CD3ζ alone in the presence of CD8αα. Interestingly, IL-2 production by BW5147 cells modified with CD28(TM)-containing scTCRs (scTCR-28-ζ and scTCR-28-ζ-Lck) decreased more sharply than that observed with cells modified with CD3ζ(TM)-containing scTCRs when the OVA257-264 peptide concentration was within the range of $10^{-4}$–$10^{-6}$M. The contribution of the costimulatory receptor CD28 on scTCR-induced T cell activation was evaluated using B7.1-transduced EL-4 (EL-4/B7.1) and EG7 (EG7/B7.1) stimulator cells. Expression of CD28 on scTCR-modified BW5147 cells led to more than a 2-fold increase in maximal IL-2 production and lowered the threshold of OVA257-264 peptide concentration needed for scTCR-induced activation to $10^{-8}$ M (IL-2: 133pg/ml, background<10pg/ml) (Fig. 7C). A similar enhancement effect of CD28 was observed in flTCR-modified BW5147 cells, but was more significant when the OVA257-264 peptide concentration was low ($10^{-10}$ M or less). To insure that B7.1 gene transfer had no direct effect on Ag presentation
capacity, fITCR-reconstituted CD28 negative BW5147 cells were cocultured with OVA_{257-264}-pulsed EL-4 or EL-4/B7.1 cells. There was no significant difference in IL-2 production using either cell line, indicating that both EL-4 and EL-4/B7.1 cells were comparable in OVA peptide presentation. Therefore, the dramatic increase in IL-2 production observed with CD28⁺ TCR-transduced BW5147 and EL-4/B7.1 cells was due to the interaction between CD28 and B7.1. Despite the dramatic enhancement effects of CD8 and CD28, none of scTCR-modified BW5147 cells were able to respond to OVA-expressing EG7 cells, whereas 2.2~7.3% (147±33~497±18 pg/ml, background <20 pg/ml) of the maximal IL-2 production (obtained by coculture of TCR-modified BW5147 cells with 10⁻⁴ M OVA peptide-pulsed EL-4 or EL-4/B7.1 cells) was observed when fITCR-reconstituted BW5147 cells were cocultured with EG7 cells.
Table 5. Summary of transgene expression on gene-modified BW5147 cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>MFI (^b) (%) (^c)</th>
<th>CD8α</th>
<th>CD28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>4.0± 0.1 (0)</td>
<td>3.7± 0.3 (2)</td>
<td>3.9± 0.2(2)</td>
</tr>
<tr>
<td>scTCR-ζ</td>
<td>179±4.4 (92)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>scTCR-ζ+CD8</td>
<td>158±3.2(98)</td>
<td>574±15(98)</td>
<td>ND</td>
</tr>
<tr>
<td>scTCR-ζ+CD8+CD28</td>
<td>163± 3.6(94)</td>
<td>596±22(98)</td>
<td>100± 8.2(92)</td>
</tr>
<tr>
<td>scTCR-ζ+CD3ζ</td>
<td>138±1.8(90)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>scTCR-ζ+CD3ζ+CD8</td>
<td>143±5.4(98)</td>
<td>624±41(96)</td>
<td>ND</td>
</tr>
<tr>
<td>scTCR-ζ-28-Lck</td>
<td>60±2.2(93)</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>scTCR-ζ-28-Lck +CD8</td>
<td>74±3.8(96)</td>
<td>269± 20(86)</td>
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<tr>
<td>scTCR-Cys-ζ+CD8</td>
<td>127±9.8(97)</td>
<td>689±27(99)</td>
<td>ND</td>
</tr>
<tr>
<td>scTCR-28-ζ+CD8</td>
<td>95±5.2(90)</td>
<td>667±19(98)</td>
<td>ND</td>
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<tr>
<td>scTCR-28-ζ-Lck</td>
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<td>ND</td>
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<td>scTCR-28-ζ-Lck +CD8</td>
<td>53±1.2(88)</td>
<td>281±45(84)</td>
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</tr>
<tr>
<td>flTCR+CD8</td>
<td>43±3.2(90)</td>
<td>685±12(100)</td>
<td>ND</td>
</tr>
<tr>
<td>flTCR+CD8+CD28</td>
<td>62±1.3(96)</td>
<td>503± 34(100)</td>
<td>72± 4.4(75)</td>
</tr>
</tbody>
</table>

\(^a\) BW5147 cells were transduced with either a TCR construct alone or a TCR construct plus one or two accessory genes. Transgene expression was determined by flow cytometry as describe in Methods and Materials. Results presented are mean ± SD of 3 experiments.

\(^b\) MFI: Mean fluorescence intensity.

\(^c\) Percentage of positive cells as shown in bracket.

\(^d\) ND: not determined.
Figure 7. IL-2 production by TCR-modified BW5147 cells. scTCR and flTCR-modified BW5147 cells were compared for IL-2 production after stimulation with various concentrations of OVA<sub>257-264</sub> peptide-pulsed EL-4 cells. (A). IL-2 release was evaluated either in the presence (■) or absence (○) of CD8α. Effect of complete CD3 complex on scTCR-induced IL-2 production was also determined after restoration of CD3δζ BW5147 cells with CD3ζ and δ genes. scTCR-Cysζ and scTCR-28ζ were compared in the presence of CD8α. (B). Full-length CD3ζ and δ chain expression in CD3ζδ-transduced BW5147 cells was determined by RT-PCR. β-actin was used as an internal control. (C). To investigate the effect of CD28 on IL-2 production, scTCR-ζ and flTCR-modified T cells were transduced with CD28 gene and cocultured with either EL-4 cells (△) or B7.1-transduced EL-4 cells (EL-4/B7,▲) as mentioned in Methods and Materials. The data are presented as mean ± SD of three independent experiments.*: p<0.05
TCR-transduced primary T cells are functional

The ultimate goal of this methodology is to use TCR gene-modified T cells for adoptive immunotherapy. Thus it's critical to test the functional efficiency of these TCR molecules (both scTCRs and fITCRs) expressed on primary T cells. Cell surface expression of OVA-specific scTCRs and fITCR on transduced primary T lymphocytes was determined using PE-anti-Vα2 mAb (Fig. 8). In mock-transduced T cells, the percentage of Vα2⁺ T cells was 30%, whereas more than 90% of transgenic OT1 T cells expressed Vα2. Transgenic expression of both scTCR and fITCR constructs varied from 10% to 40% as estimated by subtraction of the background value (30%) from the actual level of Vα2 expression. Similar to the IL-2 production observed in BW5147 cells, significant IFN-γ production by scTCR-transduced primary T cells was observed after OVA257-264 peptide stimulation (Table 6). However, the Ag threshold of IFN-γ production by scTCR-modified primary T cells was 2-logs lower than that of IL-2 release by BW5147 cells, although scTCR expression on primary T cells was lower (Fig. 8). Similar to scTCR-modified BW5147 cells, scTCR (except scTCR-ζ-28-Lck)-transduced primary T cells failed to respond to the stimulation by EG7 cells. In contrast, fITCR-modified primary T cells produced significant amounts of IFN-γ, comparable to OT1 T cells after stimulation with a low concentration of OVA peptide (10⁻¹⁰ M) or EG7 cells. The cytotoxic activity of TCR-modified splenic T cells was also tested (Fig. 9). fITCR-transduced primary T cells demonstrated a level of cytotoxicity against OVA-loaded EL-4 cells similar to OT1 CTL, but were less efficient in killing EG7 target cells. Of all the tested scTCRs, scTCR-ζ-28-Lck was the most effective in allowing
transduced T cells to lyse OVA-pulsed EL-4 cells, whereas other scTCRs endowed primary T cells with only moderate cytolytic capacity even when the Ag density on target cells was high ($10^6$ M). In addition, IFN-γ production was correlated with the level of cytotoxic activity in TCR-transduced T cells.

**TCR-modified T cells stain poorly with OVA peptide–loaded H-2Kb-Ig dimmers**

To gain insight into the affinity of transduced TCR molecules for the H-2Kb-OVA Ag complex, an OVA peptide–loaded H-2Kb-Ig dimer was used. Interestingly, although flTCR-transduced primary T cells expressed a high percentage of transgenic TCR (>40%) and produced large amounts of IFN-γ in response even to low concentration of Ag stimulation, the H-2Kb-Ig dimer staining of these cells was negative as compared with that of transgenic OT1-derived T cells (Fig.10). Similar results were obtained with scTCR-modified primary T cells.
Figure 8. scTCR and fITCR expression on primary T cells. Three days after G418 (0.6 mg/ml) selection, TCR-transduced primary T cells were then stimulated with OVA\textsubscript{257-264} (10\textsuperscript{-5} M) peptide-pulsed, mitomycin-C treated B6 splenocytes for an additional 3 days. Viable cells were isolated by centrifugation on Lympholyte-M, and stained with either PE-anti-V\textalpha\textalpha2 mAb (shaded histogram) or isotype control (open histogram). The FACS data is representative of three comparable experiments.
Table 6. IFN-γ production by TCR-modified primary T cells

<table>
<thead>
<tr>
<th>Responder Cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IFN-γ release (pg/ml)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EL-4 pulsed with OVA peptide (M)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>10&lt;sup&gt;-10&lt;/sup&gt;</td>
<td>10&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mock</td>
<td>111±26</td>
<td>120±13</td>
<td>128±10</td>
<td>127±11</td>
</tr>
<tr>
<td>scTCR-ζ</td>
<td>710±59</td>
<td>690±64</td>
<td>1518±25</td>
<td>3300±54</td>
</tr>
<tr>
<td>scTCR-ζ-28-Lck</td>
<td>868±24</td>
<td>2416±60</td>
<td>6998±32</td>
<td>7202±44</td>
</tr>
<tr>
<td>scTCR-Cys-ζ</td>
<td>96±8</td>
<td>87±27</td>
<td>225±16</td>
<td>315±128</td>
</tr>
<tr>
<td>scTCR-28-ζ</td>
<td>162±18</td>
<td>201±18</td>
<td>474±32</td>
<td>1087±21</td>
</tr>
<tr>
<td>scTCR-28-ζ-Lck</td>
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<td>95±5.1</td>
<td>134±64</td>
<td>202±32</td>
</tr>
<tr>
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<td>3121±340</td>
<td>1040±409</td>
<td>213127±17591</td>
<td>219096±6656</td>
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<tr>
<td>OT1</td>
<td>561±96</td>
<td>2957±2773</td>
<td>356487±555</td>
<td>351387±15028</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primary T cells modified with either fITCR or scTCR genes were used as responder cells. Mock-infected T cells served as a negative control, whereas OT1 T cells were used as a positive control.

<sup>b</sup> Stimulator cells are either OVA<sub>257-264</sub>-pulsed EL-4 cells or native OVA-expressing EG7 cells. A representative result (mean ± SD of triplicates) of three independent experiments was shown. Positive results (>2-fold background and 200 pg/ml) are shown in bold.
Figure 9. Specific lysis of target cells by TCR-modified primary T cells. After stimulation by OVA peptide for 3 days, viable effector T cells were cocultured with target cells EG7 or EL-4 either pulsed with OVA peptide or left unpulsed, at ratio of 10:1 in a 5-h LDH release assays. The data are presented as mean ± SD of 2 representative results from 4 independent experiments.
Figure 10. Dimer staining of TCR-modified primary T cells. G418-resistant TCR-modified T cells were stained with either OVA_{257-264} or control peptide Trp2 -loaded H-2K^{b}-Ig dimer, followed by staining with secondary mAb A85-1-PE and anti-CD8-FITC as described in Methods and Materials. OT1 T cells were used as a positive control, whereas mock-infected primary T cells served as negative controls. The presented data is representative of 4 comparable experiments.
3.4 DISCUSSION

An increased understanding of TCR recognition of the MHC-peptide complex, and the subsequent signal transduction events, has permitted a rational design of TCR-based chimeric receptors for immunotherapy. In theory, each of the three parts of 3D-scTCRs (Ag recognition region, hinge region and signal transduction domain) could be optimized for efficient functions. In this study, our major focus is on the optimization of signal transduction domains. In terms of efficiency and safety, a promising 3D-scTCR candidate should meet three requirements. First, it should be efficiently expressed on the cell surface. Second, it should recognize the MHC-peptide complex with fine antigenic specificity and transduce signals efficiently. Third, it should be not be highly immunogenic (74). Therefore, when used in vivo, no significant immune responses should be elicited, which might compromise the efficiency of scTCR-modified T cells. In the present study, our focus was on the exploration of the relationship between scTCR structure and function as well as surface expression.

Two scTCR constructs that contained the TCR Cβ region as the TM region had poor cell surface expression on the non-lymphoid NIH-3T3 cell-based packaging cell, GP+E86. This observation may be because the TM region of the TCR is responsible for assembly with the CD3 complex (which is not expressed in non-lymphoid cells) for surface expression (79,80). In contrast, scTCRs with the TM region derived either from CD3ζ or CD28 could be efficiently expressed on GP+E86 cells as well as on the CD3ζ–ζ− T cell line BW5147, indicating that the complete CD3 complex was not indispensable for membrane expression of such scTCRs. Such a CD3 complex-independent nature
allows scTCR constructs to be expressed at high levels after multiple transductions, whereas transgenic full-length TCR expression on BW5147 cells was not significantly increased due to its dependence on the presence of other components of the CD3 complex. This finding may pose a potential problem for the genetic modification of primary T cells from tumor patients using full-length TCRs because these T cells generally have reduced levels of CD3ζ expression (82), thereby affecting transgenic TCR expression. Fusion of the CD28 cytoplasmic tail to the C-termini end of CD3ζ dramatically reduced cell surface expression. A similar finding has been reported previously (67,70). However, inefficient surface expression could be completely rescued by addition of the Lck kinase to the carboxyl end of the aforementioned molecule. Although the TM region of B7.1 has been shown to be efficient in directing cell surface expression (160), no significant advantage over that seen with CD3ζ or CD28 was observed.

