Herpes virus ICP47 and US11 suppression of CTL activity adapted for use in gene therapy

by

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For the Major Program

Thank you

Dr. Link for polishing my scientific skills.
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ABSTRACT

A major stumbling block encountered in gene therapy clinical trials has been the rapid destruction of the therapeutic product by the host's immune response. The goal of this work is to diminish the host Cytotoxic T Lymphocyte (CTL) response to gene therapy transgenes and transduced cells by incorporating immunosuppression genes into gene therapy vectors. The processing and presentation of antigens through the class I Major Histocompatibility Complex (class I MHC) pathway is known to be an early response to viral infection. Disruption of the class I MHC pathway results in a decrease of the $CD8^+$ CTL response and prolongs viral survival in the host. Two viral immune suppression genes that interfere with the class I MHC pathway, the herpes virus ICP47 encoding gene and the human cytomegalovirus US11 gene, were cloned and each incorporated into an LXSN retroviral vector. The vectors were used to transduce multiple cell lines including human, rat, and dog. The US11 gene effectively decreases the class I MHC cell surface expression in all cell lines tested whereas the ICP47 gene functions in all but the rat cells. *In vitro* cytotoxicity experiments show the level of class I MHC reduction correlates with a reduction of specific lysis by anti-tumor primed CTL populations. Three separate applications of ICP47 and US11 suppression of CTL activity are reported. First, the reduction of class I MHC expression in target cells was used to characterize the ability of a clinically relevant anti-tumor cell line to select tumor targets. Second, class I MHC cell surface expression was reduced in two promising anti-tumor cell lines which are currently being used for allogeneic transplant in cancer treatments. Third, US11 class I MHC down regulation in murine cells was used in an attempt to prolong the survival of a chemically generated tumor model cell line *in vivo*.

CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

The combination of genetic defects being identified and gene target/delivery methods being developed has led to an explosion in the number of gene therapy clinical protocols [1]. Increasingly, a common problem encountered in gene therapy protocols involves the host's immune response to the proteins encoded by the genes inserted [2-4]. The majority of protocols utilize viral gene delivery and thus face similar obstacles to those encountered by wild type virus [5, 6]. Of the many types of immune responses, the CTL response to class I MHC restricted antigens is known to play an early and key role in host response to viral infection (Figure 1). As expected, host CTL response to transgenes and viral proteins has been observed in viral based gene therapy protocols [7-9]. Viruses have evolved numerous mechanisms to circumvent host immune responses [10-12]. Many of these mechanisms provide clues that help explain the pathogenesis of these organisms and in turn, suggest therapeutic treatments to augment host defenses. Traditionally, this ability of viruses has posed significant problems for the treatment of infected individuals. For gene therapy, viral evasion of host immune response offers an opportunity to prolong the persistence of therapeutic transgenes and the cells they have transduced.

The earliest specific host response to viral challenge involves the stimulation and propagation of $CD8^+$ CTL that kill cells presenting viral derived peptides on class I MHC molecules (Figure 2). The response involves very specific recognition of short peptides in conjunction with the appropriate class I MHC molecule by a CTL possessing the corresponding T Cell Receptor (TCR). Genes encoding both class I MHC and TCR are polygenic, polymorphic, and expressed co-dominant thereby generating the vast number of

peptide presenting and recognizing combinations needed to identify viral protein fragments. An individual CTL expresses a single variant of TCR and thus is said to be restricted by its corresponding class I MHC/viral peptide complex. The viral peptides are generated from viral proteins present in the cytoplasm that are degraded by the proteasome (Figure 3). Peptides 8-9 amino acids long are then transported to the endoplasmic reticulum (ER) by the Transporter associated with Antigen Processing (TAP) [13, 14], loaded onto an immature class I MHC molecule, and sent through the golgi apparatus to be displayed on the cell surface. Although all cytoplasmic proteins are subject to this process, CTL recognizing self peptides are eliminated in the thymus and thus do not generate a proliferative response. Most tissues are susceptible to viral infection and all mammalian tissue types except cornea [15], brain [16], and testis [17] present class I MHC antigens on their cell surface. The ubiquitous expression of class I MHC, its key role in host response to viral challenge, and recent characterization of class I MHC presentation inhibitors are factors making it an attractive target for generating gene therapy vectors that defeat this aspect of the immune response.

Several wild type viruses have evolved means to avoid the class I MHC mediated host immune responses. Early in the study of viral pathogenesis it was noted that both adenoviruses [18] and members of the Herpes Simplex Virus (HSV) family fail to generate CTL responses during portions of their life cycles [19]. Specifically HSV type I and Human Cytomegalovirus (HCMV, Human Herpesvirus 5) both fail to stimulate CTL proliferation and infected cells present little or no class I MHC on their cell surface. In HSV type I, the protein ICP47 has been shown to block transport of peptides from the cytoplasm to the ER by blocking the cytoplasmic peptide binding site of the TAP (Figure 3) [20-22]. Without peptide, immature class I MHC molecules are retained in the ER and do not get displayed on

the cell surface. HCMV uses at least five proteins to interrupt the presentation of class I MHC on the cell surface [23]. One of the more effective proteins is encoded by the US11 gene [24, 25]. It has been shown to bind mature class I MHC in the ER and rapidly dislocate it back into the cytoplasm. In the cytoplasm the class I MHC is degraded and thus does not make it to the cell surface. For the work presented herin, both HCMV US11 and the HSV type I gene encoding ICP47 were cloned and incorporated into a murine derived retroviral vector system (LXSN) commonly used to deliver therapeutic transgenes in human clinical protocols.

