1-1-1998

Retroviral-mediated gene transfer of Il-1β?cDNA into human activated T cells

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RETROVIRAL-MEDIATED GENE TRANSFER OF IL-1β cDNA INTO HUMAN ACTIVATED T CELLS

by

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DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

1998

MAJOR: MOLECULAR BIOLOGY AND GENETICS

Approved by:

Katrina T. Treasor 9/18/98

Adviser Date

DEDICATION

This work is dedicated to the late Dr. Robert H. Rownd,

in honor of his true dedication and commitment to all his students,

who by example, taught me how to love my family and live my life

with wisdom, strength, and courage

ACKNOWLEDGMENTS

I would like to express my sincerest thanks to my adviser, Dr. Katrina Trevor for her understanding, guidance, patience, and support over the last several years. Even more important than anything related to molecular biology or gene therapy, Tina taught me how to handle the unexpectancies of daily life and how to become more confident and successful in my research. I would also like to thank my committee members, Dr. Anton Scott Goustin, Dr. John A. Kamholz, Dr. Wei-Zen Wei, Dr. Mary Murray, and the late Dr. Robert H. Rownd. In addition, I would like to say a special thank-you to Ruth Siebenlist, for the many hours of technical assistance and for being such a terrific friend. Last, but not least, I would like to thank my husband, Tom, for his support and patience through a most trying time. The best is yet to come!

PREFACE

The main objectives of my thesis work were to improve retroviral transduction and proviral IL-1β gene expression in activated, primary human T cells. Herein, several methodologies that may result in optimal retroviral gene transfer and expression in lymphocytes are examined. An important question is addressed regarding the relationship between heightened T cell activity and the modulation of proviral gene expression. Finally, re-activation of T cell activity and proviral gene expression is explored as an important consideration for the success of adoptive immunotherapy approaches for cancer using gene-modified T cells.

Several of the findings presented in my dissertation have been recently published: T Cell Activation Modulates Retrovirus-Mediated Gene Expression. *Human Gene Therapy* **9**:1457-1467, 1998. In this article, we have shown that over a 14-day period, retrovirus gene-modified T cells downregulate expression of an introduced transgene along with endogenous cytokines that are indicators of T cell activity. A second manuscript is in preparation regarding the re-activation of transduced T cells using a bispecific antibody (anti-CD3 X anti-CEA) molecule. By cross-linking the CD3 receptor with tumor surface antigen, we have shown that bispecific antibodies induce *in vitro* anti-tumor activity and enhanced cytokine production, including augmented expression of the introduced IL-1β transgene.

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ABBREVIATIONS

ABMT, autologous bone marrow transplant;

ADA, adenosine deaminase;

Ag-MHC, antigen-major histocompatibility complex;

AICD, activation-induced cell death;

AIDS, acquired immune deficiency syndrome;

AP-1, activating protein-1;

APC, antigen presenting cell;

ATC, activated T cell;

ATCC, American Type Culture Collection;

BDIS, Becton-Dickinson Immunocytometry Systems;

bp, base pair;

BsAb, bispecific antibody;

cAMP, cyclic adenosine monophosphate;

cDNA, complementary DNA;

CEA, carcinoembryonic antigen;

CFU/ml, colony forming units per milliliter;

CM, conditioned media;

cpm, counts per minute;

CSF, colony stimulating factor;

CTLs, cytotoxic T lymphocytes;

DHFR, dihydrofolate reductase;

DMEM, Dulbecco's modified Eagle's medium;

ELISA, enzyme-linked immunosorbent assay;

EMCV, encephalomyocarditis virus;

env, envelope gene;

ER, endoplasmic reticulum;

E:T, effector:target cell ratios;

FACS, fluorescence activated cell sorting;

FasL, Fas ligand;

FBS, fetal bovine serum;

FITC, flourescein isothiocyanate;

GALV, gibbon ape leukemia virus;

GLVR-1, gibbon ape leukemia virus receptor-1;

GLVR-2, gibbon ape leukemia virus receptor-2;

GTI, Genetic Therapy, Inc.;

ICE, interleukin-1 beta converting enzyme;

IFN-γ, interferon gamma;

IL-1α, interleukin-1 alpha;

IL-1β, interleukin-1 beta;

IL-1Ra, interleukin-1 receptor antagonist;

IL-1R-AcP, interleukin-1 receptor-accessory protein;

IL-2, interleukin-2;

iOK/IL-2; immobilized OKT3 plus 100 IU/ml IL-2;

IRAK, interleukin-1 receptor associated protein kinase;

IRES, internal ribosome entry site;

IU/ml, international units per milliliter;

kD, kilodalton;

LAK, lymphokine-activated killer cell;

LTR, long terminal repeat;

mAb, monoclonal antibody;

MAP kinases, mitogen-activated protein kinases;

MEM, Eagle's minimum essential medium;

MFI, mean fluorescence intensity;

MMLV, Moloney murine leukemia virus;

MuSV, Moloney murine sarcoma virus;

MOI, multiplicity of infection;

mRNA, messenger RNA;

NIH, National Institutes of Health;

NeoR, neomycin phosphotransferase gene, or neomycin resistant;

NK, natural killer cell;

non-MHC, non-major histocompatibility complex;

OKT3, anti-CD3 monoclonal antibody;

PBL, peripheral blood lymphocyte;

PBMC, peripheral blood mononuclear cells;

PBS, Dulbecco's phosphate-buffered saline;

PCR, polymerase chain reaction;

PE, phycoerythrin;

PKC, protein kinase C;

PMA, phorbol myristate acetate;

PT, post-transduction;

rGH, rat growth hormone;

RMGT, retrovirus-mediated gene transfer;

RT, reverse transcriptase;

RT-PCR, reverse transcription-polymerase chain reaction;

SA, splice acceptor;

SD, splice donor;

SEM, standard error of the mean;

TAA, tumor associated antigen;

TCM, tissue culture medium;

TCR, T cell receptor;

TGF-β, transforming growth factor-β;

TIL, tumor infiltrating lymphocyte;

TNF-α, tumor necrosis factor-alpha;

UTR, untranslated region;

1 B/C, 1 OKT3/9.3-coated bead-per-cell;

3 B/C, 3 OKT3/9.3-coated beads-per-cell;

9.3, anti-CD28 monoclonal antibody;

[^{3}H]-thy, tritiated thymidine;

[^{51}Cr], radiolabeled chromium

CHAPTER I

INTRODUCTION

Adoptive immunotherapy has emerged as a new form of cancer treatment because conventional therapies, such as surgery, chemotherapy, and radiation, have failed to significantly enhance survival rates of cancer patients or their quality of life. Patients continue to die from residual metastatic disease and/or toxicities associated with the standard modalities of treatment. The success of cancer immunotherapy depends on the augmentation of the host's immune system and the activation of tumor-specific effector cells that will effectively reject established tumors. To improve the clinical results observed with immunotherapy, current protocols rely on 1) administration of genetically modified autologous tumor cells that may immunize the host against the parental tumor (tumor vaccine), and 2) the introduction of gene-modified immune cells to improve cytotoxic responses against tumor (for review, see Hwu, 1997; Roth and Cristiano, 1997). Yet, although the field of gene therapy shows great promise for the treatment of genetic disease, the Panel to Assess the National Institutes of Health (NIH) Investment in Research on Gene Therapy concluded that the success of these gene therapy strategies require both stable, efficient gene transfer into target cells and high, therapeutic levels of transferred gene expression (Orkin and Motulsky, 1996).

Herein, I report on retroviral transduction and expression of a complementary DNA (cDNA) encoding human interleukin-1 beta (IL-1β) in activated T cells (ATC). IL-1β, which is produced primarily by activated monocytes and macrophages, possesses a wide spectrum of metabolic, hematopoietic, and immunological properties that have been the

subject of extensive reviews (Dinarello, 1989; Platanias and Vogelzang, 1990; Dinarello, 1991; Dinarello and Wolff, 1993; Dinarello, 1994; Dinarello, 1996; Dinarello, 1997). My interest in using IL-1β gene-modified ATC for cancer gene therapy is based on the ability of this cytokine to promote not only inflammatory immune responses and hematopoiesis, but also anti-tumor effects. Because required therapeutic levels of infused IL-1β result in systemic toxicities (Redman et al., 1994; Peplinski et al., 1995b; Curti and Smith, 1995; Dinarello, 1996), gene-modified ATC that secrete IL-1β could be used as vehicles for delivery of high, local concentrations of the cytokine in more confined sites, such as primary tumor and micrometastases. More importantly, T cells secreting IL-1β could serve to recruit additional inflammatory cells (monocytes, neutrophils) which can also mediate tumor cell killing and assist in establishing immunologic memory. In addition, ATC that express IL-1β could improve engraftment in cancer patients who receive autologous bone marrow transplants (ABMT).

While pursuing aspects of retroviral-mediated gene transfer and expression in ATC, I became interested in the relationship between T cell activity and the level of proviral gene expression. I asked whether gene-modified T cells could maintain high, therapeutic levels of proviral gene expression over time in expanded cultures. This is an important consideration because of the time required to generate large numbers of genetically modified T cells for future reinfusion into patients. As a result, a major portion of my thesis research is devoted to this aim.

ATC as a Vehicle for Augmenting Anti-Tumor Responses

Previous adoptive immunotherapy approaches have relied on lymphokine-activated

killer (LAK) cells or tumor-infiltrating lymphocytes (TIL) (for review, see Chang and Shu, 1996). Grimm et al. (1983) defined LAK cells as lymphoid cells stimulated *in vitro* by exposure to high doses of interleukin-2 (IL-2). Subsequent studies using human peripheral blood lymphocytes (PBL) demonstrated that LAK cells are activated natural killer (NK) cells that possess the ability to lyse fresh tumor cells or NK-resistant tumor cell lines in a non-major histocompatibility complex (non-MHC) restricted fashion (Chang and Shu, 1996). Although anti-tumor responses were confirmed in murine models following the adoptive transfer of LAK with or without the addition of exogenous IL-2 (Mulé et al., 1985; Mulé et al., 1986), human clinical trials revealed equivalent response rates (15%) for both LAK cell therapy or IL-2 therapy alone (Lotze et al., 1986). Because LAK cell infusion did not improve response rates compared to IL-2 therapy alone, and because of the technical difficulty of expanding large numbers of LAK cells *ex vivo*, systemic use of this form of immune cell has since been abandoned (Chang and Shu, 1996).

Unlike LAK cells, the *in vitro* anti-tumor cytolytic activity of TIL is MHC class I restricted (Rosenberg et al., 1986). TIL are $CD3^+$ lymphocytes that are derived from enzymatically digested tumor tissue and expanded in conditioned media supplemented with IL-2. *In vivo* mouse models have demonstrated that TIL are 50-100 times more potent than LAK cells in mediating established tumor regression (Rosenberg et al., 1986; Speiss et al., 1987; Cameron et al., 1990). However, the main drawback of TIL or LAK therapy is the clinical toxicities related to high doses of IL-2, which are required for the *in vivo* efficacy of TIL and LAK tumor cell killing (Lotze et al., 1985; Peace and Cheever, 1989; Higuchi et al., 1991). Moreover, in a 5-year retrospective study of TIL therapy for the treatment of melanoma, breast, colon, and renal cell cancers, Rosenberg and colleagues conclude that the

success of TIL therapy is limited by the inability of TIL to efficiently traffic to tumor sites *in vivo*, the technical difficulties associated with growing highly specific TIL from a relatively small number of tumor-residing lymphocytes, and the loss of biologic activity and tumor specificity after extended periods of *ex vivo* culture, which are required for expansion of therapeutic numbers of TIL (Yannelli et al., 1996). As a result of these limitations, several groups are now attempting to create more therapeutic TIL by growing TIL in the presence of tumor antigen or tumor-specific peptides (MART-1) to generate antigen-specific TIL (Curti, 1997).

As an alternative approach for adoptive immunotherapy, our laboratory has focused on the use of activated T cells. This type of immune effector cell possesses the LAK-like activity described for conventional LAK cells, but is considered to be a more potent LAK cell (Ting et al., 1988; Anderson et al., 1988). ATC are readily generated from murine and human peripheral blood lymphocytes and expanded in the presence of low-dose IL-2 and anti-CD3 monoclonal antibody (mAb), which crosslinks the T cell receptor (TCR) inducing T cell activation and proliferation (Ochoa et al., 1987; Anderson et al., 1988; Ting et al., 1988; Ochoa et al., 1989; Yoshizawa et al., 1991). These cells are primarily T cells ($CD3^+$) that exhibit non-MHC restricted cytotoxicity directed at NK-sensitive (K562 cells) and NK-resistant (Daudi cells) targets at levels equivalent to LAK (Ochoa et al., 1987; Anderson et al., 1988; Ochoa et al., 1989; Uberti et al., 1994). Murine and human studies show that ATC grown in low dose IL-2 are non-toxic (Culver et al., 1991a; Blaese et al., 1993; Walker et al., 1993; Jin et al., 1995) and can affect *in vivo* tumor regression (Yoshizawa et al., 1991; Katsanis et al., 1991; Loeffler et al., 1991; Murphy et al., 1993; Curti et al., 1993; Saxton et al., 1997). Similar to LAK and TIL therapies, patients infused with ATC receive systemic

infusions of low dose IL-2 treatment to maintain ATC viability *in vivo*. Further studies are required to determine the anti-tumor efficacy of ATC in humans.

Interleukin-1 Beta: A Pleiotropic Effector

1. Interleukin-1 (IL-1) Gene Family.

There are three members of the IL-1 gene family: IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1Ra) (Dinarello, 1991). The IL-1α and IL-1β proteins are products of separate genes found on human chromosome 2. Although these two forms of IL-1 share only 26% amino acid similarity, they are structurally related at the three-dimensional level, recognize the same cell surface receptors, and share similar biologic functions (Dinarello, 1991). IL-1α and IL-1β are initially synthesized as 31 kilodalton (kD) precursor molecules. Unlike most secreted proteins, these precursor molecules are synthesized without signal sequences that target the nascent proteins to the rough endoplasmic reticulum (ER)/Golgi apparatus for efficient processing and secretion of the mature peptide (Dinarello, 1991). While the mature 17.5 kD IL-1β protein is secreted by stimulated monocytes, most IL-1α remains intracellular in a precursor form, where it may function as an autocrine factor by intracellular binding to receptor (Dinarello, 1991; Dinarello and Wolff, 1993; Siders and Mizel, 1995). The third member of the IL-1 gene family, IL-1Ra, is a true receptor antagonist that inhibits the biological activities of IL-1β and IL-1α by competing for occupancy of shared, cell surface receptors. In this respect, IL-1Ra is considered to play a natural role in limiting inflammation (Dinarello, 1991; Dinarello and Thompson, 1991). IL-1Ra is a secreted, glycosylated protein of 25 kD that has a classic secretory leader sequence for secretion via the Golgi pathway (Dinarello and Thompson, 1991).

2. Gene Expression and Synthesis of IL-1β.

Typical of inducible genes, the promoter region of the IL-1β gene contains a TATA box motif (Dinarello, 1996). Kinetic analysis of endotoxin stimulation of human peripheral blood mononuclear cells (PBMC) demonstrate that peak levels of IL-1β messenger RNA (mRNA) are observed 3 or 4 hours following stimulation and the levels are sustained for 6 to 8 hours. IL-1β mRNA levels then rapidly decrease, most likely due to the synthesis of a transcriptional repressor (Dinarello, 1991). The regulatory regions controlling IL-1β gene transcription are distributed over several thousand basepairs (bp) from the transcriptional start site. Using a reporter gene transfected into human and mouse macrophage cell lines, two independent enhancer regions have been defined that appear to act cooperatively; one contains a cyclic adenosine monophosphate (cAMP) response element, and the other contains a combined cAMP response element-NFIL-6 element (Tsukada et al., 1994; Shirakawa et al., 1993). In addition, an NFκB-like site and an activating protein-1 (AP-1) site also participate in inducible IL-1β gene expression by endotoxin (Dinarello, 1996). Recent evidence indicates that the abundant tissue-specific gene expression of IL-1β by activated monocytes is dependent on the hematopoietic transcription factor Spi-1/PU.1, which may also prove important for expression of IL-1α and IL-1Ra (Kominato et al., 1995).

After inflammatory stimuli such as the complement component C5a, hypoxia, or adherence to surfaces, numerous cell types (including endothelial cells, fibroblasts, keratinocytes, smooth muscle cells, astrocytes) synthesize IL-1β mRNA but do not secrete active IL-1β (for review, see Dinarello, 1997, and references therein). Although these cell types are fully capable of IL-1β gene transcription, a further signal is required to stimulate

translation. As a result, most of the IL-1β mRNA produced following inflammation is degraded. The addition of bacterial endotoxin to cells already primed with high levels of IL-1β mRNA results in augmented translation (Schindler et al., 1990). Due to the potential devastating effects of secreted IL-1β protein both locally and systemically, strict levels for regulating IL-1β production are required, including the IL-1β gene promoter, regulation of mRNA splicing processes, phosphorylations of proteins required for translation, and stabilization of the 3' untranslated region (UTR) (Dinarello, 1997).

3. IL-1β Processing and Secretion.

The first translation product of IL-1β is the 31 kD precursor, designated pro-IL-1β (Dinarello, 1989). Unlike the IL-1α precursor, the IL-1β precursor remains in the cytosol until it is cleaved for optimal biologic activity. Although tissue damage or injury can result in the extracellular release of pro-IL-1β with conversion to the 17.5 kD active form by a number of extracellular proteases, activated peripheral blood monocytes are the primary source of secreted, mature IL-1β during inflammatory events (Dinarello, 1989; Kostura et al., 1989; Dinarello, 1991). The release of mature IL-1β by these cells appears to be linked to cleavage of pro-IL-1β at the aspartic acid-alanine position (amino acids 116-117) by IL-1β-converting enzyme (ICE) (Black et al., 1988; Kostura et al., 1989; Cerretti et al., 1992; Wilson et al., 1994), although the pathway for IL-1β secretion is unclear (Dinarello, 1996). ICE is a member of a newly defined class of cysteine proteases (Ced-2/ICE) that have been implicated in apoptosis (Kumar et al., 1994). Mice that lack ICE due to gene targeting are overtly normal but resistant to endotoxic shock; the ICE-deficient monocytes are defective in secretion of mature IL-1β but appear to undergo normal apoptosis (Li et al., 1995).

4. IL-1 Receptors and Signal Transduction.

The binding of IL-1β to receptors on a number of cell types triggers local cellular and tissue alterations, resulting in IL-1β-mediated systemic effects. Two cell-specific receptors have been identified: IL-1RI (80 kD), found on nearly all cells but most prominently on T cells, fibroblasts, keratinocytes, endothelial cells, synovial lining cells, and hepatocytes and IL-1RII (68 kD), restricted to B cells, monocytes, and neutrophils (Dinarello, 1996). In addition, an IL-1 receptor-accessory protein (IL-1R-AcP) has been identified (Greenfeder et al., 1995). The extracellular domains of both IL-1 receptors and the IL-1R-AcP are members of the Ig superfamily, possessing three extracellular IgG-like domains (Sims et al., 1988; McMahan et al., 1991). Although there is 28% amino acid similarity between the extracellular portions of the type I and type II receptors, the two receptors differ in their cytoplasmic regions with the IL-1RI domain consisting of 215 amino acids and the IL-1RII of only 29 amino acids (Dinarello, 1991; Dinarello, 1994). Based on antibody studies that block receptor binding, it has since become clear that the type I receptor is the primary signal transducer (Heguy et al., 1993). The IL-1RII receptor, which binds IL-1β with high affinity but lacks the signal transducing cytosolic domain present in the IL-1RI, appears to act as a "decoy" receptor and prevents IL-1β-mediated signal transduction (Dinarello, 1996).

Most cell types express less than 500 receptors per cell, while some primary cells, such as T lymphocytes, may express less than 50 receptors per cell (Shirakawa et al., 1987). IL-1RI signaling events are complex, dependent on the cell type, and remain less well-defined than the signal cascades of other cytokines, such as IL-2. The mechanism(s) for IL-1β signal transduction include the coupling of receptors to guanine nucleotide binding

proteins (G protein coupling), generation of second messengers (cAMP, diacylglycerol, inositol trisphosphate, calcium), hydrolysis of phosphotidalcholine and activation of sphingomyelinase (for reviews, see O'Neill, 1995; Dinarello, 1996). Following low affinity binding of IL-1β to the IL-1RI, a structural change may occur in IL-1β which allows the IL-1R-AcP to bind to the IL-1RI/IL-1β complex with high-affinity (Dinarello, 1996). Within minutes, several cell-type specific biochemical changes occur within the cell (Dinarello, 1996). The most frequently reported phosphorylations of cellular proteins are on serine and threonine residues, which in the case of IL-1β signaling, is due to phosphorylation by the IL-1 receptor associated protein kinase IRAK (Martin and Falk, 1997). This serine/threonine specific protein kinase rapidly associates with the cytoplasmic domain of the IL-1RI and becomes activated as a result of auto- or cross-phosphorylation (Martin and Falk, 1997). The phosphorylation events induced by IL-1β signaling activate several G protein molecules which in turn serve to amplify downstream signaling events by activating mitogen-activated protein kinases (MAP kinases) (O'Neill, 1995; Dinarello, 1996; Martin and Falk, 1997). MAP kinases activate the transcription factor NF-κB, as well as induce c-jun and c-fos expression (Dinarello, 1991; Kracht, 1993). The expression of a variety of genes in different cell types is increased by IL-1β, reflective of new transcription or enhanced mRNA stabilization. These include IL-1β itself, IL-2 through IL-8, IL-2Rα, tumor necrosis factor-α (TNF-α) and TNF-β, colony stimulating factors (CSFs), complement, acute-phase proteins, cell adhesion molecules, plasminogen activator inhibitor, proteases, amyloid A and collagenases (Dinarello, 1991). Also, IL-1β causes the suppression of genes such as albumin, cytochrome P450, lipase, aldosterone, insulin and its own type I receptor (Dinarello, 1989; Dinarello, 1991).

5. Systemic Effects of IL-1β.

Systemically, IL-1β is a central mediator of numerous metabolic and physiological changes that reflect an inflammatory response, as well as certain inflammatory disease states (sepsis syndrome, rheumatoid arthritis, ulcerative colitis) (Dinarello and Wolf, 1993). Because of the complexity of the numerous cytokine networks involved in inflammation, the exact direct and indirect effects of IL-1β (via induction of other growth factors or cytokines) have been debated. Moreover, IL-1β shares biological properties with IL-6 and TNF-α, both of which are expressed by monocytes during inflammation, and together these inflammatory molecules can act synergistically (Dinarello, 1989; Dinarello, 1994). Based on experimental animal and human studies, multiple organs and systems are affected by IL-1β administration, including the central nervous system (fever, sleep, decreased appetite), liver (increased synthesis of clotting factors and complement), the vascular system (increased leukocyte adhesion and infiltration, procoagulation, hypotension, pulmonary congestion, depressed myocardial function), and the immune system (neutrophilia, T cell and B cell activation, NK cell activation, increased cytokine production, hematopoiesis) (Dinarello, 1989; Platanias and Vogelzang, 1990; Dinarello, 1994; Patarca and Fletcher, 1997). In humans, IL-1β can be toxic; intravenous administration of 1-10 ng/kg induces fever, sleepiness, anorexia, headache, and muscle pain. Doses of 100 ng/kg or higher cause more severe symptoms including rapid hypotension, neutrophilia and gastrointestinal disturbances (Dinarello, 1991; Dinarello, 1994; Curti and Smith, 1995).

Immune and Hematopoietic Effects of IL-1β

IL-1β is a central mediator of both inflammatory immune responses and

hematopoiesis. In macrophages and monocytes, IL-1β induces increased synthesis of itself, the production of other cytokines such as TNF-α and IL-6 (Platanias and Vogelzang, 1990), and increased cytotoxicity to certain tumor cell lines (Onozaki et al., 1985). The migration of both monocytes and neutrophils is caused by IL-1β, most likely due to IL-1β induction of the chemotactic cytokine, IL-8 (Dinarello, 1991). Recent data suggests that IL-1β is a co-stimulator of both naive and memory T cell activation, indicating its synergistic effects with IL-2 in the induction and generation of autologous tumor-specific effector cytotoxic T cells (CTLs) (Baxevanis et al., 1994). IL-1β is known to act synergistically with IL-6 in the activation of T cells, as well as B cells, inducing growth, differentiation, immunoglobulin synthesis (Dinarello, 1991; Platanias and Vogelzang, 1990). In the bone marrow, IL-1β induces CSFs (GM-CSF, G-CSF, M-CSF) and, together with these CSFs and other cytokines, IL-1β stimulates erythroid and myeloid progenitors (Dinarello, 1994; Aoki et al., 1995). Also, IL-1β has myelo-protective effects from cytotoxic agents, including radiotherapy and chemotherapy (Dinarello, 1994; Aoki et al., 1995; Proietti et al., 1993). When combined with IL-2, Interferon-α (IFN-α) or IFN-γ, IL-1β activates NK cells, including their differentiation into LAK cells, which mediate inflammation and are involved in anti-tumoricidal activities (Platanias and Vogelzang, 1990).

IL-1β in Cancer Immunotherapy

In syngeneic rodent models, significant tumor regression occurs after injection of recombinant IL-1β protein directly into the tumor area or when given systemically at distant subcutaneous sites (North et al., 1988; Belardelli et al., 1989a; Belardelli et al., 1989b; Ebina and Ishikawa, 1989; Pezzella et al., 1990; Neville et al., 1990; Ebina and Murata,

1991; Rice and Merchant, 1992; Peplinski et al., 1995a). A number of different malignant cell lines respond to IL-1β therapy *in vivo*: Friend erythroleukemia, B16 melanoma, fibrosarcoma cell lines, a Balb/c lung cancer cell line, RT-2 rat glioma, murine C3HBA breast cancer, and PAN02 pancreatic tumor cells (Belardelli et al., 1989a; Ebina and Ishikawa, 1989; Pezzella et al., 1990; McCune and Marquis, 1990; Ebina and Murata, 1991; Rice and Merchant, 1992; Peplinski et al., 1995b; Peplinski et al., 1996). The required systemic therapeutic levels of IL-1β are toxic as evidenced by wasting and/or death (Pezzella et al., 1990; McCune and Marquis, 1990; Rice and Merchant, 1992; Peplinski et al., 1995b). Also, systemic administration is less effective in tumor regression than is injection of IL-1β into the tumor site (Ebina and Ishikawa, 1989; Pezzella et al., 1990; Peplinski et al., 1995b). When IL-1β is combined with IL-2 (Ciolli et al., 1991; Belardelli et al., 1989b) and injected into the tumor, enhanced anti-tumor effects are elicited.

In vitro studies have shown that IL-1β directly inhibits growth or kills at least some tumor cell lines (Onozaki et al., 1985; Kilian et al., 1991; Raitano and Murray, 1993); one possible mechanism is increased IFN-γ receptor expression and IFN-γ binding (Raitano and Murray, 1993). Yet, not all tumor cell lines that regress as a tumor in IL-1β-treated animals are growth inhibited *in vitro* (Belardelli et al., 1989a; Peplinski et al., 1995b). The *in vivo* mechanism(s) of IL-1β tumor inhibition appear to be immune cell-mediated. Cytotoxic T cells are considered to play a direct role in tumor rejection in IL-1β-treated animals; neutrophil and macrophage infiltrations are also seen at the tumor site, indicative of an inflammatory-like process that most likely contributes to tumor rejection (North et al., 1988; Neville et al., 1990; Ebina and Murata, 1991; Ciolli et al., 1991; Zoller et al., 1992). Moreover, IL-1β can serve as an adjuvant in tumor vaccines of irradiated tumor cells and

initiate immune responses to tumors at distant sites from the IL-β-injected tumor or to micrometastases in the liver and spleen (North et al., 1988; Belardelli et al., 1989b; McCune and Marquis, 1990; Ebina and Murata, 1991).

The promising results of IL-1β therapy in animal models prompted several human clinical trials (for review, see Curti and Smith, 1995). Phase I trials of both IL-1α and IL-1β demonstrate that they result in similar systemic toxicities, including fever, chills, and hypotension (Smith et al., 1992). In a phase I trial of recombinant human IL-1β, a response rate of 33% was observed in melanoma patients given up to 100 ng/kg of IL-1β (Starnes et al., 1991). Yet, in a phase II trial for metastatic renal cell carcinoma, a dose of 50 ng/kg given intravenously for 5 days in two cycles did not result in any clinical responses (Redman et al., 1994). Only mild toxicities were observed with increases in WBC counts, primarily neutrophils, and serum IL-6 levels. The authors concluded that potentially a higher dose (up to 300 ng/kg) of IL-1β would be required for a significant anti-tumor effect (Redman et al., 1994). A more recent phase II clinical trial of IL-1α combined with indomethacin to treat patients with metastatic malignant melanoma demonstrated that although high dose IL-1α infusions resulted in significant hypotension (Grade 3), the observed hypotension could be associated with better response rates (Janik et al., 1996). A phase I trial combining IL-1β and IL-2 has been carried out with potentially beneficial results in patients with colorectal cancer, renal cell cancer and melanoma (5-50 ng/kg/day IL-1β plus fixed dose of 100 $\mu g/mm^2$/day IL-2) (Triozzi et al., 1995). Beyond the potential anti-tumor effects of IL-1β, its hematopoietic stimulatory and protective properties make it suitable for combinations with cancer therapies (radiation/chemotherapy) that are often immunosuppressive or for patients receiving bone marrow transplantation (Crown et al.,

1991; Proietti et al., 1993; Redman et al., 1994; Aoki et al., 1995).