A recent study by Holler et al (161) has clearly shown that CD8 has significant synergistic effects on TCR-MHC-peptide interactions when the affinity of the TCR is within low or medium ranges. Two roles for CD8 have been suggested in promoting T cell activation (162). First, CD8 binds to MHC although with a very low affinity (Kd: 50–200μM). Second, a Src-family kinase Lck, which is constitutively associated with the CD8α chain, is recruited to the TCR:CD3 complex after initial triggering, thereby enhancing TCR triggering by stabilizing the TCR:peptide-MHC interaction and/or amplifying the signalling pathway. Previous studies have shown that scTCR molecules have lower affinity compared with parental full-length TCRs (86,88). Therefore, CD8
might be more helpful in this case. The observation that co-expression of CD8αα and scTCR led to a 30–300% increase in maximal IL-2 production supports this hypothesis. In addition, CD8αα also promoted the function of scTCR-ζ-28-Lck and scTCR-28-ζ-Lck constructs, containing the Lck module, suggesting that the second function of CD8 is not the only one mediating the enhancement effect.

CD28 provides very potent costimulation, but requires stimulator cells to express B7, whose expression is restricted to professional antigen presenting cells (APCs). Combining the CD28 signalling domain with the CD3ζ chain in a single immunoreceptor allows for T cell activation though both primary and secondary signals (67,70,97). This strategy avoids the requirement for B7 expression by stimulator cells. Our results showed that the CD28-containing scTCRs had a 50–100% increase in maximal IL-2 production in the presence of CD8αα. In view of the lower expression of these constructs compared with scTCR-ζ (26–53%) on BW5147 cells, the costimulation effects of the CD28 moieties might be quite significant. However, CD28 when expressed separately, could provide more potent costimulation to scTCR- as well as full-length TCR-induced stimulation, suggesting that integration of CD28 moieties into chimeric immunoreceptors might not totally replace the function of wild type CD28. Compared with the weak affinity between CD8αα and MHC, the binding of CD28 to B7.1 is much stronger (Kd: ~4μM) (162). Whether the adhesion function of CD28 plays a role in enhancement of scTCR-induced T activation needs be determined using CD28 mutants whose signalling domains have been deleted.

One interesting finding was that CD28(TM)-containing scTCRs (scTCR-28-ζ and
-28-ζ-Lck) and scTCR-Cys-ζ (containing a cysteine residue, Cys240, at the TCR Cβ hinge region) in BW5147 cells demonstrated significant decreases in IL-2 production when the OVA peptide concentration decreased from 10^{-4} to 10^{-6} M. No similar finding was found with primary T cells. A previous study (163) has shown that a chimeric receptor with the TM region derived from the CD3ζ chain, when transferred to primary T cells, could form both homodimers and heterodimers of the chimeric receptor with the endogenous ζ chain. Since BW5147 cells do not express endogenous CD3ζ or CD28, if CD3ζ(TM)- and CD28(TM)-containing scTCRs exist in a dimer form, it will be only as homodimers. Distinct dimer forms of scTCR on primary T cells and BW5147 cells might play a role in the differential sensitivity of scTCR-modified T cells to antigen stimulation. Other factors, such as a higher sensitivity of IFN-γ release by CTL than IL-2 production by BW5147 cells, and exclusive expression CD8αβ rather than the less effective CD8αα on splenic T cells (164), might also be involved in lower activation thresholds in scTCR-modified primary T cells.

Transfer of flTCR genes to primary T cells has been shown to be an effective approach to bestow specificity and function to transduced T cells. Our results, demonstrating that flTCR-modified T cells could respond to antigen stimulation at a sensitivity comparable to natural CTL, were in agreement with these findings. One significant drawback of using flTCR is that the competition between the transgenic and endogenous TCR might reduce the percentage of correct chain pairings, thus limiting the avidity and specificity of the resulting TCR-transduced cells (74,165). This problem cannot be completely overcome by increasing the gene transfer efficiency and could be more significant when antigen density on target cells is low (165). Although a 3D-
scTCR would allow coordinate expression of TCR Vα and Vβ chains on the same molecule regardless of the presence of endogenous TCRs, the affinity of MHC-peptide recognition by such chimeric receptors may not be optimal as suggested by the low efficiency of scTCR compared to fITCR. It has been shown that TCR affinity for MHC/peptide complex dictates the threshold of activation (161). T cells with a high affinity TCR require low antigen density to be activated, whereas low affinity TCR-expressing T cells require high antigen concentrations to do so. The response threshold of the scTCRs in response to OVA peptide stimulation is much higher (2~3-logs) than that seen in fITCR-modified T cells, suggesting that it's likely that the affinity of scTCRs in this study is lower than that of fITCR.

To determine whether inefficient recognition of H-2Kb-OVA by scTCRs was due to low TCR affinity, an OVA257-264–loaded H-2Kb-Ig dimer was used to stain the TCR-transduced T cells. Surprisingly, dimer staining of fITCR-transduced primary T cells was poor or negative although these cells were functional and expressed a high percentage of transgenic TCR. In addition, fITCR-transduced BW5147 cells also stained negative with the dimer. These facts suggest that factors other than TCR affinity are involved in dimer staining, as transgenic OTI T cells were essentially 100% dimer-positive. As discussed before, the competition between the transgenic and endogenous TCR might reduce the relative density of correct chain pairings. This hypothesis has been supported by the facts that only a small portion (20~30%) of transgenic fITCR+ primary T cells stained positive for tetramers (74,165). This result also indicates that the use of H-2Kb-Ig dimers to identify antigen-specific T cells is limited. Negative staining of scTCR-modified T cells by H-2Kb-Ig dimer might represent the low affinity of
scTCR to MHC-peptide complex. But we cannot exclude the possibility that the low sensitivity of H-2K\(^b\)-Ig dimer is primarily responsible for the negative staining. Recently, Mallet-Designe et al (166) have shown that incorporation of tetramers into liposomes allowed low-affinity T cells to be effectively detected. In the future, we may try a similar strategy to identify the differential affinity between fITCR and scTCR. Holler et al (167) have demonstrated that the low affinity of scTCR molecules could be dramatically increased by an \textit{in vitro} selection system though site-directed mutagenesis. Derivation of scTCR with moderate affinity (\(K_d: 1\sim10\mu M\), as compared to classic TCR) might be more appropriate in our case however, because high affinity TCR (\(K_d: 10\sim100nM\)) could lead to autoimmunity (168,169). Primary T cells modified with the scTCR-\(\zeta\)-28-Lck could lyse low concentrations (\(10^{-10}\) M) OVA peptide-pulsed EL-4 cells (E:T=10:1), although at a marginal level. After a moderate improvement of TCR affinity by the \textit{in vitro} selection system mentioned above, a mutagenized scTCR-\(\zeta\)-28-Lck could be expected to be a promising candidate in \textit{in vivo} study.

In summary, a series of scTCR molecules containing various TM regions and cytoplasmic signalling domains have been constructed to explore the effect of scTCR structure on T cell function. Our results showed that transfer of TCR genes (either scTCRs or fITCR) into T cells bestowed the resulting T cells with both specificity and functional activity. TM regions and signalling domains had significant effects on the expression and function of TCR-modified T cells. However, despite many optimizations, scTCRs were less efficient than fITCR in response to low concentrations of antigen stimulation. This low efficiency of scTCR appears to be due to the inherent low-affinity binding of the receptor.
4.1 OVERVIEW

One of the major advantages of adoptive therapy is the ability to transfer Ag-specific CTL of known avidity and specificity. The transfer of Ag-specific T cells has been applied in the therapy of viral infectious diseases and virus-associated malignancies (189). Successful adoptive immunotherapy has been achieved for the control of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) infection complicating haematopoietic stem cell transplantation (SCT). The adoptive transfer of CMV-specific CTL clones has reduced the incidence of CMV disease (190). Similarly, infusions of donor-derived EBV-specific T cell lines following T cell-depleted SCT have prevented EBV-triggered lymphoproliferative disorders (191, 192) and reduced the viral load in patients with high titre EBV DNA post-transplant (192).

As described in chapters 1 and 3, TCR gene transfer bestows the recipient T cells with functionality and specificity. The sources of TCR could be derived from autologous, allogeneic or even murine T cells. To bypass self-tolerance to the human tumor Ag mdm2, CTL were isolated from HLA-A2 transgenic mice (75). Murine HLA A2-restricted CTL were generated by immunizing the mice with a human mdm2 peptide.
epitope differing by a single amino acid to the naturally presented murine peptide. A high-avidity TCR was cloned from murine CTL, partially humanized and retrovirally transferred into human T cells to produce CTL capable of high-avidity killing of mdm2-expressing cells. Both avidity and Ag-specificity were reconstituted in the human T cells after retroviral transduction of TCR genes. Such therapeutic TCR transfer provides a method of rescuing the self-MHC-restricted human T cell repertoire by producing high-affinity, broad-spectrum tumor reactive TCRs that are usually deleted by tolerance mechanisms. Alternative approaches of generating high-affinity receptors for tumor-associated peptide epitopes have been described recently. For example, in vitro mutagenesis followed by tetramer selection can be used to isolate TCRs with improved Ag-specific binding affinity (31). This technology can be used to convert low-affinity TCRs specific for tumor-associated self-peptides into high-affinity TCRs, and thus improve tumor cell recognition (193). Taken together, these novel approaches provide a platform to overcome one of the major limitations of autologous immune responses against tumors, namely low-avidity T cell responses against tumor-associated Ags.

In addition to TCR transfer into mature T cells, it is also possible to target the haematopoietic stem cell. It has been demonstrated that TCR transfer into murine haematopoietic stem cells produced mature T cells that responded to Ag-specific challenge in vivo (194). A significant advantage of this approach is the potential to continually generate Ag-specific T cells from a self-renewing stem cell pool.

To date, very little is known about functional activity of scTCR-modified T cells in vivo. Although, in our previous study (Chapter 3), scTCR-modified T cells in vitro
required higher concentrations of Ag to be activated, it’s likely that higher-avidity scTCR-modified T primary cells might be selectively expanded due to potential \textit{in vivo} selection mechanisms (high-avidity scTCR-modified T cells preferentially proliferate when the Ag concentration is low which is the case \textit{in vivo}).

In order to explore the possibility of \textit{in vivo} selection for high-avidity scTCR-modified T cells and test the efficacy of Ag-specific \textit{flTCR}-modified primary T cells on tumor growth, B6 mice were subcutaneously implanted with EG7 tumors followed by \textit{i.v} infusion of TCR-modified T cells. \textit{In vivo} tumor growth was determined by measurement of tumor volume. Additionally, the expansion capacity of genetically modified T cells was evaluated after G418 selection followed by weekly Ag stimulation.
4.2 METHODS AND MATERIALS

Cell preparations

OVA-specific T cells were obtained from the spleens of OT-1 TCR-transgenic mice. Activation of OT1 T cells was performed by stimulation of red blood cell-depleted OT1 splenocytes (2×10^6/ml) with ConA (2.5 μg/ml) in 20 ml of RPMI-10 in 75cm^2 T-flasks for 2 days. Activated T cells were then collected and expanded in ConA-free, IL-2 (25 U/ml human IL-2, Roche, Nutley, NJ)-containing media for an additional 5 days. Cell numbers were checked every day and cell density was adjusted to be within the range of 0.3~1.0×10^6/ml by adding fresh RMPI-10 plus IL-2 (25 U/ml). Small aliquots of expanded OT1 T cells were tested for phenotypic analyses and functional activity as described in the Methods and Materials section of Chapter 2. Using this expansion protocol, more than 90% of T cells were CD8^+Vα2^+ and OVA peptide-H-2K^b^-Ig-dimer positive (Figures 8 and 10). The remaining OT1 T cells were frozen in liquid nitrogen using ORIGEN DMSO freeze media (IGEN International, Inc., Gaithersburg, MD) at a density of 10×10^6 cells/ml for future experiments. Two or three weeks before the in vivo study, OT1 T cells were thawed and cultured in RPMI-10 plus 25 U/ml IL-2 at a density of 1×10^6/ml. After 3 days, cells were washed twice in PBS and stimulated weekly with mitomycin-C treated, OVA peptide (10 μM)-pulsed B6 spleen cells (1×10^6 cells/ml) at a ratio of 1:5. Viable cells were isolated by density centrifugation using Lympholyte-M (Cedarlane Laboratories, Hornby, Canada).

Two or three weeks before adoptive cell transfer, the primary T cells were modified with concentrated retroviral vectors encoding either the scTCR-ζ-28-Lck or fITCR
according to the retroviral transduction protocol in Methods and Materials (Chapter 2). Mock-infected primary T cells (negative control) were obtained by using supernatant from empty vector LXSN-transduced GP+E86. After selection in G418 (600 μg/ml) for 5 days, dead T cells were removed using Lympholyte-M. Viable T cells were specifically expanded in the same way as OT1 T cells. Both CD8 and TCR Vα2 expression were analyzed by FACS in all groups of T cells before adoptive transfer.