Several gene delivery methods are currently in clinical use to deliver therapeutic genes. Each system has advantages and disadvantages that make it suitable for a given task (Figure 4). Molony murine leukemia virus (MoMLV) has been the predominant method used in clinical trials to introduce therapeutic genes [26, 27]. With a minimal number of genes encoded by the vector, host immunological challenge can be minimized. Transduction of most mammalian cell types is possible using vector packaged with amphotropic receptor and vector integration leads to stable gene expression [28]. For these reasons, LXSN was chosen as the vector system to deliver the ICP47 and US11 class I MHC suppression genes. The drawbacks for use of this system in gene therapy are that LXSN only integrates in dividing cells and packaging systems produce low titers relative to other delivery vectors. For *in vitro* experimentation, the ability to choose rapidly dividing cell lines and select transduced populations effectively negates these disadvantages.

To generate supernatants containing viruses encoding ICP47 or US11, a two step transduction process was used (Figure 5) [28, 29]. First the LXSN derived vectors were transfected into the GP +E86 cell line which contains a stably transduced copy of the

necessary GAG, POL, and ENV genes. Only sequence from the LXSN vector contains the packaging signal and thus gets assembled into functional virus and secreted into the supernatant. The GP +E86 ENV gene encodes an ecotropic envelope protein enabling the virus produced from this cell line to transduce other murine cells. This supernatant is then used to transduce the PA317 cell line containing an ENV gene that encodes an amphotropic envelope protein. Supernatants from the PA317 cells were used to transduce the rest of the target cells that were discussed herein. This two step process is more efficient at generating transduction competent virus containing full-length LXSN sequences. Target cells are then selected for in media with G418; a neomycin phosphotransferase homologue that kills cells lacking the LXSN encoded Neo^R gene. Function of the immune suppression genes was assayed using cell surface labeling by an anti-class I MHC Ab, a fluorescent secondary Ab, and flow cytometry quantification (Figure 6).

DISSERTATION ORGANIZATION

The work presented within this dissertation focuses on the generation of LXSN vectors incorporating ICP47 and US11 to decrease cell surface presentation of class I MHC antigens. In this manner, the suppression of the class I MHC expression and the resulting CTL response occurs only in the transduced cells thus avoiding problems associated with general immune suppression. Chapter 2 of this dissertation entitled "Effective suppression of class I MHC expression by the US11 or ICP47 genes can be limited by cell type or IFNγ exposure" details the construction of vectors and transduction of a panel of cell lines representing several tissues. Cell type variability of function by ICP47 and the US11 gene product and cytokine induction of class I MHC expression are identified as factors that may limit the use of these inhibitors under specific conditions. Chapter 2 has been accepted for

publication in an upcoming issue of the journal *Human Gene Therapy*. Chapter 3 of this dissertation entitled "TALL-104 cytotoxicity correlates with the level of class I MHC expression in a nonrestricted manner" makes use of ICP47 and US11 inhibition of class I MHC expression to characterize the targeting mechanism of the clinically relevant anti-tumor cell line TALL-104. Although the work is specific to the TALL-104 cell line, recent publications indicate this may involve a novel form of tumor surveillance by a newly identified subset of T cells. This chapter will be submitted for publication to the journal *Cancer Research*. Chapter 4 of this dissertation entitled "Application of class I MHC immune suppression for use in allogeneic transplant and tumor model cell evasion of host response" reports the results of three groups of experiments designed to exploit the advantages of ICP47 and US11 within their limitations. The experiments involving inhibition of class I MHC presentation in NK-92 cells while maintaining their anti-tumor activity will be reformatted and submitted to the journal *Anticancer Research* for publication.

Thomas Radosevich was the principle investigator for the work presented in each chapter of this dissertation. Co-major professors Charles J. Link and M. Duane Enger advised the work.

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Figure 1. Review of the cells of the immune system. Non-specific responses such as responses to parasites, identification of stressed cells, and non antigen specific secretion of cytokines are regulated through basophils, eosinophils, neutrophils, monocytes, and NK cells. Humoral responses to specific antigens are mediated by B lymphocytes and antigen specific cellular responses are mediated by T lymphocytes. An effective long term strategy to evade host detection will likely require means to minimize non specific recognition of stressed cells and eliminate antigen specific B cell and T cell mediated responses. Figure adapted from