IL-1β and Cytokine Gene Therapy of Cancer

As stated earlier, two prominent gene therapy approaches for cancer treatment have been proposed. The first approach consists of vaccination with gene-modified tumor cells, and the second, adoptive immunotherapy with TIL that have been similarly genetically modified (Hwu, 1995; Hwu, 1997). Both strategies are designed to provide therapeutic levels of cytokines locally without increasing systemic toxicities. With respect to tumor vaccines, a number of cytokine genes, including IL-1β, have been inserted and expressed in tumor cells of mice and/or humans in an attempt to stimulate a stronger immune response against native tumor (Gansbacher et al., 1990; Columbo et al., 1991; Porgador et al., 1993; Dranoff et al., 1993; Hwu, 1994; Saito et al., 1994; Peplinski et al., 1995b; Hwu, 1995; Noteboon et al., 1995; Hwu, 1997). Currently, there are a vast number of ongoing human clinical trials utilizing gene modified tumor cells that express a variety of cytokine genes (TNF-α, GM-CSF, IFN-γ, IL-4, IL-7); for a recent listing, see (Human Gene Therapy **9** (10): 1998).

In preclinical mouse models, Dranoff et al. (1993) and Saito et al. (1994) compared the ability of cytokines to enhance the immunogenicity of tumor cells following *in vitro* retrovirus transduction and transplantation of irradiated, transduced cells. Dranoff et al. determined that proviral expression of GM-CSF (versus IL-2 and other cytokines) stimulated the most potent, long lasting anti-tumor immunity against non-transduced, native B16 melanoma cells; anti-tumor immunity appeared to require both $CD4^+$ and $CD8^+$ T cells (Dranoff et al., 1993). In contrast, Saito et al. found that IL-2 expression in an MBT-2

bladder tumor model was superior to GM-CSF. IL-1β-secreting MBT-2 cells were also examined, and the results indicated that IL-1β was much less effective than GM-CSF in increasing animal survival and cytotoxic T cell responses (Saito et al., 1994). An important consideration ignored by the authors is that IL-1β requires ICE for efficient processing and secretion of the mature protein. Beyond monocytes, most cell types do not normally express ICE; in IL-1β-MBT-2 tumor cells, the detectable level of secreted IL-1β was extremely low (Saito et al., 1994; Peplinski et al., 1995b).

To remedy the problem of IL-1β processing in tumor cell vaccines, Peplinski et al. (1995a; 1995b; 1996) utilized a recombinant cDNA that encodes a protein consisting of the human growth hormone signal sequence fused to the carboxy-terminus of the mature IL-1β protein. Processing and subsequent secretion of this fusion IL-1β protein is more efficient and is considered to occur via the ER/Golgi pathway. When expressed in a recombinant IL-1β-vaccinia virus, a variety of infected tumor cell lines secrete 200-fold more IL-1β than that observed by Saito et al. (Peplinski et al., 1995b; Saito et al., 1994). Moreover, tumor growth is slowed when the recombinant vaccinia virus is administered intravenously into mice bearing tumors or injected directly into the tumor site. Yet, inhibition of tumor growth is only temporary, most likely due to host-mediated immune response directed at amplifying vaccinia virus at the tumor site (Peplinski et al., 1995b). In a more recent study using a murine breast cancer (C3HBA) model, systemic anti-tumor immunity was generated following treatment with recombinant IL-1β-vaccinia virus alone in low concentrations or produced in combination with IFN-γ or GM-CSF and rechallenge with non-irradiated, parental C3HBA tumor cells. The authors suggest that the anti-tumor immunity is attributed to the induction of a non-specific intratumoral immune cell infiltrate

which increased the likelihood of generating a specific anti-tumor immune response (Peplinski et al., 1996).

Retrovirus-Mediated Gene Transfer in T Lymphocytes

Recombinant retroviral vectors are the most widely used and thoroughly tested system for introduction of foreign genes into T lymphocytes (Rosenberg et al., 1990; Morgan and Anderson, 1993; Bordignon et al., 1995; Blaese et al., 1995; Morgan and Walker, 1996; Mullen et al., 1996). In fact, retrovirus-mediated gene transfer (RMGT) has been used in greater than 60% of all clinical gene therapy protocols to date (for a recent listing see, Hum. Gene Ther. **9:** 935-976, 1998). Although recombinant retroviruses have several advantages for use as gene delivery systems including stable, site-specific integration with respect to the proviral long terminal repeats (LTRs), safety, and high efficiency gene transfer into many host cell types (Cournoyer and Caskey, 1993; Crystal, 1995), there are several potential limitations that can depend both on target cell type and the gene of interest. These limitations include: 1) an absolute requirement of cell division for infection and integration of the provirus, 2) a limited gene insert size of approximately 8 kilobases (kb), 3) random insertion into the host cell genome which could result in insertional mutagenesis or disruption of a tumor suppressor gene, and 4) transient and/or low level gene expression (Miller, 1992; Mulligan, 1993; Crystal, 1995).

During the normal life cycle of wild-type retroviruses, viral RNA is reverse transcribed to yield a double-stranded DNA species that readily integrates into the host cell DNA (Smith, 1995). As a result, the proviral genome stably passes to all progeny cells through normal division and replication of the host cell (Varmus, 1988). The

majority of retroviral vectors are Moloney murine leukemia virus (MMLV) derivatives (Miller, 1992). These vectors are considered to be replication-incompetent due to the fact that all the structural genes necessary for the normal life cycle of the retrovirus have been deleted. The deleted genes encode *gag*, the group specific antigens or viral core proteins involved in encapsidation; *pol*, the reverse transcriptase and integrase proteins; and *env*, the viral envelope protein (for review see, Smith, 1995). Only the *cis*-acting elements of the virus remain intact, including the 5' and 3' viral LTRs and the packaging signal (ψ^+). In this fashion, the structural proteins can be replaced by therapeutic and selectable marker genes (Smith, 1995), such as human IL-1β and NeoR (neomycin phosphotransferase), respectively.

To produce infectious virus particles, the missing structural proteins must be provided in *trans* by a packaging cell line (Fig. 1). Packaging cells are generally mouse fibroblast cell lines created by stably transfecting the sequences for the retroviral *gag*, *pol*, and *env* structural genes without the presence of a packaging signal (Miller, 1992). These sequences are contained either on a single plasmid or divided onto multiple plasmids to reduce the possibility of production of replication-competent retrovirus as a result of recombinatorial events between the sequences (Miller and Buttimore, 1986). Two widely used packaging cell lines that express MMLV *gag* and *pol* proteins are PA317 and PG13 (Miller and Buttimore, 1986; Miller et al., 1991). The only significant difference between these two cell lines is the type of *env* protein expressed, which determines the target specificity of the virus (tropism). PA317 cells produce retrovirus with the amphotropic MMLV *env* while PG13 cells create retrovirus particles expressing the gibbon ape leukemia virus (GALV) *env* (Miller and Buttimore, 1986; Miller et al., 1991). Infectious

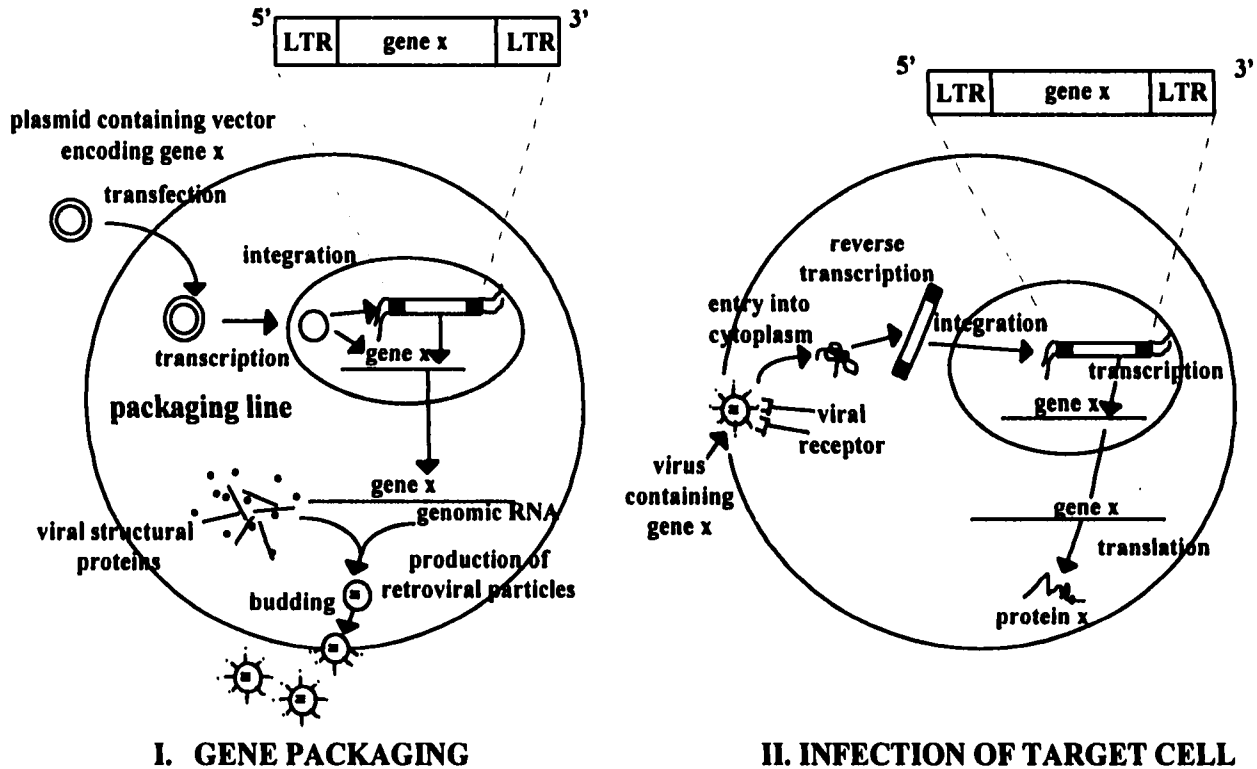

Figure 2. Replication-Incompetent Retroviruses. Packaging cell lines encode the viral structural proteins *gag*, *pol*, and *env*. Production of infectious virus particles requires introduction of plasmid DNA encoding the retroviral genome. Following DNA transfection, a full length viral transcript containing the Ψ^+ packaging sequence is initiated from the 5' LTR. The Ψ^+ sequence is recognized by the structural capsid proteins and the genome is packaged into infectious virus particles which bud from the cell. Infection of a target cell occurs through specific interactions between the virus envelope and host cell receptors. Following infection, the viral genome is reverse transcribed into a double-stranded DNA species that integrates into the host cell genome. Host proteins are responsible for transcription of a full length viral genome initiated from the 5' LTR of the virus. Because the viral genome does not encode any structural proteins, the cell cannot produce infectious virus. Retroviral sequences are passed to all progeny cells during normal cell division. Adapted from Cepko, 1992.

virus particles are produced following introduction of plasmid DNA carrying the recombinant retroviral genome into the packaging cell line, and packaging of the full-length viral transcript which contains the ψ^+ packaging sequence (Cepko, 1992).

First generation recombinant retroviruses originally developed by Miller and Rosman (1989) utilize an MMLV LTR for expression of the proviral genome, and an internal SV40 promoter for expression of the NeoR selectable marker gene. These vectors are referred to as LN-type retroviruses (L, LTR; N, NeoR). Because these first generation retroviruses can be subject to down regulation in a number of cell types (Palmer et al., 1991; Scharfmann et al., 1991; Challita and Kohn, 1994; Lu et al., 1996), I generated an MFG-based retroviral vector for expression of human IL-1β in ATC. This vector, designated MFGIL-1βEN, is designed to drive the gene of interest from the 5' MMLV LTR similar to the LN-type vectors. Unlike LN-type vectors, MFG-based vectors retain the wild-type MMLV splice acceptor (SA) in an effort to provide more efficient mRNA processing via an upstream splice donor (SD) site. In addition, the IL-1β cDNA is inserted in-frame with the normal ATG of the viral *env* gene which places the gene of interest in a more native, viral context. MFGIL-1βEN also contains an internal ribosome entry site (IRES) located 5' of the NeoR gene. The IRES allows expression of a single, polycistronic mRNA species transcribed from the MMLV LTR (Morgan et al., 1992) from which both IL-1β and NeoR may be translated.

Retrovirus infection of a target cell occurs by the specific interaction of a normal cell surface protein and the viral envelope proteins (Varmus, 1988) (Fig. 1). To date, several retroviral receptor cDNAs have been identified, including the murine amphotropic virus receptor (GLVR-2/RAM-1) (Miller et al., 1994), the gibbon ape leukemia virus

receptor (GLVR-1) (O'Hara et al., 1990), and the receptor for the human immunodeficiency virus (CD4) (Dagleish et al., 1984). Kavanaugh et al. (1994) reported that both GLVR-1 and GLVR-2 serve as sodium-dependent phosphate transporters, present on a wide range of mammalian cells. Moreover, this group demonstrated that although GLVR-2 expression is more prevalent in liver and heart tissue, the highest levels of GLVR-1 expression are found in the bone marrow (Kavanaugh et al., 1994). As a result of these findings, retrovirus produced using the PG13 packaging cell line has been applied in transduction studies utilizing bone marrow or cells of hematopoietic lineage (Miller et al., 1991).

Gene-modified T lymphocytes have applicability to a variety of diseases, including cancer, acquired immune deficiency syndrome (AIDS), and congenital disorders, such as adenosine deaminase (ADA) deficiency and $CD18^{-}$ leukocyte adhesion deficiency (for reviews see, Morgan and Anderson, 1993; Cournoyer and Caskey, 1993; Dropulic and Jeang, 1994; Crystal, 1995; Hwu, 1995). However, the primary limitation to using retroviral vectors for genetic modification of T lymphocytes has been the inefficiency of gene transfer (Wilson et al., 1990; Woffendin et al., 1994; Mavilio et al., 1994). Recent efforts, including our own, have focused on enhancing gene transfer and expression in T lymphocytes (Coutre et al., 1994; Mavilio et al., 1994; Bunnell et al., 1995; Lam et al., 1996; Rudoll et al., 1996; Plavec et al., 1997; Pollock et al., 1998; Agarwal et al., 1998; Quinn et al., 1998).

Differential Methods of T Cell Activation: iOK/IL-2 versus 3B/C

To be transduced with murine recombinant retroviruses, target cells must be

proliferating (Springett et al., 1989; Miller et al., 1990). Common *in vitro* methods for T cell activation employ mAbs to cell surface receptors that stimulate immune regulatory signals reminiscent of *in vivo* T cell interactions with specific antigen-major histocompatibility complexes (Ag-MHCs) on antigen-presenting cells (APCs) (Weiss and Imboden, 1987; June et al., 1994; Boise et al., 1995b). One classically defined method for T cell activation utilized in many gene therapy protocols is stimulation using anti-CD3 mAb (OKT3) and IL-2 (Blaese et al., 1995; Bunnell et al., 1995; Morgan and Walker, 1996). Cross-linking the CD3 portion of the TCR with anti-CD3 mAbs induces polyclonal T cell proliferation and acquisition of T cell effector functions, such as cytokine production and cytolytic activity (Weiss and Imboden, 1987; Ochoa et al., 1987; Anderson et al., 1988; Uberti et al., 1994). A series of intricate signal transduction events occur, including tyrosine phosphorylation of cytoplasmic and membrane proteins, hydrolysis of membrane inositol phospholipid, and increases in both intracellular calcium concentrations and protein kinase C (PKC) activity. Signal transduction events culminate in expression of a number of genes including certain transcription factors (c-fos and c-myc), cytokines (IL-2, IFN-γ, and GM-CSF), and growth factor receptor genes (transferrin receptor, IL-2 receptor) (Weiss and Imboden, 1987).

Co-stimulation of the accessory CD28 receptor pathway with anti-CD28 mAbs in conjunction with anti-CD3 mAbs provides additional, distinct signals for optimal T cell activation and proliferation (June et al., 1990; Schwartz, 1992; June et al., 1994; Bluestone, 1995). The interaction of the CD28 molecule on activated T cells with its ligands CD80 (B7-1) on APCs is known to enhance the production of certain type I cytokines (Th1/Tc1) including IL-2, IFN-γ, TNF-α, and GM-CSF through both transcriptional and post-

transcriptional mechanisms (Thompson et al., 1989; June et al., 1994; Bluestone, 1995). Moreover, anti-CD3/anti-CD28 co-stimulation augments the *in vitro* survival of activated T cells by not only increasing production of IL-2, a regulator of T cell growth, but also by enhancing the expression of survival factor Bcl-x_L which confers resistance to apoptosis (Boise et al., 1995a).

Recently, Levine et al. reported a novel *cis* method for T cell activation using magnetic beads carrying co-immobilized anti-CD3 (OKT3) and anti-CD28 (9.3) mAbs (Levine et al., 1995; Levine et al., 1996; Levine et al., 1997). Stimulation of the T cell CD28 receptor pathway with soluble anti-CD28 mAbs in conjunction with anti-CD3 mAbs (*trans* co-stimulation) provides secondary signals that enhance T cell activation, similar to *in vivo* CD28 ligation by the APC B7 counter-receptors (Linsley and Ledbetter, 1993; June et al., 1994). Co-stimulation induces cytokines more effectively when both signals are provided together in *cis*, which is considered more realistic of T cell/APC interactions (Linsley and Ledbetter, 1993; Levine et al., 1995). In purified $CD4^+$ or $CD8^+$ T cell populations, *cis* OKT3/9.3 bead co-stimulation at an optimized ratio of 3 beads per one T cell (3B/C) induces extremely high levels of Th1/Tc1 type cytokines, including IL-2, IFN-γ and TNF-α (Levine et al., 1995; Levine et al., 1996). Furthermore, unlike soluble anti-CD28 mAb, co-activation of $CD4^+$ T cells with bead-immobilized antibodies results in an HIV-1 virus-resistant state (Levine et al., 1996; Carroll et al., 1996). Considering the unique immunological features induced by bead co-stimulation, T cell populations generated by this procedure could prove effective in adoptive immunotherapy for cancer or AIDS and serve as cell targets for gene therapy.

CHAPTER II

MATERIALS AND METHODS

Cell Lines and Cell Culture.

All cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD): human melanoma line A375.S2 (ATCC CRL 1872), mink lung epithelial line Mv1Lu (ATCC CCL 64), human colon adenocarcinoma line LS 174T (ATCC CL 188), human metastatic fibrosarcoma cell line Hs 913T (ATCC HTB 152), murine fibroblast line NIH 3T3 (ATCC CRL 1658), the MMLV *env* amphotropic packaging cell line PA317 (ATCC CRL 9078), and the GALV *env* packaging cell line PG13 (ATCC CRL 10686). The A375.S2 and Mv1Lu cell lines were maintained in Eagle's minimal essential medium (MEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Logan, UT), L-glutamine (2 mM), sodium pyruvate (1 mM), and gentamicin sulfate (30 μg/ml). The LS 174T and Hs 913T cell lines were maintained in RPMI-1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), and L-glutamine (200 mM). All other cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL) supplemented with 10% heat-inactivated FBS, L-glutamine (2 mM), sodium pyruvate (1 mM), and gentamicin sulfate (30 μg/ml). Cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Recombinant Retroviral Vector Constructs.

All subcloning procedures were performed according to standard methods

(Sambrook et al., 1989). The retroviral vector plasmids LXSN and LIL-1βSN were kindly provided by G. McGarrity (Genetic Therapy, Inc., Bethesda, MD). The IL-1β cDNA was derived as a 551 base pair Nco I/Bam HI fragment from the LIL-1βSN plasmid. The MFGIL-1βEN retroviral vector was constructed by insertion of the Nco I/Bam HI fragment into the Nco I/Bam HI sites of the MFG retroviral vector (provided by P. Robbins, University of Pittsburgh, Pittsburgh, PA), which placed the ATG start codon of the cDNA in-frame with the unique viral ATG start codon of the vector (Byun et al., 1996; Krall et al., 1996). In addition, a Bam HI fragment consisting of an IRES from the encephalomyocarditis virus placed 5' of the NeoR gene (Treisman et al., 1995) was obtained by digestion of the pSAMEN vector (provided by J. Treisman, St. Luke's Medical Center, Milwaukee, WI) and inserted into the Bam HI site of the MFG vector. A control vector, MFGEN, that lacks the IL-1β cDNA was constructed by blunt-ended ligation of the IRES-NeoR Bam HI fragment into the Nco I/Bam HI site of the MFG vector.

Standard Production of Retrovirus Supernatant.

For virus production, $2x10^5$ packaging cells (PA317 or PG13) were plated onto a 60 mm^2 dish and incubated overnight at 37°C. The cells were transfected the following day using a calcium phosphate precipitation method (Pear et al., 1993). Prior to transfection, fresh medium containing 25 μM chloroquine was added to the cells. Retroviral vector DNA (10 μg) in 500 μl of water containing 250 mM $CaCl_2$ was precipitated by drop-wise addition of 500 μl 2X HBS (50 mM HEPES pH 7.05, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na_2HPO_4) and added directly to the

culture medium. Following incubation at 37°C for 10 hours, the cells were rinsed, fed with fresh medium, and incubated overnight at 37°C. The next day, transfected cells were plated at 10^3, $5x10^4$, and 10^5 cells/100 mm^2 dish in fresh media. Geneticin® (G418, Gibco BRL) was added 18-24 hours later at a final concentration of 400 μg/ml. Cells were fed every 2 days with fresh medium containing G418. After 7-10 days, G418 resistant producer colonies were isolated by ring cloning, transferred to a 24-well plate, and allowed to grow to confluency (approximately 2-3 days).

Supernatants from producer cell clones were analyzed for secreted IL-1β protein to identify clones producing the highest amount of retrovirus. Individual clones were removed with 0.05% trypsin/EDTA (Gibco BRL), counted, and replated in a 24-well plate at $2x10^5$ cells per well. The clones were incubated at 37°C for 24 hours. Following incubation, media containing virus was removed from each clone, centrifuged at 1000xg for 5 minutes to pellet cell debris and stored at -80°C. In addition, clones were frozen at -80°C in their respective wells following trypsinization, centrifugation (1000xg for 10 minutes), and resuspension in freezing medium (DMEM containing 20% FBS and 10% dimethyl sulfoxide). Supernatant samples were thawed and analyzed for vector-derived human IL-1β using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine™ Kit; R&D Systems). For large scale production of virus stock, the producer cell clone that secreted the highest amount of human IL-1β was thawed at 37°C, plated onto 150 mm^2 dishes and allowed to grow to 80% confluency at 32°C. Fresh media was added to the cells, and virus containing supernatant was harvested from the confluent monolayers every 48 hours, filtered through a 0.45 μm filter and stored at -80°C.

Standard Virus Titration.

The number of infectious virus particles or colony forming units per milliliter (CFU/ml) was quantitated based on the ability of the virus to confer G418 resistance to the appropriate target cell lines. For PA317-produced virus, $1x10^5$ NIH 3T3 target cells were plated onto a 60 mm^2 dish. The following day, 10 µl or 1 µl of viral supernatant in 2 ml of media containing polybrene (8 µg/ml) was added, and the cells were incubated for 4 hours at 37°C. The viral supernatant was removed and replaced with fresh media, and the cells were incubated overnight. G418 was then added at a final concentration of 400 µg/ml, and the cultures were subsequently fed with fresh G418-containing media every other day. Ten days later, G418-resistant colonies were stained using a modification of the Wright Stain technique (Diff-Quik®, Baxter, McGraw Park, IL). The virus titer in CFU/ml was determined by counting the total number of colonies and dividing by the dilution of virus applied in milliliters. For PG13-packaged virus, $2.5x10^5$ Mv1Lu target cells were plated onto each well of a 6-well dish according to Olsen et al. (1995) and cells were infected as described previously in the presence of polybrene (8 µg/ml). Twenty-four hours after virus infection, the cells were trypsinized and replated in equal amounts onto duplicate 100 mm^2 dishes and cultured in media containing 800 µg/ml G418. The cells were fed with fresh media containing G418 every 2 days for a total of 10 days and then stained as described previously. The titer (CFU/ml) was determined by counting the number of colonies on both plates and dividing by the dilution of virus applied (ml). For experiments directly comparing PA317 and PG13 retrovirus stocks, PG13-packaged virus was titered on both target cells to ensure similar titers between the virus stocks.

Superinfection of PG13 Packaging Cells.

In an attempt to increase the titer of virus produced by the PG13 (GALV *env*) packaging cells, PG13 cells were superinfected with PA317 amphotropic retrovirus supernatant (Parente and Wolfe, 1996). Briefly, PG13 packaging cells ($5x10^4$) were plated in a 6-well tissue culture dish and incubated overnight at 37°C. The following day, 2 ml of either PA317-packaged MFGEN or MFGIL-1βEN retrovirus supernatant containing polybrene (8 μg/ml) was added to the cells. The cells were centrifuged at 1000xg for 90 minutes at 32°C and incubated for 4 hours at 32°C. Following the incubation, the cells were fed with fresh media, incubated overnight, and reinfected with retrovirus supernatant the following day. On the third day, the cells were split 1:2, and the infection cycle was repeated for a total of four exposures to retroviral supernatant. Following the last exposure to virus, the cells were counted and 10^3, 10^4, $5x10^4$, and 10^5 cells were plated onto 100 mm^2 dishes in DMEM containing 400 μg/ml G418. Seven to ten days later, individual G418-resistant producer colonies were isolated, media containing virus was removed from each clone and frozen at -80°C, and individual clones were frozen at -80°C as described previously. High titer retrovirus-producing clones were identified by RT-PCR analysis of retroviral supernatant based on the procedure by Quinn and Trevor (1998). Large scale production of virus stock from a high-titer producer clone was performed as described previously. Virus was harvested every 48 hours, filtered through a 0.45 μm filter and stored at -80°C.

Isolation and Cell Culture of T Lymphocytes.

Peripheral blood mononuclear cells (PBMC) were isolated from blood of normal

healthy donors by Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation. Interface PBMC were washed three times in Dulbecco's phosphate-buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA) supplemented with glucose (2 g/L) and 5% FBS. Cultures were initiated at a concentration of $1x10^6$ cells/ml in 24-well dishes in tissue culture medium (TCM), consisting of RPMI-1640 containing 10% FBS, penicillin (100 U/ml), streptomycin sulfate (100 mg/ml), and L-glutamine (200 mM). For plastic-immobilized anti-CD3 activation of PBMC, wells were precoated by incubation for 4-8 h at 37°C with 1 ml of a 2 μg/ml PBS solution of the anti-CD3 mouse mAb OKT3 (Ortho Pharmaceuticals, Raritan, NJ) (Uberti et al., 1994). The wells were washed with PBS, and PBMC were plated in TCM containing 100 international units (IU)/ml recombinant IL-2 (Cetus Corp., Emeryville, CA). PBMC were co-stimulated with anti-CD3/anti-CD28 (OKT3/9.3) mAb-coated Dynal beads (provided by B. Levine and C. June, Naval Research Institute, Bethesda, MD) by the direct addition of beads at bead-to-cell ratios of 1:1 (1B/C) or 3:1 (3B/C). Soluble anti-CD28 co-stimulation was performed by the addition of the mouse mAb 9.3 (provided by J. Ledbetter, Bristol-Myers Squibb, Seattle, WA) at 1 μg/ml to PBMC plated on immobilized OKT3-coated wells. Following activation, cultures were continuously grown in media containing 100 IU/ml IL-2. Viable cell numbers were determined by dye exclusion using 0.4% trypan blue (Sigma Chemical, St. Louis, MO).

Transduction and G418 Selection of Lymphocytes.