Subcutaneous tumor establishment

Syngeneic C57BL/6 mice were injected subcutaneously with EG7 tumor cells (1×10^6 cells) in 100-μl volumes of sterile PBS. Tumor cells for injection were recovered from log phase in vitro growth (viability>95%) and were injected into the right flank of recipient mice. Tumors were clearly palpable after 1 week in an encapsulated fashion.

Tumor measurement

Tumors were measured three times a week in two perpendicular axes using a caliper. Tumor volumes were calculated using the formula: Volume (mm^3) = 0.5xlengthxwidth^2 (178), and are presented as the mean of 6-10 identically treated mice ± SEM.

Adoptive transfer of T cells

A total of 5x10^6 effector cells were injected in 200-μl of PBS. Injections were delivered once via the tail vein 7 days after tumor implantation.

Statistical analysis

The data were analyzed to determine any significant differences among the experimental groups using a nonpaired Student’s t test. A value of p≤0.05 was
considered significant. All statistical analysis was performed using Excel 2000 Software (Microsoft, Redmond, CA).
4.3 RESULTS

TCR-modified T cells efficiently expand in G418 selection followed by Ag stimulation

After an initial stimulation with ConA, spleen cells proliferated rapidly. Even after 5 days of G418 selection, cell numbers increased by a 9-20-fold in one week (Fig. 11A). However, the growth rates decreased by the second week. Ag stimulation led to a further 4-5-fold expansion of TCR-transduced T cells. Before adoptive transfer, transgenic TCR expression was measured using an anti-TCR Vα2 mAb as described in Chapter 3. As shown in Figure 11B, before G148 selection, the percentage of splenocytes which expressed significant amounts of transgenic scTCR or flTCR, were approximately 10% and 30%, respectively. These percentages increased up to 35 and 55%, respectively, after 5 days of G418 selection.

Effects of adoptive transfer of TCR-modified T cells on in vivo tumor growth

In our previous studies (in vitro), TCR (either scTCR or flTCR) -modified primary T cells demonstrated cytotoxic activity against EG7 or OVA peptide-pulsed EL-4 cells. Whether these TCR-transduced T cells would be functional in vivo was a very interesting question to answer. The capacity of the flTCR and scTCR-ζ-28-Lck to stimulate T cell anti-tumor function against EG7 tumor targets was evaluated in adoptive transfer assays using syngeneic B6 mice. Transduced flTCR and scTCR-ζ-28-Lck cells (5 ×10⁶) were injected i.v. into mice 7 days after s.c. inoculation of OVA⁺ EG7 tumor. As shown in Figure 12A, mock-infected T cells were not capable of eradicating the implanted EG7 tumor. All six inoculated mice developed tumors without
signs of significant tumor shrinkage or growth arrest. However, the positive control OT1 T cells (OVA-specific T cells) mediated an effective anti-tumor response, with the complete eradication of 2 of 10 EG7 tumors, significant tumor shrinkage or growth arrest in another 5 EG7 tumors and no significant effects in the remaining 3 tumors after adoptive transfer. The average tumor growth in the OT1 transfer group was significantly slower than that observed in mock-infected T cell transfer group (Fig.12A). In comparison, $\text{flTCR}$-modified T cells demonstrated less effective anti-tumor effects with the complete eradication of only one out of 10 tumors, and a partial tumor growth arrest in another mouse. Although 2 out of 9 EG7 tumors underwent temporary shrinkage after transfer of scTCR-$\zeta$-28-lck-modified T cells, no complete eradication of tumors was observed. When the data were plotted as mean ± SEM, no statistical significance among the groups of mice treated with flTCR-, scTCR- and mock-infected T cells was observed (Fig. 12 B).
Figure 11. In vitro expansion of genetically modified T cells. (A) Mock-(■), flTCR-(▲) and scTCR-ζ-28-Lck-(□) modified T cells were expanded for 2 days after transduction in the presence of 25 U/ml IL-2, and then selected in G418(600μg/ml) for additional 5 days. Viable cells were stimulated with mitomycin-C-treated, OVA peptide pulsed-B6 spleen cells for another week as described in the Methods and Materials. Cell counts were determined weekly. (B) Transgenic TCR expression on gene-modified T cells either in the absence (□) or presence (▲) of G418 was estimated using anti-TCR Va2 mAb. *: p<0.05
Figure 12. Effects of adoptive transfer of TCR-modified T cells on in vivo tumor growth. B6 mice were injected subcutaneously with $1 \times 10^6$ EG7 tumor cells. Seven days later mice received either $5 \times 10^6$ of OT1, mock-infected, scTCR-$\zeta$-28-Lck or fITCR–transduced T cells. Tumor sizes were measured three times weekly. Tumor volumes were plotted either individually (A) or based on the average values (B) of each group. The error bars represent SEM. *: $p<0.05$. 
4.4 DISCUSSION

The average frequency of Ag-specific T cells in naïve animals is expected to be less than 1 in $10^4$. Our results showed that at least 50% of flTCR- and 35% of scTCR-modified T cells expressed the transgenic TCR after 5 days of G418 selection. The difference in frequency is about 5000-fold. Theoretically, it would take at least 12 additional cell divisions ($5000 = 2^{12} - 2^{13}$) for the frequency of Ag-specific T cells to increase to that extent (35–50%). In reality, it will require more than 12 divisions to achieve this goal due to the presence of activation induced T death (AICD) in a fraction of Ag-specific T cells (170) and the proliferation of non-specific T cells (171). However, T lymphocytes generally have a limited life span. During long-term culture, T cells proliferate for a restricted number of cell divisions, after which the cells cease to proliferate and become senescent (172). In addition, our data showed that it only took a very limited time (1–2 weeks) for TCR-transduced primary T cells to expand to sufficient numbers for adoptive transfer. Therefore, genetic modification of T cells with Ag-specific TCR genes provides a tremendous advantage over traditional in vitro T cell expansion. An additional advantage of TCR gene transfer is that tumor-specific TCRs do not necessarily need to be derived from the patients themselves, they can be obtained from HLA-mismatched individuals.

Our results demonstrated that TCR (either flTCR or scTCR)-modified primary T cells were effective to some extent in controlling the growth of well-established EG7 tumors, and even eradicating some tumors. However, most TCR-modified T cell recipient mice didn’t show any significant signs of anti-tumor effects. Several factors might affect the
anti-tumor effects of TCR-modified T cells after adoptive transfer. First, the number of transferred T cells in relation to tumor burden plays a critical role in dictating the immunological consequences after adoptive T cell transfer. For the transferred T cells and host immune system to be efficient in controlling tumor growth, the tumor burden should not be too large. In the in vivo experiments, EG7 tumors were well-established 7 days after implantation. Adoptive transfer of $5 \times 10^6$ OT1 T cells (positive control) only led to the complete eradication of tumors in 20% of mice and 30% of mice did not show any significant response. These results suggest that one round of adoptive transfer of $5 \times 10^6$ cells might not be sufficient. In the future, we plan to transfer T cells at multiple times and/or in greater numbers. Second, immunogenicity of the tumor cells is another limiting factor that affects the recognition of tumor targets by T cells. Like the parental EL-4 cells, EG7 cells express high levels of H-2K$^b$ (137). The endogenous over-expressed OVA Ag renders the EG7 cells susceptible to lysis by CTLs such as OT1 T cells. However, tumor cells can downregulate the expression of Ags that are recognized by the immune system as a mechanism for tumor escape (195), Whether such an “Ag loss” mechanism plays a role in the regrowth of tumor after initial shrinkage needs to be further investigated. Third, T cells must have the capacity to home to the tumor, a process that is controlled by the expression of tumor Ag receptors, homing receptors (adhesion molecules) and chemokine receptors. Expression of these receptors is generally dependent on the T-cell expansion conditions (196). Fourth, duration of transgenic TCR expression in TCR-modified T cells after administered in vivo is also critical for these T cells to function efficiently. It has been reported that Moloney-based
vectors are sometimes sensitive to promoter silencing \textit{in vivo} (83), TCR-modified T cells might not sustain long-term memory due to the loss of expression of the transgenic TCR. This problem can be solved by development of silencing-insensitive retroviral vectors and serial infusion of previously frozen TCR-transduced T cells (\textit{i.e.}, T cell “banking”).

A limitation associated with \textit{in vivo} application of scTCR-transduced T cells is the potential immunogenicity of scTCRs. Fusion sites between different fragments and the (G4S)$_3$ linker might be recognized as foreign. It remains to be seen however, whether scTCR immunogenicity will be a significant problem.

Two types of problems might be associated with adoptive transfer of TCR-transduced T cells. First, autoimmunity either derived from incorrect TCR pairings or ubiquitous expression of tumor Ags on normal tissues could lead to untoward consequences following infusion of autologous tumor-reactive T cells (196). Second, in rare cases, some retrovirally transduced T cells might be transformed resulting in leukemia (197). The most common strategy is to provide a control mechanism to abort T-cell responses or eliminate the transformed T cells via the transduction of a regulated suicide function. Expression of herpes simplex virus thymidine kinase (\textit{hsv}TK), which confers sensitivity to the pro-drug ganciclovir (GCV), provides an effective means to delete the modified T cells (198).

Several other aspects of TCR gene transfer may also need to be optimized. Besides construction of an scTCR to avoid formation of heterodimers consisting of endogenous and exogenous chains, an alternative way to minimize the formation of mixed
heterodimers may be the remodeling of the TCRαβ interface, whose proof of principle has been demonstrated in construction of immunoglobulin heterodimers (173). For tumor lineage Ags for which no high affinity TCRs may be present in vivo due to self-tolerance, TCR gene transfer may be used to introduce tumor-specific TCRs that have been optimized in vitro by either yeast or retroviral TCR display (74).

In summary, the ability to genetically engineer primary T cells creates new prospects for the investigation of T-cell biology, tumor immunity and cancer immunotherapy. The transduction of T cells with genes that encode TCRs enables the recognition of Ags that are either poorly immunogenic or ignored by the immune system. Tumor targeting with genetically enhanced T lymphocytes provides an important key for better understanding the molecular and cellular requirements for effective tumor immunity.
CHAPTER 5

SING: A NOVEL STRATEGY FOR IDENTIFYING TUMOR SPECIFIC, CTL-RECOGNIZED TUMOR ANTIGENS

5. 1 OVERVIEW

Identifying T-cell recognized tumor antigens (Ags) is a critical step in studying tumor immunity and designing tumor vaccines for cancer immunotherapy. Clinically successful specific cancer immunotherapy depends on the identification of tumor-rejection Ags. Tumor Ags have been identified by analyzing either T-cell or antibody responses of cancer patients against autologous cancer cells (115). Thanks to the landmark studies by Boon and Rosenberg, the once suspect hypothesis that human cancers express Ags that can be targeted specifically by cellular immunity has now become a scientifically justifiable rationale for the design and clinical testing of novel Ag-specific cancer immunotherapies (114,115).

Four major strategies have been applied for identifying tumor Ags. These methods include transfection of recombinant tumor cDNA libraries and HLA molecules into target cells ("genetic approach") (104, 105); elution of peptides from the binding cleft of tumor HLA molecules ("peptide-elution approach") (106, 107); serological analysis of recombinant tumor cDNA expression libraries (SEREX approach) (108); and deduction of peptide sequences from known oncogenes or tumor-associated proteins using known HLA-anchor motifs ("reverse immunology approach") (109, 110).
The classic method of identifying T cell-recognized tumor Ags was developed by Boon et al (111). This method requires the screening of at least 1000 pools of tumor cell-derived cDNAs using Ag-specific CTLs. However, the transfection of >1000 pools of plasmids, and the subsequent T cell assays for the cytokines produced by CTLs are time-consuming and expensive tasks. The disadvantages of other three approaches include being technical demanding ("Peptide elution" approach), being based on tumor-specific antibody production not CTL generation (SEREX approach), and the requirement for multiple steps to validate the immunogenicity of candidate tumor Ags ("reverse immunology" approach).

In an attempt to simplify the traditional approaches, we have designed a novel and direct strategy, "SING" (Signal transduction molecule-mediated, NFAT-controlled, GFP expression), for cloning T-cell recognized tumor Ags. The SING system is an artificial Ag presentation system. It was established by transducing a mouse T cell line BW5147 with a gene coding for a chimeric H-2K^b receptor (the cytoplasmic tail of the wild type H-2K^b was replaced with signal transduction domains) and a NFAT-controlled GFP expression vector. The resultant BW5147 cells were named BS cells. According to our hypothesis, after stimulation through the chimeric H-2K^b molecule, the NFAT pathway is activated and NFAT-controlled GFP expression is induced. Our results showed that BS cells could respond to external signals, such as phorbol ester (PMA) plus ionomycin, anti-H-2K^b mAb cross-linking and stimulation by Ag-specific TCR, by expressing GFP. Although the efficiency of GFP induction by endogenous Ag-expressing BS cells after TCR engagement was not high, our results suggested the possibility of using the SING system to display tumor Ags and to subsequent retrieve the genes coding the Ags by PCR using the genomic DNA derived from GFP^+ BS cells.
5.2 METHODS AND MATERIALS

Construction of chimeric H-2K\textsuperscript{b} molecules

The H-2K\textsuperscript{b}-CD28-ζ-Lck plasmid was constructed by combining the H-2K\textsuperscript{b} extracellular and transmembrane domains with a combination of T cell signal transduction domains in a single molecule. Briefly, the Bgl II-Sph I H-2K\textsuperscript{b}-CD28 fragment from plasmid 12A (a gift from Dr. T.L. Geiger, St Jude Children's Research Hospital, Memphis, TN) and the Sph I-Sal I CD3ζ-Lck fragment from 7BC18 (also from Dr. T.L. Geiger) were ligated into the retroviral vector, pLNCX2 (Clontech, Palo Alto, CA).