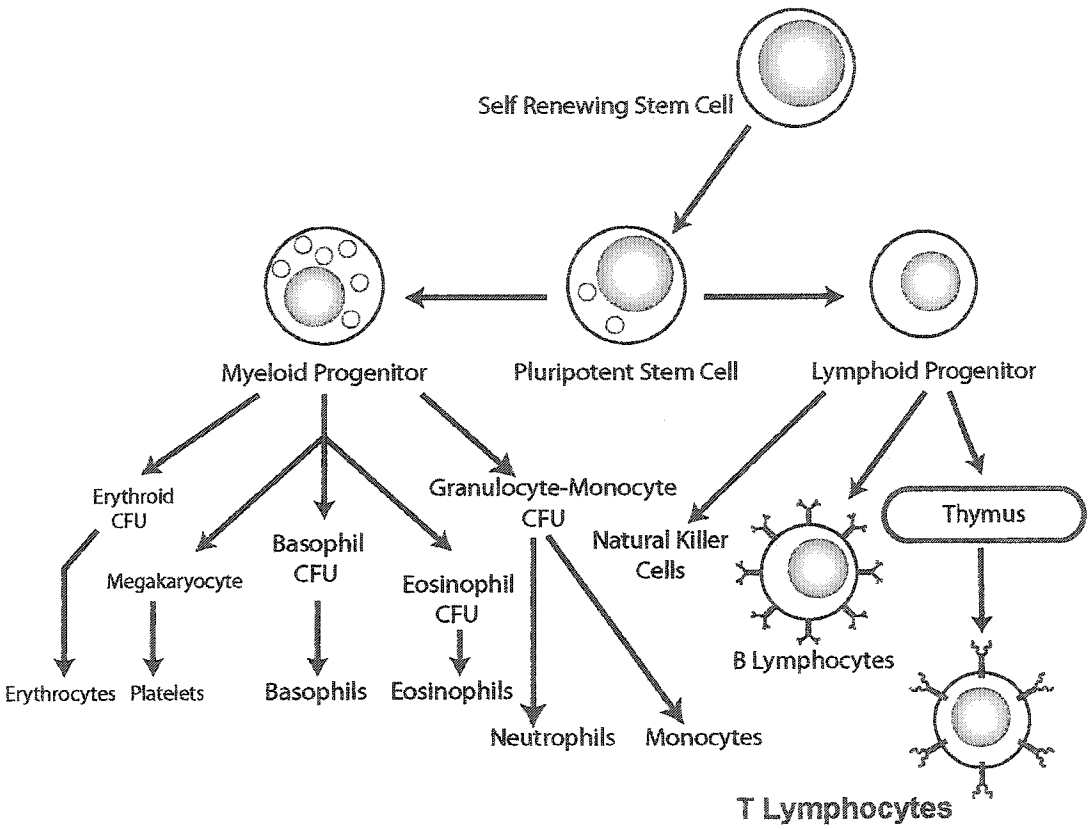

Self Renewing Stem Cell
Myeloid Progenitor
Pluripotent Stem Cell
Lymphoid Progenitor
Erythroid CFU
Megakaryocyte
Basophil CFU
Eosinophil CFU
Granulocyte-Monocyte CFU
Natural Killer Cells
Thymus
Erythrocytes
Platelets
Basophils
Eosinophils
Neutrophils
Monocytes
B Lymphocytes
T Lymphocytes

Figure 2. A typical time course of various aspects of the immune response. After viral infection, non specific responses by cells secreting interferons and other cytokines peak within 3-5 days. The first antigen specific response by CTL peaks between 8-12 days and leads to the decrease of viral titers. Primary Ab response, likely from antigens processed as viral infected cells are killed, peaks around day 15 and the more substantial secondary response after additional challenge peaks at 40-45 days.