Following 48-72 hours of plastic-immobilized OKT3 or OKT3/9.3 bead co-stimulation, PBL were transduced with recombinant retrovirus based on modifications of

a previously described protocol (Bunnell et al., 1995). Briefly, $5x10^5$ cells were placed in each well of a 6-well dish. MFGIL-1βEN and MFGEN retrovirus supernatants (PA317 or PG13-packaged) were added to the cells at multiplicities of infection (MOIs) of 0.2 to 5.0 and supplemented with IL- 2 (100 IU/ml) and protamine sulfate (8 μg/ml). The cells were centrifuged at 1000xg for 60-90 minutes at 32°C followed by incubation for 10-12 hours at 32°C. At the end of the transduction period, cells were pelleted, washed twice, and resuspended at $1x10^6$ cells/ml in equal parts of fresh media and conditioned media from non-transduced, activated cultures containing IL-2 (100 IU/ml). Cultures were then incubated at 37°C overnight. This process was repeated twice more over the following 2 days unless otherwise indicated.

G418-selected populations were generated by removing a portion of the transduced lymphocytes at 3 days post-transduction (PT) and culturing in media containing 0.6 mg/ml G418 for 6 days. Viable cells were isolated by pelleting through a 35% Percoll (Pharmacia LKB, Uppsala, Sweden) density gradient at 500xg for 10 minutes. Cells were washed in PBS and resuspended ($1x10^6$ cells/ml) in media containing 100 IU/ml IL-2.

Analysis of Transduction Efficiency.

Retroviral vector transduction efficiencies were determined by comparative polymerase chain reaction (PCR) analysis using NeoR positive reference standards. Routinely, $3\text{-}5x10^5$ transduced cells were centrifuged, washed in PBS, pelleted and stored at -80°C. Aliquots were thawed and lysed for 1 hour at 56°C in PCR lysis buffer containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Tween-20,

0.5% Triton X-100, and 500 μg/ml Proteinase K, followed by 10 minutes at 95°C. NeoR positive standards were prepared by combining PCR lysates of transduced NIH 3T3 cells carrying approximately 1 copy/cell of the MFGIL-1βEN provirus with lysates from non-transduced control T cells to generate mixtures containing known percentages of NIH 3T3-transduced cell equivalents. For all samples, $2x10^3$ total cell equivalents were submitted to PCR analysis using NeoR primers. This primer pair hybridizes to nucleotides 140-162 and 726-748 of the NeoR gene and yields a 610 bp product: upper strand primer (neo1up), 5'-TTTTGTCAAGACCGACCTGTCC-3'; lower strand primer (neo2dn), 5'-CGGGAGCGGCGATACCGTAAAG-3'. To normalize PCR lysates prepared from transduced lymphocytes, an equivalent amount of each lysate was separately PCR amplified using primers specific for the endogenous human IL-1β gene that recognize sequences within introns D and E, respectively (Bensi et al., 1987). This primer pair yields a 909 bp product: upper strand primer (4193), 5'-CTACTGGTGTTGTCATCAGACTT-3'; lower strand primer (5077), 5'-TTCAAAGTCAGCAGCTTAGTTG-3'. PCR reactions (25 μl) were performed in 1X PCR buffer (Gibco BRL) containing 0.5 μM primers and 60 Units/ml Taq DNA polymerase (Gibco BRL). After denaturation at 94°C for 5 min, samples were amplified with 30 cycles of 94°C, 20 seconds; 65°C, 45 seconds; 72°C, 45 seconds followed by a final 10 minute extension at 72°C. Routinely, 6.25 μl of each PCR reaction were separated by electrophoresis on a 2% agarose gel containing Tris/Acetate/EDTA buffer and 0.5 μg/ml ethidium bromide and visualized by UV transillumination (Sambrook et al., 1989). Amplimer intensities were quantitated by densitometric analysis (Gel Doc 1000, Bio-Rad, Hercules, CA) with the appropriate software (Molecular Analyst, Bio-

Rad) and normalized to PCR products generated using intronic primers specific for the endogenous IL-1β gene. Linear regression analysis was performed on the NeoR positive reference standards. Transduction efficiencies were assessed by comparison of amplimer intensities to the NeoR standards.

RNA analysis.

Total cellular RNA was isolated by the RNAzol™ Method (Tel-Test, Inc., Friendswood, TX). RNA samples (0.5 μg) were reverse transcribed with MuLV reverse transcriptase according to the manufacturer's directions using random hexamers (GeneAmp® RNA PCR Kit, Perkin Elmer, Branchburg, NJ). Each cDNA was amplified by PCR in a reaction (25 μl) containing 1X PCR buffer (Gibco BRL), 0.2 μM of each primer, and 60 Units/ml Taq DNA polymerase (Gibco BRL). Retroviral vector-derived transcripts were detected using the previously described NeoR primers and cycle parameters. To normalize for amounts of RNA utilized in the reverse transcriptase (RT)-PCR reactions, primers specific for the human β-actin cDNA were used (Stratagene, La Jolla, CA). The primers amplified a 661 bp product: upper strand primer (1038u), 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3'; lower strand primer (1876d), 5'-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3'. Primers specific for human IFN-γ cDNA (Clontech, Inc., Palo Alto, CA) amplified a 427 bp product: (upper) 5'-GCATCGTTTTGGGTTCTCTTGGCTGTTACTGC-3', and (lower) 5'-CTCCTTTTTCGCTTCCCTGTTTTAGCTGCTGG-3'. Primers specific for human IL–2 cDNA (Clontech, Inc.) amplified a 310 bp product: (upper) 5'-CATTGCACTAAGTCTTGCACTTGTCA-3', and (lower) 5'-

CGTTGATATTGCTGATTAAGTCCCTG-3'. PCR reactions were performed by heat denaturation at 94°C for 5 minutes and 35 cycles of amplification: 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 2 minutes. This was followed by a single 10 minute extension at 72°C. As a control for potential cellular DNA contamination, all RNA samples were submitted to identical reaction conditions in the absence of reverse transcriptase followed by PCR analysis. No DNA contamination was detected in the experiments shown.

GLVR-1/Ram-1 Receptor Studies.

Following 72 hours of stimulation using plastic-immobilized anti-CD3 plus 100 IU/ml IL-2 (iOK/IL-2) or anti-CD3/anti-CD28 coated beads at bead-to-cell ratios of 1B/C or 3B/C, total cellular RNA was isolated from activated PBL cultures and reverse transcribed using random hexamers as detailed in the previous section. Each cDNA was used in a PCR reaction (25 µl) containing primers specific for GLVR-1 and GLVR-2 (Bauer et al., 1995) which amplified a 230 bp product: (upper) 5'-TTCCAGTTCCTGCAGGTCCT-3', and (lower) 5'-TCCTTCCCCATGGTCTGGAT-3'. The upper primer recognizes both receptor RNA species, but differs by one base each from the GLVR-1 (A to G at base 16) and GLVR-2 (T to G at base 6) cDNAs, whereas the lower primer is identical to GLVR-1 and GLVR-2 sequences. PCR reactions were performed with cycling parameters of 94°C for 1 minute; 5 cycles of 94°C for 15 seconds, 55°C for 15 seconds, and 72°C for 15 seconds; and 30 cycles of 94°C for 15 seconds and 60°C for 30 seconds. This was followed by a single 5 minute extension at 60°C. PCR reaction products were immediately digested with Hae III for 2 hours at

37°C, ethanol precipitated, electrophoresed on an 8% polyacrylamide gel, and quantitated by densitometric analysis (Gel Doc 1000, Bio-Rad) with the appropriate software (Molecular Analyst, Bio-Rad).

Cytokine Measurements.

Unless otherwise indicated, cells were cultured ($1x10^6$ cells/ml) in fresh media containing IL-2 (100 IU/ml) and incubated for 24 hours at 37°C. Following incubation, supernatants were harvested from the wells, centrifuged at 1000xg for 5 minutes to pellet cell debris and stored at -80°C. Samples were thawed and analyzed for human IL-4, IFN-γ, TNF-α, IL-1β, and IL-2 in supernatant samples using commercially available ELISA kits (R&D Systems). As indicated by the manufacturer, sensitivities in buffer diluent were 3.0 pg/ml for IL-4 and IFN-γ , 4.4 pg/ml for TNF-α, < 1 pg/ml for IL-1β, and < 7 pg/ml for IL-2. All reported values were determined using a series of dilutions that yielded readings within the linear portion of a standard curve. Results are expressed as the mean ± standard error of the mean (SEM). The SEM was never greater than 20 percent.

Bioactivity of Human IL-1β.

The bioactivity of human IL-1β was determined based on its cytotoxicity to human A375 melanoma cells (Nakai et al., 1988). This assay compared the cytotoxicity of recombinant human IL-1β (R&D Systems, Minneapolis, MN) and retroviral vector-derived human IL-1β. A375 cells ($2x10^3$) were plated in duplicate or triplicate wells of a

flat-bottom 96-well dish. Supernatant was collected from MFGEN and MFGIL-1βEN-transduced lymphocyte cultures that had been previously cultured ($1x10^6$ cells/ml) for 24 hours at 37°C in the presence of fresh media containing 100 IU/ml IL-2. Supernatants were serially diluted in TCM and added to each well. ELISA analysis (Quantikine™ Kit; R&D Systems) determined that MFGIL-1βEN-transduced T cell supernatants contained approximately 800 pg/ml of vector-derived IL-1β. Based on this calculation, serial dilutions of recombinant human IL-1β protein (R&D Systems) were prepared and served as controls in this assay. Following a 4 day incubation period, nonadherent cells were removed by rinsing the wells with PBS and the remaining cells were stained using Diff-Quik® (Baxter) and counted.

Monoclonal Antibodies and Cell Labeling.

For staining of cell surface antigens, transduced and non-transduced control cells were washed twice and resuspended in PBS at $1x10^7$ cells/ml. Aliquots of cells (0.5-$1x10^6$) were incubated for 30 minutes at room temperature (in the dark) with fluorescein isothiocyanate (FITC)-conjugated or phycoerytherin (PE)-conjugated monoclonal antibodies (Becton-Dickinson Immunocytometry Systems (BDIS), San Jose, CA). Following incubation, the cells were washed with 2 ml PBS and resuspended in 0.5% paraformaldehyde (0.3 ml). If sample data were not acquired immediately, the tubes were covered with parafilm and stored for up to 24 hours at 4°C in the dark. Monoclonal antibodies utilized for these studies were: FITC-conjugated anti-CD3 (Leu-4, IgG1), FITC-conjugated anti-CD4 (Leu-3a, IgG1), FITC-conjugated anti-CD8 (Leu-2a, IgG1), FITC-conjugated anti-CD25 (IL-2R, IgG1), FITC-conjugated IgG1, and PE-conjugated

IgG2a (Simultest™ Control, BDIS).

Fluorescence Activated Cell Sorting (FACS) Analysis.

All cytometric analysis was performed by R. Siebenlist (William Schuette Cell Biology Facility, St. Luke's Medical Center, Milwaukee, WI) on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA) according to the procedures detailed in the FACScan and Lysis II Manuals (Becton Dickinson). At least 10,000 events per sample were acquired and analyzed using the Lysys II and CELLQuest software package (BDIS).

Proliferation Assays.

To determine the proliferation of activated PBL populations, $1.5x10^5$ PBMC were plated onto 96-well dishes in 0.2 ml and activated with either iOK/IL-2 or OKT3/9.3 mAb-coated beads at 1B/C or 3B/C. Following incubation for three days, 1 μCi of [^{3}H]-thymidine (ICN Pharmaceuticals, Inc., Irvine, CA) was added to each well. Cells were harvested after 18-20 hours using a FilterMate™ cell harvester (Packard Instrument Co., Meriden, CT), and [^{3}H]-thymidine incorporation was measured using a Matrix 96™ Direct Beta Counter (Packard Instrument Co., Meriden, CT). To assay proliferation of transduced T cell populations, $1.5x10^5$ cells were plated in 96-well dishes in 0.2 ml of fresh media plus IL-2. [^{3}H]-thymidine incorporation was assessed as described above for PBMC and expressed as the mean counts per minute (cpm) ± SEM of triplicate wells.

Anti-CD3 X Anti-Carcinoembryonic Antigen (CEA) Bispecific Antibody.

The anti-CD3 X anti-CEA bispecific antibody (BsAb) utilized in these experiments was developed by K. Knox and M. Sen (unpublished results, St. Luke's Medical Center, Milwaukee, WI). Anti-CD3 and anti-CEA monoclonal antibodies were produced using commercially available hybridoma cell lines obtained from ATCC, and protein heteroconjugation and purification was performed according to Anderson et al. (1992).

MFGIL-1βEN and MFGEN-transduced T lymphocytes were resuspended at $20x10^6$ cells/ml in ice-cold RPMI containing 2% FBS and pre-coated (armed) with BsAb for 1 hour at 4°C with gentle agitation every 15 minutes. Armed and unarmed cells were washed, resuspended in RPMI-10% FBS and added to each well at the appropriate effector cell to target cell (E:T) ratios.

BsAb-Redirected ^{51}Chromium [^{51}Cr] Release Assay.

For the cytotoxic [^{51}Cr]-release assay, $2x10^4$ LS 174T (CEA-positive) target cells or $2x10^4$ Hs 913T (CEA-negative) target cells were plated in triplicate wells of a flat-bottom 96-well dish and incubated overnight at 37°C. The following morning, the adherent target cells were washed and labeled with [^{51}Cr] (2 μCi/well $Na_2{}^{51}CrO_4$; Amersham Life Science, Arlington Heights, IL) for 4 hours at 37°C. The labeled target cells were then washed and 100 μl RPMI-10% FBS was added to each well. BsAb-armed and unarmed transduced T lymphocytes were added to each well at the appropriate E:T ratios, and the cells were incubated for 20 hours at 37°C. After incubation, the plate was centrifuged and the radioactivity in 100 μl culture supernatant from each well was

determined using a Cobra II Auto-Gamma® Counting System (Packard Instrument Company, Meriden, CT). The percentage of specific lysis was determined as: (cpm, experimental release - cpm, spontaneous release)/(cpm, maximal release - cpm, spontaneous release) x 100. Maximum [^{51}Cr] release was determined by lysis of target cells with 10% SDS, and spontaneous release was measured in the absence of effector cells. The [^{51}Cr]-release assay shown in Figure 19 was performed by M. Sen.

BsAb-Mediated Cytokine Production.

For determination of BsAb-mediated proviral IL-1β and endogenous IFN-γ cytokine production, LS 174T and Hs 913T target cells ($2x10^4$) were plated in triplicate wells of a flat-bottom 96-well dish and incubated overnight at 37°C. The following morning, MFGIL-1βEN and MFGEN-transduced lymphocytes, armed and unarmed with BsAb, were plated at the appropriate effector-to-target cell ratios in the presence of 100 IU/ml IL-2, and incubated for 20 hours at 37°C. Supernatants were collected and combined from the triplicate wells, centrifuged at 1000xg for 10 minutes to pellet cellular debris, and frozen at -80°C. Samples were thawed and analyzed as described previously using commercially available ELISA kits (R&D Systems).

CHAPTER III

STRATEGIES FOR IMPROVED IL-1β RETROVIRUS GENE TRANSDUCTION AND EXPRESSION IN ACTIVATED T LYMPHOCYTES

INTRODUCTION

The success of T lymphocyte-based gene therapy approaches will depend on both efficient gene delivery and persistent high-level gene expression in primary, human T cells. In the following experiments, T cells were transduced with a retroviral vector encoding a modified human IL-1β cDNA. Several parameters important for improved gene transfer and increased proviral gene expression were evaluated early after virus exposure (72 hours post-transduction). Firstly, I applied the MFG retroviral vector, which is known to be superior in both virus titer and gene expression compared to alternative internal promoter-containing retrovirus vectors (Byun et al., 1996). Secondly, I investigated whether substitution of the GALV-derived retrovirus packaging cell line PG13 for the amphotropic PA317 cell line might improve transduction efficiency in T cell cultures. Thirdly, I developed an optimized transduction protocol and utilized higher titer retrovirus supernatants to transduce T cells. Finally, because infection of target cells with retroviruses requires actively dividing cells (Springett et al., 1989; Miller et al., 1990), I compared the efficiency of retrovirus-mediated gene transfer in T cells stimulated with beads carrying co-immobilized anti-CD3 and anti-CD28 mAbs, or activated by the more standard method of immobilized anti-CD3 plus IL-2. The improved transduction protocol developed in the laboratory routinely yields gene transfer efficiencies of 50-75% in activated PBL.

RESULTS

Application of an Alternative Retroviral Vector.

Several years ago, preliminary transduction studies performed by our laboratory group focused primarily on a retroviral vector designated LIL-1βSN, which was provided by Dr. Gerard McGarrity of Genetic Therapy, Inc. (GTI, Gaithersburg, MD). The LIL-1βSN retroviral vector is comprised of the LN-type plasmid backbone originally developed by Miller and Rosman (1989) (Fig. 2). This type of retroviral vector is designed to have the MMLV LTR promote transcription of the gene of interest (human IL-1β, in this case) with the internal SV40 early region promoter driving expression of the NeoR gene. The NeoR gene allows for selection of stably transduced cells in the presence of the neomycin analogue G418. In the plasmid, the 5' LTR differs from the 3' LTR in that it is derived from the Moloney murine sarcoma virus (MuSV). After reverse transcription and synthesis of the DNA proviral genome, the 3' LTR is copied to the 5' end of the viral genome, resulting in a 5' MMLV LTR that promotes proviral IL-1β gene transcription. An extended packaging region (ψ^+) was left intact during subcloning for efficient packaging of viral transcripts during recombinant virus production. In addition, the LN-type vector possesses two engineered alterations that were considered to allow efficient expression of a subcloned gene: 1) a start codon for the viral Pr65 *gag* protein that lies within the ψ^+ region was mutated from ATG to TAG to prevent a false translational start for the subcloned gene; and 2) the 3' SA that is utilized in the normal splicing of wild-type viral mRNAs was removed with the idea that this would be advantageous in cells that for some reason would not efficiently splice

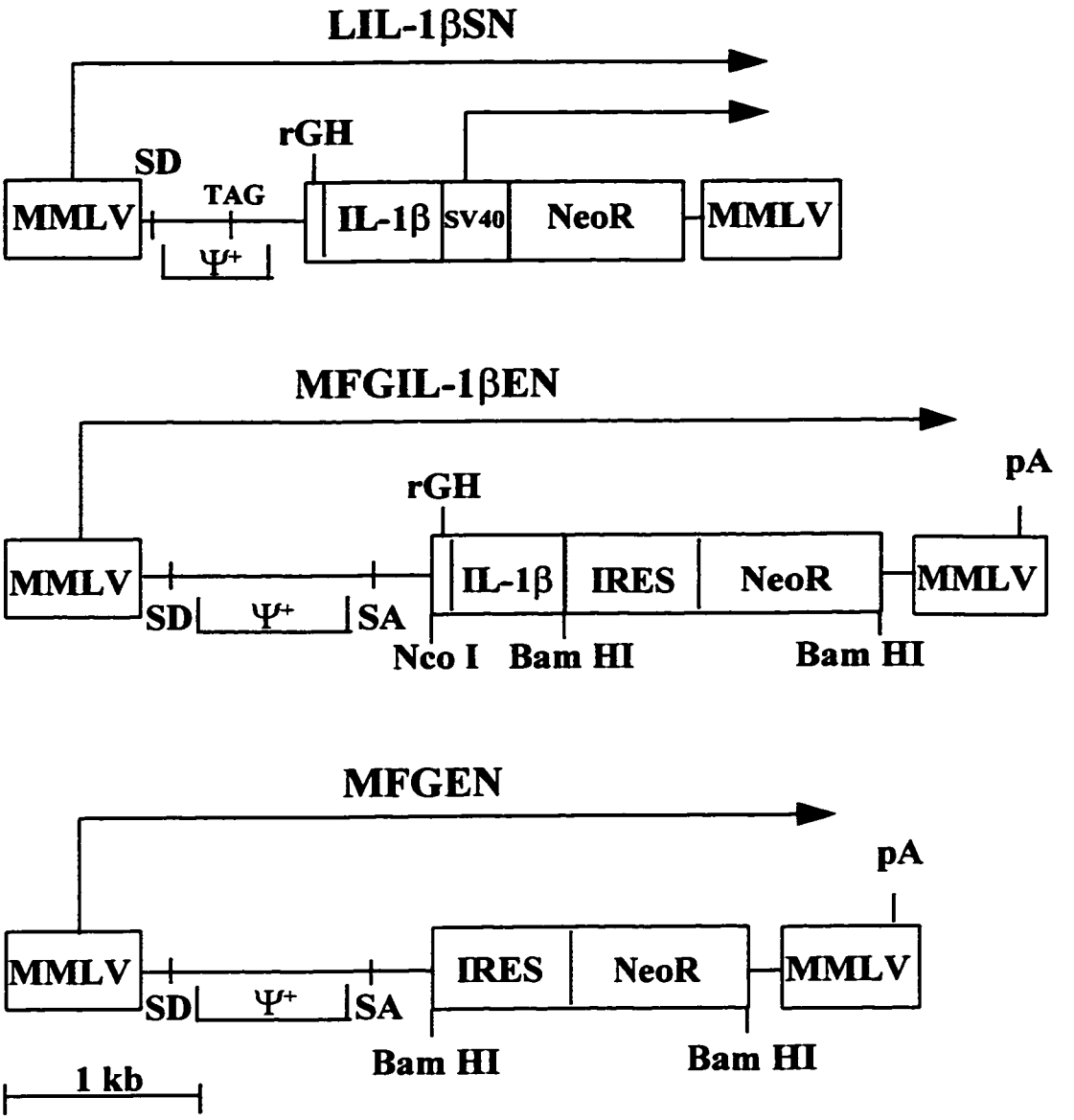

Figure 2. Schematic Diagram of LN and MFG-Based Retroviral Vectors. A fusion construct of the rat growth hormone signal sequence and the full length human IL-1β cDNA was removed from the LIL-1βSN vector and cloned into the Nco I/Bam HI site of the MFG vector. In addition, a Bam HI fragment containing an internal ribosome entry site fused to the NeoR gene was inserted into the Bam HI site to yield MFGIL-1βEN. The MFGEN vector does not contain the IL-1β cDNA. Arrows indicate transcriptional start site and direction of transcription. Abbreviations: MMLV, Moloney murine leukemia virus LTR; SD, splice donor; SA, splice acceptor; ψ^+, extended packaging signal; TAG, original Pr65*gag* ATG that was mutated to TAG; rGH, rat growth hormone signal sequence; IL-1β, modified human IL-1β cDNA; IRES, internal ribosome entry site; NeoR, neomycin phosphotransferase gene; pA, polyadenylation site. All vectors are drawn to scale.

retroviral-like mRNA (Miller and Rosman, 1989).

LIL-1βSN encodes a fusion protein of the rat growth hormone (rGH) signal sequence upstream of the mature 17.5 kD form of the IL-1β protein. Although processing of the mature IL-1β protein is dependent upon the activity of ICE, the pathway for IL-1β secretion is unclear (Dinarello, 1996). The purpose of this fused cDNA construct is to lead to efficient secretion of the mature IL-1β protein from transduced ATC via the rough ER/Golgi pathway. This construct is similar to that used by Peplinski et al. (1995a; 1995b) in studies of recombinant vaccinia vector expression of IL-1β in tumor cells; however, this group utilized the human GH signal sequence. Studies of a human GH-IL-1β fusion construct in Chinese hamster ovary cells indicated efficient secretion of a biologically active 22 kD glycosylated form which was attributed to ER/Golgi processing (Pecceu et al., 1991). In addition, this cDNA lacks the 3' untranslated region of the IL-1β cDNA that contains AU-rich elements, which are thought to function in destabilizing the mRNA of several different cytokines (Bohjanen et al., 1991; Chen and Shyu, 1995).

Although LN-type retrovirus vectors pseudotyped with the murine amphotropic envelope are capable of infecting human cells, transduction generally results in low levels of gene transfer and extremely low levels of secreted proviral-encoded protein product (Kasid et al., 1990; Culver et al., 1990; Culver et al., 1991; and Morgan et al., 1994). Because of these limitations and our lack of success using the LIL-1βSN vector in preliminary transduction studies (data not shown), I developed an alternative retroviral vector, MFGIL-1βEN, based on the MMLV vector MFG. The MFG vector was originally developed for the transfer of cytokine genes into irradiated tumor cells (Jaffee et al., 1993; Dranoff et al., 1993). However, MFG possesses two modifications not present in

the LN-series vectors: 1) the normal 5' and 3' splice sites used in the splicing of wild-type viral mRNA are present to provide more efficient mRNA splicing with enhanced gene expression; and 2) the vector is designed with a 5' Nco I site such that subcloning into the Nco I site would position the initiation codon of the cDNA in-frame with the normal ATG of the viral *env* gene. The combination of these modifications in the RNA splicing elements were considered to increase levels of subgenomic transcripts, resulting in higher levels of vector-derived gene expression. As depicted in Figure 1, MFGIL-1βEN was constructed by insertion of the rGH-IL-1β human cDNA (originally encoded by the LIL-1βSN retroviral vector) into the 5' Nco I site of the MFG vector. Because there are indications that an internal promoter can interfere with LTR-driven transcription (Emerman and Temin, 1984; Emerman and Temin, 1986; Treisman et al., 1995; Byun et al., 1996), a Bam HI fragment was inserted downstream of IL-1β which contains an IRES from the encephalomyocarditis virus (EMCV) fused to the NeoR gene (Treisman et al., 1995). The IRES sequence allows cap-independent translation of internal ATGs, resulting in a polycistronic full-length proviral transcript from which both IL-1β and NeoR are translated. A control vector, MFGEN, that lacks the IL-1β cDNA, was constructed by insertion of the IRES-NeoR Bam HI fragment into the Bam HI site of the MFG vector (Fig. 1).

Several groups have compared the transduction efficiency and expression levels of different retroviral vectors and have found the MFG vector to be superior. Krall et al. (1996) reported a 5-fold greater level of spliced RNA generated by an MFG-type vector versus an LN-type, which corresponded to a 7-fold higher level of detected protein product. Maintenance of the normal ATG start site did not appear to be a major factor for

product translation (Krall et al., 1996). In addition, Byun et al. (1996) demonstrated that MFG was superior with respect to both the relative level of gene expression and the generation of higher viral titer from a virus producer cell line when several LN-type vectors and MFG-based vectors were compared. As a result of our preliminary data and that of others, LN-based retroviral vectors are no longer utilized in our laboratory for transduction of ATC.

Optimization of Transduction Conditions for Retroviral-Mediated Gene Transfer in T Lymphocytes.

1. Retrovirus Packaging Cell Lines

As these studies were being initiated, one report demonstrated improved transduction frequencies in hematopoietic cell lines using PG13-packaged retrovirus, while a second study suggested a 2-fold higher transduction frequency of human PBL using PG13-packaged virus relative to PA317-produced virus (Bauer et al., 1995; Bunnell et al., 1995). To determine whether PG13-derived retrovirus supernatant might improve transduction efficiencies in activated T cells, PBL from two normal donors (PBL_{CJ} and PBL_{MW}) were activated for three days using iOK/IL-2. The cells were exposed for two consecutive days to MFGIL-1βEN virus (MOI 0.5) packaged either using PG13 or PA317 producer cells. The titers of both retrovirus supernatants were quantitated using Mv1Lu target cells, which are readily infected by both GALV-derived and murine amphotropic-derived viruses. Three days after the last exposure to virus, cells were replated in fresh media containing 100 IU/ml IL-2. After 24 hours, proviral IL-1β protein

production was evaluated by ELISA analysis of supernatants and cells were lysed for PCR analysis of proviral integrants (transduction frequency). Using primers specific for the NeoR gene, PCR results shown in Figure 3A indicate that exposure of PBL_{CJ} cultures to PG13-MFGIL-1βEN retroviral supernatant resulted in transduction frequencies of approximately 6% relative to the NeoR PCR standard. Exposure of the same cells to PA317-MFGIL-1βEN virus resulted in approximately 3-fold lower transduction frequencies (2%), corresponding with decreased levels of proviral-encoded IL-1β secretion (Fig. 3A, compare lanes 1 and 2). PBL_{MW} cells transduced with PG13-MFGIL-1βEN virus examined 3 days post-transduction resulted in transduction frequencies of approximately 17% relative to the NeoR PCR standard (Fig. 3B). A similar transduction frequency was observed in cultures transduced with PG13-MFGEN control virus. Exposure of cells to PA317-packaged MFGIL-1βEN virus resulted in transduction frequencies of approximately 3% (Fig. 3B, compare lanes 2 and 3). Taken together, these data demonstrate a 3- to 6-fold higher transduction frequency for virus packaged with the GALV envelope (PG13 producer) relative to the amphotropic envelope (PA317 producer). Although a direct correlation was not observed between proviral copy number and amounts of secreted IL-1β, approximately 10-fold higher levels were detected in cultures receiving PG13 retrovirus.