Structure of the NFAT-responsive reporter vector SIN-(NFAT)\textsuperscript{6}-GFP

The SIN-(NFAT)\textsuperscript{6}-GFP retroviral vector was a gift from Dr. H. Spits (18) (Netherlands Cancer Institute/ Antoni van Leeuwenhoek Hospital, Amsterdam, The Netherlands). This self-inactivating (SIN) retroviral construct carries six NFAT-binding sites, followed by the minimal IL-2 promoter and the reporter gene, GFP (Green Fluorescent Protein). Because the 3' LTR of the retroviral vector carries a deletion in the U3 region, the promoter activity of this LTR is abrogated upon integration into the genome of the transduced cell. The expression of GFP is then dependent upon binding of NFAT to the multiple NFAT-binding sites (Fig. 13).
In virus-producing cells:

\[
\begin{align*}
&\text{U3} \quad \text{R} \quad \text{U5} \\
&\text{NFAT}^6 \quad \text{IL-2} \\
&\text{GFP} \\
\end{align*}
\]

Transcription and packaging of RNA

\[
\begin{align*}
mRNA & \Delta U3 \\
\end{align*}
\]

Transduction and integration in T cells

\[
\begin{align*}
&\text{U3} \quad \text{R} \quad \text{U5} \\
&\text{NFAT}^6 \quad \text{IL-2} \\
&\text{GFP} \\
\end{align*}
\]

Translation

\[
\begin{align*}
e\text{GFP expression} \Delta U3 \\
\end{align*}
\]

Figure 13. The SIN-(NFAT)\textsubscript{6}-GFP retroviral construct (18). The SIN-(NFAT)\textsubscript{6}-GFP-retroviral vector contains EBNA sequences to maintain high copy numbers of the transfected construct in the GP-293 cells, enabling the production of high titer viral supernatants. Transduction of BW5147 cells with the retroviral vector, SIN-(NFAT)\textsubscript{6}-GFP, ensures that expression of the reporter gene is dependent on binding of transcription factors to the multiple NFAT-binding sites, because an introduced deletion in the U3 region of the 3' LTR (which, on integration, will function as the upstream LTR) prevents promoter activity of this LTR. Thus, only activation of the T cell will lead to expression of GFP.
Construction of LXSN-OVA

The LXSN-OVA retroviral vector was constructed by inserting the full-length gene coding for chicken ovalbumin (ova). Briefly, poly (A)+ mRNA was isolated from MO5 (OVA-stably transfected mouse melanoma cell line B16) cells using an Oligotex mRNA kit (Qiagen, Valencia, CA) according to manufacturer’s instruction. RT-PCR was performed to amplify the full-length ova gene using the ProStar™ Ultra HF RT-PCR System kit (Stratagene, La Jolla, CA). The primers were designed according to the ova gene sequence data (GenBank database, accession number: V00383) and synthesized by Sigma-Genosys (Woodlands, TX). The primers were as follows: 5’-AGCGAATTC GCC GCCACCATGGGCTCCATCGGC (sense); 5’-GCGTCTAGATTAAGGGAAACAC ATCTGC (antisense). In order to facilitate the cloning process, an EcoR I site was inserted into the sense primer as indicated by the underline. An optimal Kozak sequence (italic bold) was also included. The 1.2 kb oval/EcoR I-blunt end fragment was then ligated with the EcoR I-Hpa I LXSN vector (a gift from Dr. A.D. Miller, Fred Huchison Cancer Center, Seattle, WA). The sequence was confirmed by sequencing with an ABI PRISM® 377 DNA Sequencer performed by The University of Arizona DNA Sequencing Service.

Construction of LXSN-CD8a

First, LXSN was modified to contain an additional Clal site in the multiple cloning sites. Briefly, LXSN was digested with BamHI followed by a fill-in reaction (performed by Klenow fragment) and self-ligation. The resultant plasmid was named LXSN-ClaI. Next, the plasmid PBS-CD8a (kindly provided by Dr. Dan R. Littman, New York
University, NY) was cut with Xbal, filled in with Klenow fragment and cut again with ClaI. The 0.8kb ClaI-blunt CD8a cDNA fragment was then isolated and ligated with LXSN-ClaI (cut with ClaI and Hpal).

Construction of LNCX2-mCD80 (B7.1)

The mouse CD80 gene was amplified by PCR using the plasmid LL218 (ATCC 63369). The primers were as follows: 5’- TCGGAAGCTTGCCACCATGGCTTGCAATTGTCAG (sense); 5’-GGACAGGCCTCTAAAGGAAGACGGTCTGTTCA (anti-sense). A HindIII site and a StuI site were included in the sense and anti-sense primers, respectively (shown underlined). The HindIII/StuI CD80 fragment was then ligated with the linearized LNCX2 vector (cut with HindIII and StuI).

Construction of pFB-mCD28

The full-length gene encoding mouse CD28 was obtained by PCR amplification using the mouse CD28 cDNA (gift of Dr. James P. Allison, University of California at Berkeley, Berkeley, CA) as a template. The primers were designed to include an XhoI site and a Kozak sequence at the 5’ end, and a BamHI site just before the stop codon, TGA. The PCR fragment was digested with XhoI and BamHI, and then ligated with the large fragment of the XhoI/BamHI doubly cut pFB-neo-LacZ (Stratagene, La Jolla, California).

Construction of NIP

The plasmid, NIP, is the SIN-(NFAT)6-GFP vector modified by insertion of an internal ribosome entry site (IRES) element followed by the gene encoding for
puromycin-resistance (puromycin N-acetyl-transferase, \textit{pac}) downstream of the \textit{GFP} gene. This modification allows BS cells to become transiently puromycin-resistant as well as EGFP$^+$ after T cell stimulation. The NIP vector was constructed as follows: SIN-(NFAT)$_6$-GFP was cut with \textit{BamHI}, blunted with Klenow fragment and ligated with the \textit{PstI-XbaI} IRES-Puromycin fragment (blunted by Klenow fragment at both termini) from pVpack-10A1 (Stratagene, La Jolla, CA).

**Viral vector production and transduction of BW5147 cells**

The above retroviral constructs were co-transfected with a pVSV-G plasmid (encoding an envelope glycoprotein from the vesicular stomatitis virus) into a pantropic packaging cell, GP-293, using Fugene-6 (Roche Diagnostics, Nutley, NJ), according to the manufacturer's protocols. Two and three days post-transfection, virus supernatants were collected, filtered (0.45 \( \mu \)m), and then used to cross-infect the 10A1-pseudotyped packaging cell, PT67. This approach allows a rapid and high titer production of retrovirus with titers of over \( 1 \times 10^6 \) CFU/ml. When SIN-derived viral vectors are used to infect target cells, the 3' U3 deletion is transferred to the 5' LTR, resulting in the transcriptional inactivation of the provirus in the infected cell. Therefore the viral supernatants from SIN vectors were obtained by transient transfection rather than from stable virus-producing cells.

On day 1, \( 2 \times 10^5 \) BW5147 cells were cultured in pantropic virus supernatants from GP-293/SIN-(NFAT)$_6$-GFP in the presence of polybrene (8 \( \mu \)g/ml) at 37°C. Six hours later, cells were washed and cultured in fresh media. Infection was repeated on days 2 and 3. On day 5, the BW5147/ SIN-(NFAT)$_6$-GFP cells were then infected with virus
supernatants from PT67/K\(^b\)-CD28-\(\zeta\)-Lck. The resulting cells were named BW5147/SING (BS) cells. In the retroviral vector K\(^b\)-CD28-\(\zeta\)-lck, a selection marker, the \textit{neo} gene, was driven under the control of the 5' LTR. BW5147 cells transduced with the vector K\(^b\)-CD28-\(\zeta\)-lck were selected with G418 at a concentration of 0.8 mg/ml for 7 days to kill the untransduced cells. There is no selection marker in the integrated form of the retroviral vector SIN-(NFAT)\(_6\)-GFP. Therefore, the transduction efficiency of the vector SIN-(NFAT)\(_6\)-GFP is dependent on the viral titers without selection. In the backbone of the nonintegrated form of SIN-(NFAT)\(_6\)-GFP, EBV sequences coding for EBNA/OriP are included, allowing for episomal amplification of the vector. High efficiency (20-40%) transduction of human primary T cells have been similarly obtained using viruses from SIN-(NFAT)\(_6\)-GFP transiently transfected packaging cells (18).

\textbf{Flow cytometry}

BS Cells were analyzed using an Epics XL flow cytometer (Beckman Coulter, Inc., Miami, FL). GFP expression was determined 24 h after stimulation with PMA plus ionomycin or anti-H-2K\(^b\) mAb cross-linking. Chimeric H-2K\(^b\) expression on BS cells was determined using a PE-labelled anti-H-2K\(^b\) mAb, AF6-88.5 (Pharmingen, San Diego, CA). Cells were incubated with either the PE-labeled antibody or an irrelevant isotype control (Pharmingen) for 30 min at 4°C. The cells were washed twice, and fixed in PBS containing 2% paraformaldehyde.
Determination of GFP expression in BS cells after stimulation with PMA plus ionomycin or anti-H-2K<sup>b</sup> mAb cross-linking

When BS cells are stimulated by anti-H-2K<sup>b</sup> antibody cross-linking or T cell mitogen (PMA plus Ionomycin), phosphorylated NFAT (the active form) is rapidly exported from the nucleus and binds to NFAT binding sites which function as enhancers. Therefore, EGFP expression, which is initiated by the minimal IL-2 promoter and enhanced by the occupied NFAT binding sites, should be greatly induced after stimulation. BS cells were tested for GFP expression after overnight stimulation with PMA (10 ng/ml) plus ionomycin (500 ng/ml) as a positive control, as this stimulation protocol stimulates all transduced cells. In order to determine whether the H-2K<sup>b</sup>-ζ-CD28-Lck fusion molecule could transduce signals intracellularly, BS cells were stimulated with the purified anti-H-2K<sup>b</sup> mAb, AF6-88.5. Briefly, the antibody (20 μg/ml in PBS or indicated concentrations) was coated on 24-well non-tissue culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in 0.4 ml per well at 37°C. Two hours later, the unbound antibody was removed and the plate was blocked with 2% BSA in PBS at room temperature for an additional 30 min. The plates were then washed twice with PBS, and 5×10<sup>5</sup> BS cells in 1 ml of DMEM-10 (DMEM plus 10% fetal bovine serum) were added to each well. Twenty-four hours after stimulation, cells were collected for FACS analysis of GFP expression. To determine GFP expression 24 h after stimulation with PMA plus ionomycin or anti-H-2K<sup>b</sup> mAb, cells were collected, washed and cultured in regular DMEM-10 media. Samples were collected and fixed every 24 or 48 h up to 7 days after stimulation.
Isolation of BS clones with high sensitivity to non-specific stimuli and low background fluorescence using limiting dilution

To obtain BS cells with high sensitivity and low background, cells were sorted twice using a FACStar (Becton Dickinson, San Jose, CA) flow cytometer. First, the highest (most sensitive) 10% of GFP^+ BS cells after stimulation with mAb AF6-88.5 were sorted. Five days later, the GFP^- BS cells without stimulation (lowest background) were sorted. The doubly-sorted BS cells were then cloned by limiting dilution. Briefly, BS cells were serially diluted and dispensed into wells of 96-well plates at a concentration of 0.3 cells/per well in 100 µl media. Five days later, 100 µl media was added to each well. Cell growth was determined using an inverted microscope. Based on the Poisson distribution, growth in less than 26% of the wells indicated that an average of more than 0.3 cell/well was plated, and hence colonies growing in the wells were considered to be true clones (141). Two weeks later, cloned BS cells were tested for response to PMA plus ionomycin and mAb AF6-88.5 stimulation. A BS clone, BS-4, with low background fluorescence but maximal response to PMA plus ionomycin were chosen for use in the study.

Functional test of BS-4 cells for response to H-2K^b engagement

In order to determine whether BS-4 cells were capable of responding to TCR stimulation, an H-2K^b-restricted, OVA-specific T cell hybridoma (B3Z) was used. Briefly, BS-4 cells were pulsed with various concentrations of OVA_{257-264} peptide (SIINFEKL, synthesized by Sigma-Genosys, Woodlands, TX) at a density of 2×10^6 cells/ml for 3 h. The cells were then washed three times with PBS and OVA peptide-
pulsed BS-4 cells were incubated with B3Z cells in round-bottom, 96-well plates (0.2 ml/well). The ratio of B3Z to BS-4 cells varied from 2:1 to 10:1 with the constant density of cell mixtures at 1x10^6 cells/ml. After 24 h of incubation, the cells were fixed and stained for CD8 α chain expression using PE-labeled anti-CD8a mAb (Clone: 53-6.7, Pharmingen, San Diego, CA). The B3Z cells could be readily distinguished from the BS cells during flow cytometric analysis due to their expression of CD8α and negative expression of GFP. In order to make sure that GFP expression by BS-4 cells is a specific response to H-2K^b engagement, a control peptide, the B16 melanoma-specific mTRP2 (VYDFFVWL) peptide, was used to pulse BS-4 cells under the same conditions. The OVA-encoding retroviral vector LXSN-OVA transduced BS-4 cells (BS-4/OVA) were used to demonstrate the interaction between BS cells and Ag-specific T cells at physiological conditions.