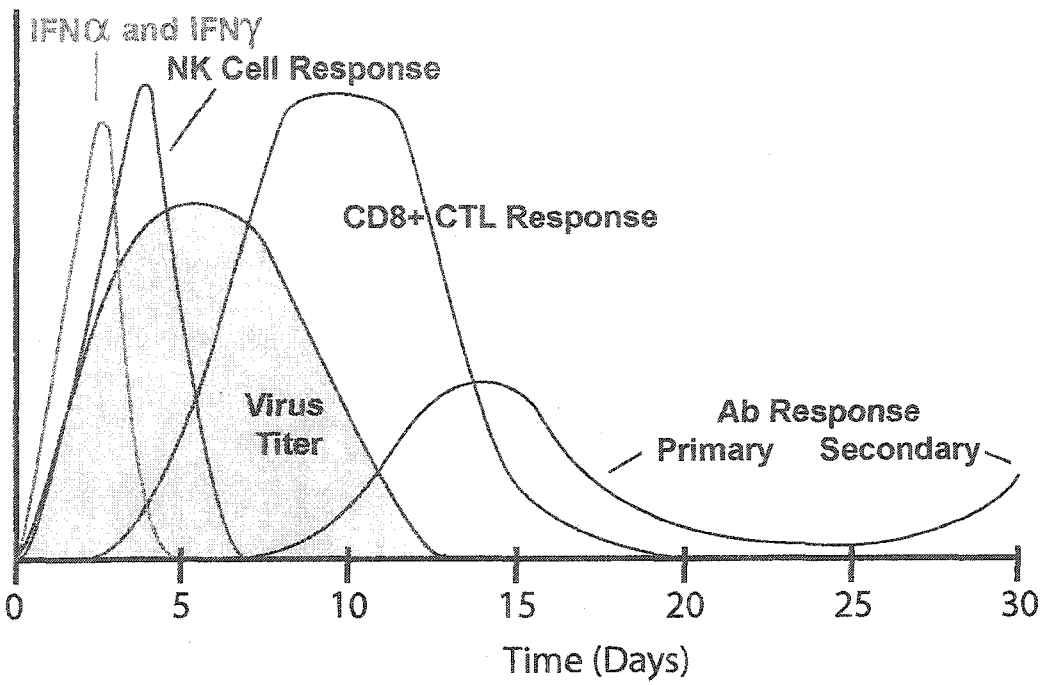

IFNα and IFNγ
NK Cell Response
CD8+ CTL Response
Virus Titer
Ab Response
Primary
Secondary
0
5
10
15
20
25
30
Time (Days)

Figure 3. Class I MHC processing and presentation pathway. Cytoplasmic proteins are processed into 8-10 AA peptides by the proteasome and transported into the ER. Mature class I MHC molecules are assembled and transported through the golgi complex and secretory vesicles to the cell surface where they are displayed for recognition by CTL. HSV ICP47 and HCMV US11 inhibit different steps in the pathway.

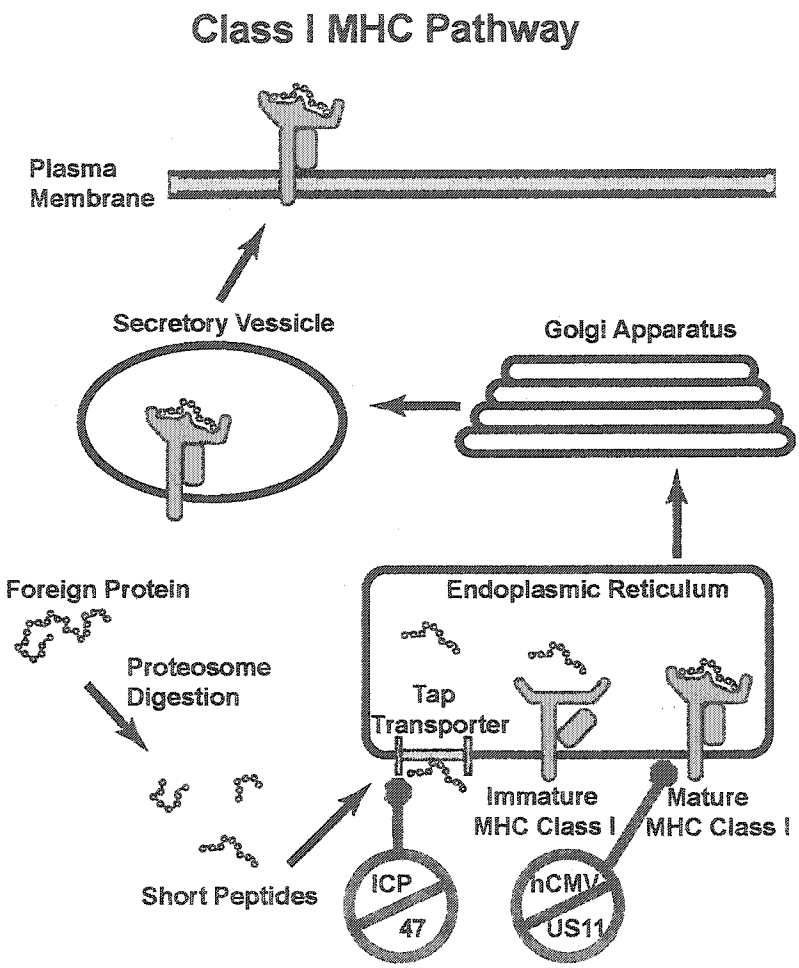

Class I MHC Pathway
Plasma Membrane
Secretory Vessicle
Golgi Apparatus
Foreign Protein
Endoplasmic Reticulum
Proteosome Digestion
Tap Transporter
Immature MHC Class I
Mature MHC Class I
Short Peptides
ICP 47
hCMV US11

Figure 4. Advantages and disadvantages of the different gene delivery systems in clinical use.

Vector Class	Expression	Advantages	Disadvantages
Murine Molony Leukemia Virus (retrovirus)	Stable	Low Immunogenicity against viral vector, Integrates	Only integrates in dividing cells, Relatively low viral titers
Lentivirus (Retrovirus)	Stable	Transduces non-dividing cells, Can target CD4+ cells, integrates	Unknown safety issues
Adenovirus	Transient	High viral titer, Transduces most cell types	Highly immunogenic, Transient expression
Adeno-Associated Virus	Stable	Minimal Immunogenicity against viral vector, Integrates	Low Transduction efficiency
Herpes Simplex Virus (HSV)	Transient	Ability to package large sequences, Transduces most cell types	Non-integrating
Vaccina Virus	Transient	Infects most cell types, Packages large sequences	Induces strong T-cell responses
Cationic Lipids and Liposomes	Transient	No vector immune response	Low transfection rates