To investigate the basis for improved T cell transduction efficiency using virus produced from PG13 packaging cells, the relative amounts of GLVR-1 and GLVR-2 retrovirus receptor RNA were determined from total cellular RNA by RT-PCR and Hae III digestion according to Bauer et al. (1995). In this experiment, PBL were exposed either to iOK/IL-2 or OKT3/9.3-coated beads at ratios of 1 bead-per-cell (1B/C), or 3

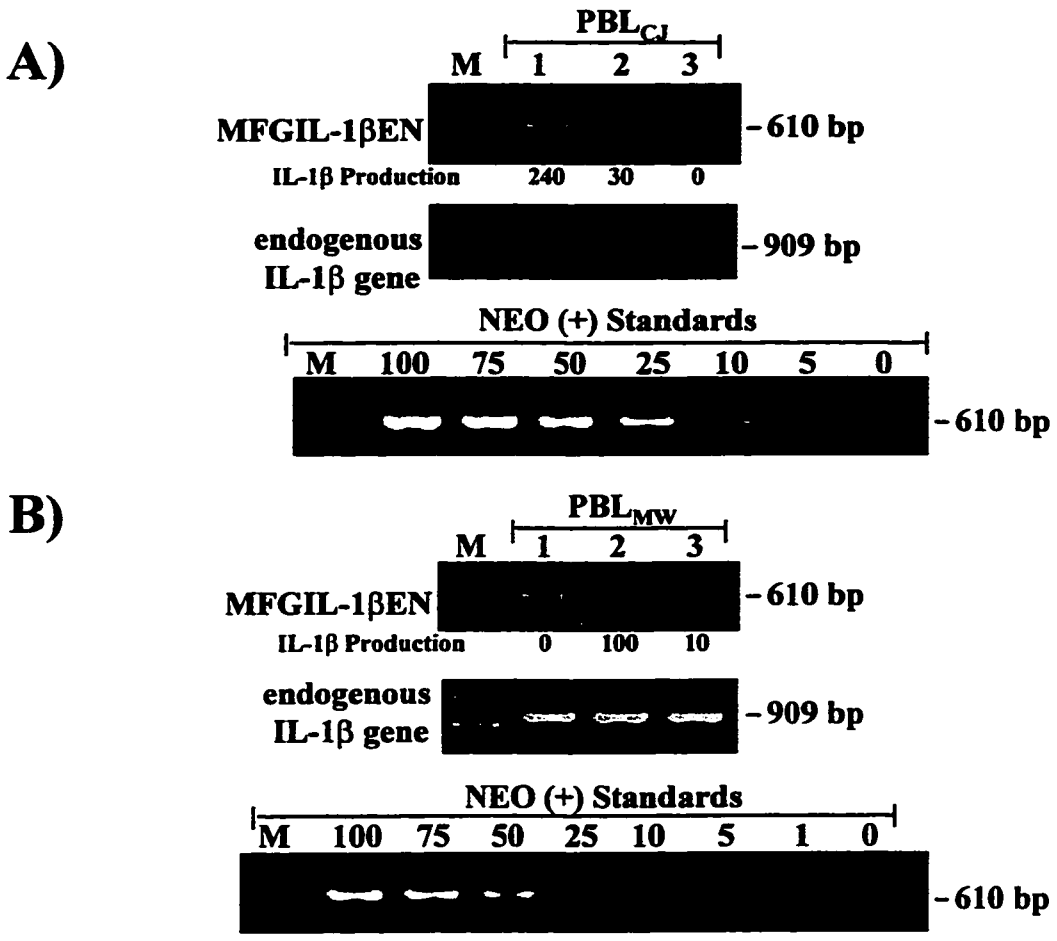

Figure 3. T Cell Transduction and Proviral Gene Expression 3 Days Post-Transduction. PBL were activated using iOK/IL-2 and transductions were performed as described in Materials and Methods. Quantitation of transduction efficiency was performed 3 days after the last exposure to virus following DNA amplification with primers specific for the NeoR gene. (A) Transduction efficiency of PBL_{CJ} cultures. Lane 1, PG13-packaged MFGIL-1βEN virus; lane 2, PA317-packaged MFGIL-1βEN virus; lane 3, mock-transduced. M, λ Hind III/ ϕX174 Hae III molecular size marker. (B) Transduction efficiencies of PBL_{MW} cultures. Lane 1, PG13-packaged MFGEN virus; lane 2, PG13-packaged MFGIL-1βEN virus; lane 3, PA317-packaged MFGIL-1βEN virus. M, λ Hind III/ ϕX174 Hae III molecular size marker. NeoR positive reference standards are indicated in both panels. Densitometric comparisons of the NeoR amplimer standard intensities with that of the samples allowed approximation of the percentage of cells containing the provirus. Samples were normalized by comparisons of band intensities derived from amplification of the endogenous IL-1β gene. For determination of IL-1β production, cultures were washed, plated in fresh media containing IL-2 (100 IU/ml) and incubated for 24 hours prior to ELISA; values are reported as pg/ml/10^6 cells/24 hours.

beads-per-cell (3B/C) for 3 days prior to RNA isolation. Total RNA was isolated from each culture, reverse transcribed and amplified using PCR primers designed to amplify sequences for both GLVR-1 and GLVR-2. The PCR amplimers were digested with Hae III, which cuts at different positions within each gene (Fig. 4A). The digested products were electrophoresed on a 12% polyacrylamide gel to separate the fragments. The results shown in Figure 4B indicate that regardless of the mAb stimulation method, 3-day PBL possess approximately 5-fold higher levels of GLVR-1 relative to GLVR-2.

2. Improved Method of T Cell Transduction.

In an attempt to improve the efficiency of retroviral-mediated gene transfer into human lymphocytes, PBL were transduced with recombinant retrovirus based on modifications of a previously described protocol (Bunnell et al., 1995). Figure 5 outlines the current transduction protocol developed in the laboratory. Briefly, this protocol combines exposure of 2 or 3-day activated PBL to high titer retrovirus supernatants (MOI 0.2-5.0), centrifugation at 1000xg for 60-90 minutes at 32°C, low temperature incubation of cells and virus for 10-12 hours at 32°C during the transduction period, and multiple exposures to retrovirus supernatant.

Further confirmation of this type of protocol comes from a study by Rudoll et al. (1996) in which two parameters important for efficient gene transfer in $CD4^+$ T lymphocytes were identified: 1) use of a centrifugation step, and 2) multiple exposures of cells to retroviral supernatant. In addition, this group reported that incubation of $CD4^+$ lymphocytes with viral supernatant 48 hours after mAb stimulation resulted in a 3- to 5-

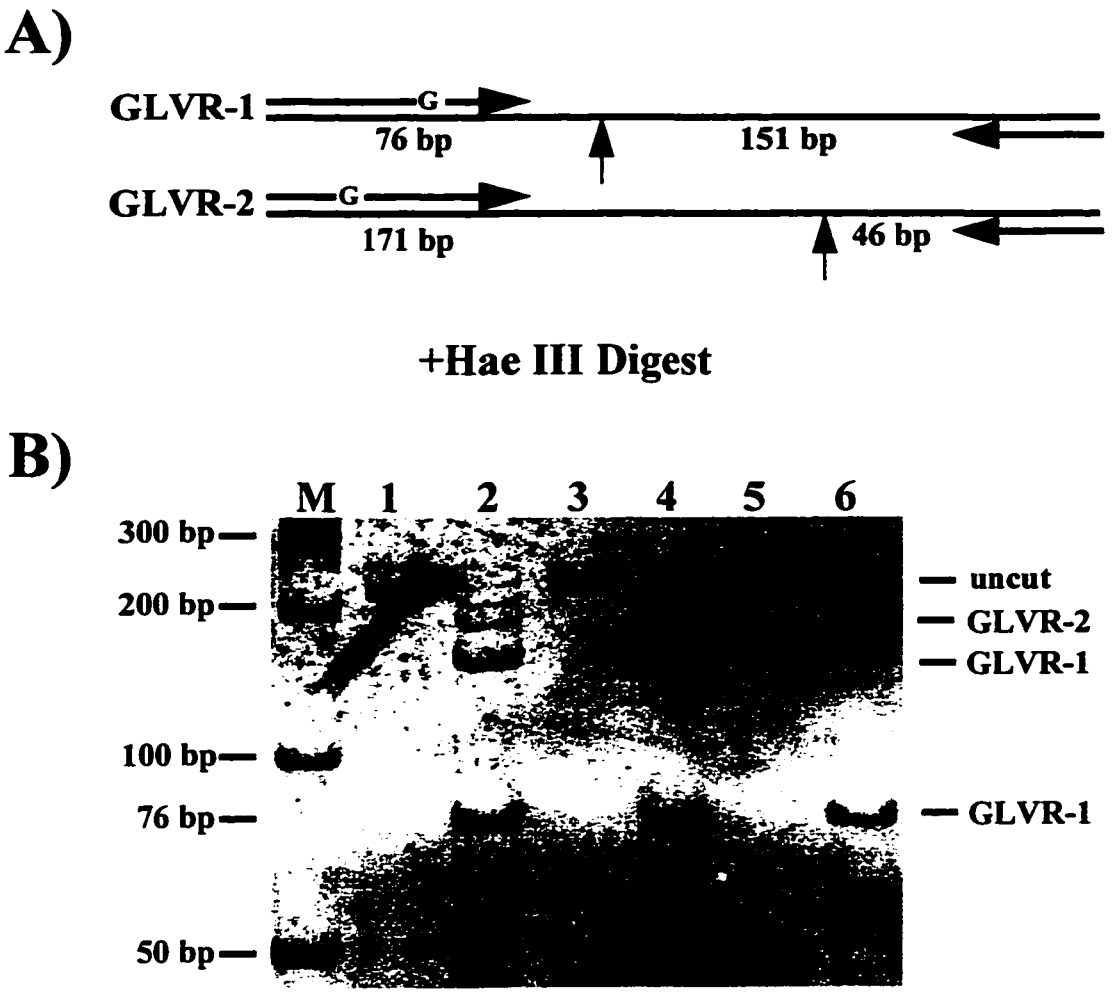

Figure 4. Analysis of GLVR-1 and GLVR-2 Receptor mRNA Expression. PBL were activated with iOK/IL-2, 1B/C, or 3B/C. RT-PCR was performed on total cellular RNA isolated from T cells following 3 days of mAb stimulation. RNA (0.5 μg) was reverse transcribed using random hexamers, and the cDNA was submitted to PCR amplification using primers designed to amplify both GLVR-1 and GLVR-2 (Bauer et al., 1995). (A) Schematic diagram depicting the nucleotide differences in the sequences of the respective receptor RNAs relative to the forward primer; the reverse primer is identical for GLVR-1 and GLVR-2 sequences. Following PCR amplification, the amplimers (230 bp) were digested with Hae III, which cuts at a different position within each gene indicated by the vertical arrows. (B) The Hae III digested products were electrophoresed on a 12% polyacrylamide gel, stained with ethidium bromide, and quantitated by densitometric analysis. Lane 1 and 2, iOK/IL-2-activated, uncut and Hae III digest, respectively; Lane 3 and 4, 1B/C-activated, uncut and Hae III digest, respectively; Lane 5 and 6, 3B/C-activated, uncut and Hae III digest, respectively. M, 50-2000 bp Ladder DNA.

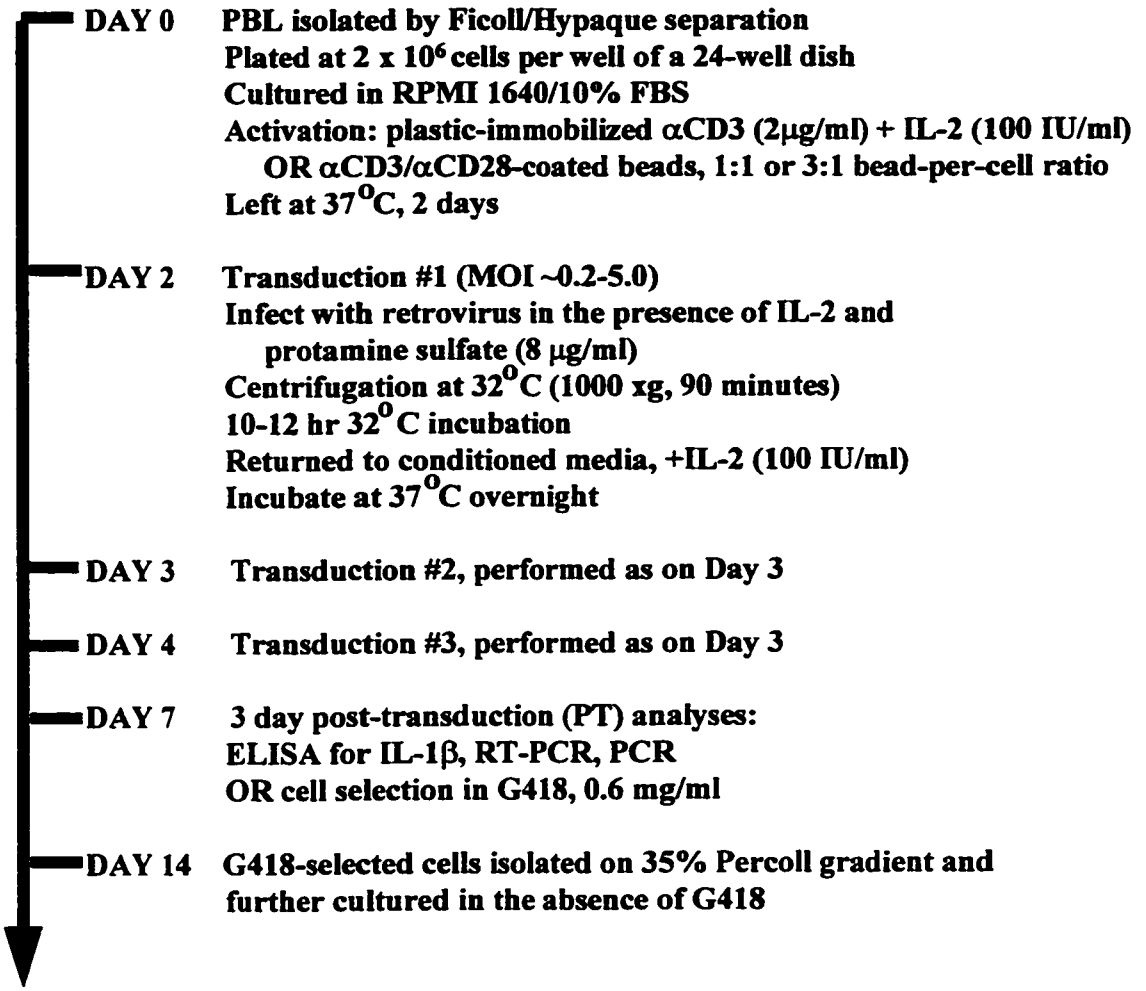

Figure 5. Protocol for Retroviral Transduction and G418 Selection of ATC. For complete protocols, refer to Materials and Methods (Isolation and Cell Culture of Lymphocytes).

fold increase in gene transfer efficiency compared to earlier and later time points (Rudoll et al., 1996). Because these findings were based on soluble mAb stimulation of purified $CD4^+$ cells, experiments were performed to compare transduction efficiencies between 48 and 72-hour whole PBL populations stimulated under our conditions with anti-CD3 and anti-CD28 mAbs co-immobilized on beads. In these studies, PBL from two normal donors were stimulated for either 48 or 72 hours (2 or 3 days, respectively) with 3B/C and subsequently transduced by multiple exposures to PG13-MFGEN retrovirus supernatant (Fig. 6A). [^{3}H]-thymidine incorporation was determined following 2 or 3 days of mAb stimulation, and PCR analysis using primers specific for the NeoR gene was performed 3 days following the last exposure to retrovirus. Figure 6B and Table 1 demonstrate that a 2- to 5-fold increase in transduction frequency was observed when transduction was initiated 48 versus 72 hours following 3B/C stimulation. Higher transduction frequencies observed in the PBL_{NG} populations may be due to higher MOI (5.0 versus 0.5 for PBL_{RS}). Proliferation analysis indicated that there was no direct correlation between [^{3}H]-thymidine incorporation and transduction efficiency. Based on the SEM between triplicate wells in these experiments (not greater than 10%), both the day 2 and the day 3 cultures showed similar rates of proliferation, although day 2 cultures show the highest levels of gene transfer.

3. Production of High-Titer Retrovirus Supernatant.

High titer retrovirus can improve transduction efficiency by enabling infection of target cells to be carried out in the presence of increased numbers of infectious virus particles per cell target (defined as the multiplicity of infection, or MOI). Historically, the

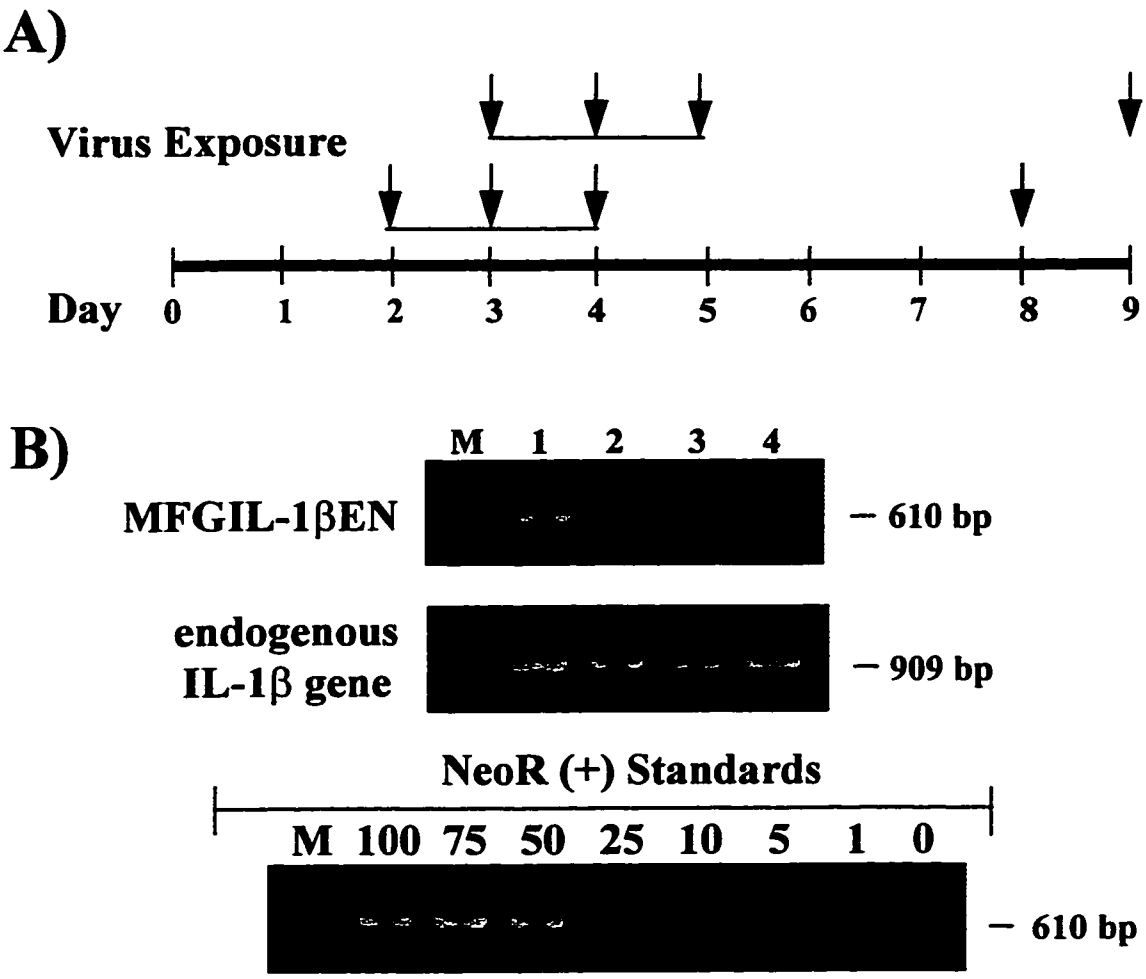

Figure 6. Comparison of Day 2 Versus Day 3 for Initiation of Transduction. (A) Schematic diagram of T cell transduction performed with PBL from 2 normal donors, PBL_{NG} and PBL_{RS}. PBL were activated for either 2 or 3 days with 3B/C and transductions were performed for 3 consecutive days as described in Materials and Methods using PG13 MFGEN retrovirus supernatant. For each culture, transduced cells were lysed for PCR analysis of proviral integrants 72 hours following the last exposure to virus. Vertical arrows indicate each exposure to retrovirus supernatant and the time of PCR analysis for each culture following virus exposure. (B) PCR analysis of transduction frequencies performed 72 hours post-transduction using primers for the NeoR gene. Lanes 1 and 3: PBL_{NG} and PBL_{RS} cultures, respectively, transduced with MFGEN retrovirus starting on day 2 following 3B/C stimulation. Lanes 2 and 4: PBL_{NG} and PBL_{RS} cultures, respectively, transduced with MFGEN retrovirus starting on day 3 following 3B/C stimulation. M, λ Hind III/ ϕX174 Hae III molecular size marker. Quantitations were determined as described in Figure 3.

Table 1. Transduction Efficiency Following 3B/C Stimulation Using PG13-MFGEN Supernatant

Experiment[a]	Activation Method	Day of Initiation	%Transduction (PCR)[b]	Proliferation (^{3}H-thy cpm)[c]
PBL_{NG}	3B/C	2	40.0	16,435
		3	20.0	20,695
PBL_{RS}	3B/C	2	5.0	16,990
		3	<1.0	27,785

[a]Summary for two independent experiments. PBL_{NG}: MOI 5.0; PBL_{RS}: MOI 0.5.
[b]PCR analysis was performed 3 days post-transduction using primers for the NeoR gene. Band intensity values were derived by densitometric scanning of gel electrophoresed, PCR products stained with ethidium bromide. Values were first normalized to the endogenous IL-1β gene as described in Materials and Methods.
[c]±Standard Error of the Mean (SEM) not greater than 10%.

most common method for production of infectious retrovirus has been by stable transfection of packaging cell lines (Miller and Rosman, 1989). PG13 and PA317 packaging cells were transfected with the viral MFGIL-1βEN DNA plasmid and individual G418-resistant clones were isolated. Approximately $2x10^5$ cells of each clone were plated onto each well of a 24-well dish. The clones were grown to confluency (2-3 days) and 24 hour supernatant samples were collected. Virus titer was determined by Mv1Lu NeoR titration and the amount of secreted IL-1β present in the supernatant was determined by ELISA.

Although infectious virus is produced by cells within 48 hours following DNA transfection, the virus titer generated by these cells is generally low. Therefore, in an attempt to create higher titer retrovirus stocks, I applied a superinfection protocol introduced by Parente and Wolfe (1996). Because infection of cells with retrovirus supernatant is much more efficient than DNA transfection, superinfection using retrovirus of another tropism (e.g. PA317) may result in the introduction of multiple proviral copies of the vector into a clone of packaging cells (PG13). For comparison, a PA317-transfected clone capable of producing high-titer retrovirus was grown to confluency in 150 mm^2 dishes. Virus supernatant was collected every 48 hours, filtered, and stored at -80°C. PG13 producer cells were superinfected with the PA317 virus stocks, plated at low density and selected in G418, and individual clones were isolated and expanded. Recombinant virus levels were determined by Mv1Lu NeoR titration and secreted levels of IL-1β were determined by ELISA.

Table 2 summarizes the relative Mv1Lu titers and the IL-1β ELISA values for the individual PG13-MFGIL-1βEN clones produced either by DNA transfection or by

Table 2. IL-1β Secretion and Virus Production in PG13-MFGIL-1βEN Producer Clones.

PG13 Clones	Mv1Lu Titer (CFU/ml)	IL-1β Concentration[a] (pg/ml/10^6 cells/24 h)
Transfected		
MFGIL-1βEN.1	1.0×10^4	43.0
MFGIL-1βEN.2	5.0×10^4	86.0
MFGIL-1βEN.4	1.0×10^5	61.0
MFGIL-1βEN.7	1.0×10^5	156.0
Superinfected		
MFGIL-1βEN.2	5.0×10^5	17,916
MFGIL-1βEN.5	4.0×10^5	32,146
MFGIL-1βEN.6	1.0×10^6	24,568
MFGIL-1βEN.11	1.0×10^6	22,893

[a]24-hour culture incubated in fresh media.

superinfection. Superinfection of PG13 cells resulted in clones capable of producing approximately 10-fold higher virus titers (10^6 versus 10^5 CFU/ml). Moreover, secretion of IL-1β was approximately 300-fold higher in the superinfected clones. These results demonstrate that superinfection is capable of introducing multiple proviral copies of the vector into a clone of producer cells, resulting in increased transcription and translation of the proviral genome. Increases in viral titer comparable to the increases in secreted IL-1β were not observed following superinfection due to the fact that there may be an upper limit of virus packaging function in these producer cells. However, 10-fold increases in viral titer were critical for optimized T cell transduction and were utilized for transduction studies under conditions of increased MOI (see discussion below).

To demonstrate that increased viral titer (and thus increased MOI) results in enhanced retroviral transduction efficiency, PBL from two normal donors (RS and NG) were activated for 2 days with 3B/C and exposed to PG13-MFGEN retrovirus stocks at two different MOIs. Transduction was performed as previously described by multiple exposures to virus over the course of three days. PCR analysis shown in Figure 7 using NeoR specific primers indicates that by 72 hours post-transduction, cultures transduced at the higher MOI of 5.0 showed transduction efficiencies of 60-70% relative to the NeoR PCR standards. This value was approximately 5- to 6-fold higher than cultures transduced at the lower MOI of 0.2. To support the PCR results, PBL_{NG} populations were cultured for an additional 6 days in G418 (0.6 mg/ml) to select for MFGEN-transduced T cells carrying the NeoR gene (Table 3). As expected, cultures transduced at an MOI of 5.0 survived better in G418 versus cultures transduced at an MOI of 0.2, shown by an increase in total viable cell expansion (52.0×10^6 versus 14.0×10^6 cells).

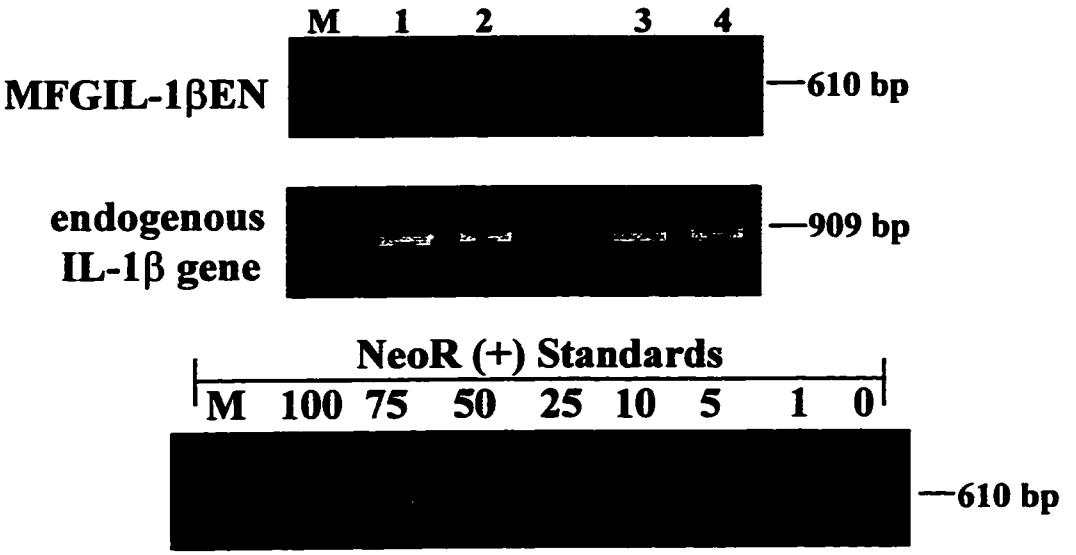

Figure 7. PCR Analysis of Transduction Frequencies in 3B/C-Activated PBL Transduced at Different Multiplicities of Infection. PBL from two normal donors (PBL_{RS} and PBL_{NG}) were activated for 48 hours with 3B/C and exposed to PG13-packaged MFGEN retrovirus stocks at MOIs of 5.0 (high) and 0.2 (low). This was achieved by the addition of 10^5 CFU and 2.5 x 10^6 CFU of virus, respectively, to 5 x 10^5 T cells. Transduction was performed as previously described by multiple exposure to virus at these MOIs over the course of 3 days. PCR analysis of transduction efficiencies was performed at 72 hours post-transduction using primers for the NeoR gene. Lanes 1 and 3: PBL_{RS} and PBL_{NG} cultures, respectively, transduced with MFGEN retrovirus at an MOI of 5.0; lanes 2 and 4; PBL_{RS} and PBL_{NG} cultures, respectively, transduced with MFGEN retrovirus at an MOI of 0.2. M, λ Hind III/ ϕX174 Hae III molecular size marker. Quantitation of transduction efficiencies was determined as described in Fig. 3.

Table 3. Transduction Efficiency in PG13-MFGEN Transduced T Cells Following High and Low MOI Viral Infection.