**Determination of the capacity of BS-4 cells to present endogenously produced H-2K^b-restricted OVA peptides**

Detection of OVA peptide presentation was performed using the B3Z T cell hybridoma. Activation of B3Z cells was measured by lacZ activity, as previously described (85). Briefly, 5x10^5 OVA gene-transduced BS-4 cells (BS-4/OVA) were mixed with 5x10^5 B3Z cells in 24-well culture plates (1 ml/well). A total of 16 to 20 h later, the supernatant was removed, the cultures were washed with PBS, and the cells were fixed with 2% formaldehyde/0.2% glutaraldehyde for 10 min at 4°C. The cells were washed again with PBS and overlaid with 250 µl of 0.5 mg/ml X-gal (5-bromo-4-
chloro-3-indoyl-β-D-galactopyranoside; Fisher Biotech, Pittsburgh, PA). The blue cells in the cultures were examined by microscopy after a 2- to 4-h incubation at 37°C.

**Titration of the threshold concentration of OVA peptide needed for induction of GFP expression in BS-4 cells after coculture with B3Z cells**

To assess the sensitivity of the SING system in response to Ag, OVA peptide was titrated from $10^{-10}$ to $10^{-4}$ M and pulsed on BS-4 cells. GFP expression was then evaluated by FACS analyses after 24 h of coculture with B3Z T cells.

**Determination of GFP expression in BS-4 cells after coculture with concanamycin A-treated OVA-specific CTLs (OT1 T cells)**

Before coculture with peptide-pulsed or OVA-expressing BS-4/CD28 cells, OT1 T cells were pretreated with an inhibitor of granule exocytosis (and thus, lysis), concanamycin A (CMA, 100 nM, Sigma, St. Louis, MO), at 37°C for 2h. BS-4/CD28 cells were pulsed with either OVA or control Trp2 peptides ranging from $10^{-10}$ to $10^{-4}$ M at 37°C for 3h, washed with media three times and then cocultured with OT1 T cells in the presence of CMA (100 nM) at a ratio of 1:2 for 24 h. Cell mixtures were stained with PE-anti-CD3 mAb. The percentage of GFP$^+$ BS-4/CD28 cells was calculated as $(\%\text{GFP}^+\%\text{CD3}^+) \times 100$.

**Analysis of the enrichment capacity of modified BS (mBS) cells after stimulation followed by transient puromycin selection**

Like the original BS cells, mBS cells were prepared in a similar procedure except that the NIP vector rather than the SIN-(NFAT)6-GFP vector was used. The mBS cells contain a stably integrated pac gene downstream of the NFAT-GFP and an IRES.
effective pac gene expression is dependent on activation via the NFAT pathway. Since the inducible expression though the NFAT pathway is transient, the selection protocol using puromycin is different from routine selection procedures, which are optimized and based on constitutive expression of the pac gene. The protocol for determination of enrichment capacity of OVA-expressing mBS cells after puromycin selection was as follows. Either mBS/CD28 or ova gene-transduced mBS/CD28 (mBS/CD28+OVA) cells (1×10⁴/ml) were pre-mixed with BW5147 cells at a ratio of 1:100, and then the cell mixtures were cocultured with B3Z/B7.1 (1×10⁶) in 200 µl DMEM-10 without PMA on U-bottom 96-well plates. Twenty-four hours later, the cells were collected and selected in puromycin at the indicated concentrations for an additional 24 or 48 h. The cells were then washed twice in PBS and cultured in puromycin-free media for 5 days. The percentage of mBS/CD28 or mBS/CD28+OVA in cell mixtures was determined by FACS analysis of GFP⁺ cells after stimulation with PMA plus ionomycin. The enrichment capacity was calculated based on the following formula.

\[
\text{Enrichment Capacity Index (ECI)} = \frac{\% \text{GFP}^+ \text{mBS/CD28+OVA}}{\% \text{GFP}^+ \text{mBS/CD28}}
\]

**Statistical Analysis**

The data were analyzed to determine any significant differences among the experimental groups using a nonpaired Student’s t test. A value of \( p \leq 0.05 \) was considered significant. All statistical analysis was performed using Excel 2000 Software (Microsoft, Redmond, CA).
5.3 RESULTS

Structure of the chimeric H-2K^{b} molecule

Similar to the construction of scTCRs, the chimeric H-2K^{b} molecule H-2K^{b}-28-ζ-Lck was also made in a cassette fashion. The amino acid sequence of each component is shown in Figure 14A. Additional amino acids added at the junction between components came from the insertion of restriction enzyme sites required for construct synthesis. The extracellular and transmembrane domains of this receptor are identical to the wild type H-2K^{b} molecule. The cytoplasmic domain of this chimeric receptor is composed of the cytoplasmic tails of the CD28 and CD3ζ chain, and p56Lck37-509 (Fig. 14B).

Expression of the chimeric H-2K^{b} molecule on BS cells

To determine chimeric H-2K^{b} expression, BS cells were stained with an anti-H-2K^{b} mAb, AF6. As shown in Figure 15A, 80% of BS cells expressed the transgenic H-2K^{b} receptor. In untransduced wild type BW5147 (H-2^{k}) cells, no H-2K^{b} expression was observed. However, 2.5% of BS cells constitutively expressed GFP without stimulation.
Figure 14: Structure of chimeric H-2K\textsuperscript{b} molecule. Amino acid sequence of each component is shown (A). Constructs were created by linking components in a cassette fashion (B). Additional amino acids added at the junction between components result from the insertion of restriction enzyme sites required for construct synthesis. Extracellular and transmembrane domains of this receptor are derived from the MHC class I H-2K\textsuperscript{b} molecule. The cytoplasmic domain is composed of cytoplasmic tails of CD28 and CD3\textgreek{z} chain and p56\textsuperscript{Lck} 37-509.
BS cells express GFP in response to stimulation with PMA plus ionomycin and anti-H-2K\textsuperscript{b} mAb cross-linking

The efficiency of transduction with SIN-(NFAT)\textsubscript{6}-GFP containing retroviruses was estimated from the GFP expression after overnight stimulation with PMA and ionomycin. After a single transduction with the SIN-(NFAT)\textsubscript{6}-GFP vector, more than 70% of BS cells expressed GFP (Fig.15B). As expected, BS cells also responded to immobilized anti-H-2K\textsuperscript{b} mAb. The response rate (42%) of BS cells to antibody was 60% of that observed when using PMA plus ionomycin.

Cloning and Screening of BS cells

Although BS cells responded well to various stimuli, problems could arise due to high background fluorescence (2~2.5%) and heterogeneous transgene expression of chimeric H-2K\textsuperscript{b} molecule. Background fluorescence even as low as 2% could be significant when the ratio of tumor Ag-expressing BS cells is low or the sensitivity of the assay is limited. Low H-2K\textsuperscript{b} expression on a subpopulation of BS cells could lead to compromised responses in BS cells after stimulation though the chimeric H-2K\textsuperscript{b} receptor. In order to overcome these problems, cloning and screening of BS cells for low background fluorescence prior to stimulation and high signal upon stimulation was necessary.

When cloning, based on the Poisson distribution, the theoretical value of cell growth when an average of 0.3 cells/well are plated is equal to 0.26 (=1-e\textsuperscript{-\mu}, \mu=0.3). Upon plating 384 wells, we observed 54 wells showing cell growth. The positive rate was 0.14, which was less than the theoretical value. Therefore, colonies growing in the wells
could be considered to be true clones. BS clones were then screened for response to
PMA plus ionomycin stimulation. Three representative clones are shown in Figure 15.
Clones #4 and #23 were chosen for study due to low background fluorescence without
stimulation and high signal upon stimulation (Fig. 16A). Clone #32 was not chosen due
to high background fluorescence. Also, clone #4 was almost 100% positive for H-2K$^b$
expression and responded well to immobilized H-2K$^b$ stimulation.

Duration of GFP expression in BS-4 cells after withdrawal of stimuli

An important feature of inducible vectors is the ability to switch off gene expression
after withdrawal of stimuli. Therefore it’s necessary to ascertain whether fluorescent
BS-4 cells could shut down GFP expression with time after various stimuli are
withdrawn. As shown in Figure 17, GFP expression decreased with time after stimuli
were withdrawn. By day 5, GFP expression decreased to background levels. There was
no significant difference in the decreasing rate of GFP expression after withdrawal of
stimulation by either PMA plus ionomycin or anti-H-2K$^b$ mAb.
Figure 15. FACS analysis of chimeric H-2K\textsuperscript{b}-CD28-ζ-Lck expression on BS cells and GFP expression after stimulation. (A) The H-2K\textsuperscript{b} expression on untransduced BW5147 and BS cells was determined using PE-labeled anti-H-2K\textsuperscript{b} mAb or an isotype control. (B) GFP expression was analyzed in BS cells stimulated with immobilized anti-H-2K\textsuperscript{b} mAb or PMA plus ionomycin.
Figure 16. Cloning and screening of BS cells. The basal and inducible expression of GFP in three representative clones #4, #23 and #32 is shown in (A). The H-2K\textsuperscript{b} expression and H-2K\textsuperscript{b} mAb-induced GFP expression in clone #4 was also determined by flow cytometry (B).
Figure 17. Duration of GFP expression in BS-4 cells after stimuli were withdrawn. GFP expression in BS cells was determined by FACS. Cells stimulated by either PMA plus ionomycin (■) or immobilized anti-H-2K\textsuperscript{b} mAb (▲) were washed 24 h after stimulation and cultured in regular media. Non-stimulated BS cells served as negative control. At the indicated time the cells were analyzed by FACS for GFP expression. The result is presented as mean ± SD of 3 independent experiments.
Titration of the anti-H-2K<sup>b</sup> mAb necessary to induce significant GFP expression in the clone BS-4

As shown in Figure 18, immobilized anti-H-2K<sup>b</sup> mAb AF6-induced GFP expression in BS-4 cells was dose-dependent when the antibody concentration was within the ranges of 0.1 to 1 μg/cm<sup>2</sup>. The stimulation threshold was close to 0.1 μg/cm<sup>2</sup>. Above 1 μg/cm<sup>2</sup>, stimulation was maximal in terms of GFP expression.

OVA peptide-pulsed BS-4 cells express GFP after engagement with OVA-specific T hybridoma cells

The most important aspect of the SING system is whether the H-2K<sup>b</sup> chimeric molecule could present Ag peptides and respond to stimulation by Ag-specific TCR. To assess the GFP response after low-affinity engagement of the chimeric H-2K<sup>b</sup>, an OVA-specific H-2K<sup>b</sup>-restricted T hybridoma, B3Z, was used to stimulate OVA<sub>257-264</sub> peptide-pulsed BS-4 cells. After binding of OVA peptide to the extracellular (H-2K<sup>b</sup>) domain of the chimeric molecule, the B3Z TCR can engage and stimulate the chimeric H-2K<sup>b</sup>-OVA peptide complex. As shown in Figure 19, 24% of the cell mixture of B3Z and BS-4 cells {~44% of BS-4 cells: =0.24/(0.24+0.31)} became GFP<sup>+</sup>, whereas in control mTrp2 peptide-pulsed groups, only background GFP expression was observed. This finding demonstrated the functionality and specificity of the chimeric H-2K<sup>b</sup> molecule.
Figure 18. Titration of the concentration of anti-H-2K\textsuperscript{b} mAb necessary to induce significant GFP expression in the clone BS-4. Either the anti-H-2K\textsuperscript{b} or control mAb (anti-CD16/CD32) was coated on non-tissue culture-treated 24-well plates at the indicated concentrations as described in Methods and Materials. 5x10\textsuperscript{5} BS-4 cells were added to each well in 1ml DMEM-10 media and incubated for 24 h before FACS analysis. The results are presented as mean ± SD of 3 independent experiments.
Figure 19. GFP expression in OVA peptide-pulsed BS-4 cells after coculture with B3Z cells. BS-4 cells were pulsed with 5×10^{-5} M of either OVA peptide (B) or control peptide TRP2 (A) as described in Methods and Materials. After washing 3× with media, peptide-loaded BS-4 cells were cocultured with B3Z cells in U-bottom 96-well plates at a ratio of 1:2 for 24 h. The cocultures were then collected, stained with PE labeled anti-CD8α antibody and subjected to 2-color FACS analysis.
Sensitivity and kinetics of the SING assay after H-2K^b engagement

In order to determine the sensitivity of the SING system, peptide titration was performed. The peptide concentrations varied from 10^{-10} to 10^{-4} M. The responses of peptide-pulsed BS-4 cells to H-2K^b were dose-dependent (Fig. 20A). Unfortunately, the sensitivity of SING assay was not very high. The lowest OVA peptide concentration which stimulated BS-4 cells to express significant amounts of GFP (>2-fold above background) was approximately 10^{-7} M. Kinetics of GFP expression in BS-4 cells in response to H-2K^b engagement were also determined. At the indicated time points, cells were collected and analyzed. The percentage of GFP expressing BS-4 cells increased over time within 24 h (Fig. 20B).
Figure 20. Sensitivity and kinetics of the SING assay system after H-2K\textsuperscript{b} engagement. (A) BS-4 cells were pulsed with either OVA (\textbullet) or control Trp2 peptides (\textcircled{O}) ranging from \(10^{-10}\) to \(10^{-6}\) M and then cocultured with B3Z cells at ratio of 1:5 for 24 h. Cell mixtures were stained with PE-anti-CD8 mAb. The percentage of GFP\textsuperscript{+} BS cells was calculated as \((\%\text{GFP}^+/\%\text{CD8}^-) \times 100\). (B) Kinetics of GFP expression was determined by mixing two cells at ratio of 1:2 (BS-4: B3Z) at the indicated times. The relative GFP expression was calculated by setting the GFP expression at 24h as the maximal level (100\%). The data represents the mean ± SD of 3 independent experiments. The maximal % of GFP expression at 24 h was 30–40\%. 
Effects of the CD8 coreceptor and costimulatory molecules on GFP expression in BS-4 cells after coculture with B3Z T cells