Compiled from: *The Scientist* 14[19]:28, Oct. 2, 2000

Figure 5. Two step process for the generation of virus with amphotropic envelope proteins. Plasmid DNA is first transfected into GP +E86 cells where it is reverse transcribed, migrates to the nucleus, and stably integrates. GAG, POL, and ENV genes are already integrated and function to produce empty viral capsids. The packaging signal on the LXSN vectors directs the cell to package full-length retroviral RNA into the capsids that are secreted into the supernatant. The ecotropic envelope protein encoded by the GP +E86 cells enables transduction of other murine cells and the supernatant is used to transduce the PA317 cell line. The process is repeated with the exception that the PA317 ENV gene encodes an amphotropic receptor enabling the transduction of any mammalian cell.

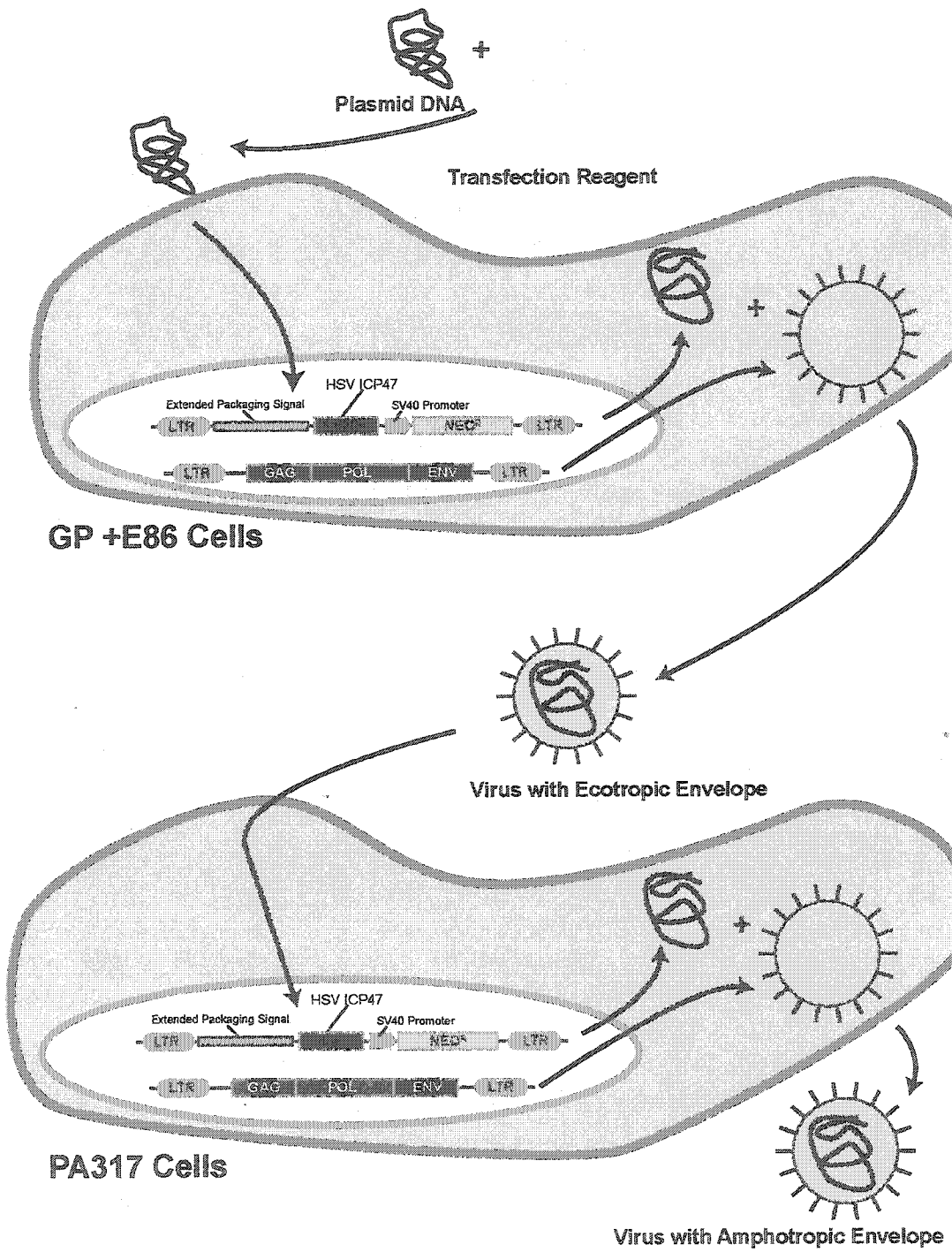
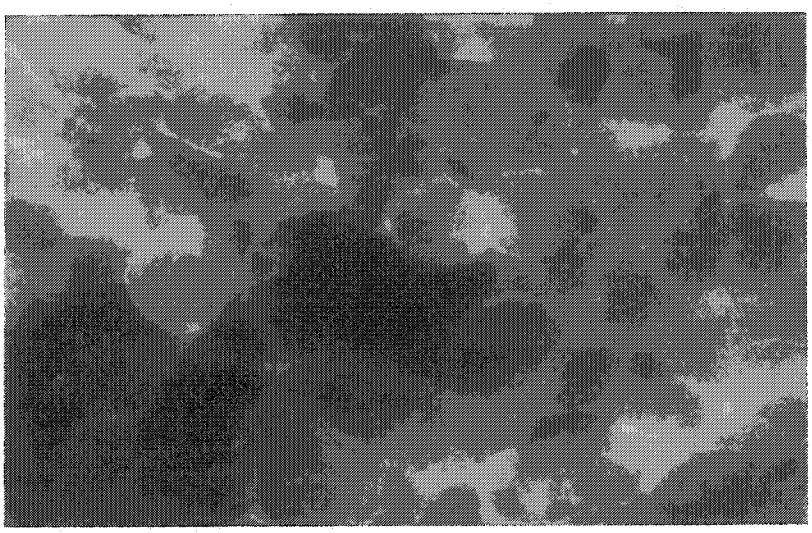
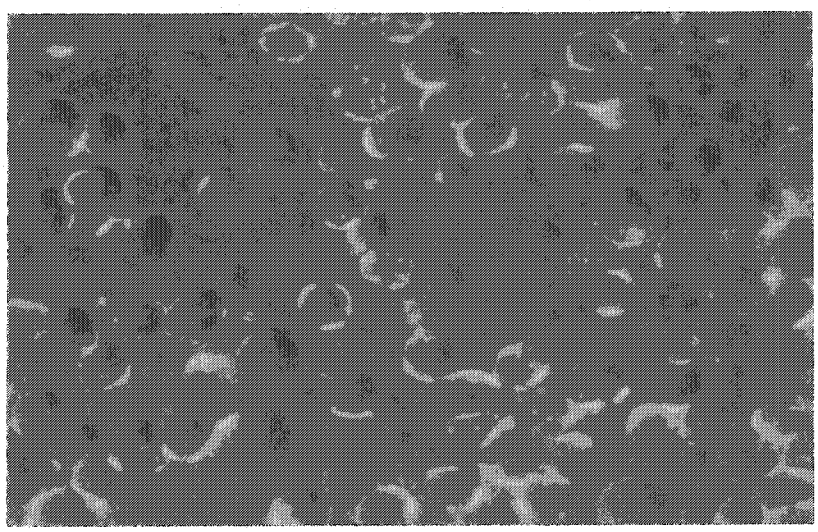