Samples		% Transduction (PCR)[a]	Total Viable Cells (G418 Selection)[b]
PBL_{RS}			
	5.0 MOI	69.0	nt
	0.2 MOI	14.0	nt
PBL_{NG}			
	5.0 MOI	61.0	52.0×10^6
	0.2 MOI	9.8	14.0×10^6

[a]PCR analysis was performed 3 days post-transduction using primers for the NeoR gene. Band intensity values were derived by densitometric scanning of gel electrophoresed, PCR products stained with ethidium bromide. Values were first normalized to the endogenous IL-1β gene as described in Materials and Methods.

[b]Three days post-transduction, cells were cultured in the presence of G418 (0.6 mg/ml) for 6 days. The total number of viable cells was calculated by trypan blue exclusion.

Alternative Strategies for T Cell Activation.

1. Direct Comparison of Proviral IL-1β Gene Transduction and Expression in Differentially Activated T Cell Cultures.

To assess the effects of the different T cell stimulation methods retrovirus gene transduction and proviral IL-1β expression, PBL_{CJ} and PBL_{MW} (previously activated for 3 days with iOK/IL-2 as shown in Figure 3) were co-stimulated with OKT3/9.3 coated-beads at a ratio of 3 beads-per-cell (3B/C). In separate experiments, PBL from 3 other

normal donors (PBL_{BS}, PBL_{RS}, and PBL_{NRJ}) were stimulated for 3 days with 3B/C or the more standard method of immobilized OKT3/IL-2. The activated PBL populations were transduced with PG13-MFGEN and PG13-MFGIL-1βEN retroviruses as previously described by multiple exposures to retrovirus supernatant (MOIs ranged from 0.2-5.0). Seventy-two hours after the last exposure to virus (3 days post-transduction), proviral IL-1β expression was evaluated by ELISA analysis of supernatants to determine the relative amounts of secreted IL-1β protein, and cells were lysed for PCR analysis of proviral integrants. As summarized in Table 4, relatively high levels of IL-1β were detected in all 3B/C-stimulated T cell cultures on day 3 post-transduction. The levels were 2- to 6-fold higher than those observed in populations stimulated with iOK/IL-2. No IL-1β was detected in supernatants derived from the same cultures transduced with the control MFGEN retrovirus (data not shown). Unexpectedly, equivalent transduction frequencies were observed when comparing iOK/IL-2 and 3B/C-activated cultures.

2. Characteristics of Activated T Cells Following mAb Stimulation.

Cultures were examined for Th1/Tc1 cytokine expression, mitogenic activity and cell viability following mAb stimulation with either iOK/IL-2, 1B/C, or 3B/C. Because proliferation can influence transduction frequencies, I was interested in determining the proliferation rates at a time when transduction is usually initiated. In these experiments, [^{3}H]-thymidine incorporation was analyzed following 3 days of mAb activation. In addition, it was important to determine phenotypic variation between normal donors and between cultures stimulated by the respective methods.

Table 5 shows the accumulated amounts of cytokines produced by 3-day

Table 4. Proviral IL-1β Production 3 Days Post-Transduction

Experiment[a]	Activation Method	% Transduction (PCR)[b]	IL-1β Concentration[c] (pg/ml/10^6 cells/24 h)
PBL_{CJ}	iOK/IL-2	6.0	240.0
	3B/C	5.0	500.0
PBL_{MW}	iOK/IL-2	17.0	100.0
	3B/C	20.0	570.0
PBL_{BS}	iOK/IL-2	10.0	90.0
	3B/C	10.0	310.0
PBL_{RS}	iOK/IL-2	40.0	1830.0
	3B/C	40.0	3160.0
PBL_{NRJ}	iOK/IL-2	25.0	860.0
	3B/C	25.0	1900.0

[a]PBL were transduced with PG13-MFGEN and MFGIL-1βEN retroviruses. No IL-1β was detected in supernatants derived from MFGEN-transduced populations.
[b]PCR analysis was performed 3 days post-transduction using primers for the NeoR gene. Band intensity values were derived by densitometric scanning of gel electrophoresed, PCR products stained with ethidium bromide. Values were first normalized to the endogenous IL-1β gene as described in Materials and Methods.
[c]24-hour culture plated 3 days after the last exposure to virus in fresh media plus IL-2.

stimulated, non-transduced PBL populations obtained from three normal donors. Co-stimulation with 3B/C results in extremely high levels of secreted Th1/Tc1 cytokines (IL-2, IFN-γ, and TNF-α) as compared to stimulation with iOK/IL-2 or 1B/C. The inability of the lower bead-to-cell ratio (1B/C) to induce higher cytokine secretion levels is most likely a consequence of less surface exposure between mAb-coated beads and cells, which would decrease the overall strength of co-stimulatory signaling. In addition, there were slightly elevated levels of the Th2/Tc2 cytokine IL-4 following 3B/C stimulation. These values are similar to those seen by Levine et al. in purified $CD4^+$ and $CD8^+$ cultures exposed to 3B/C (Levine et al., 1995; Levine et al., 1996). Moreover, although cytokine levels varied dramatically with the method of mAb stimulation, no overt differences were observed in proliferation rates when monitored by [^{3}H]-thymidine incorporation on day 3 of activation (Table 5). Cell viabilities for each population were equivalent by trypan blue exclusion (80-90% viable).

RT-PCR analysis for IL-2 transcripts present in 3-day stimulated PBL demonstrated significantly greater amounts of IL-2 RNA in 3B/C-stimulated cultures, corresponding with increased IL-2 secretion (Fig. 8, lane 5). PBL populations activated with iOK/IL-2 and 1B/C displayed similar levels of IL-2 transcripts that were substantially lower than the 3B/C-activated culture (Fig. 8, lanes 2 and 4). By day 3 of activation, no IL-2 was detected in PBL activated with immobilized OKT3 alone (Fig. 8, lane 1), and the corresponding IL-2 RNA was barely detectable. Co-stimulation with iOKT3 and soluble 9.3 appeared to enhance IL-2 expression, but the amount was much less than that observed with 3B/C (Fig. 8, lane 3).

Table 5. Characteristics of 3-Day Stimulated PBL.

PBL Population	Activation Method	3-day Cytokine Accumulation (pg/ml/10^6 cells)				Proliferation (^{3}H-thy cpm)[b]
		IL-2	IFN-γ	TNF-α	IL-4	
PBL_{MW}	iOK/IL-2[a]	170	12,400	8,035	0	22,000
	1B/C	180	19,000	2,300	20	18,185
	3B/C	33,900	107,300	11,400	80	20,734
PBL_{NRJ}	iOK/IL-2	100	38,400	4,100	0	22,257
	1B/C	10	9,100	800	0	18,946
	3B/C	16,000	173,500	11,500	300	25,170
PBL_{RS}	iOK/IL-2	70	42,000	4,400	0	29,048
	1B/C	10	21,700	1,000	0	18,503
	3B/C	11,800	139,300	9,000	70	31,526

[a]iOK/IL-2 cultures were initiated in the presence of 100 IU/ml IL-2.
[b]±SEM not greater than 10%.

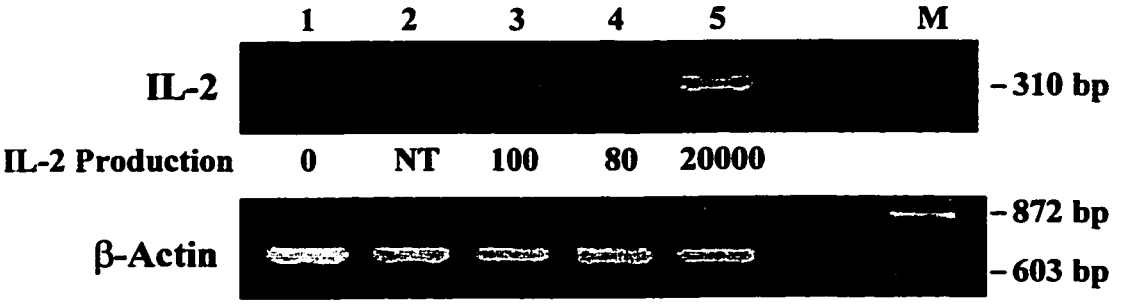

Figure 8. Analysis of IL-2 Expression in 3-Day PBL Cultures Activated by Different mAb Stimulation Methods. RT-PCR analysis was performed on total cellular RNA isolated from activated PBL_{MW} cultures using PCR primers specific for human IL-2 or β-actin cDNA. β-actin RNA was RT-PCR amplified for the purpose of normalization. PBL were activated using: lane 1, immobilized OKT3 only; lane 2, iOK/IL-2; lane 3, immobilized OKT3 and soluble 9.3 mAb (1 mg/ml); lane 4, 1B/C; lane 5, 3B/C. M, λ Hind III/ ϕX174 Hae III molecular size marker. ELISA determinations for IL-2 protein were performed using 3-day accumulated supernatants. Values are reported as pg/ml/10^6 cells. NT, not tested.

CHAPTER IV

FATE OF PROVIRAL GENE EXPRESSION IN ACTIVATED T LYMPHOCYTES

INTRODUCTION

The success of gene therapy strategies targeting T lymphocytes depends not only on the percentage of cells that can be transduced *ex vivo*, but also on stable, long-term expression of the exogenous gene in physiologically relevant quantities. A further consideration is the potential effect of biological T cell activity on the expression status of the introduced proviral gene. In the following studies, retrovirus-mediated gene transfer and the fate of proviral gene expression were evaluated in human T cells activated using: 1) immobilized anti-CD3 mAb plus IL-2, or 2) co-stimulation using beads carrying co-immobilized anti-CD3 and anti-CD28 mAbs. By crosslinking the CD3 and CD28 receptors, these mAbs mimic *in vivo* signaling events, leading to cytokine production and proliferation. Several biological characteristics in differentially activated T cell cultures were examined to determine whether proviral-encoded IL-1β expression corresponded with the activation status of the transduced T cell.

RESULTS

Proviral IL-1β Gene Expression in Differentially Activated T Cells.

To assess the effects of the different T cell stimulation methods on proviral gene expression early after virus exposure and in long term cultures, PBL were stimulated for 3 days with iOK/IL-2 or OKT3/9.3-coated beads at bead to cell ratios of 1B/C or 3B/C.

The activated PBL populations were transduced by multiple exposures to PG13-packaged MFGEN or MFGIL-1βEN retroviruses over a subsequent 3-day period. The fate of proviral IL-1β gene expression was evaluated by ELISA analysis of supernatants at various times post-transduction to determine the relative levels of secreted proviral IL-1β protein. In addition, IL-1β protein expression was determined for activated T cells that had undergone selection in G418 for enrichment of MFGEN and MFGIL-1βEN-transduced cells.

Table 6 shows ELISA data for two experiments of retrovirus-transduced PBL (MOI 0.5) from the same normal donor, PBL_{MW}. IL-1β levels were analyzed on days 3 and 12 following the end of the transduction period (days 7 and 15 following culture initiation, respectively). Flow cytometry for the T cell marker CD3 has routinely indicated that greater than 95% of the PBL are $CD3^+$ by day 7 after activation, regardless of the mAb stimulation method. Similar to the data presented in Chapter III (Table 4), relatively high levels of secreted IL-1β were observed in the 3B/C-stimulated T cell cultures by day 3 post-transduction. The levels were 5- to 10-fold higher than those observed in transduced cultures exposed to iOK/IL-2 or 1B/C. No IL-1β was detected in supernatants derived from populations that were transduced with the control MFGEN retrovirus. By day 12 post-transduction, all non-selected (-G418) cultures showed a decrease in IL-1β production with the most dramatic decline observed in the 3B/C-activated cultures. Based on IL-1β ELISA data derived from populations cultured with and without G418, varying degrees of transduced cell enrichment occurred after G418 selection.

The decline in IL-1β expression observed in cultures stimulated with iOK/IL-2

and 3B/C was more carefully examined by analyzing transduced PBL supernatants for IL-1β expression at several time points post-transduction. In this experiment, cells derived from two other normal donors, PBL_{NG} and PBL_{JK}, were transduced (MOI 4.0) and supernatants were analyzed by ELISA for IL-1β expression at 24-hour intervals over the first 4 days following transduction and on day 12 (Table 7). For both donor populations, the highest levels of IL-1β production were seen at the earliest time point, day 1. Although both stimulated populations showed a loss in protein expression over time (40-70% by day 4 and 80-90% by day 12), overall higher levels of proviral expression were again observed in the early 3B/C-activated cultures.

Table 6. Proviral IL-1β Production Post-Transduction

Experiment[a]	Activation Method	IL-1β Concentration[b] (pg/ml/10^6 cells/24 hours)		
		Day 3	Day 12 (-G418)	Day 12 (+G418)
#1 PBL_{MW}	iOK/IL-2	200	125	370
	1B/C	100	50	220
	3B/C	990	130	1820
#2 PBL_{MW}	iOK/IL-2	240	80	720
	1B/C	440	80	1450
	3B/C	2400	95	1030

[a]PBL were transduced with PG13-MFGEN and MFGIL-1βEN retroviruses. No IL-1β was detected in supernatants derived from MFGEN-transduced populations.
[b]24-hour culture plated on indicated day in fresh media plus IL-2 (100 IU/ml).

Table 7. Decline in Proviral IL-1β Expression Post-Transduction

PBL Population[a]	Activation Method	IL-1β Concentration[b] (pg/ml/10^6 cells/24 hours) Day 1	Day 2	Day 3	Day 4	Day 12
PBL_{NG}	iOK/IL-2	4,900	2,950	1,560	1,480	670
	3B/C	13,500	11,000	7,010	6,690	1,950
PBL_{JK}	iOK/IL-2	3,290	1,540	1,260	1,070	380
	3B/C	5,380	4,930	4,900	3,320	460

[a]PBL were transduced with PG13-MFGEN and MFGIL-1βEN retroviruses. No IL-1β was detected in supernatants derived from MFGEN-transduced populations.
[b]24-hour culture plated on indicated day in fresh media plus IL-2 (100 IU/ml).

To approximate the percentage of T cells positive for proviral integration, PCR analysis was performed on each of the activated, transduced populations (#1 PBL_{MW}, PBL_{NG}, PBL_{JK}). In the #1 PBL_{MW} populations at 3 days post-transduction, all cultures transduced with the MFGIL-1βEN virus had comparable transduction frequencies of approximately 10 percent relative to the NeoR PCR standards (Fig. 9, lanes 2-4). In addition, PBL_{NG} and PBL_{JK} cells examined at 3 days post-transduction showed equivalent transduction frequencies (approximately 75%) irrespective of the mAb activation method (Fig. 10). The transduction frequency was much greater in these later populations most likely due to the higher MOI (4.0 versus 0.5 for #1 PBL_{MW}). In

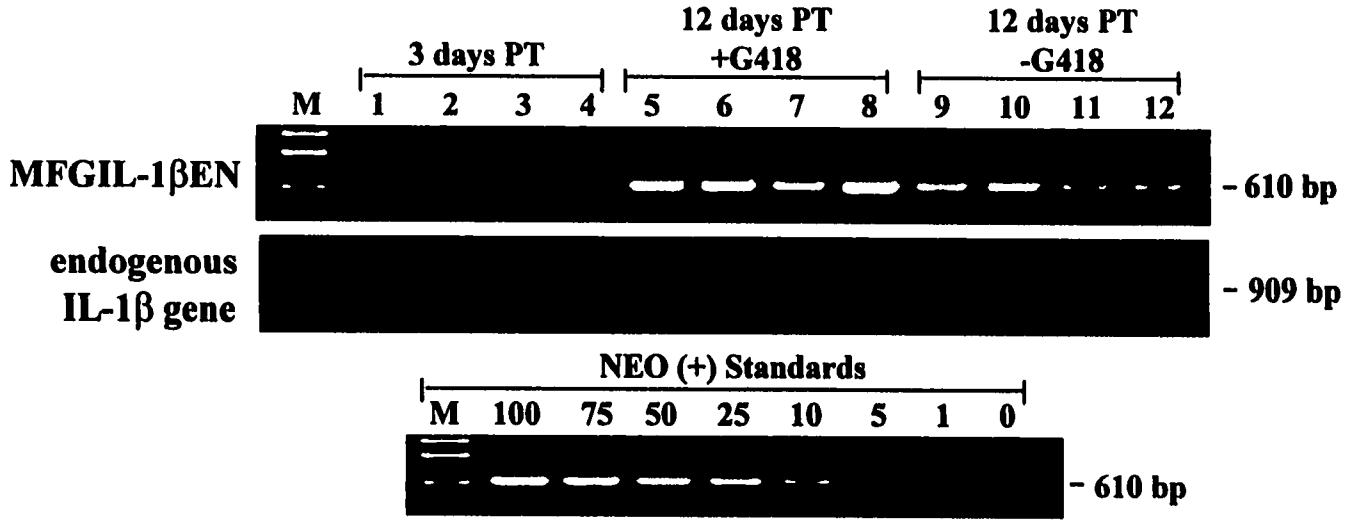

Figure 9. PCR Analysis of T Cell Transduction Efficiencies in iOK/IL-2, 1B/C, and 3B/C-Activated Cultures. Quantitation of transduction efficiency was performed following DNA amplification with primers specific for the NeoR gene. Transduction efficiencies of PBL_{MW} cultures were performed at 3 days and 12 days post-transduction. Transduced populations were cultured in the presence (0.6 μg/ml) or absence of G418, as described in Material and Methods. Lanes 1, 5, and 9: 3:1 bead-activated, MFGEN virus, lanes 2, 6, and 10: iOK/IL-2-activated, MFGIL-1βEN virus; lanes 3, 7, and 11: 1B/C-activated, MFGIL-1βEN virus; lanes 4, 8, and 12: 3B/C-activated, MFGIL-1βEN virus. M, λ Hind III/ ϕX174 Hae III molecular size marker. Quantitations were determined as described in Figure 3.

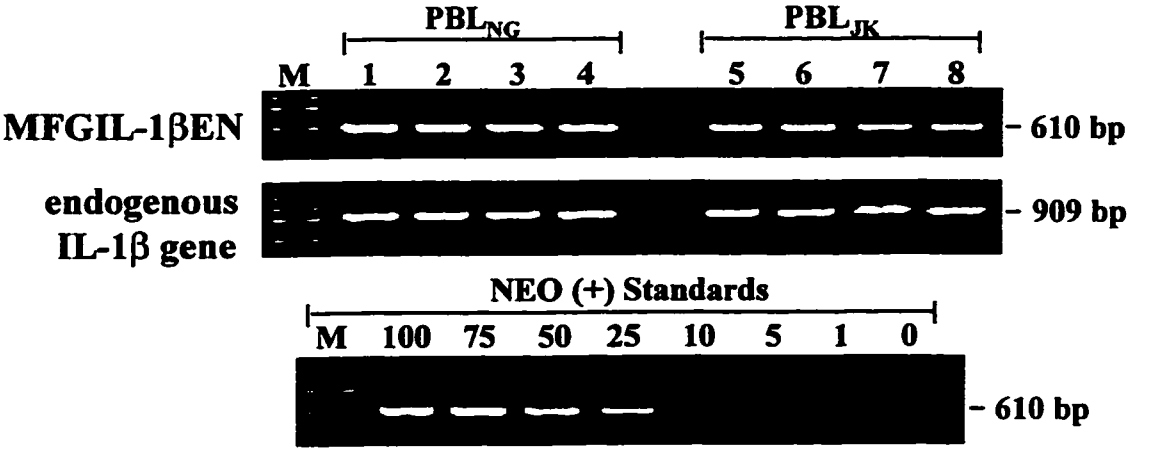

Figure 10. PCR Analysis of T Cell Transduction Efficiencies. Quantitation of PBL_{NG} and PBL_{JK} transduction efficiencies were performed at 3 days post-transduction following DNA amplification with primers specific for the NeoR gene. Lanes 1 and 5: iOK/IL-2-activated, MFGEN virus; lanes 2 and 6: iOK/IL-2-activated, MFGIL-1βEN virus; lanes 3 and 7: 3B/C-activated, MFGEN virus; lanes 4 and 8: 3B/C-activated MFGIL-1βEN virus. M, λ Hind III/ ϕX174 Hae III molecular size marker. Quantitations were determined as described in Figure 3.

addition, higher levels of secreted IL-1β were observed in the PBL_{NG} and PBL_{JK} populations relative to the PBL_{MW} cultures (compare day 3 post-transduction analyses, Tables 6 and 7), which appeared to reflect the increased number of proviral genomes. These results demonstrate that the higher levels of IL-1β protein observed in the 3B/C-activated cultures were not due to enhanced retroviral gene transfer. Moreover, a similar transduction frequency was observed in the 3B/C-activated cultures transduced with the control MFGEN vector (Figs. 9 and 10). Because PCR analysis of proviral integrants is performed using an identical number of cell equivalents, similar transduction frequencies for MFGEN and MFGIL-1βEN cultures demonstrate that expression of vector-derived IL-1β does not result in the preferential elimination of gene-modified cells.

Following 12 days of culture, non-selected (-G418) #1 PBL_{MW} populations showed an equivalent percentage of cells carrying the MFGEN or MFGIL-1βEN provirus as compared to those analyzed at 3 days post-transduction (Fig. 9, lanes 9-12). Yet, at this time point, there was a general decrease in the amounts of detected proviral IL-1β protein in these non-selected cultures with the most obvious decline occurring in the 3B/C-stimulated culture (Table 6). After G418 enrichment and culturing for 12 days, 75-100 percent of the cells in each population appeared to possess the provirus (Fig. 9, lanes 5-8). Relative to day 12 populations cultured without G418, this enrichment was accompanied by increased amounts of secreted IL-1β (Table 6). However, although G418 selection resulted in a 7- to 10-fold enrichment of vector-positive cells compared to day 3 cultures, comparable increases in IL-1β production were not observed. At most, IL-1β levels were enhanced only about 2-fold. These findings clearly demonstrate that the augmented IL-1β production observed in the 3B/C-stimulated cultures was not the result of enhanced

retroviral gene transfer. Moreover, proviral integrants appear to be maintained at similar levels over time in the respective T cell populations, but proviral protein expression declines.

Proviral IL-1β Gene Expression Corresponds With the Activation Status of the T Cell.

1. Proviral IL-1β and Endogenous IFN-γ Gene Expression at 3 Days Post-Transduction.

Early after activation and transduction, 3B/C co-stimulation resulted in extremely high levels of endogenous cytokines (IFN-γ, IL-2, and TNF-α) in 3-day activated PBL cultures and significantly increased amounts of proviral IL-1β protein at early time points post-transduction (refer to Chapter III, Tables 4 and 5). Together, the data suggested a heightened activation status early following 3B/C exposure that would be reflected in augmented expression of both the proviral IL-1β gene and endogenous cytokines. The expression levels of IFN-γ and proviral IL-1β were compared at 3 days post-transduction for the respective #1 PBL_{MW} populations denoted in Table 6. Total RNA was isolated for RT-PCR analysis and supernatants were re-examined by ELISA for the presence of secreted IFN-γ. In these studies, IFN-γ was chosen as an indicator of endogenous cytokine expression rather than IL-2 because exogenous IL-2 was supplied to all cultures to maintain cell viability and growth. Figure 11 demonstrates that at 72 hours post-transduction, 3B/C co-stimulation resulted in the production of substantially higher levels of IFN-γ which correlated with increased levels of secreted IL-1β observed in these cultures (Fig. 11, lane 4). RT-PCR analysis confirmed the augmented production of the

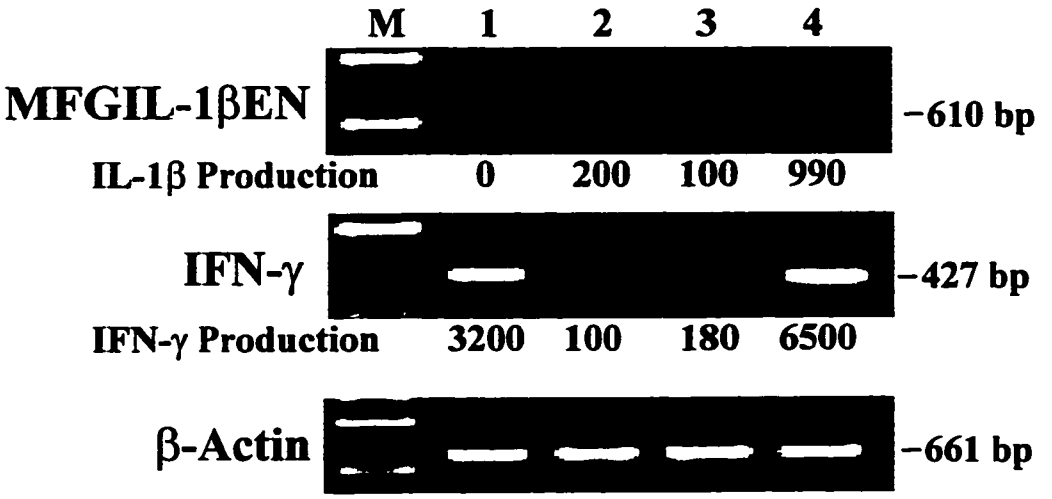

Figure 11. Expression of the MFG Provirus and Endogenous IFN-γ in Transduced T Lymphocytes at 3 Days Post-Transduction. RT-PCR was performed using total cellular RNA isolated from transduced T cells (PBL_{MW}) on day 3 following the last virus exposure. PCR primers were specific for NeoR, IFN-γ, or β-actin cDNA. Samples were normalized by RT-PCR amplification of β-actin RNA. M, λ Hind III/ ϕX174 Hae III molecular size marker. Lane 1, 3B/C activation, MFGEN virus; lane 2, iOK/IL-2 activation, MFGIL-1βEN virus; lane 3, 1B/C activation, MFGIL-1βEN virus; lane 4, 3B/C activation, MFGIL-1βEN virus. For determination of IL-1β and IFN-γ production, cultures were washed, plated in fresh media containing IL-2 (100 IU/ml) and incubated for 24 hours prior to ELISA analysis; values are reported as pg/ml/10^6 cells/24 hours.

respective cytokines corresponded with increased amounts of both MFGIL-1βEN and IFN-γ transcripts in the 3B/C cultures relative to iOK/IL-2 and 1B/C cultures (Fig. 11). Moreover, T cells activated with 3B/C and transduced with the MFGEN retrovirus, lacking the IL-1β cDNA insert, produced high amounts of IFN-γ and displayed increased RNA levels for both IFN-γ and the provirus (Fig. 11, lane 1). In addition, ELISA analysis indicated enhanced production of IL-2 at this time point for 3B/C cultures (data not shown).

2. Phenotype Analysis: Correlation with CD25 Expression.

To determine phenotypic variation between normal donors and between cultures stimulated by the respective methods, non-transduced PBL obtained from 5 normal donors were analyzed by Fluorescence Activated Cell Sorting (FACS) (Table 8). In these studies, FACS analysis was performed prior to activation on day 0 (baseline) and following 7 days of continuous culture, which is the time point equivalent to the 72 hour post-transduction samples. Although baseline (day 0) $CD3^+$ percentages vary between normal donors, all populations regardless of the method of mAb stimulation were greater than 95 percent $CD3^+$ by day 7 of culture. Also by day 7, 4/5 cultures stimulated with iOK/IL-2 showed a reduction in the number of $CD4^+$ lymphocytes and an increase in the percentage of $CD8^+$ lymphocytes compared to baseline values. These cultures, which were continuously grown in 100 IU/ml IL-2, were generally greater than 60 percent $CD8^+$ by day 7. In contrast, 5/5 cultures stimulated with 3B/C resulted in an overall increase in the percentage of $CD4^+$ lymphocytes by day 7, creating populations with nearly equal numbers of $CD4^+$ and $CD8^+$ lymphocytes. Day 14 phenotype analysis shown for #2

Table 8. Phenotype Analysis of Non-Transduced Control Cells.

PBL Population/ Surface Marker		Baseline[a] (day 0)	iOK/IL-2 (day 7)	3B/C (day 7)	iOK/IL-2 (day 14)	3B/C (day 14)
NRJ						
	CD3	59.3	99.1	98.1		
	CD4	22.7	14.0	55.2		
	CD8	51.7	86.0	44.8		
	Ratio	0.4	0.2	1.2		
BS						
	CD3	86.4	97.6	98.7		
	CD4	58.9	37.0	69.6		
	CD8	17.0	63.0	30.4		
	Ratio	3.5	0.6	2.3		
NG						
	CD3	87.4	99.9	99.7		
	CD4	50.1	30.7	52.9		
	CD8	24.4	69.3	47.1		
	Ratio	2.0	0.4	1.1		
JK						
	CD3	77.7	99.8	99.5		
	CD4	50.6	59.1	67.7		
	CD8	14.2	40.9	32.3		
	Ratio	3.6	1.4	2.1		
RS_1						
	CD3	72.8	99.6	99.6		
	CD4	43.5	34.4	62.0		
	CD8	27.9	65.6	45.9		
	Ratio	1.6	0.5	1.3		
RS_2						
	CD3	65.0	99.3	99.6	99.8	99.7
	CD4	46.9	41.6	62.6	26.3	47.1
	CD8	20.3	58.4	37.4	73.7	52.9
	Ratio	2.3	0.7	1.7	0.4	0.9

[a]Phenotype analysis was performed immediately following PBL isolation.