The effects of coreceptor (CD8) and costimulatory molecules (B7-1 and CD28) on GFP expression in BS-4 cells were investigated. The transgene expression of these molecules on B3Z and BS-4 cells is shown in Figure 21. CD8α overexpression (MFI increased by 6-7 fold) on B3Z cells increased signalling in BS-4 cells by 2-fold (Fig. 22A). Surprisingly, when the B7-1 gene was transduced to BS-4 cells, the GFP expression was dramatically decreased even in the presence of suboptimal concentrations of PMA plus OVA peptide after coculture with CD28^+ B3Z cells. In contrast, OVA peptide-pulsed CD28^+ BS-4 cells when cocultured with B7-1^+ B3Z cells gave rise to 10 to 20-fold higher GFP expression. To have a more accurate idea about the sensitivity of the improved “SING” system (containing the costimulatory signals), a peptide titration was performed. As shown in Figure 22B, CD28 gene expression on BS-4 cells significantly lowered the threshold peptide concentration (required to induce significant GFP expression in BS-4 cells) to as low as 10^{-9} M after coculture with B7^+ B3Z T cells, although the maximal percentage of GFP^+ BS cells stayed at 40%.
Figure 21. Transgene expression on B3Z, BS-4 and their derivative cells. Expression of CD8, CD28 and B7.1 on B3Z (A), BS-4 (B) and their derivative cells was determined by FACS analysis. TCR expression on B3Z and H-2K<sup>b</sup> expression on BS-4 cells were also analyzed.
Figure 22. Effects of CD8 and costimulatory molecules on GFP expression in BS cells after TCR engagement. (A). Either B3Z or gene-modified B3Z cells (CD8, CD28 or B7.1) were cocultured with BS-4 or costimulatory molecule-modified BS-4 cells in the presence of $10^{-6}$ M OVA peptide at a ratio of 5:1. Twenty-four hours later, GFP expression was determined by FACS analysis. The GFP expression in BS-4 cells after coculture with unmodified B3Z cells was set arbitrarily as 1 (2–5% GFP^+). The data are presented as mean ± SD of 3 independent experiments. (B). Sensitivity of the optimized “SING” system. BS-4/CD28 cells were pulsed with either OVA (■) or control Trp2 peptides (O) ranging from $10^{-10}$ to $10^{-4}$ M and then cocultured with B3Z/B7.1 (CD8^+) cells at ratio of 1:5 for 24 h. Cell mixtures were stained with PE-anti-CD8 mAb. The percentage of GFP^+ BS cells was calculated as (%GFP^+/%CD8^+) ×100. Statistical significance (p<0.05) between the original and modified SING systems was determined by Student’s t test. *: p<0.05.
Endogenously produced OVA peptide can be presented to T cells via the chimeric H-2K^b molecule

In order to determine whether BS-4 cells transduced with the retroviral vector LXSN-OVA (BS-4/OVA) could efficiently present the endogenously processed OVA peptides to OVA-specific T cells, BS-4/OVA were cocultured with B3Z T cells. B3Z cells have been engineered to express the LacZ gene in response to stimulation with OVA_{257-264} peptide in conjunction with the H-2K^b molecule, which allows activated cells to become blue after staining with the substrate X-gal. Twenty-four hours after coculture, cells were subjected to LacZ staining. As shown in Figure 23, LacZ^+ cells (blue cells) were observed when B3Z cells were cocultured with Ova gene-transduced BS-4 cells compared to normal BS-4 cells, suggesting that endogenously produced OVA peptide had been presented via the chimeric H-2K^b molecule to B3Z cells.
Figure 23. Presentation of H-2K\textsuperscript{b}-restricted endogenous OVA peptides by \textit{ova} gene-transduced BS-4 cells to the B3Z T cells. Either BS-4 (left panel) or \textit{ova} gene-transduced BS-4 cells (right panel) were cocultured with B3Z cells overnight at a ratio of 1:1. The cell mixtures were then subjected to LacZ staining using \( \beta \)-gal as substrate. The “blue” cells as indicated by arrows represent LacZ-expressing B3Z cells.
BS-4 cells become GFP⁺ after presenting endogenous OVA peptides to T cells

We next determined whether OVA-expressing BS-4 cells would express significant amounts of GFP after H-2Kᵇ engagement. To maximize the signal transduction mechanisms in BS-4 cells, CD28 gene-transduced BS-4 (BS-4/CD28) cells were used. As shown in Figure 24, in the absence of PMA, approximately 15.5% (=0.031/(0.169+0.031)) of OVA-expressing BS-4/CD28 cells produced GFP, whereas only 4.4% (=0.008/(0.173+0.008)) of control BS-4/CD28 cells expressed GFP. Therefore, the specific GFP expression was estimated at 11.5%. Addition of low concentrations of PMA (0.1 or 0.2 ng/ml) led to a higher background of GFP expression (~7%=0.013/(0.013+0.17)) in BS-4/CD28 cells, but was not very helpful in increasing the specific GFP expression in BS-4/CD28-OVA cells.

CTL can stimulate BS-4 cells to express GFP in the presence of the granule exocytosis inhibitor, concanamycin A (CMA)

Since non-cytotoxic Ag-specific T hybridomas are not always available, the ability to directly use Ag-specific CTLs would make the “SING” system more applicable. However, unmodified CTLs would kill BS-4 cells that expressed the cognate tumor Ags. To solve the problem, a strong inhibitor of granule exocytosis (the major mechanism of CTL-mediated cytotoxicity), concanamycin A (CMA) (183), was used. As shown in Figure 25, in the presence of CMA (100 nM), OVA peptide-pulsed BS-4/CD28 cells expressed GFP after coculture with OT1 T cells (OVA-specific CTL) at a ratio of 1:2. The threshold peptide concentration required to induce GFP expression> 2-fold above background was approximately 10⁻⁹ M. After coculture with OT1 T cells, the
endogenous OVA peptide-producing BS-4 cells also expressed GFP but at low
efficiency (percentage of specific GFP expression was approximately 5% after
subtraction of background level).

**OVA-expressing mBS cells can be enriched after short-term puromycin selection**

In mBS cells, a modified SIN-(NFAT)\textsubscript{6}-GFP vector, termed NIP, instead of SIN-(NFAT)\textsubscript{6}-GFP, was stably transduced into BW5147 cells. In NIP, an IRES plus a \textit{pac} gene was placed downstream of the \textit{GFP} gene (Figure 26A). Therefore, after activation of the NFAT pathway, not only the \textit{GFP} gene but also the \textit{pac} gene is expressed because the IRES allows cap-independent translation. This vector design provides the possibility for enrichment of low abundance Ag-expressing mBS cells after TCR engagement followed by transient puromycin selection. As shown in Figure 26B, a brief puromycin (2~10 \(\mu\text{g/ml}\)) selection either in 24 or 48 h led to significant enrichments (2~4-fold, \(p<0.05\)) of OVA-expressing BS cells. In addition, a 48 h selection was better than 24 h and 5 \(\mu\text{g/ml}\) of puromycin seemed to be optimal for the purpose of enrichment.
Figure 24. GFP expression by OVA-expressing BS-4 cells after TCR engagement. Either CD28-transduced or CD28 + ova genes doubly-transduced BS-4 cells were cocultured with B7.1+ B3Z T cells in the absence or presence of PMA (0.1 and 0.2 ng/ml) at a ratio of 1:5(BS-4:B3Z). After 24 h, cells were stained with PE-anti-CD8 mAb and subjected to 2-color FACS analysis. The result is representative of 8 independent and similar experiments.
Figure 25. Sensitivity of the SING system using concanamycin A-treated antigen-specific CTLs. BS-4/CD28 cells were pulsed with either OVA (▲) or control Trp2 peptides (■) ranging from $10^{-10}$ to $10^{-4}$ M, and then cocultured with OT1 T cells in the presence of CMA (100nM) at a ratio of 1:2 for 24 h. To determine the GFP expression in OVA-expressing BS-4 cells, OVA-transduced BS-4/CD28 cells (BS-4/CD28+OVA) were cocultured with OT1 T cells in the same way as peptide-pulsed BS-4/CD28 cells. Cell mixtures were stained with PE-anti-CD3 mAb. The percentage of GFP⁺ BS-4/CD28 cells was calculated as $(\%GFP⁺/\%CD3⁺) \times 100$. The result is representative of 2 separate experiments.
Figure 26. Enrichment capacity of OVA-expressing mBS cells after TCR engagement followed by brief puromycin selection. (A) Diagram of the modified NFAT-GFP vector, NIP. A fragment containing an IRES and a pac gene was inserted downstream of the GFP gene. (B) The enrichment capacity index (ECI) was determined by using puromycin selection at the indicated concentrations for either 24h (□) or 48h (■) as described in Materials and Methods. Compared with non-selection, brief selection with puromycin (2–10 μg/ml) led to a 2–4-fold increase in ECI (*: p<0.05).
5.4 DISCUSSION

In this chapter, a novel strategy ("SING") was described for identifying T cell-recognized tumor Ags. The diagram in Figure 27 shows the overall procedures (5 steps) by using the SING system to clone T cell recognized tumor Ags. First, a tumor-derived retroviral cDNA library (cDNAs are inserted into a retroviral vector) is made. Second, the cDNA library is transferred to BS cells via retroviral transduction. Third, tumor-specific T cells (non-cytotoxic) are cocultured with cDNA-transduced BS cells (BS/cDNAs). Fourth, GFP+ BS cells are isolated by FACS sorting and/or other selective methods. Fifth, cDNAs coding for T cell recognized Ags are retrieved by PCR using vector-specific primers and sequenced. The cloned tumor cDNAs are then confirmed by the ability to activate Ag-specific T cells after being reintroduced to APCs.

As shown in Figure 14, the chimeric H-2K\(^b\) receptor was made in a cassette fashion, in which the extracellular domain was fused in a linear order to the intracellular signalling domains derived from different signal transduction molecules. This alignment allows the chimeric molecule to efficiently respond to stimuli. Cassette construction is simple and especially useful method for making 'magic proteins' when molecular conformation is not required for function (70).

Theoretically, the very weak strength of the minimal IL-2 promoter will lead to low background GFP in the absence of the 'binding form' of NFAT. However, the IL-2 promoter along with the GFP gene will not be totally insulated if its integration site is within an active transcription region (18), where promoters and enhancers cluster. GFP expression might also be affected by surrounding cellular promoters and enhancers as well as the minimal IL-2 promoter. This hypothesis might explain the 2~4 % of BS bulk cultures that constitutively expressed GFP in the absence of stimuli.
Figure 27. Procedures of identifying T cell-recognized tumor antigens using the "SING" system. BS cells transduced with tumor cell-derived retroviral cDNA libraries are cocultured with non-cytotoxic tumor-specific T cells. GFP⁺ BS cells are isolated by FACS sorting or other selective methods. Finally, the cDNAs coding for tumor antigens are retrieved from GFP⁺ cells by PCR and sequenced.
Significant GFP expression only after specific stimulation is a checkpoint for the specificity of this system. Inducible expression of the GFP protein allows activated indicator cells to be readily separated from non-activated BS cells. Preliminary functional tests showed that chimeric H-2K^b molecule could transduce signals in response to either PMA plus ionomycin or anti-H-2K^b mAb cross-linking, and become GFP^+. In addition, GFP expression in BS cells induced by antibody-crosslinking was dose-dependent. One microgram of mAb AF6 (IgG, MW: 150 Kd) is equal to $4 \times 10^{12}$ Ab molecules. Approximately, $10^6$ BS-4 cells are required to cover an area of 1 cm² at nearly 100% confluence. Therefore, the threshold of stimulation is approximately $4 \times 10^5$ cross-linked Ab per cell {$=(0.1 \mu g \times 4 \times 10^{12} \text{molecules}/\mu g)/10^6 \text{cells}$}. This threshold amount is similar to anti-CD3 or anti-TCR induced IL-2 production in T cells (174). Compared with TCR-MHC-peptide interactions, antibody-crosslinking leads to stronger signalling, but the response threshold is much higher than that required by TCR-MHC-peptide interactions.