Plasmid DNA
Transfection Reagent
HSV ICP47
Extended Packaging Signal
SV40 Promoter
LTR
NEO
LTR
LTR
GAG
POL
ENV
LTR
GP +E86 Cells
Virus with Ecotropic Envelope
HSV ICP47
Extended Packaging Signal
SV40 Promoter
LTR
NEO
LTR
LTR
GAG
POL
ENV
LTR
PA317 Cells
Virus with Amphotropic Envelope

Figure 6. Cell surface labeling of class I MHC epitopes. Examples of (A) an attached cell line (A375) and (B) a suspension cell line (NK-92) labeled with the H58A Ab. H58A recognizes a region of class I MHC that is highly conserved across species. Fluorescence is provided by a FITC labeled secondary Ab. Characteristic cell surface staining is seen.

A.



B.



CHAPTER 2. EFFECTIVE SUPPRESSION OF CLASS I MHC EXPRESSION BY THE US11 OR ICP47 GENES CAN BE LIMITED BY CELL TYPE OR IFNγ EXPOSURE

A paper accepted by the journal *Human Gene Therapy*, 2003

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Running Title: Suppression of MHC I by the ICP47 and US11 genes

Key Words: ICP47, US11, $CD8^+$, immune response, transgene, class I MHC

Abbreviations:

HSV: Herpes Simplex Virus MHC: Major Histocompatibility Complex

CTL: Cytotoxic T Lymphocyte CMV: Cytomegalovirus

TAP: Transporter associated with Antigen Processing

LDH: Lactate Dehydrogenase

PBMC: Peripheral Blood Mononuclear Cells

ABSTRACT

An impediment encountered in many viral based gene therapy clinical trials has been the rapid destruction of the transgene by the host's immune response. The processing and presentation of antigens through the class I major histocompatibility complex (MHC) pathway is the initial specific response to viral infection. Disruption of the class I MHC pathway by Herpes Simplex Virus (HSV) or the human Cytomegalovirus (HCMV) results in a decrease of the $CD8^+$ cytotoxic T lymphocyte (CTL) response and prolongs survival of infected cells in the host. Two viral immune suppression genes that interfere with the class I MHC presentation pathway, the HSV type I ICP47 gene and HCMV US11 gene were cloned and each incorporated into a retroviral vector. HSV ICP47 and HCMV US11 transgenes were expressed in multiple cells lines and compared in their abilities to reduce antigen presentation on the cell surface by class I MHC. Retroviral supernatants were used to transduce human, canine, and rat cell lines. FACS analysis of US11 and ICP47 transduced cell lines demonstrated substantial reductions in class I MHC cell surface expression in most cell lines except in rodent cells where ICP47 is non-functional. The decrease in the level of class I MHC expression for ICP47 transduced cell lines ranged from 31% to 98% relative to negative controls. US11 decreased class I cell surface MHC by 67% to 96%. When both ICP47 and US11 are expressed in human cells, a further reduction of class I MHC was observed. Next, human A375 melanoma cells were tested to determine if the resulting reduction in cell surface class I MHC would reduce *in vitro* cytotoxicity by CTL. A375 cells expressing either ICP47 or US11 demonstrated a two to threefold reduction of specific lysis by primed $CD8^+$ CTL. These data clearly establish an ability to convey immune protection to human cells by viral genes. However, further analysis demonstrated that Interferon