PBL_{RS} was representative of results from several normal donors. These results demonstrate that the general trends observed for cultures at day 7 were also observed following 14 days of culture. In iOK/IL-2 cultures, the majority of cells were $CD8^+$ (73.7%). Alternately, after 14 days of bead-stimulation, 3B/C-stimulated cultures maintained nearly equal numbers of $CD4^+$ and $CD8^+$ lymphocytes.

Originally, I was interested in determining whether the presence of proviral-encoded IL-1β in the conditioned media of transduced MFGIL-1βEN cultures would influence the cellular phenotype of iOK/IL-2 or 3B/C-stimulated cultures. Table 9 shows the phenotype data for transduced populations from two normal donors, PBL_{NG} and PBL_{JK}. For these studies, PBL were stimulated by the respective methods and transduced as previously described by multiple exposures to PG13-MFGEN or MFGIL-1βEN retroviruses (MOI 5.0). PBL were analyzed by FACS analysis prior to mAb stimulation (day 0) and again on day 7 (72 hours post-transduction). Also on day 7, supernatants were collected and analyzed by ELISA for the presence of proviral-encoded IL-1β. In these studies, vector-derived IL-1β was present in relatively high amounts at day 7. Similar to the data presented in Table 8, PBL_{NG} and PBL_{JK} cultures stimulated with 3B/C had a higher percentage of $CD4^+$ cells relative to iOK/IL-2 populations. The ratio of $CD4^+/CD8^+$ cells was not altered by the presence of IL-1β in the respective cultures.

Also shown in Table 9 is the expression of CD25, the low affinity α-subunit of the human IL-2 receptor. Following mAb stimulation of T lymphocytes, surface expression of CD25 increases (Leonard et al., 1982; Depper et al., 1984) and becomes associated in a trimolecular complex with the β and γ subunits to create a high affinity IL-2 receptor (Greene and Leonard, 1986). In this fashion, we have used CD25 surface

protein as a marker for the activation status of T cells. In the iOK/IL-2 and 3B/C populations, IL-1β did not alter the percentage of T cells positive for CD25 nor influence the number of receptor molecules per cell as indicated by the mean fluorescence intensity (MFI). However, it was obvious that although both iOK/IL-2 and 3B/C cultures had a greater percentage of $CD25^+$ lymphocytes relative to day 0 (baseline), 3B/C cultures had approximately 20% more cells expressing CD25 and the amount per cell was 7- to 9-fold higher than iOK/IL-2 activated cells. Using surface CD25 expression as another marker for the status of T cell activation, these results demonstrate that increased IL-1β protein production correlates with the increased activation status observed in T cell cultures following co-stimulation with 3B/C.

Table 9. Phenotype Analysis of MFGEN and MFGIL-1βEN Transduced Cultures.

PBL Population/ T Cell Surface Marker	Baseline (day 0)	iOK/IL-2 (day 7) MFGEN	iOK/IL-2 (day 7) MFG IL-1βEN	3B/C (day 7) MFGEN	3B/C (day 7) MFG IL-1βEN
PBL_{NG} IL-1β Concentration[a]		0	2,948	0	11,032
CD25 (MFI)[b]	8.3 (30.0)	74.9 (91.0)	78.6 (109.0)	99.6 (932.0)	99.7 (952.0)
CD4	50.6	23.2	27.2	53.4	59.3
CD8	26.7	76.8	72.8	46.6	40.7
CD4/CD8	1.9	0.3	0.4	1.1	1.5
PBL_{JK} IL-1β Concentration[a]		0	1,540	0	4,929
CD25 (MFI)[b]	33 (30.0)	78.7 (130)	79.8 (124.0)	99.3 (862.0)	99.0 (721.0)
CD4	51.3	51.7	49.7	80.9	80.7
CD8	12.8	48.3	50.3	19.1	19.3
CD4/CD8	4.0	1.1	1.0	4.2	4.2

[a]Values are reported as pg/ml/10^6 cells/24 h at day 7 of culture (72 h post-transduction).
[b]MFI; mean fluorescence intensity. Represents the number of molecules per cell.

3. Effects of Cell Supernatants from iOK/IL-2 and 3B/C-Activated Cultures on Proviral Gene Expression.

To determine whether secreted products present in the supernatants of 3B/C-activated cultures could modulate proviral gene expression, PBL_{MW} populations were stimulated with iOK/IL-2 and 3B/C for 2 days and subsequently transduced with PG13-packaged MFGIL-1βEN retrovirus (MOI 4.0). After the last exposure to virus on day 4, transduced PBL were cultured in the presence of conditioned media (CM) obtained from non-transduced PBL_{MW} cells previously stimulated with either 3B/C or iOK/IL-2 (Fig. 12A). After a 3-day incubation in the respective CM (8 days following initial activation), several parameters were compared, including proviral IL-1β expression, the production of an endogenous cytokine (IFN-γ), and cell growth characteristics (Fig. 12B). In addition, to determine if relatively high levels of proviral IL-1β product altered characteristics of 3B/C cultures, cells activated with 3B/C and transduced with PG13-MFGEN were analyzed.

Irrespective of the supplied CM, iOK/IL-2 populations secreted relatively low levels of proviral IL-1β and endogenous IFN-γ while similarly transduced 3B/C cultures displayed high amounts of both proteins (Fig. 12B, upper panels). Furthermore, 3B/C cultures exposed to the control MFGEN virus produced increased levels of endogenous IFN-γ comparable to identical cells receiving the MFGIL-1βEN vector. PCR analysis indicated transduction efficiencies of approximately 40% for each population (data not shown).

After incubation with either CM type, transduced iOK/IL-2-activated cultures showed the greatest expansion over the 3-day incubation period, and also, possessed the

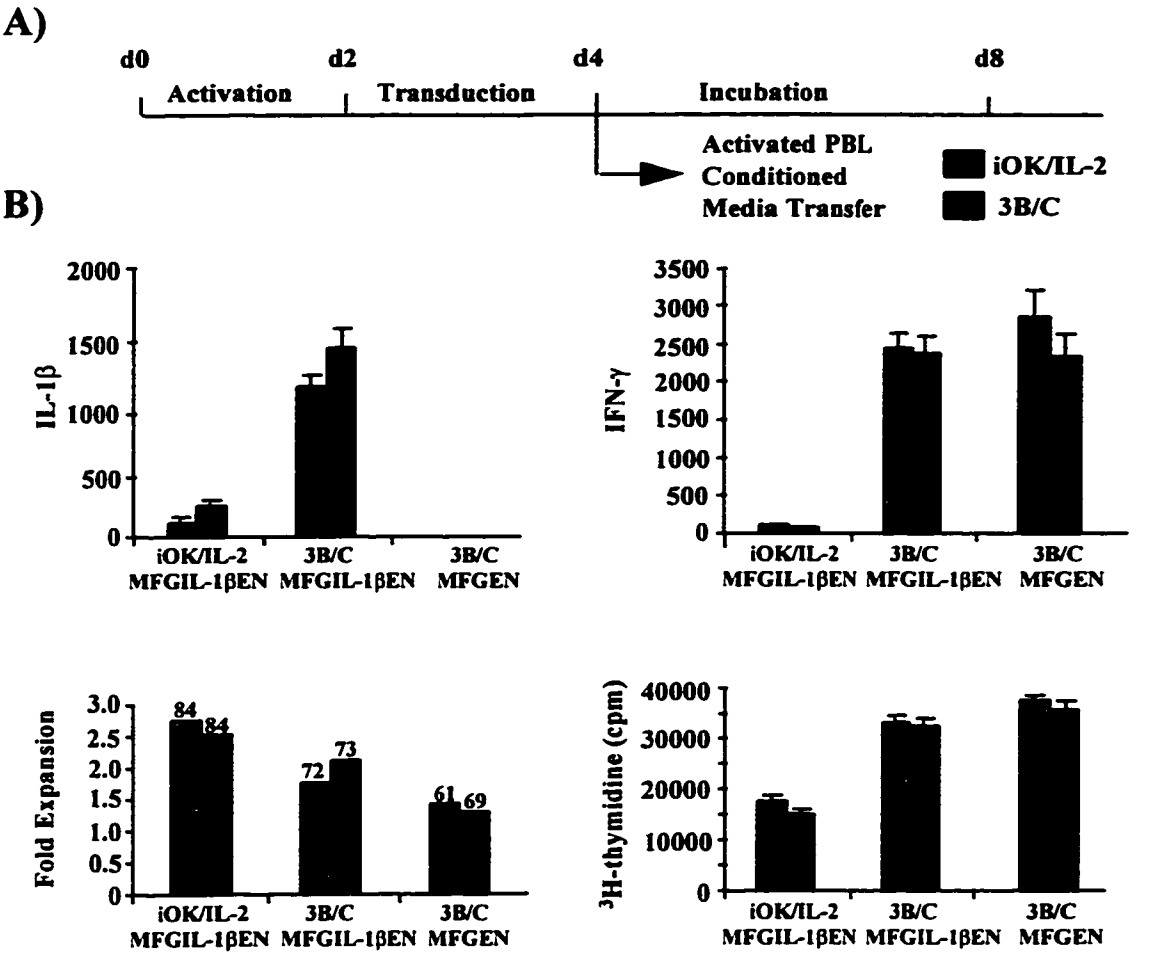

Figure 12. Lack of Effects of Activated PBL Conditioned Media (CM) on Proviral Gene Expression and T Cell Properties. (A) PBL_{MW} cells were activated for 2 days using either 3B/C or iOK/IL-2 and exposed to virus for 3 consecutive days. At the end of the transduction period (day 4), cells were replated in CM derived from 4-day cultures of non-transduced PBL_{MW} cells activated with either iOK/IL-2 or 3B/C. CM was diluted 1:2 with fresh media containing 200 IU/ml IL-2 prior to addition to the respective cultures. On day 8 (3 days post-transduction), the cultures were subjected to analysis. (B) Upper panels, ELISA determinations for IL-1β and IFN-γ production. Values are reported as pg/ml/10^6 cells/24 hours; error bars are the mean ± SEM of duplicate wells. Lower left panel, fold-expansion of individual cultures over the 4-day incubation period. Percent cell viabilities are indicated above each bar. Lower right panel, proliferation assays based on [^{3}H]-thymidine incorporation. Results are reported as the mean cpm incorporated ± SEM of triplicate wells.

highest number of viable cells (Fig. 12B, lower left panel). Although overall cell expansion was less for 3B/C-activated cells transduced with the MFGIL-1βEN or MFGEN vector, [^{3}H]-thymidine incorporation was approximately 2-fold higher regardless of either the CM or the presence of IL-1β (Fig. 12B, lower right panel). The distinct growth characteristics displayed by the differentially activated cultures contrasts with the 3-day stimulated PBL populations (prior to transduction), which showed similar growth properties (Chapter III, Table 5). Overall, the results of this CM "switch" experiment indicate that in early transduced T cell cultures neither proviral gene expression nor the patterns of cytokine expression and growth are directly influenced by paracrine effects of secreted factors. Moreover, 3B/C co-stimulation results in a higher T cell activation status as defined by higher mitogenic activity and increased endogenous cytokine gene expression and protein secretion. The expression of the proviral IL-1β gene correlates with this heightened state of T cell activation.

4. Analysis of Proviral IL-1β and Endogenous IFN-γ Production From Transduced Cultures Following Magnetic Bead Removal.

To investigate the effects of early bead removal on retroviral gene transduction and proviral IL-1β expression in OKT3/9.3-bead activated cultures, PBL_{NG} were stimulated for 2 days with iOK/IL-2 or OKT3/9.3-coated beads at a ratio of 1 bead per cell (1B/C) and transduced by multiple exposures to PG13-MFGEN and MFGIL-1βEN vectors (MOI 5.0). Transduced populations were either continuously cultured in the presence of beads, or exposed to a hand held magnet at day 5 of culture (24 hours post-transduction) to remove the beads (Fig. 13A). Proviral IL-1β and endogenous IFN-γ

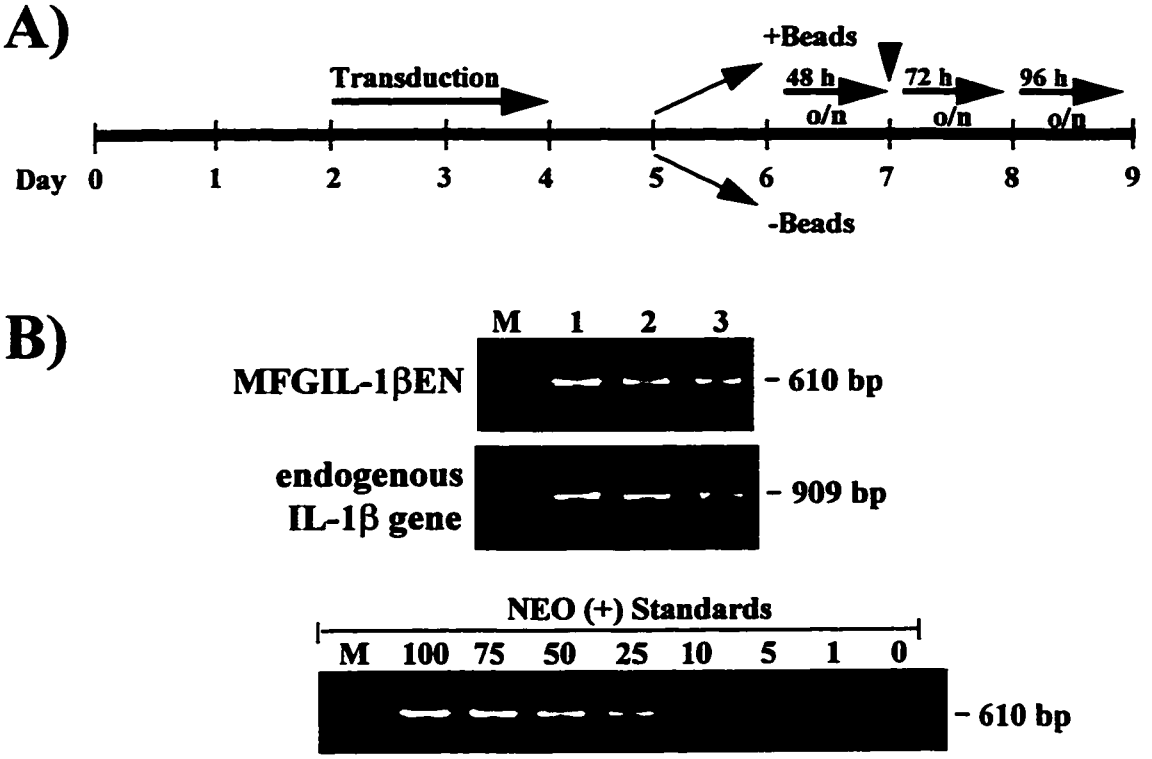

Figure 13. Comparison of T Cell Transduction With and Without Magnetic Bead Removal. (A) Timeline of events for comparison of IL-1β and IFN-γ secretion in transduced T cell cultures with and without early bead removal. Briefly, PBL_{NG} were activated for 2 days with either iOK/IL-2 or 1B/C and exposed to PG13-packaged MFGEN and MFGIL-1βEN retrovirus (MOI 5.0) daily for a total of three exposures. Twenty-four hours after the last exposure to virus (day 5), the cultures (+Beads) were split and the beads were removed from the 1B/C-stimulated cultures (-Beads) by magnetic separation with a hand-held magnet (Dynal). All cells were replated in fresh media containing 100 IU/ml IL-2. The following day, overnight samples were established by replating cells in fresh media containing 100 IU/ml IL-2. Cells were incubated for 24 hours, and supernatants were collected for ELISA. Samples were set up identically at 72 and 96 hours following the last exposure to virus. The vertical arrow indicates day 7 initiation of PCR analysis. (B) Quantitation of the transduction efficiency of PBL_{NG} populations were performed at 3 days post-transduction following DNA amplification with primers specific for the NeoR gene. Lane 1, MFGIL-1βEN virus, iOK/IL-2-activated; lane 2, MFGIL-1βEN virus, 3B/C-activated without bead removal (+Beads); lane 3, MFGIL-1βEN virus, 3B/C-activated with early bead removal (-Beads). M, λ Hind III/ ϕX174 Hae III molecular size marker. Quantitations of transduction efficiencies were determined as described in Figure 3.

protein expression were evaluated by ELISA analysis of supernatants starting at 48 hours post-transduction. PCR analysis performed at 72 hours following virus exposure was used to estimate the percentage of cells transduced by the recombinant retroviruses.

As summarized in Table 10, ELISA indicated that continuous co-stimulation by beads (+Beads) resulted in higher levels of IL-1β and IFN-γ proteins, which were maintained at higher levels through day 9 (96 hours post-transduction) relative to iOK/IL-2 populations and cultures with bead removal (-Beads). In all cultures, the highest levels of both cytokines were observed at the earliest time point after virus exposure (48 hours). However, populations cultured in the presence of beads showed lower total viable cell expansion (6.8×10^6) versus cultures with bead removal (-Beads) and iOK/IL-2 cultures (15.0×10^6 and 15.8×10^6 cells, respectively) (data not shown). To approximate the percentage of cell positive for proviral integration, PCR analysis was performed on each of the transduced cultures 3 days after virus exposure. Figure 13B demonstrates that transduction frequencies were identical (approximately 40%) in all populations regardless of the presence or absence of beads.

These results are in accordance with data from another member of our lab group, Garlie et al. (Journal of Immunotherapy, in press), who demonstrated that magnetic removal of the beads prior to day 14 of culture increases total viable cell expansion several fold. Garlie et al. also report that early bead removal results in the preferential outgrowth of $CD8^+$ lymphocytes and decreased levels of $CD25^+$ expression. Together, these results suggest that early bead removal in 3B/C-stimulated T cell cultures (prior to day 14 of culture) results in a population of cells that behave similarly to iOK/IL-2 stimulated cultures with respect to increased growth and total viable cell expansion.

Table 10. Proviral and Endogenous Cytokine Secretion With and Without Day 5 Bead Removal (24 h Post-Transduction, PT).

MFGIL-1βEN Samples	Cytokine Accumulation[b] (pg/ml/10^6 cells/24 hours)					
	iOK/IL-2		**+ Beads**		**-Beads**	
	IL-1β	IFN-γ	IL-1β	IFN-γ	IL-1β	IFN-γ
PBL_{NG} 72 h PA[a]		18,055		56,976		56,976
48 h PT	5,171	302	6,830	1,652	4,384	122
72 h PT	1,965	189	3,728	800	1,666	0
96 h PT	1,690	48	2,831	763	1,196	0

[a]Accumulated cytokine present in the supernatant of 3-day activated cultures.
[b]24-h culture plated on indicated day in fresh media plus IL-2 (100 IU/ml).

However, there is a rapid decrease in both proviral gene expression and endogenous cytokines and a loss in $CD25^+$ expression, reflecting a general decline in the T cell activation state.

5. Bioactivity of the Retroviral-Encoded IL-1β Molecule.

Previous experiments by Pecceu et al. (1991) with secreted IL-1β encoded as a fusion protein with the human GH signal sequence indicated that it retained biological activity equal to the endogenous secreted form, even though the protein was glycosylated (Pecceu et al., 1991). All transduction experiments described in this chapter resulted in the production of relatively high levels of vector-derived IL-1β. These results

demonstrated that the presence of proviral IL-1β did not alter the production of endogenous cytokines (IFN-γ), cellular phenotype, or growth characteristics of iOK/IL-2 or 3B/C-activated cultures. Because the ultimate goal of these studies was to have an active form of IL-1β secreted by retrovirus gene-modified ATC, it was necessary to assay for the bioactivity of the rat GH-IL-1β fusion protein encoded by the MFGIL-1βEN retroviral vector.

For this analysis, a sensitive bioassay was employed based on the cytotoxicity of IL-1β to human A375 melanoma cells (Nakai et al., 1988). MFGIL-1βEN-transduced T cell cultures were incubated for 24 hours in the presence of 100 IU/ml IL-2 and supernatant was collected and analyzed by ELISA to determine the relative amount of secreted IL-1β protein. ELISA analysis of supernatants prior to the bioassay indicated the presence of approximately 800 pg/ml of vector-derived IL-1β in the MFGIL-1βEN culture. Based on this value, a series of dilutions ranging from 200 pg/ml to 0 pg/ml were prepared (Fig. 14, left). In an attempt to quantitate the amount of bioactive IL-1β present in the transduced T cell supernatant, a standard curve was generated from serial dilutions of recombinant human IL-1β (rIL-1β) protein (Fig. 14, right). As a control for specificity, supernatant from MFGEN-transduced cultures that lacked the IL-1β sequences was also serially diluted (Fig. 14, center). A375 cells were subsequently incubated for 96 hours in the presence of transduced T cell supernatants or recombinant IL-1β. Figure 14 demonstrates that the amount of cell death observed following exposure of A375 cells to 50 pg/ml of vector-derived IL-1β (MFGIL-1βEN-transduced supernatant) was equivalent to the cell death observed following incubation of A375 cells with 50 pg/ml of recombinant IL-1β protein. Moreover, no significant cell death was observed when the

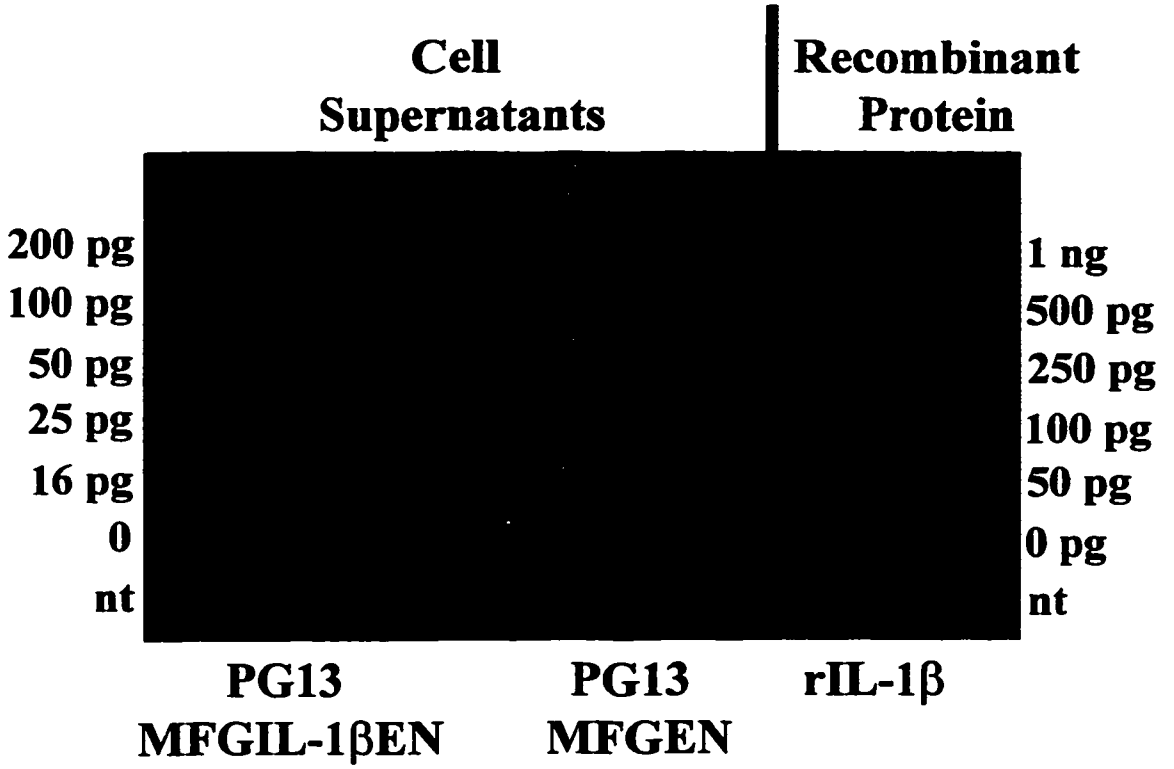

Figure 14. IL-1β Bioactivity Assay. Two weeks post-transduction, supernatants from PG13-MFGIL-1βEN and PG13-MFGEN cultures were collected, serially diluted, and added to wells that contained 2 x 10^3 A375 cells. Protein values shown on the left represent the amount of vector-derived IL-1β protein (produced by the PG13-MFGIL-1βEN cultures) added to the tumor cells. Identical serial dilutions were created using supernatants from MFGEN cultures, although these supernatants do not contain vector-derived IL-1β. Protein values shown on the right represent the amount of recombinant IL-1β protein added to the A375 cells. After four days, nonadherent cells were removed by washing and the remaining cells were stained with Diff-Quik and counted.

A375 cells were incubated with the control MFGEN-transduced T cell supernatant, indicating that the cytotoxic effect was due to the IL-1β molecule. These data regarding the cytotoxic effect of IL-1β on A375 cells demonstrate that vector-derived IL-1β is bioactive.

CHAPTER V

MODULATION OF IL-1β PROVIRAL GENE EXPRESSION IN LONG-TERM ACTIVATED T CELL CULTURES

INTRODUCTION

Several groups have demonstrated the long-term *in vivo* survival of retrovirus gene-modified T lymphocytes along with maintained expression of introduced genes, including ADA and NeoR (Rosenberg et al., 1990; Morecki et al., 1991; Fauser, 1991; Bordignon et al., 1995; Blaese et al., 1995; Mullen et al., 1996). However, the results presented in Chapter IV clearly demonstrate that long-term T cell cultures fail to maintain a heightened activation status, as characterized by the downregulation of proviral-encoded IL-1β gene expression and endogenous cytokines. *In vitro*, T cell cultures appear to assume a more quiescent state which is reminiscent of memory cell generation *in vivo* following antigen-specific T cell responses (Sprent, 1997).

Based on the rapid decline in T cell activity and proviral gene expression, I wanted to further examine the relationship between proviral gene regulation and T cell activation in long-term transduced cultures. In the following studies, transduced ATC were re-activated using two different approaches to determine whether proviral gene expression was permanently suppressed or could be re-induced by a second exposure to activating signal. Firstly, long-term transduced T cells were re-activated with fresh iOK/IL-2 and 3B/C mAbs. Secondly, a BsAb was utilized to establish a more clinically relevant model for re-activation of proviral gene expression and T cell activation. Adoptive immunotherapy strategies for the treatment of refractory malignancies now

include utilization of bispecific antibodies (BsAb). Typical BsAbs are created using one mAb with specificity for an activation molecule on an immune effector cell ($CD4^+$ and $CD8^+$ T lymphocytes, NK cells, or polymorphonuclear cells) and the other mAb with specificity for a tumor associated antigen (TAA) (Kroesen et al., 1998). By cross-linking the CD3 or CD28 receptors with tumor surface membrane antigens, investigators have shown that BsAbs can activate T cells, bypassing the requirement for a specific TCR/Ag-MHC recognition (Kroesen et al., 1998). BsAb-mediated effects include augmented tumor cell lysis and induction of endogenous cytokine production (Fig. 15) (Renner and Pfreundschuh, 1995). Production of cytokines such as IFN-γ and IL-2 enhance the cytolytic ability of the T cell, function as growth factors to sustain the life of the effector cell, directly inhibit tumor cell growth, and upregulate MHC molecules on relatively non-immunogenic tumors (Kroesen et al., 1998).

RESULTS

Effects of Fresh mAb Re-Stimulation on Proviral Gene Expression.