As shown in Figure 17, GFP expression in our SING system was transient. Activated BS cells shut down GFP expression within 5 days after withdrawal of stimuli. Although reversible GFP expression makes it easier to screen BS clones with low background GFP expression without stimulation but high GFP expression after stimulation, NFAT-induced persistent (non-reversible) expression of selective markers might be more optimal for subsequent screening and selection. In the future, we may combine our SING system with the Cre/Loxp system (175), which allows for the generation of non-reversible genetic markers.
Whether peptide-loaded BS clones can efficiently respond to stimulation by Ag-specific TCR is a key to this technology. A high percentage of GFP\(^+\) cells in the tumor-Ag bearing BS clones after H-2K\(^b\) engagement is preferable. The intensity of T cell responses to Ag stimulation is determined by the cumulative interactions between TCR molecules and their ligands (174, 176, 167). Likewise, OVA peptide-pulsed or OVA-expressing BS cells are expected to respond to H-2K\(^b\) engagement by B3Z cells in a similar fashion. In theory, there are three major factors could affect the ability of chimeric MHC molecules to present endogenous Ags and respond to TCR engagement. First, the ability of chimeric MHC molecule to present endogenous peptides and direct efficient surface expression is critical to Ag presentation. High surface expression of the chimeric MHC molecule is expected to increase densities of both Ags on the cell surface and intracellular signalling domains. This aim could be partially achieved by multiple transductions of the retroviral vector containing chimeric H-2K\(^b\) receptor, followed by cell sorting. Second, a chimeric MHC molecule with multiple signalling domains, which allow transduction of both primary and secondary signals as well as the efficient recruitment of the kinases Zap70 and Lck is preferable (174). Third, Ag expression is another limiting factor dictating the Ag density in the context of chimeric MHC. The higher the expression of a tumor Ag is in BS cells, the greater the density of cognate MHC-peptide will be on cell surface. Therefore, after coculture with Ag-specific T cells, stronger interactions between BS cells and T cells are expected to induce greater GFP expression in BS cells. Of course, the response intensity of BS cells
after H-2K<sup>b</sup> engagement could also be affected by many other parameters (*i.e.*, ratios of BS cells to T cells, density of TCR molecule, time of incubation, *etc.*).

CD28-B7 is the best well-known costimulation system in T cell activation. According to the "dual signal" theory, the costimulation (second signal) delivered by CD28 can significantly increase the overall signals in conjunction with the first signal elicited by TCR ligation (92, 93, 98). In the SING system, transduction of the CD28 gene into BS cells was hypothesized to be better than CD28 transduction into B3Z cells because coexpression of CD28 and the chimeric H-2K<sup>b</sup> molecule allows the conveyance of both signals intracellularly. Thus, in this case, the B7.1 gene encoding the CD28 ligand must be transferred to the B3Z cells. Our results showed the advantageous effects of the use of CD28 as a signal amplifier. Surprisingly, we observed that B7.1<sup>+</sup> BS-4 cells after stimulation by CD28<sup>+</sup> B3Z cells, in conjunction with suboptimal concentrations of PMA, showed lower level of GFP expression, even below the "background level" induced by the suboptimal concentration of PMA. This result suggested that B7-1 crosslinking might be responsible for the dampened GFP expression. This observation is quite interesting because no similar report has been made in terms of the signalling function of the B7 molecule. To confirm that the role of B7.1 as a negative signal transducer when expressed on T cells, further testing is needed.

However, despite multiple optimizations, only ~11% ova gene-transduced BS-4 cells expressed GFP after coculture with B3Z cells. A low frequency of GFP expressing-BS-4 cells would pose a potential problem for subsequent screening since the frequency of any one cDNA encoding a specific Ag in a tumor cell-derived cDNA library should be
generally low (<1/10^4) (178). Therefore, a second selection marker puromycin-resistance gene (pac), was added along with the GFP gene in an attempt to simplify the selection procedure for isolation of GFP^+ BS cells (Fig. 28A). The result showed that the IRES-puromycin fragment downstream of the GFP gene allowed OVA-expressing mBS cells to be enriched, although not very dramatically after a single round of selection.

We chose to use an IRES rather than a second promoter to direct pac gene expression for three reasons. First, the use of bicistronic vectors with a selectable marker downstream of an IRES sequence effectively eliminates false positives in which transfected cells express drug resistance but not the protein of interest, as can occur with conventional dual cassette vectors (179,180). Second, expression can be maintained over long periods in culture by maintaining the selective pressure. Third, high levels of first gene expression can be achieved using this system in conjunction with increased levels of drug selection (179). Puromycin as a selectable marker has several advantages over other such drugs as G418 or hygromycin B. It acts quickly to kill non-transfected cells within 24-48 h, reducing the problem of overgrowth of cells during selection. It is active at low concentrations (1-10 μg/ml) (181). There have also been reports that the neo gene can down-regulate transcription of adjacent genes in expression vectors, an effect that has not been seen with pac (182). Placing the resistance gene second can also take advantage of any inefficiency of initiation by the IRES sequence as more of the combined message would be needed to overcome a given level of selection pressure leading to higher levels of message for the first protein of interest.
Figure 28. Modification of the SING system for facilitated isolation of antigen-expressing BS cells. (A). The \( \text{pac} \) (puromycin-resistance) gene was inserted downstream of the \( \text{GFP} \) gene with an IRES element in between allowing the co-expression of \( \text{GFP} \) and \( \text{pac} \) genes simultaneously. \( \text{GFP}^+ \) BS (expressing antigen) cells could be enriched by brief puromycin selection. (B) In another improved SING system, recombinase \( \text{Cre} \) is controlled by NFAT-IL-2 promoter. Two \( \text{lox} \) \( P \) sites (recognized by \( \text{Cre} \)) are placed next to a fragment containing the \( \text{GFP} \) gene and a translation STOP signal in a \( \text{GFP} \)-on-off vector. In the absence of stimuli, no significant amount of \( \text{Cre} \) can be produced and BS cells constitutively express \( \text{GFP} \) but not the \( \text{pac} \) gene. After activation of the NFAT-pathway, \( \text{Cre} \) recombinase is produced and works on the \( \text{LoxP} \) sites leading to the loop-out of in-between genes. Therefore, the resulting BS cells irreversibly lose the \( \text{GFP} \) expression but acquire the puromycin-resistance.
Until now, either the original or modified SING system has been based on the NFAT-induced transient expression of GFP or/and puromycin-resistance. The transient features of the system makes it somewhat difficult to sort out and select Ag-expressing BS cells when the relative amount of Ag cDNA is low. To solve the problem, we plan to combine our SING system with an elegant Cre/LoxP system (175) to achieve the goal of persistent expression of genetic marker after activation of BS cells. As shown in Figure 29B, expression of the Cre recombinase is controlled by the (NFAT)$_6$-IL-2 promoter. The GFP gene plus a translational STOP cassette that is flanked by loxP sites (the target for the Cre recombinase) is placed between the 5' LTR and the coding sequence of the puromycin-resistance gene. In the absence of Cre (i.e., BS cells are not activated), the GFP-on/off vector is expected to constitutively express GFP but not the puromycin-resistance gene due to the presence of the STOP cassette. When Cre is present (i.e., BS cells are activated), the STOP cassette and GFP gene are looped out from the integrated provirus. The constitutive expression of GFP is lost and the protein product of the puromycin-resistance gene is made.

Since OVA-specific CTLs would lyse ova-expressing BS or mBS cells after TCR engagement, a modified protocol is needed to inhibit cytolysis mediated by CTLs while leaving TCR engagement intact. An inhibitor of vacuolar type H+-ATPase, concanamycin A (CMA), which inhibits perforin-based cytotoxic activity (183), meets the abovementioned criteria. An alternative method is the use of glutaraldehyde (0.05%)-fixed T cells as stimulators (184,185). Use of such modified CTL lines or clones in the SING system will make this novel technology more applicable.
In the future, the SING system will be tested for re-isolation of the model ova gene. If the ova gene is effectively identified using this novel technology, we plan to apply this system to the identification of unknown tumor Ags. The next step after identification of unknown Ags is a computer search for sequence homology using the BLAST program available in the GenBank database. The next critical step in studying these Ags is the identification of T cell epitopes. Various computer-assisted algorithms have been designed to assist in this process by predicting potential MHC-binding epitopes, based either on the analysis of natural MHC ligands or on the binding properties of synthetic peptides. Two epitope prediction programs are now available free of charge on interactive websites: BIMAS (http://www-bimas.dcrtr.nih.gov/molbio/hla_bind) and SYFPEITHI (http://www.syfpeithi.de). Recently, a new technique, called tetramer-guided epitope mapping (TGEM), has emerged (186,187). In this method, a panel of overlapping peptides spanning the protein(s) of interest is divided into pools, with each pool containing five to ten peptides. Each peptide pool is loaded onto soluble MHC molecules to generate pooled-peptide tetramers, and used to stain Ag-specific T cells. Pooled tetramers that give positive staining are identified by FACS. Peptides from positively staining pooled-peptide tetramers are then loaded individually onto the MHC molecules and the staining repeated. Tetramers that positively stain in the second round of FACS analysis enable the identification of functional MHC-restricted epitopes. A combination of TGEM with predictive computer algorithms should allow the rapid identification of T-cell epitopes from large proteins.
In order to verify that the predicted peptides are generated during Ag processing in vivo, as well as their immunogenic potential, several experimental approaches must be conducted to show stimulation of primary T cell responses against the predicted peptides and subsequent testing of the recognition pattern towards target cells that express the Ag. Mass spectrometry-based approaches have been used to detect predicted peptides among isolated natural ligands. The functional analyses which provide verification of the T cell activation capacity of predicted peptides include cytotoxicity assays, cytokine release assays and tetramer staining (188). Although a combination of computational prediction and experimental methods provides a rapid method of screening T cell epitopes as potential tools for therapeutic and diagnostic purposes, the identified epitopes still must pass the ultimate test: they have to prove to be useful in the in vivo situation (i.e., immunotherapy).
CHAPTER 6
SUMMARY AND CONCLUSIONS

Retrovirus vectors can efficiently transduce murine primary T cells, as well as T cell lines. A combination of use of ecotropic vectors, high virus titer (>10^7 CFU/ml) and infection within 24 hours of stimulation is required for efficient transduction (>30% gene transfer efficiency) of murine primary T cells. Our findings may help to establish a model protocol for transduction of murine primary T cells.

To systematically investigate the impact of scTCR structure on T cell function, a series of scTCR molecules containing various TM regions and cytoplasmic signalling domains were constructed. The results showed that transfer of TCR genes (either scTCRs or fITCR) into T cells bestowed the resulting T cells with both specificity and functional activity. TM regions and signalling domains had dramatic effects on the expression and function of TCR-modified T cells. Expression of scTCRs was CD3 complex-independent. The coreceptor CD8 and costimulatory receptor CD28, but not the CD3 complex, could significantly enhance scTCR-induced T cell activation. However, despite many optimizations, scTCRs were less efficient than fITCR in response to low concentrations of Ag stimulation. This low efficiency of scTCR appears to be due to the inherent low-affinity binding of the receptor.

TCR-modified primary T cells could efficiently expand after 5 days of G418 selection followed by Ag stimulation. The effects of adoptive transfer of TCR-modified T cells on in vivo tumor growth were also determined. The results demonstrated that TCR (either fITCR or scTCR)-modified primary T cells were effective to some extent in controlling the growth of well-established EG7 tumors and sometimes in complete
eradication of tumors. Unfortunately, most TCR-modified T cell recipient mice didn’t show any significant signs of anti-tumor effects. This result suggests the possible application of scTCR- as well as flTCR-modified T cells for adoptive immunotherapy, but additional optimization of this approach needs to be done in future animal experiments.

An artificial Ag presentation system, “SING”, which could be used as a direct strategy for cloning T-cell recognized tumor Ags, was established. In the SING system, a mouse T cell line BW5147 was manipulated to respond to various signals, such as PMA plus ionomycin, anti-H-2K^b mAb cross-linking and stimulation by Ag-specific TCR (the resultant BW5147 cells were termed BS cells). Upon activation with the above-mentioned stimuli, BS cells become transiently fluorescent (Green fluorescence protein, GFP^+). The interaction between BS cells and Ag-specific T cells could be enhanced by introduction of the CD28 gene into BS cells. Currently, BS cells have been optimized to sense TCR ligation after being pulsed with the relevant peptides at concentrations as low as 10^{-9} M. Endogenous Ag-expressing BS cells could also become fluorescent after coculture with Ag-specific T cells. In a modified SING system, a puromycin-resistance gene, pac, was used. Brief puromycin selection after coculture with T cells led to a more than 4-fold enrichment of endogenous Ag-expressing BS cells. This result provides a proof of principle for using the SING system to identify tumor Ags (provided that Ag-specific T cells are available).