gamma (IFNγ) could reverse part or all of the down-regulation of class I MHC induced by the ICP47 or US11 genes. The ICP47 and US11 genes when expressed in target cells decrease class I MHC presentation and as such might be employed in strategies to create local immunosuppression against transgenes or allografts.

OVERVIEW SUMMARY

Host immune response to vector encoded proteins and therapeutic transgenes is a major impediment to attaining long term gene expression in gene therapy applications. Several members of the herpes virus family have developed unique strategies for overcoming host immune response. ICP47 and the US11 gene products are two viral proteins that inhibit presentation of class I MHC on the cell surface. Incorporation of these genes into gene transfer vectors may induce local immune suppression by this mechanism. Herein, two potential problems with this approach are addressed. First, cell type specific variability of function of the viral genes was observed that may limit immune protection for some cell types. Secondly, host cytokine release after transgene exposure may upregulate class I MHC expression and thus complicate the use of this approach *in vivo*.

INTRODUCTION

A common problem encountered in gene therapy protocols involves the host's immune response to the proteins encoded by the genes inserted. Transgenes delivered by viral vectors face similar host immune surveillance and elimination mechanisms as those encountered by wild type virus. The CTL response to class I MHC restricted antigens is known to play an early and key role in host response to viral infection. As expected, host CTL response to therapeutic transgene proteins and/or viral proteins has been observed in gene therapy protocols employing viral vectors to deliver transgenes (Yang *et al.*, 1995). Host elimination of viral vectors (Lochmuller *et al.*, 1996; Riddell *et al.*, 1996; Smith *et al.*, 1996), and specific T cell response to a transgene (Poller *et al.*, 1996; Tripathy *et al.*, 1996) have been well documented. Overcoming this host CTL response and prolonging vector survival is now recognized as a major goal for many gene therapy models. Optimally, the host immune response would be suppressed against only the transduced cells expressing the transgene in order to avoid general immune suppression and its complications.

This work employs two viral genes delivered by a retroviral gene therapy vector to reduce class I MHC expression. Several classes of viruses have evolved proteins that specifically suppress the CTL response in order to prolong their own survival (Wold and Gooding, 1991; Beersma *et al.*, 1993; McFadden and Kane, 1994; Fruh *et al.*, 1995). Both the ICP47 protein and the US11 gene product have been characterized in detail and each inhibits a different step in the antigen processing and presentation pathway. ICP47 is a 88 amino acid immediate early protein that was identified by its ability to block the cell surface expression of class I MHC restricted HSV peptides (York *et al.*, 1994; Fruh *et al.*, 1995). The N-terminal 35 amino acids of ICP47 bind to the peptide-binding site on the cytosolic

side of the transporter associated with antigen processing (TAP) (Galocha *et al.*, 1997). This ICP47/TAP association prevents the 8-10 amino acid peptides derived from proteosome degraded proteins from entering the endoplasmic reticulum (ER). This leads to the retention and degradation of immature class I MHC molecules and reduces their transport through the Golgi to the cell surface (Ahn *et al.*, 1996; Tomazin *et al.*, 1996). The HCMV US11 protein affects a different step in the antigen processing and presentation pathway (Hengel *et al.*, 1996; Wiertz *et al.*, 1996). The US11 gene encodes a 215 amino acid transmembrane glycoprotein which dislocates the class I MHC heavy chain via a polyubiquitination mediated mechanism from the ER to the cytosol where it is rapidly degraded (Kikkert *et al.*, 2001; Shamu *et al.*, 2001). In cells where US11 is expressed, newly synthesized class I MHC proteins have a short half-life and are not presented on the cell surface (Beersma *et al.*, 1993).

The 267 base pair ICP47 and 645 base pair US11 genes can fit easily within the packaging constraints of most retroviral gene therapy vectors with additional room for another transgene. Their size makes them ideal candidates to suppress immune responses when long term gene expression is desired. A direct comparison of ICP47 function to US11 in multiple cell lines was conducted to quantitatively assess reductions of class I MHC expression. In varying gene therapy approaches, foreign transgenes have been expressed in a variety of cell types and tissues (Cox *et al.*, 1993; Engelhardt *et al.*, 1993; Bagnis *et al.*, 1994; Philip *et al.*, 1994). The ICP47 and US11 genes were evaluated to determine if the strategy can function independent of cell type. Our experiments demonstrate significant variability of ICP47 and US11 in reducing class I MHC across the human cell lines. In A375 melanoma cells reduction of class I MHC on the cell surface after ICP47 or US11 expression resulted in an