As shown in Tables 6 and 7 (Chapter IV), the detectable levels of IL-1β protein secreted by each of the transduced populations declined with continued culturing, irrespective of the activation method. To further examine the relationship between proviral gene regulation and T cell activation, long-term transduced cultures were re-activated to determine whether proviral gene expression was permanently suppressed or could be re-induced by a second exposure to the respective activating signals. Previously, Levine et al. (1995) reported that re-stimulation of $CD4^+$ T cells with 3B/C enhanced

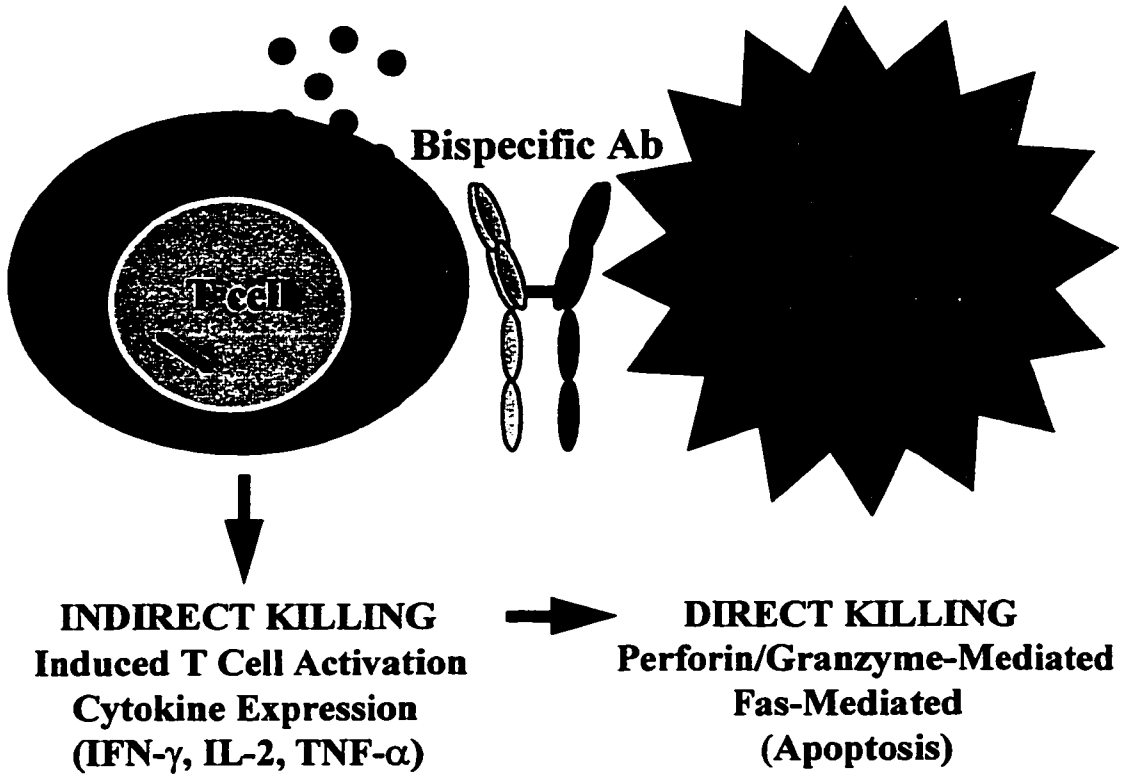

Figure 15. Coordinated Strategy For Enhancing T Cell Activity and Proviral Gene Expression Using a Bispecific Antibody (BsAb).

both IL-2 and IFN-γ production.

Transduced T cell cultures derived from donor PBL_{MW} and grown in the absence of G418 (#1 PBL_{MW}, Table 6) were re-activated on day 17 post-transduction. Following a 3-day re-stimulation with iOK/IL-2 or 3B/C, RT-PCR analysis was used to determine MFGIL-1βEN and endogenous IFN-γ transcript levels, and IL-1β and IFN-γ protein production was determined by ELISA analysis of supernatants. In both the iOK/IL-2 and 3B/C cultures, RT-PCR analysis demonstrated increased transcript levels for both IFN-γ and the MFGIL-1βEN vector following re-stimulation (Fig. 16). Proviral IL-1β protein production in the re-activated iOK/IL-2 and 3B/C cultures increased approximately 25- and 12-fold, respectively (Fig. 16). In distinct contrast to early transduced cultures at 3 days post-transduction (Table 6), IL-1β secretion was not significantly greater in the 3B/C re-activated culture relative to the re-stimulated iOK/IL-2 population. In fact, the amount of secreted IL-1β was approximately 4-fold less. Yet, re-stimulation with 3B/C did induce substantially higher levels of IFN-γ. In separate experiments, 3B/C re-activation up-regulates IL-2 secretion along with IFN-γ (data not shown). Similar to early activated T cells, transduced cultures re-exposed to 3B/C were less viable than cells re-activated with iOK/IL-2 (Fig. 16).

Re-stimulation of proviral IL-1β and endogenous IFN-γ protein expression was more carefully examined by analyzing transduced T cell supernatants for IL-1β and IFN-γ expression following increased exposure to fresh mAbs. In this experiment, iOK/IL-2-activated PBL_{NG} cells grown in the absence of G418 for 12 days (refer to Table 7, Chapter IV) were re-activated by exposure to 3B/C for 24, 48, or 72 hours. Following re-stimulation, each of the cultures were washed, replated in fresh media containing 100

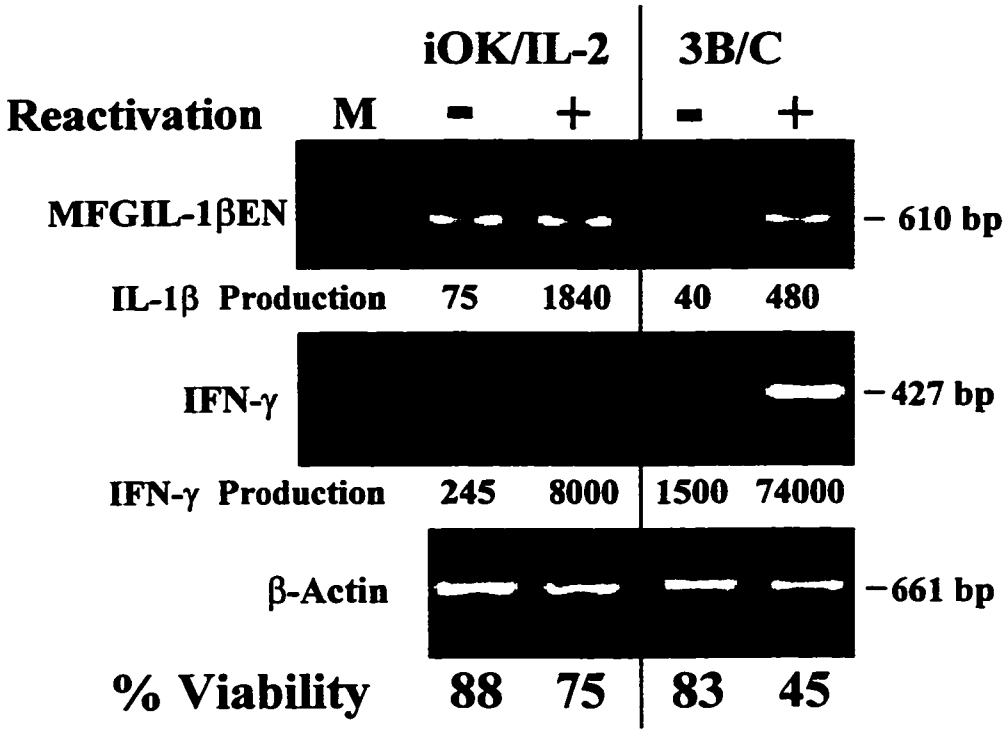

Figure 16. Induction of Proviral IL-1β and Endogenous IFN-γ Gene Expression Following T Cell Re-activation. Individual MFGIL-1βEN-transduced PBL_{MW} populations were cultured for 17 days following virus exposure and subsequently plated for 3 days in the absence (-) or presence (+) of iOK/IL-2 or 3B/C. RT-PCR was performed using total cellular RNA isolated from the respective populations. PCR primers were specific to NeoR, IFN-γ, or β-actin cDNA. RT-PCR amplification of β-actin RNA served to normalize sample loading. M, λ Hind III/ ϕX174 Hae III molecular size marker. For IL-1β and IFN-γ production, cells were washed, replated in fresh media containing 100 IU/ml IL-2 and cultured for 24 hours prior to ELISA analysis. Values are reported as pg/ml/10^6 cells/24 hours. The percentage of viable cells in each culture was calculated by trypan blue exclusion analysis and is indicated below.

IU/ml IL-2 for 24 hours, and secreted levels of IL-1β and IFN-γ proteins were determined by ELISA analysis. Similar to the data presented in Figure 16, proviral IL-1β protein production increased approximately 25-fold relative to non-restimulated cultures (23,861 pg/ml versus 890 pg/ml) following 72 hours of mAb re-stimulation (Table 11). The highest levels of both IL-1β and IFN-γ were observed following 72 hours of re-stimulation with fresh beads (3B/C). Although production of both proteins continued to increase following extended exposure to activation signal, re-exposure of transduced cells to 3B/C for greater than 24 hours resulted in a dramatic decrease in the total number of viable cells (Table 11).

Table 11. Proviral IL-1β and Endogenous Cytokine Secretion Following Restimulation With Fresh mAbs.

iOK/IL-2 Population	Re-stimulation[a]	IL-1β Concentration[b] (pg/ml/10^6 cells/24 h)	IFN-γ Concentration[b] (pg/ml/10^6 cells/24 h)	% Viability[c]
24-hours	+	1680	24,850	78.0
	-	640	20	85.0
48-hours	+	9873	25,912	52.0
	-	836	20	86.0
72-hours	+	23,861	34,833	56.0
	-	890	20	87.0

[a]iOK/IL-2 populations were re-stimulated with fresh beads (3B/C) for the indicated number of hours. Non-restimulated (-) cultures were maintained in 100 IU/ml IL-2.
[b]24-hour overnight samples were established in the presence of 100 IU/ml IL-2.
[c]Total number of viable cells was determined by trypan blue exclusion as previously described.

Reactivation of Proviral IL-1β Gene Expression Using Bispecific Antibody.

To investigate the effects of the BsAb molecule on T cell activation and proviral IL-1β gene expression, PBL_{RS} cells were activated using iOK/IL-2 and transduced by multiple exposures to PG13-MFGEN and MFGIL-1βEN retroviruses (MOI 0.2). Following 21 days of culture (day 18 post-transduction), non-G418 selected populations were pre-coated (armed) with OKT3 x CEA BsAb and incubated in the presence of a CEA-positive colon carcinoma cell line (LS 174T) starting at a 10:1 E:T ratio. After 20 hours, BsAb-redirected tumor cell lysis and supernatant accumulation of IL-1β and IFN-γ was determined. PCR analysis prior to this experiment indicated that approximately 5% of the cells were positive for the proviral genome (data not shown). Figure 17 shows that at a T cell (effector) to LS 174T cell (target) ratio of 10:1, both the armed MFGIL-1βEN and MFGEN-transduced cells showed a dramatic increase in their ability to lyse LS 174T target cells relative to the same cells that were not armed with BsAb (Fig. 17A). ELISA analysis for IFN-γ indicated that both T cell populations induced high levels of IFN-γ (400-500 pg/ml) when armed with BsAb and exposed to tumor cells (Fig. 17B). The armed MFGIL-1βEN-transduced T cells showed a more modest increase in the secretion of IL-1β following incubation with the LS 174T cells (approximately 3-fold relative to unarmed or incubation in the absence of tumor cells). As compared to IFN-γ, the induction of IL-1β was not as dramatic, possibly because so few of the armed cells carried the proviral gene (5%).

I wanted to confirm these results using PBL from another normal donor. For this experiment, PBL_{DI} cells were activated with iOK/IL-2, transduced as previously described with MFGEN and MFGIL-1βEN retroviral vectors (MOI 5.0), and armed with

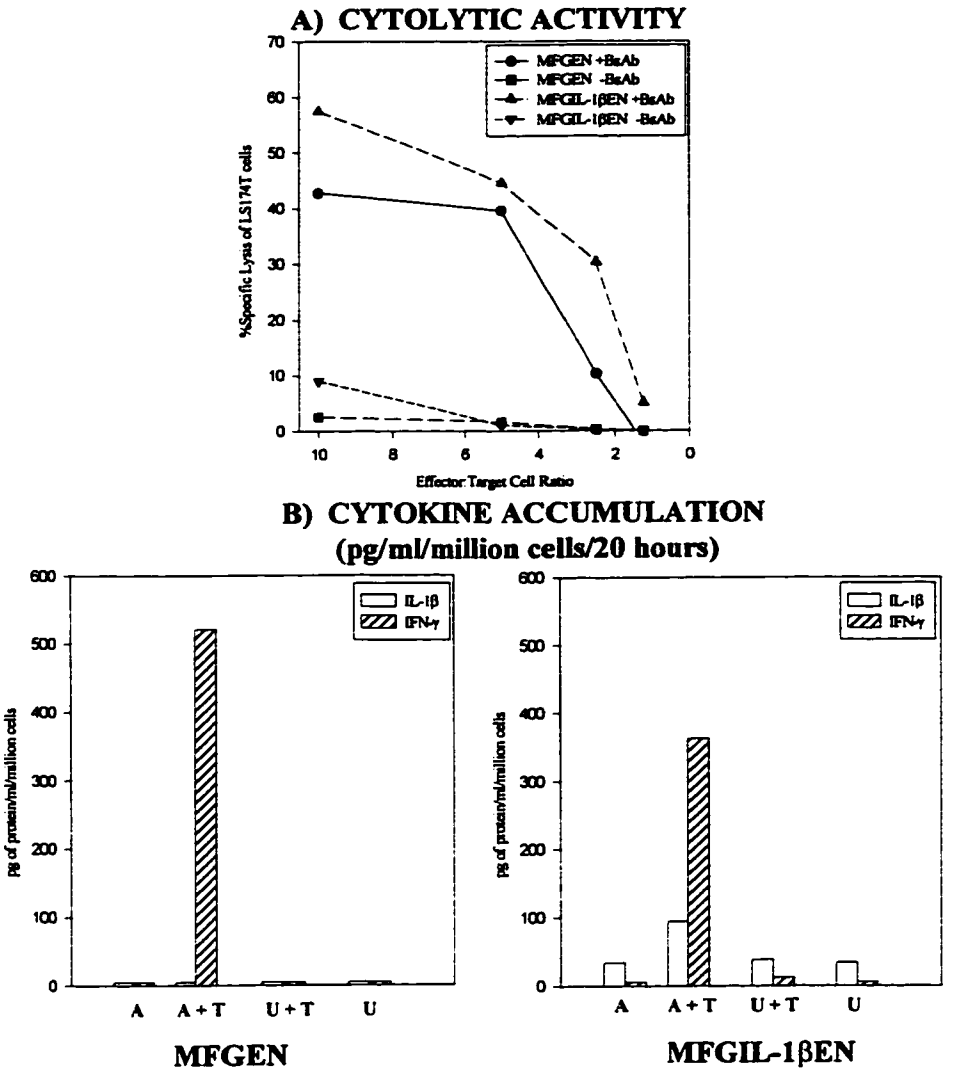

Figure 17. Re-Activation of Transduced PBL_{RS} Using a BsAb. (A) BsAb-redirected cytolysis of LS 174T tumor cells was analyzed at different E:T ratios in a 20 hour [^{51}Cr]-release assay. $2x10^4$ target cells were plated in triplicate wells of a flat-bottom 96-well dish, incubated overnight at 37°C, and labeled with [^{51}Cr] (2 μCi/well) for 4 hours at 37°C. MFGEN and MFGIL-1βEN-transduced cells originally activated with iOK/IL-2 and cultured for 21 days were resuspended at $20x10^6$ cells/ml and armed with 5 ng of BsAb per $1x10^6$ ATC at 4°C for 1 hour. Armed and unarmed cells were washed, resuspended in RPMI-10% FCS, added to each well at an E:T ratio of 10:1, 5:1, 2.5:1, and 1.25:1. Cells were incubated for 20 hours at 37°C. Abbreviations: +BsAb, T cells armed with BsAb; -BsAb, unarmed T cells. (B) Accumulation of IL-1β and IFN-γ in transduced ATC armed or unarmed with BsAb. $2x10^4$ LS 174T target cells were plated in triplicate wells of a flat-bottom 96-well dish and incubated overnight at 37°C. MFGEN and MFGIL-1βEN-transduced lymphocytes, armed or unarmed with BsAb as described above, were plated at a 10:1 effector to target ratio, and incubated for 20 hours at 37°C. Following incubation, supernatants were collected, combined from the triplicate wells, and used for ELISA. Abbreviations: A, armed T cells alone; A + T, armed T cells plus LS 174T cells; U + T, unarmed T cells plus LS 174T cells; U, unarmed T cells alone.

OKT3 x CEA BsAb on day 14 of culture (day 11 post-transduction). Armed and unarmed cells were incubated in the presence of LS 174T cells at the appropriate E:T ratios. As a control for BsAb re-directed specificity to the CEA antigen, armed transduced T cells were also incubated in the presence of a CEA-negative tumor cell line, Hs 913T. In addition, transduced T cells were pre-coated with unconjugated parental OKT3 mAb. After 20 hours, BsAb-redirected tumor cell lysis and supernatant accumulation of IL-1β and IFN-γ was determined. PCR analysis prior to this experiment indicated that approximately 50% of the cells were positive for the proviral genome (data not shown). Figure 18 is a representative experiment illustrating the BsAb-mediated targeting (rosetting) of ATC to CEA-positive and CEA-negative tumor cell lines at a T cell(effector) to LS 174T cell (target) ratio of 10:1. When viewing these cultures by microscopy, armed iOK/IL-2 cells demonstrated an obvious adhesion to LS 174T tumor cells versus unarmed iOK/IL-2 cells (Fig. 18A, top panels). In contrast, no adhesion was observed when armed transduced cells were incubated in the presence of CEA-negative Hs 913T cells (Fig. 18A, middle panels). As a further control, transduced ATC pre-coated with parental OKT3 mAb did not rosette or adhere to the LS 174T colon carcinoma cells (Figure 18B, lower left panel), demonstrating that both arms of the BsAb molecule are required to form the physical bridge between the armed ATC and the tumor cell.

Figure 19A demonstrates that at a 30:1 effector to target cell ratio, both the armed MFGIL-1βEN and MFGEN-transduced cells showed enhanced target cell lysis relative to the same cells that were not armed with BsAb. ELISA analysis of supernatants collected from cultures that were incubated for 20 hours at an effector to target cell ratio of 2:1 demonstrated that both T cell populations induced high levels of IFN-γ (1900-2200

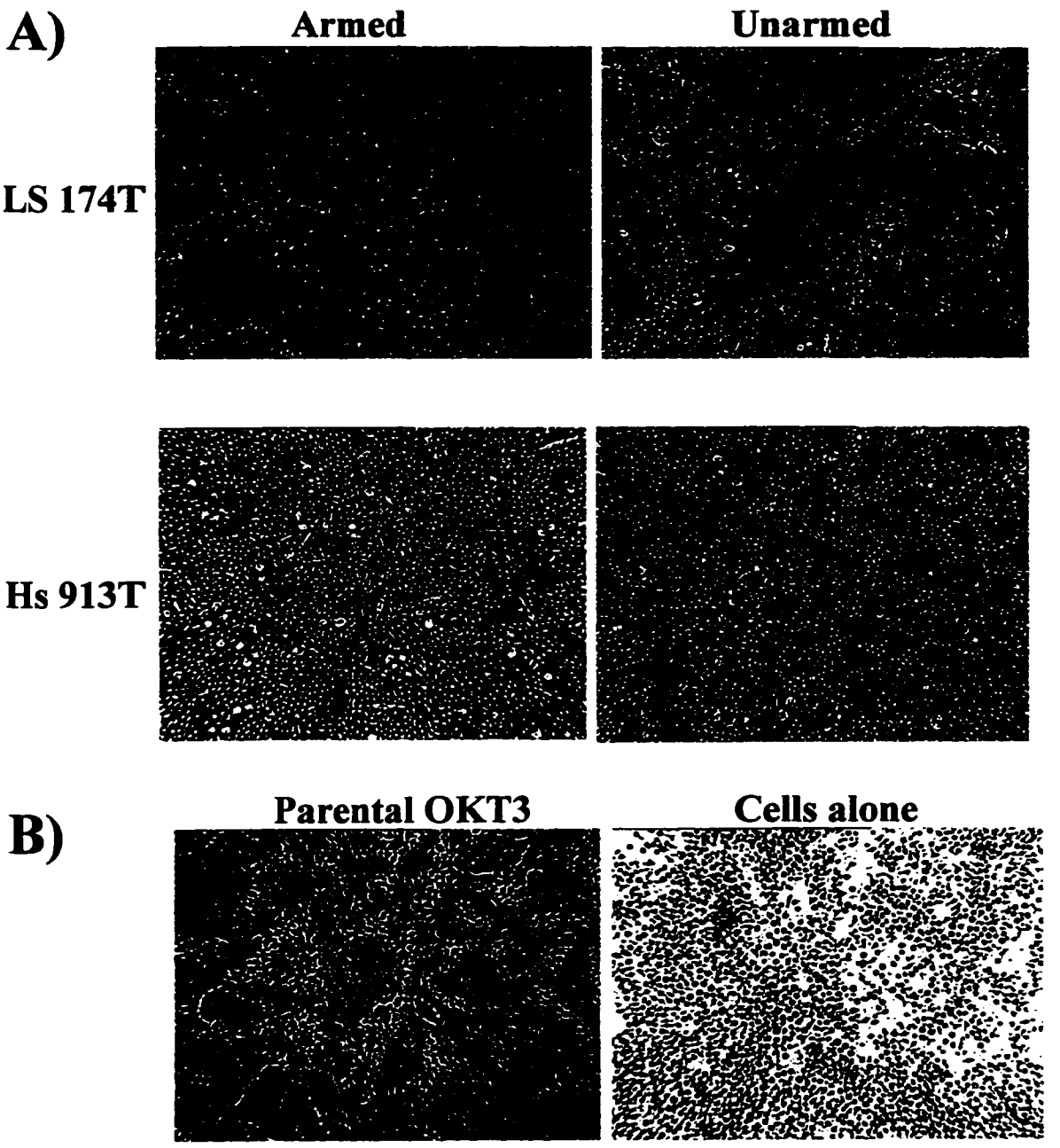

Figure 18. Bispecific Antibody Re-Directed Rosetting Assay of Armed and Unarmed Activated T Lymphocytes. MFGIL-1βEN-transduced cultures originally activated with iOK/IL-2 were armed with 50 ng of OKT3 x CEA BsAb per $1x10^6$ ATC at 4°C for 1 hour according to Materials and Methods. Identical results were obtained for non-transduced and MFGEN-transduced control populations from several normal donors. (A) Following a 20-hour incubation, armed and unarmed MFGIL-1βEN-transduced cells were analyzed for their ability to adhere to either LS 174T (CEA-positive) or Hs 913T (CEA-negative) tumor cells. (B) MFGIL-1βEN-transduced cells were also armed with an identical concentration of parental OKT3 mAb and incubated in the presence of LS 174T cells for 20 hours. The right panel shows MFGIL-1βEN-transduced cells alone.

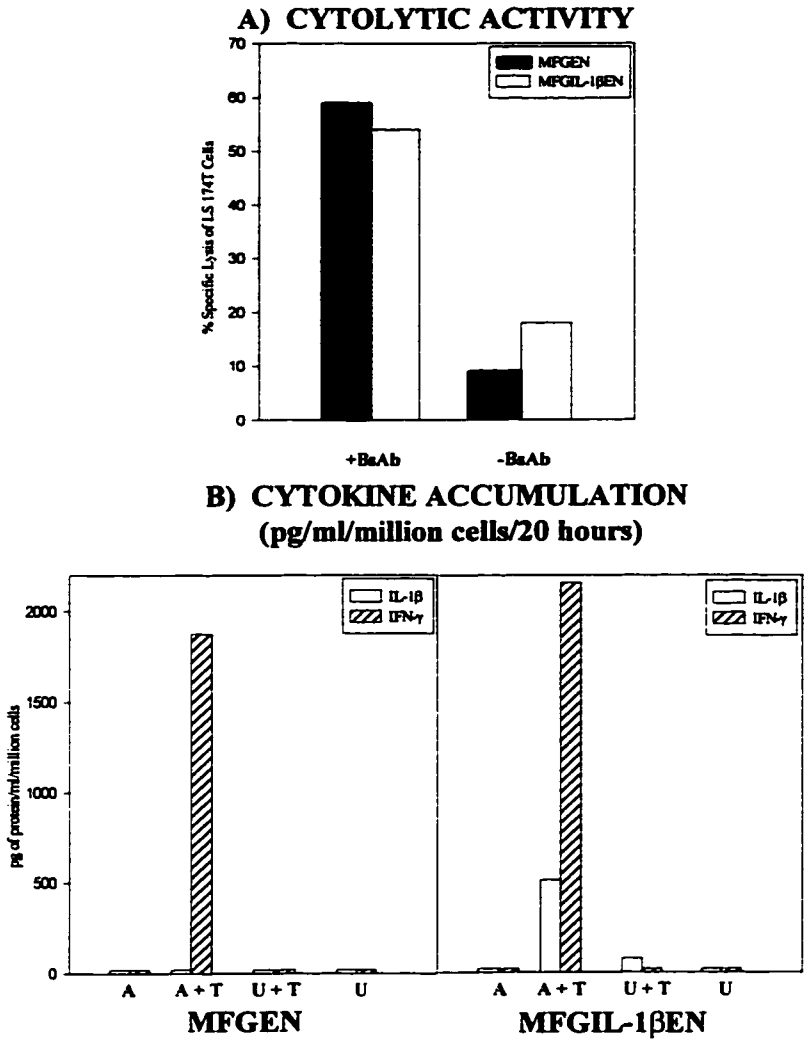

Figure 19. Re-Activation of Transduced iOK/IL-2-Activated PBL Using a BsAb. (A) BsAb-redirected cytolysis of CEA-positive LS 174T tumor cells was analyzed at different effector cell to target cell (E:T) ratios in a 20 hour [^{51}Cr]-release assay. 2x10^4 target cells were plated in triplicate wells of a flat-bottom 96-well dish, incubated overnight at 37°C, and labeled with [^{51}Cr] (2 μCi/well) for 4 hours at 37°C. MFGEN and MFGIL-1βEN-transduced cells originally activated with iOK/IL-2 and cultured for 21 days were resuspended at 20 x 10^6 /ml and armed with 50 ng of BsAb per 1 x 10^6 ATC at 4°C for 1 hour. Armed and unarmed cells were washed, resuspended in RPMI-10% FCS, added to each well at the appropriate E:T ratios (30:1), and incubated for 20 hours at 37°C. Abbreviations: +BsAb, T cells armed with BsAb; -BsAb, unarmed T cells. (B) Accumulation of IL-1β and IFN-γ in transduced ATC armed or unarmed with BsAb. 2 x 10^4 LS 174T target cells were plated in triplicate wells of a flat-bottom 96-well dish and incubated overnight at 37°C. MFGEN and MFGIL-1βEN-transduced lymphocytes, armed or unarmed with BsAb as described above, were plated at a 2:1 effector to target ratio, and incubated for 20 hours at 37°C. Following incubation, supernatants were collected, combined from the triplicate wells, and used for ELISA. Abbreviations: A, armed T cells alone; A + T, armed T cells plus LS 174T cells; U + T, unarmed T cells plus LS 174T cells; U, unarmed T cells alone.

pg/ml) when armed with BsAb and exposed to tumor cells (Fig. 19B). Moreover, the armed MFGIL-1βEN-transduced T cells showed an increase in secreted IL-1β following incubation with the LS 174T cells (approximately 7-fold relative to unarmed or incubation in the absence of tumor cells). Compared to IFN-γ, the induction of IL-1β was not as dramatic, possibly because only 50 percent of the armed transduced T cells carry the proviral gene. Similar ELISA results were obtained when transduced T cells were incubated at a 10:1 effector-to-target cell ratio.

CHAPTER VI

DISCUSSION

One of the major challenges for cancer therapy is overcoming an inadequate host-mediated immune response against residual, metastatic tumor. Deficient anti-tumor immunity can be attributed to poor immunogenicity of the tumor cells, lack of tumor-encoded co-stimulatory molecules necessary for optimal T cell activation, tumor cell production of T cell immunosuppressive factors such as transforming growth factor (TGF)-β, and MHC downregulation resulting in poor TAA processing and presentation by tumor cells (for reviews, see Zier and Gansbacher, 1995; Speiser and Ohashi, 1998). Over the years, adoptive cellular immunotherapies have focused on the *ex vivo* expansion, generation, and reinfusion of highly activated immune effector cells to induce tumor immunity in refractory cancer patients. In contrast to other cellular therapies including LAK or TIL, large numbers of autologous ATC can be readily generated from peripheral blood and polyclonally expanded *ex vivo* by activation in the presence of anti-CD3 plus low doses of IL-2 (Ochoa et al., 1987; Ting et al., 1988; Ochoa et al., 1989). Although early ATC cultures proliferate, produce cytokines, and exhibit non-MHC restricted killing properties (Ochoa et al., 1987; Ochoa et al., 1989), infusion of highly activated, anti-CD3 stimulated T lymphocytes does not result in the induction of significant tumor responses in patients suffering from malignant melanoma, renal cell carcinoma, or gastrointestinal carcinomas (Curti et al., 1993; Curti et al., 1996).