Taken together, two novel approaches were designed in an attempt to simplify the general procedures for identifying T cell-recognized tumor Ags (the SING system) and to broaden the use of immunotherapy for cancers. Both approaches have taken
advantage of the recent developments in T cell-based signal transduction and molecular engineering. Applications of the “SING” system to identifying T cell-recognized tumor Ags will eventually be beneficial to the field of specific immunotherapy since the active immunotherapy requires the knowledge of tumor Ags. However, the efficacy of immunotherapy sometimes has been hampered by the difficulty in growing T cells against tumor cells and lack of signaling events after TCR engagement despite the presence of known Ags on tumors that are recognized by T cells. Therefore, our second approach of genetic modification of primary T cells with chimeric TCR molecules containing multiple T cell-derived signal transduction domains could potentially solve the latter problem as demonstrated using both in vitro and in vivo models.
APPENDIX A

Complete RPMI (RPMI-10)

To 500ml of RPMI (Scientific Irvine, Santa Ana, CA) add:
50ml of Heat Inactivated Fetal Bovine Serum (Gibco-BRL, Grand Island, NY)
5ml of 1000u/ml Penicillin + 1000μg/ml Streptomycin (Gibco-BRL, Grand Island, NY)
5ml of 100mM sodium pyruvate (Gibco-BRL, Grand Island, NY)
5ml of 100mM Non-essential amino acids (Gibco-BRL, Grand Island, NY)
250μl of 5mg/ml of Gentamycin (Sigma, St. Louis, MO)
5μl of β-mercaptoethanol (Sigma, St. Louis, MO)

Complete DMEM (DMEM-10)

To 500ml of RPMI (Scientific Irvine, Santa Ana, CA) add:
50ml of Heat Inactivated Fetal Bovine Serum (Gibco-BRL, Grand Island, NY)
5ml of 1000u/ml Penicillin + 1000μg/ml Streptomycin (Gibco-BRL, Grand Island, NY)
5ml of 100mM sodium pyruvate (Gibco-BRL, Grand Island, NY)
5ml of 100mM Non-essential amino acids (Gibco-BRL, Grand Island, NY)
250μl of 5mg/ml of Gentamycin (Sigma, St. Louis, MO)
5μl of β-mercaptoethanol (Sigma, St. Louis, MO)

50 x TAE (1 Liter)

242.0 g Tris base
57.1ml glacial acetic acid
100ml 0.5 M EDTA, PH 8.0

Luria Broth (LB) (pH 7.0, 1 Liter)

10.0 g NaCl
10.0 g Tryptone
5.0 g Yeast extract
Adjust pH value to 7.0 with 10N NaOH
SOC Medium (250ml)

5.0 g Tryptone
1.25 g Yeast extract
0.15 g NaCl
0.05 g KCl
245 ml H₂O, pH to 7.0

Autoclave and add the following:
2.5 ml 2 M Mg²⁺ solution (1 M MgSO₄, 1 M MgCl₂)
2.5 ml 2 M glucose

FACS Buffer

1X PBS
1% FBS
0.02% azide
APPENDIX C

BD™ DIMER-X

- Application: Detection of antigen-specific T-cells and epitope mapping.
- Structure: three extracellular domains of MHC class I molecules (H-2K\(^b\)) fused to the N-termini of VH region of the mouse IgG1.
- Production:
  - Co-transfection of H-2K\(^b\)-Ig construct with human β2-microglobulin gene into a myeloma cell line (deficient in immunoglobulin heavy chain but retains the expression of immunoglobulin light λ chain).
  - Configuration of final product: a three-chain complex molecule.
    - (a) A recombinant H-2K\(^b\)-Ig fusion chain (heavy chain).
    - (b) An Ig light chain disulphide bonded to the heavy chain.
    - (c) A non-covalently associated human β2m.
- Advantages of structures:
  1) The bivalent nature of peptide-binding sites of the DimerX molecules increases the avidity and results in stable binding to antigen-specific T-cells.
  2) The hinge region in the immunoglobulin scaffold of DimerX provides a more flexible access for T-cell binding.
APPENDIX D
(DNA sequences)

Sequences of OVA-specific TCRs

TCR α chain

V-GENE: TRAV14 (TCR Vα2), J-GENE: TRAJ30 (J23)

Signal peptide:

M D K I L T A T F L L L G L H L A G V N G
ATG GAC AAG ATC CTG ACA GCA ACG TTT TTA CTC CTA GTC GCA CTA GCT GGG GTG AAT GGC

Translation of mature protein

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TCR β chain

V-GENE: TRBV12 (TCR Vβ5.2), J-GENE: TRBJ2-3 (TCR Jβ2.3)

Signal peptide

| M | S | N | T | V | L | A | D | S | A | W | G | I | T | L | L | S | W | V | T | V | F | L | L | G |

ATG TCT AAC ACT GTC CTC GCT GAT TCT GCC TGG GGC ATC ACC CTG CTA TCT TGG GTT ACT GTC TTT CTC TTG GGA ACA AGT S A TCA GCA

Translation of mature protein

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GAC AAA GGA TTC CTA CCC ... AGC AGA TTC TCA GTC CAA CAG TTT ... GAT GAC TAT CAC TCT GAA
Sequence of scTCR part of various scTCR constructs

**Signal peptide**

```
1 ATGGACAAGATC CTGACAGC AAC GTTTTTACTC CTAGGC CTTC AC CTAGCTGGGGTGAATG
21 11 21 31 41 51 6
J
31 (+i) MDK I L T A L L G L H L L A G V N Q Q
41 14
44 24 (+1) Q E K R D Q Q Q V R Q S P Q S L T V W E G T A
53 141 (+1) IL H C S Y E D S T F N Y F P W Y Q Q F P G E
63 211 63 (+1) G P A L L I S I R S V D K K E D G R F T I F
73 281 211 (+1) F H K K K L S L N I T D S Q P G D S A T Y F
83 351 118 (+1) C A A S T N A Y K V I F G K G T H L H V L P N
93 421 141 (+1) E L G G G G S G G S G G G S R D S G V
103 491 164 (+1) V Q S P R H I I K E K G G R S V L T C I P I S G
113 561 188 (+1) H S N V W Y Y Q Q T L G K E L K F L I H Y E
123 631 211 (+1) K V E R D K G F L P S R F S V Q Q F D D Y H S
133 701 701 (+1) E H N M S A L E E D S A R Y F C A T S D R S S
143 771 258 (+1) A E T L Y F G S G T R L T V E D L R N V T P
153 841 281 (+1) P K V S L F E P S K A E I A N K Q K A T L V C
163 911 304 (+1) L A R G F F P D H V E L S U V W N G K E V H S G
183 1051 301 (+1) V S T D P Q A Y K E S N Y S Y C L S S R L R V
193 1121 1051 (+1) S A T F W H P R N H F R C Q V Q F H G L E
203 1191 374 (+1) E D K U W E P G S P K F V T Q N I S A E A W G R A
213 1261 398 (+1) D k
```

*: There is an extra cysteine residue in the construct, scTCR-cys-ζ.
Sequences of CD3ζ chain in scTCR constructs

Extracellular part

```
1 21 31 41 51 61
1 AAGCTCAGAGCTTTTGCTGCAGATCCCAAACCTCTGCTACTTGCTAGATGGMTCCTCTTCATCTACG
1(+1) K I L Q S F G L L D P K L C Y L L D G I L F I Y
71 GAGTCATCACTCAGACCCTGTAACCTGAGAGCAAATTAAGAGGATGACAGACTGCGCCAACCTGCA
24(+1) G V I I T A L Y L R A K F S R S A E T A A N L Q
141 GGGCCCACACAGCTCTACAATGAGCTCAATCTAGGGCAGAGAGGAGGAGAGGAGCCACACAG
48(+1) D P N Q L Y N E L N L G R R E E Y D V L E K K
211 CGGCTCAGAGGATGAGGAGGAAACAGCAGAGGAGGAGGAGCCAGGAGCAGAGCAG
71(+1) R A R D P E M G G K Q Q R R R N P Q E G V Y N
281 CACTGCAGAAAGACAAAGAAATGAGGAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGG
94(+1) A L Q K D K M A E A Y S E I G T K G E R R R R G K
351 GGGGCACAGATGGCCTTTACCAAGGTCAGCTGCCACGACAGGCTGAGTGGCTGCTGCATGGCAG
118(+1) G H D G L Y Q G L S T A T K D T Y D A L H M Q
421 ACCCTGGCCCTCGGC
141(+1) T L A P R
```

*: TM: transmembrane domain

#: CYP: Cytoplasmic domain

In some scTCR constructs (scTCR-ζ, scTCR-Cys-ζ, scTCR-ζ-28 and scTCR-ζ-28-Lck), the CD3ζ chain contains the extracellular part, transmembrane domain and cytoplasmic region.

In the rest of scTCR constructs (scTCR-Cβ-ζ, scTCR-Cβ-ζ-28-Lck, scTCR-28-ζ, scTCR-28-ζ-Lck and scTCR-B7.1-ζ), only the cytoplasmic region was used.
Sequences of CD28 chain in scTCR constructs

In the scTCR constructs: scTCR-28-ζ and scTCR-28-ζ-Lck, the TM plus CYP regions were used. Whereas in constructs: scTCR-ζ-28, scTCR-ζ-28-Lck and scTCR-Cβ-ζ-28-Lck, only the cytoplasmic region was used.
In the scTCR-28-ζ-Lck, the Lck region starts from phe\textsubscript{37}, whereas in scTCR-ζ-28-Lck, the Lck region begins with III\textsubscript{34}. 
Sequence of murine B7.1 gene

The full-length murine B7.1 gene was inserted into the retroviral vector LNCX2 as described in the Chapter 3.

In the scTCR-B7.1-ζ, only the hinge region plus TM and CYP domains of the B7.1 were used.
Alignment of OVA gene Sequences

There is a minor difference (several nucleotides) between the Genbank sequence (accession #: V00383) and the OVA gene cloned from the MO5 cells (OVA gene transfected B16 cells). But both gene products contain the H-2K\(^\text{b}\)-restricted OVA peptide SIINFEKL.
Full-length sequences of CD3δ and ζ genes

CD3δ

1
ATGGAAACACACGCGGATCTGGACTGATAGATTGCTGCTCCTCCTCCTCCACGGGACCCCTTCAAGA
1 (+1) MEHSGLASLILIAVLPQGSPFK

71
TACAAGTGACCAATATAGGACAAAGTATTATGTGACCTGCAATACCAAGCTGCTGCAATCTGACAGAC
24 (+1) IQVTEYEDKVFVTCNTSVMHLDGT

141
GGTGGAAAGGATGTTTTCAGAAGAAAAATACACTCAATTGCGAAAAGGCTCTCTGAGACCACAGGATA
48 (+1) VEGWFPAKRNKTLNLGKGVLDPRGI

211
TATCTGTCTATTGGAACAGCAAGCTGCTGCAAGCTGCAATCTGGAGATATGCAACATAGCCCTGACC
71 (+1) YLCNGTEQLAKVSSVQHYRRC

281
AGAAGCCTGCTGAGCTGCAATCTGGAGATATGCAACATAGCCCTGACC
94 (+1) QNCVHELDSGTMAVGVIDLIATL

351
CCTGGCTTTGCGCTCTACTCTGCTTTCAGAAGCACATGAGACCAGGAAAAGGCTCTCTGAGACCACAGGATA
118 (+1) LAGVYCFAGHETGRPSGAEEVQ

421
GCACCTGCTGAGAGCCAGCTGCAATCTGGAGATATGCAACATAGCCCTGACC
141 (+1) ALKLNEQPLQDRDREDTQYSRRL

491
GAGGAAACTGGCCCGGAAACAAATATCT
164 (+1) GGNWPRNKKKS

CD3ζ

1
ATGAAGTGGAAAGTGTCTGTTCTCGCCTGCATCCTCCACGTGCGGTTCCCAGGAGCAGAGGCACAGAGCT
1 (+1) HKWKVSVLACILHVRFPGAQAQS

71
TTGGTCTGCTGATCCAAACTCTACTCTGCTTATGGGACTTCTCAGATCGAGATAGGATGATATCCTGCTGG
24 (+1) FGLLDPKCYLLDGILFIYGVIIT

141
AGCCTGTCTACCTGAGAAATATTGAGGAAAGCTGCTGCAATCTGGAGATATGCAACATAGCCCTGACCAGG
48 (+1) ALYLRAKFSRSAETANLQDPNQ

211
CTCTCAATATGAGCTACATCTGCTGAGAGCTGCTGCAATCTGGAGATATGCAACATAGCCCTGACC
71 (+1) LYNELNLGLRRHEEYDVLEKRRAD

281
CAGAGATGGCAGAACAGCCAGGAGGAAAGGAGGAGAACAGCCAGAGGAGGAGAGGAGGAGGACAGCGGATGGC
94 (+1) PEMGGKQCARQQRNPQEQGVYNAQLKD

351
CAAGATGGCAGAACAGCTACATGAGATGCCAACAAAAAGCCGAAGGGGAGGAGGAGGAGGAGGACAGCGGATGGC
118 (+1) KMAEAYSEIGTKGERRRRKGHDG

421
CTTTACCAGGCTTCAGACCTGCTGACACCCACAAAGGACAACATCAGATCCCTGCTGATATAGCAGAACCCTGGCCCTC
141 (+1) LYQGGLSTATKDITYDHALHMQLTLP

491 GC
164 (+1) R
### APPENDIX E

#### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CMA</td>
<td>Concanamycin A</td>
</tr>
<tr>
<td>ConA</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>fITCR</td>
<td>full-length T cell Receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorter</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
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<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
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<td>IL-4</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
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<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
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<tr>
<td>i.v.</td>
<td>Intravenous</td>
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<tr>
<td>$K_d$</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>LTR</td>
<td>Long terminal repeat</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MuLV</td>
<td>Murine leukemia virus</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>RPMI-10</td>
<td>Complete RPMI media supplemented with 10% FBS</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
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<tr>
<td>scTCR</td>
<td>Single chain T cell receptor</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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APPENDIX F

Animal Use Approval

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted Final Approval according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:
#00-105 - “Gene Therapy of Cancer”

PRINCIPAL INVESTIGATOR/DEPARTMENT:
David T. Harris, PhD - Microbiology & Immunology

GRANTING AGENCY:
Cord Blood Bank Discretionary Funds

SUBMISSION DATE: June 6, 2000

APPROVAL DATE: November 8, 2000
APPROVAL VALID THROUGH*: November 7, 2003

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: November 9, 2000

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell, PhD, MS
Vice President for Research

DATE: November 9, 2000

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.
REFERENCES


132. Zhou P, Lee J, Moore P and Brasky KM. High-efficiency gene transfer into rhesus macaque primary T lymphocytes by combining 32 degrees C centrifugation and


146. Andreadis ST, Brott D, Fuller AO and Palsson BO. Moloney murine leukemia virus-derived retroviral vectors decay intracellularly with a half-life in the range of 5.5 to 7.5 hours. *J. Virol.* 1997; 71:7541-7548.