To enhance ATC-mediated tumor cell killing and anti-tumor responses, T lymphocytes are being genetically modified to encode cytokines or other immune

regulatory proteins including IL-2 and TNF-α (for review, see Schmidt-Wolf and Schmidt-Wolf, 1995, and references therein; Hwu, 1997). In addition, gene-modified T lymphocytes are being applied to the treatment of infectious diseases and genetic disorders of the immune system. Although the safety and feasibility of administering gene-modified cells has been demonstrated in clinical trials using retrovirally transduced TIL and activated PBL (Rosenberg et al., 1990; Blaese et al., 1995), their utility in clinical medicine has been limited by low levels of gene transfer (Wilson et al., 1990; Woffendin et al., 1994; Mavilio et al., 1994). Because therapeutic efficacy of genetically modified T lymphocytes may depend on persistent, high-level expression of the introduced gene (Hwu, 1997), recent efforts have focused on enhancing retrovirus gene transfer and modifying retroviral vectors for improved T cell expression (Coutre et al., 1994; Mavilio et al., 1994; Bunnell et al., 1995; Lam et al., 1996; Rudoll et al., 1996; Pollock et al., 1998; Agarwal et al., 1998).

The improved method of T cell transduction developed in the laboratory combines: 1) the MFG retroviral vector; 2) PG13 packaging cells for the production of infectious retrovirus particles with the GALV envelope; and 3) an optimized protocol for retroviral transduction, which includes initiation of transduction 48 versus 72 hours following T cell activation and multiple exposures to higher titer virus. Application of these methodologies (Fig. 5) routinely results in transduction frequencies ranging from 50-75%. These values are equivalent to the highest levels of retrovirus-mediated gene transfer reported to date for primary human T lymphocytes (Bunnell et al., 1995; Pollock et al., 1998), suggesting that transduction by this optimized protocol generates a population of transduced cells that does not require selection to enrich for transduced

cells. This may be important for several reasons. Firstly, by eliminating the requirement for G418 selection following retroviral infection, the length of time that is required for the *ex vivo* expansion of therapeutic transduced cell numbers is lessened. This may be clinically relevant as precipitating mAb responses to the lipoprotein fraction of fetal calf serum and other culture components have been demonstrated in one ADA-deficient patient following the third transfusion of gene-corrected T lymphocytes (Muul et al., 1996; Muul et al., 1998). Secondly, the development of cytotoxic T cell responses to the NeoR transgene have been demonstrated in highly immunodeficient HIV^+ positive patients (Muul et al., 1998). If enrichment for transduced cells by selection is no longer required, re-designed retroviral vectors without the NeoR gene can be utilized for delivery of corrective genes.

In activated PBL populations, higher transduction frequencies were observed following infection with PG13-packaged retrovirus (containing the GALV envelope) versus PA317-packaged retrovirus (containing the amphotropic envelope). Moreover, 3-day activated PBL expressed higher levels of RNA encoding the GALV envelope receptor (GLVR-1) versus the amphotropic envelope receptor (GLVR-2), suggesting that at the time of transduction initiation, T lymphocytes may have higher surface expression of GLVR-1 to bind retrovirus with the GALV envelope. Shortly after completing these studies, Lam et al. (1996) confirmed these findings for activated human PBL and TIL. Using Northern analysis, 8- to 19-fold higher mRNA levels of GLVR-1 were reported as compared to GLVR-2. Moreover, the increased expression of GLVR-1 correlated with a 4- to 18-fold higher transduction frequency for PG13-packaged virus comparable to my own data (Lam et al., 1996).

Similar to the findings of Rudoll et al. (1996) in a study based on soluble mAb stimulation of purified $CD4^+$ T cells, initiation of transduction on day 2 following PBL activation resulted in higher gene transfer efficiencies compared to transduction on day 3 post-activation (Fig. 6). As the efficiency of retrovirus gene transfer is cell cycle dependent (Springett et al., 1989; Miller et al., 1990), one likely explanation for the increased transduction observed following 2 days of activation is due to higher proliferation rates. However, [^{3}H]-thymidine incorporation was similar between the two cultures. Improved transduction at this earlier time point is most likely due to increased expression of GLVR-1 receptor on the cell surface. In a recent study, the expression levels of both GLVR-1 (PiT-1) and GLVR-2 (PiT-2) receptor mRNA were analyzed in 5 human cultured hematopoietic cell lines (Sabatino et al., 1997). Using Northern analysis, the investigators demonstrated that both receptor mRNAs were induced following polyclonal activation with phorbol myristate acetate (PMA), and the maximum levels of each receptor mRNA were present following 12-24 hours of activation. Although the authors do not directly analyze receptor RNA expression in ATC, it is reasonable to hypothesize that there may be greater surface expression of GLVR-1 receptor at day 2 versus day 3 of activation. Further analysis is required to investigate the kinetics of the GLVR-1 receptor expression early after T cell activation.

Production of higher titer PG13 retrovirus supernatant allowed for infection of lymphocytes to be carried out in the presence of an increased number of infectious virus particles per target cell (defined as MOI). Under conditions of higher MOI (5.0 versus 0.2), transduction was increased as indicated by PCR analysis and cell survival was enhanced in the presence of the neomycin analogue, G418. Single infection of target cells

with retrovirus generally results in approximately 1 copy/cell of the proviral genome. However, multiple exposures to higher titer recombinant retrovirus results in improved retroviral-mediated gene transfer (Bunnell et al., 1995; Rudoll et al., 1996), most likely due to introduction of multiple proviral copies per cell. In support of this hypothesis, Pollock et al. (1998) recently reported that co-localization of retrovirus and T lymphocytes on specific adhesion domains of fibronectin (FN) resulted in transduction frequencies ranging from 80 to 90% following 2 to 3 days of consecutive FN-mediated virus exposure. Moreover, the authors demonstrated that FN-mediated gene transfer resulted in greater than 5 copies of the proviral genome per cell (Pollock et al., 1998). Our laboratory has begun to investigate the utility of FN-mediated gene transduction of ATC, which could result in a greater number of proviral genomes per cell and thereby, result in overall higher levels of proviral gene expression.

While pursuing aspects of retrovirus gene transfer and proviral gene expression in ATC, I observed increased levels of the vector-derived IL-1β product in early transduced T cell cultures activated with an optimized 3:1 ratio of OKT3/9.3 beads-to-cells (3B/C). These studies suggested that bead co-stimulation might improve retroviral gene transfer relative to iOK/IL-2 activation. However, the percentage of cells possessing proviral genomes in 3B/C cultures is similar to those activated with iOK/IL-2. The equivalent transduction frequencies observed in these cultures are attributed to the comparable proliferation rates of the activated PBL populations at the time of transduction initiation (Table 5). In addition to heightened proviral gene expression, co-stimulation with 3B/C induces more potent costimulatory signals that result in augmented production of Type 1 (Th1/Tc1) cytokines, including IFN-γ and IL-2. Taken together, these data suggest that

early after T cell activation and transduction (3 days following the last exposure to virus), 3B/C-stimulated cultures possess a heightened state of T cell activation, as characterized by augmented endogenous cytokine production and proviral IL-1β gene expression.

Further analyses of iOK/IL-2 and 3B/C gene-modified T cells demonstrate that expression of the introduced proviral IL-1β gene is modified by the activation status of the transduced cell. At 3 days post-transduction, co-stimulation with 3B/C induces the highest levels of proviral IL-1β and endogenous IFN-γ gene transcription, correlating with augmented production of the corresponding cytokines (Fig. 11). Moreover, surface expression of CD25 (low affinity IL-2 receptor α) is greater in 3B/C-activated cultures, as characterized by both an increased number of cells expressing the T cell activation marker, and an increased number of receptor molecules per cell (Table 9). These results are in accordance with Plavec et al. (1997) who previously reported that high-level proviral gene expression (Lyt-2 or RevM10) from various retroviral vectors correlates with increased T cell activity.

It would appear that the presence of significantly higher numbers of $CD4^+$ lymphocytes in the 3B/C co-stimulated cultures (versus iOK/IL-2 cultures) could account for the observed increase in proviral IL-1β gene expression in these cultures (Table 9). Although I did not perform transduction experiments utilizing purified $CD4^+$ or $CD8^+$ lymphocyte populations, several groups have demonstrated equivalent gene transfer into both T cell subsets (Morecki et al., 1991; Mavilio et al., 1994). In addition, recent studies have shown that both $CD4^+$ and $CD8^+$ T lymphocytes are responsive to 3B/C *cis* costimulation resulting in enhanced production of Th1/Tc1 cytokines, including IL-2 and IFN-γ (Levine et al., 1995). Re-activation of long-term iOK/IL-2 cultures using fresh

mAbs (iOK/IL-2 and 3B/C) demonstrates that these populations are capable of producing significant amounts of both proviral IL-1β and endogenous IFN-γ even though these cultures are predominantly $CD8^+$ lymphocytes by this time in culture (data not shown). Together, these results demonstrate that overall higher levels of T cell activity and proviral gene expression observed in 3B/C-stimulated cultures are due to activation and co-stimulatory signaling events rather than the differential contribution of $CD4^+$ or $CD8^+$ lymphocytes in these cultures.

The conditioned media "switch" experiment (Fig. 12) demonstrates that although early activated 3B/C cultures express relatively high levels of endogenous cytokines (IFN-γ and IL-2), the extrinsic cellular factors present in the conditioned media of these cultures are not responsible for the differences in proviral gene activity and T cell behavior observed between the iOK/IL-2 and 3B/C-stimulated populations. Irrespective of the supplied conditioned medium, 3B/C-stimulated cultures showed higher amounts of both proviral IL-1β and endogenous IFN-γ cytokines. The modulation of transgene expression is an important consideration in these studies. Several groups have examined the inhibitory effects of certain cytokines, and found them to be cell-type dependent (Harms and Splitter, 1995; Qin et al., 1997; Ghazizadeh et al., 1997). In myocytes, Qin et al. found that both IFN-γ and TNF-α caused inhibition of transgenes under the control of different viral promoters while Ghazizadeh et al. in studies of human keratinocytes found that IFN-γ but not TNF-α repressed MMLV LTR transgene expression.

The physiological role of IFN-γ remains highly controversial in activated T cell populations. Depending on the experimental system, different effects are observed, including the induction of proliferation in early activated T cell cultures, induction of

apoptosis in longer-term cultures, as well as blocking activation-induced apoptosis in other cultures (Novelli et al., 1996). These varying results may relate to the amounts of IL-2 present in the cultures during activation as well as the expression levels of IFN-γ receptor α and β chains in the respective T cell populations. Together with IL-2, IFN-γ and its receptor appear to play a role in cell death following re-stimulation. IFN-γ appears to mediate apoptosis via the up-regulation of FasL gene expression (Novelli et al., 1996; Novelli et al., 1997). In light of this recent literature, it was important to demonstrate that soluble factors, such as IFN-γ, do not affect the growth characteristics or cytokine production of gene-modified T cell cultures. The results from this supernatant "switch" experiment expand the understanding of MMLV LTR regulation by demonstrating that in activated T lymphocytes, IFN-γ does not play a role in the down-regulation of MMLV LTR proviral gene expression.

Rather than modulation by extrinsic cellular factors, the distinct cellular properties of the differentially activated cultures appear to be the result of the initial T cell stimulation method. Previous studies have demonstrated that cytokine expression is augmented upon *trans* co-stimulation with immobilized anti-CD3 and soluble anti-CD28 mAbs via specific mRNA stabilization and transcriptional up-regulation (Lindsten et al., 1989; Thompson et al., 1989; June et al., 1994). In these transduction experiments, proviral RNA stabilization would not be attributed to the IL-1β coding sequences as 3B/C co-stimulation also increases the RNA levels of the control MFGEN vector. In addition to increased mRNA stability, CD3/CD28 co-stimulation results in the augmentation of certain cytokines (such as IL-2) via up-regulation of gene transcription. The promoter region for the IL-2 gene contains a composite elements called the CD28RE (responsive

element) which binds a number of transcription factors, including NF-κB/c-Rel, and AP-1 (June et al., 1994). These transcription factors are known to be induced upon CD3/CD28 co-stimulation (Cantrell, 1995), and most likely play a major role in endogenous cytokine induction in the 3B/C-stimulated cultures.

Previous studies have demonstrated that wild-type retroviruses are highly dependent on host cell proteins for viral replication, proviral gene transcription, processing, and translation of viral structural proteins (Varmus, 1988). Although the exact interplay between LTR regulatory elements and T cell-specific transcription factors is poorly defined (Short et al., 1987; Losardo et al., 1989; Speck et al., 1990; Coutre et al., 1994; Mavilio et al., 1994), the MMLV LTR is known to have a preferential activity in T cell lines and primary cultures. Most likely, the increased expression of the MMLV LTR-driven IL-1β by anti-CD28 co-stimulation is due to a similar modulation of transcriptional factors that are readily available following T cell activation. Simple sequence analysis of the MMLV LTR does not reveal any obvious CD28RE/AP-1 elements nor NF-AT sites. However, there are consensus sites for numerous other transcription factors, including the T cell-specific factor Tcf-1 and NF-1. Other motifs include those found in GM-CSF, IFN-γ and the IL-6 genes (Coutre et al., 1994). More recently, the RNA-dependent protein kinase PKR (protein kinase RNA regulated) has been implicated in transcriptional regulation of many genes, including genes expressed by activated T lymphocytes (Clemens and Elia, 1997). PKR is thought to exert its effects, in part, by the ability of the kinase to activate members of the NF-κB family of transcription factors resulting in transcriptional upregulation of IFN-α, IFN-β, MHC I molecules, and the Fas antigen (Clemens and Elia, 1997).

Translational regulation may also be an important control for gene regulation following T cell activation. In murine T cell clones, Garcia-Sanz et al. (1998) reported that although resting and activated Ag-specific T cell clones show similar levels of functional mRNA transcripts, differential polysome loading was observed between the clones with the most translationally repressed mRNAs being devoid of ribosomes. In human primary T cell cultures stimulated using several polyclonal activators including immobilized anti-CD3 mAb, it was demonstrated that IL-2 gene expression can be translationally regulated (Garcia-Sanz and Lenig, 1996). Further analyses must be performed to determine whether transgene expression is being regulated at the transcriptional level, the level of mRNA stability, or at the level of translation.

Of further consideration are my findings that 3B/C-activation results in less viable cell populations that expand slower following *in vitro* culture, and yet, show enhanced proliferation by [^{3}H]-thymidine incorporation and higher amounts of the CD25 activated T cell marker (Table 9 and Fig. 12). Using non-transduced PBL derived from several normal donors, T cell expansion is routinely less efficient in 3B/C-stimulated cultures than PBL exposed to iOK/IL-2 (data not shown). Moreover, the behavior of the transduced 3B/C population is not related to the presence of proviral IL-1β protein as identically activated, MFGEN-transduced cultures show similar characteristics. One important difference between the two mAb stimulation methods, is that in contrast to iOK/IL-2 activation, the OKT3/9.3-coated beads remain in the culture throughout the entire expansion period. Although the beads get diluted and most likely lose potency over time, chronic bead co-stimulation may account for higher levels of proviral IL-1β, endogenous cytokines, and surface CD25 expression in early transduced cultures (day 3

post-transduction). These data suggest that repeated or prolonged stimulation with 3B/C results in an elevated state of T cell activation, but also induces death of activated cells by a process of apoptosis, now known as activation-induced cell death (AICD) (Winoto, 1997).

Initially, the effects of 3B/C activation appear contradictory. In naive T cells and freshly isolated PBL, anti-CD3/anti-CD28 co-stimulation is considered to be the "second signal" for optimal T cell activation and results in the rescue of cells from apoptotic cell death by increasing expression of the bcl-x_L cell survival gene (Boise et al., 1995; Van Parijs et al., 1996). However, in previously activated cells or cells experiencing repeated antigenic stimulation, CD28 co-stimulation does not rescue the cells from cell death, but rather results in the increased susceptibility to AICD especially in the presence in increased levels of secreted IL-2 (Van Parijs et al., 1996). *In vivo*, AICD is important for the death of autoreactive lymphocytes and the induction of tolerance to certain foreign antigens (Van Parijs and Abbas, 1998). In $CD4^+$ T lymphocytes, AICD usually occurs via interaction of Fas (CD95) and Fas ligand (FasL), which are co-expressed on activated cells (Abbas et al., 1997). Prior phenotype analysis of 7-day activated cultures demonstrated that 3B/C activation results in populations of T cells that have a higher percentage of $CD4^+$ lymphocytes versus iOK/IL-2 cultures. Moreover, high concentrations of IL-2 are known to enhance the expression of FasL on activated $CD4^+$ T cells and the development of sensitivity to Fas-mediated apoptosis. These results suggest that following prolonged mAb stimulation *in vitro*, the activated lymphocytes could be killing each other via Fas receptor-mediated interactions.

Rapid expansion of therapeutic numbers of 3B/C-stimulated T cells is difficult

because of the increased cell death that occurs in these populations. Because this finding causes concern for the *in vivo* use of adoptively transferred T cells, we monitored proviral gene expression and T cell activity following magnetic removal of the CD3/CD28-coated beads. Although early bead removal (day 5 of culture) in 3B/C-stimulated T cell cultures results in a population of cells that exhibit increased growth and total viable cell expansion similar to iOK/IL-2 stimulated cultures, there is a rapid decrease in both proviral gene expression and endogenous cytokines, reflecting a general decline in the T cell activation state (Table 10). This data demonstrates that although T cell activation (3B/C and iOK/IL-2) induces relatively high-level proviral IL-1β gene expression, the expression was only transient.

Regardless of the T cell activation method, we have demonstrated that over a 14-day *ex vivo* expansion period retrovirus-gene modified T cells downregulate expression of the introduced proviral gene along with endogenous cytokines that are indicators of T cell activity (Quinn et al., 1998). Close kinetic analysis of both iOK/IL-2 and 3B/C-transduced T cell cultures demonstrated that the highest levels of proviral IL-1β production were observed at the earliest time point following transduction, day 1 (Table 7). The values rapidly declined over time in expanded cultures with greater than 40-70% loss of protein expression by day 4, and greater than 80-90% loss by day 12 post-transduction. Moreover, the CD25 expression is increased early after mAb stimulation but declines in long-term cultures, similar to the observed loss of endogenous cytokines (data not shown). In these studies, loss of proviral gene expression could not be attributed to the loss of proviral integrants as the percentage of cells possessing proviral integrants remained the same following continued, long-term culture. At later stages of *in vitro*

activation, the T cell cultures appear to assume a more quiescent state, which is reminiscent of memory cell generation *in vivo* following antigen-specific T cell responses (Sprent, 1997).

Downregulation of retrovirus gene expression has been described in detail throughout the years utilizing various retrovirus constructs in multiple target cell types. In a very famous study, Emerman and Temin (1986) were the first to describe internal promoter interference using an MMLV LTR-containing retroviral vector. The authors demonstrated that expression of one gene under the control of the viral LTR promoter is suppressed when there is selective pressure for expression of the second gene transcribed from an internal promoter (Emerman and Temin, 1986). The authors concluded that downregulation was based on changes in the topological nature of the DNA integration site of the provirus; establishment of an active transcription unit at one promoter changes the chromatin structure at the other transcription unit and causes inefficient transcription from the second transcription complex (Emerman and Temin, 1984; Emerman and Temin, 1986). Additional studies using transplanted, retrovirally-transduced rat or mouse primary fibroblasts demonstrated a gradual inactivation of the retrovirally-transferred genes *in vivo* (Palmer et al., 1991; Scharfmann et al., 1991). Although vector sequences could be detected in transplanted tissue, no detectable LTR or SV40-promoted transcripts were observed (Palmer et al., 1991). Scharfmann and colleagues compared the CMV immediate-early gene enhancer/promoter with a more stable, constitutive dihydrofolate reductase (DHFR) promoter. These authors concluded that the CMV promoter is not a good choice for long term expression *in vivo* as the CMV is an inducible promoter and requires actively growing cells (Scharfmann et al., 1991). Moreover, downregulation of

MMLV-LTR transcriptional inactivity was attributed to methylation of proviral sequences (Palmer et al., 1991). Subsequent studies confirmed the presence of inactive, methylated proviral sequences, although it is now thought that methylation of many genes is a secondary event after downregulation of expression has already occurred (Challita and Kohn, 1994). My results demonstrate that down-regulation of gene expression is not specific to the MMLV LTR promoter-driven transcripts. Rather, in late passage cultures, overall T cell activity declines resulting in lower levels of gene transcription for both endogenous cytokines and proviral IL-1β. Because long-term T cell cultures down-regulate overall gene expression, any transferred gene could assume a more basal activity irrespective of the vector design or accompanying promoter/enhancer sequences.

Several groups have reported similar findings related to down-regulation of proviral gene expression over time in expanded gene-modified T lymphocyte cultures. A recent study by Bunnell et al. demonstrated that non-stimulated gene-marked $CD4^+$ cultures obtained after apheresis of rhesus monkeys showed undetectable levels of anti-HIV transgene expression; proviral gene expression was only evident by RT-PCR analysis following *in vitro* culture of transduced lymphocytes in the presence of IL-2 (Bunnell et al., 1997). Moreover, in AIDS studies using primary $CD4^+$ T cells, Plavec et al. identified a similar problem in maintaining expression of the RevM10 anti-HIV gene when expressed from an MMLV LTR (Plavec et al., 1997). For AIDS, improved transgene expression in non-activated T cells could prove important in developing effective anti-HIV gene therapies. Also, there is concern for the development of vectors for hematopoietic stem cell gene therapy because many of the subsequent differentiated progenitors retain a quiescent state. One possibility is the development of retrovirus

vectors containing scaffold attachment regions (SARs) or locus control regions (LCRs). SARs/LCRs are AT-rich sequences that bind to the nuclear matrix and function to maintain open chromatin domains that are less influenced by changes in the cellular differentiation state of the infected cell. In addition, these sequences may protect the provirus from methylation following long-term culture (Dang et al., 1998). In fact, Plavec et al. are pursuing SAR vectors in RevM10 anti-HIV studies to sustain expression in quiescent T cell progenitors following gene-modification of hematopoietic stem cells (Plavec et al., 1998).

Because of the downregulation of T cell activity, infusion of gene-modified T cells for delivery of high, local concentrations of cytotoxic cytokines seems impractical without some type of *in vivo* mechanism to re-induce high-level proviral gene expression. As a model, fresh iOK/IL-2 and 3B/C mAbs were utilized to re-stimulate long-term T cell cultures (17 days post-transduction). In these experiments, both T cell activation and proviral gene expression were re-induced following a second exposure to activating signal (Fig. 16). However, in contrast to primary activation, 3B/C re-stimulation was a less effective inducer of proviral gene activity than re-exposure to iOK/IL-2. Moreover, closer kinetic analysis revealed that although production of both the proviral IL-1β and endogenous IFN-γ proteins continued to increase following extended exposure to activation signal (3-day restimulation), re-exposure of transduced cells to 3B/C for greater than 24 hours resulted in a dramatic decrease in the total number of viable cells (Table 11), similar to that observed in the early 3B/C-activated cultures. The lower levels of IL-1β transgene expression after *in vitro* re-activation may be a result of the enhanced cell death that is occurring in these cultures (Fig. 16).

The results presented herein provide additional insights into the effects of T cell activation on proviral gene expression. Without re-stimulation, only basal levels of proviral gene expression occurs in expanded T lymphocyte cultures *in vitro*. Moreover, following patient reinfusion, most of the gene-altered T cells would presumably retain basal activity because the resulting population is polyclonal and not specific for any one, particular Ag-MHC that would induce a general re-activation. In certain situations, such as T cell ADA deficiency, where only minimal amounts of gene product are required for clinical benefit, low level vector activity may suffice. However, for cancer adoptive immunotherapy, the use of combined experimental approaches for *in vivo* re-activation of gene-modified T cells may be required. Recent molecular and genetic interventions include: 1) bispecific antibodies that would activate gene-modified T cells via cross-linking of CD3 and CD28 receptors with tumor cell-associated antigens (Renner and Pfreundschuh, 1995) and 2) engineering of T lymphocytes that express both the gene of interest and an antibody-type chimeric receptor gene designed to redirect antigen specificity against particular antigens and induce T cell activation (Roberts et al., 1994; Hwu, 1995; Eshhar et al., 1996; Altenschmidt et al., 1997).

Herein, we have proposed a coordinated strategy of BsAb-arming of retrovirus gene-modified T cells for targeted delivery of a potentially beneficial, tumoricidal molecule to the tumor site. In my studies, I have focused primarily on a BsAb that targets the T cell to surface carcinoembryonic antigen (CEA), which is present on the vast majority of breast, colorectal, gastric, and pancreatic carcinomas (Thompson et al., 1991). IL-1β gene-modified T cells armed with BsAb (OKT3 x CEA) can be specifically re-directed to tumor cells expressing the CEA surface antigen as demonstrated by adherence

to LS 174T colon carcinoma cells. Moreover, arming MFGEN and MFGIL-1βEN-transduced T cells results in a dramatic increase in their ability to lyse CEA-positive tumor cells and also results in the specific induction of both IFN-γ and proviral IL-1β protein production. These results suggest that a BsAb molecule can be employed to induce T cell activation as characterized by enhanced cytolytic activity, the induction of endogenous cytokine production, and expression of provirus encoded transgenes.

Although several investigators have shown the preclinical and clinical applications of BsAb (for review see, Kroesen et al., 1998), the *in vivo* application of murine BsAb is limited for a number of reasons. Probably the most obvious problem is the immunogenic nature of non-human, murine sequences, which after multiple infusions can induce strong antibody responses referred to as human anti-mouse antibody (HAMA) responses (Kroesen et al., 1998). In addition, these molecules are generally quite large and are not able to penetrate bulky solid tumor very efficiently. This leads to the requirement for direct intratumoral injection of BsAb or BsAb-coated cells, eliminating the possibility of systemic treatment. With the caveat in mind that bispecific antibodies on T cells may allow only transient responses *in vivo*, our group is also working on expressing novel chimeric receptors (T-bodies) in T cells. Similar to the BsAb molecules, these receptors would potentially target the transduced T cell to tumor and induce activation together with the expression of introduced cytokine genes (like IL-1β) or chemokine genes. Activation-induced expression of a chimeric receptor in ATC could generate more potent and more specific cytotoxic T cells that may be critical for the elimination of residual tumor cells and induction of tumor-antigen specific immune responses.

In summary, my work has highlighted some of the problems pertaining to the use of gene-modified T lymphocytes for the treatment of certain genetic disorders, including cancer. When contemplating approaches for gene introduction and expression in T cells, one must consider the physiology of T cell activation *in vivo* and its effects on the overall levels of proviral gene expression. Use of a coordinated genetic approach for the delivery of beneficial, tumoricidal molecules to the tumor site may be required.

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ABSTRACT

RETROVIRAL-MEDIATED GENE TRANSFER OF IL-1β cDNA INTO HUMAN ACTIVATED T CELLS

by

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DECEMBER 1998

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Autologous activated T cells (ATC) are being utilized in clinical trials as potential mediators of anti-tumor cytotoxicity in refractory cancer patients who suffer from hematologic malignancies or solid tumors. ATC are readily generated from peripheral blood and possess non-major histocompatibility complex (non-MHC) restricted killing properties. ATC are also powerful targets for genetic modification and hold promise for treatments of cancer, AIDS and T cell disorders.

Retrovirus-mediated gene transfer and the fate of proviral gene expression were evaluated in human T cells using 1) immobilized anti-CD3 monoclonal antibody (mAb) plus interleukin-2 (IL-2), or 2) *cis* costimulation using beads carrying co-immobilized anti-CD3 and anti-CD28 mAbs. By cross-linking the CD3 and CD28 receptors, these mAbs mimic *in vivo* signaling events, leading to cytokine production and proliferation. A modified human interleukin-1β (IL-1β) cDNA inserted into the MFG retroviral vector served as an indicator gene in these studies. Optimized methodologies resulted in T cell

transduction frequencies of approximately 50-75%.

An important consideration for the success of T lymphocyte-based gene therapy is the relationship between T cell activity and the level of proviral gene expression. Early after mAb stimulation and virus exposure, proviral gene expression was greater at the RNA and protein levels in optimized anti-CD3/anti-CD28 bead-activated cultures, corresponding with augmented endogenous cytokine responses and mitogenesis. Proviral gene expression was not regulated by extrinsic cellular factors present in activated T cell supernatants. Regardless of the mAb stimulation method, proviral IL-1β expression declined in long-term cultures concomitant with a decrease in cellular cytokines. Restimulation of transduced T cells by either mAb method reinduced both T cell activity and vector expression. The finding that proviral gene regulation is downmodulated in the absence of T cell signaling events suggests that *in vivo*, high-level proviral gene expression will not be maintained. To overcome the loss of activity in gene-modified T lymphocytes, we have applied a bispecific antibody (BsAb) to redirect cytotoxicity to tumor and enhance T cell activity. For successful cancer adoptive immunotherapy, a coordinated approach of BsAb arming of retrovirus-gene-modified T cells can be utilized for delivery of beneficial, tumoricidal molecules to the tumor site.

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