expected decrease in specific lysis by $CD8^+$ CTL. Although these results are encouraging, the immune response is clearly more complex and often involves a host cytokine response (Harms and Splitter, 1995; Zhai *et al.*, 1996; DeMatteo *et al.*, 1999). The potential antagonistic effect of the cytokine IFNγ was therefore assessed. IFNγ has been shown to upregulate transcription of genes in the class I MHC locus (Weber and Rosenberg, 1988). Culturing cells in IFNγ substantially reduces or eliminates the class I MHC decrease induced by either ICP47 or US11. This would have the effect of 'unmasking' the transgene in response to any local immune challenge that leads to the release of IFNγ thus compromising long-term immune suppression. Therefore, our data suggests a more complex interplay will be required to adapt these immunomodulating genes for application in transgene or transplant therapies.

MATERIALS AND METHODS

Vector and cell line construction

Polymerase chain reaction (PCR) amplification of the ICP47 (Sense: GCAAGCTTGAAGCGCGTATGTCGTGGGCCCTGGAAATGGCG, Antisense: GCAAGCTTTCAACGGGTTACCGGATTACGGGG) gene from purified HSV DNA (Sigma-Aldrich Corp., St. Louis, MO) and the US11 gene (Sense: GCAACGTTGAAGCGCGTATGAACCTTGTAATGCTTATTCTA, Antisense: GCAAGCTTTCACCACTGGTCCGAAAACATCCA) from HCMV DNA (Sigma-Aldrich Corp.) was performed and the products were cloned into the T/A cloning vector PCR 3.1 (Invitrogen Inc., Carlsbad, CA). Sample clones were sequenced to confirm their identity (data not shown). ICP47 and US11 were excised from PCR3.1 using the restriction endonuclease EcoR I and each sub-cloned into the LXSN vector at the EcoR I site(Miller and Rosman, 1989). To express US11 from a second vector with a different selection marker, US11 was subcloned into the MSCVp vector that employs the puromycin resistance gene. The US11 in MSCVp vector was constructed by excising the Eco RI fragment from the PCR3.1 construct and ligating it into the Eco RI site in the multicloning region of the MSCVp vector (Clontech Inc. Palo Alto, CA). Vectors in the proper orientation were selected (Fig. 1A, 5A). The LISN, LUSN, US11 in MSCVp, and control LXSN vectors were each transfected into the GP+E86 cell line (Hesdorffer *et al.*, 1990). Supernatants were filtered (0.2μm) and used to transduce PA317 amphotropic vector producer cells (VPC) (Miller and Buttimore, 1986).

The following cell lines were transduced and selected in the neomycin homologue G418 (1mg/ml) for 2 weeks: A375 human malignant melanoma (Giard *et al.*, 1973); SW620

human colorectal adenocarcinoma (Fogh *et al.*, 1977); A549 human lung carcinoma (Giard *et al.*, 1973); 293 adenovirus transformed human fetal kidney (Graham *et al.*, 1977); IGROV human ovarian carcinoma (Benard *et al.*, 1985); OVCAR-3 human ovarian adenocarcinoma (Hamilton *et al.*, 1983); DU145 human prostate carcinoma (Stone *et al.*, 1978); 9L rat brain (Lampson *et al.*, 1992); D17 dog osteosarcoma (Riggs *et al.*, 1974); MOLT-3 human acute lymphoblastic leukemia (Minowada *et al.*, 1972); VA13 SV40 transformed human fetal fibroblast (Girardi *et al.*, 1966).

Northern analysis

Total RNA was purified from the transduced mixed A375 cell lines (RNeasy®, Qiagen Inc., Germantown, MD). The RNA was electrophoresed on a 1.2% agarose gel, blotted overnight onto nylon membrane (.45 μm) and UV cross-linked. Random primed ^{32}P radiolabeled DNA probes were made for the full length ICP47, the full length US11, and a 680bp fragment of the neomycin phosphotransferase gene. Probes were incubated with the membrane for 12 hr at 42° in hybridization solution (Hybrisol® I Hybridization Solution, Oncor, Gathersberg, MD) and washed 5 min 2 times in 20x SSC, 1% SDS at room temperature. The membrane was then exposed to Kodak XAR-5 film for 30 sec and developed.

Class I MHC Expression assay

Transduced, mixed populations of cells were briefly trypsinized and labeled with anti-MHC class I monoclonal Ab H58A (VMRD Inc., Pullman, WA) at 1.5 μg/ml/0.5×10^6 cells in serum free media at 37° for 1 hr (Davis *et al.*, 1987). Cells were washed 5 min in Hank's

buffer and a 1:64 dilution of goat anti-mouse FITC labeled secondary Ab (Sigma-Aldrich Corp.) in serum free media was added for 30 min at 37°. Cells were again washed 5 min in Hank's buffer and resuspended in Phenol red free/serum free media (OptiMEM®, Invitrogen Corp., Inc.Grand Island, NY) for fluorescent analysis. All fluorescent analysis experiments were performed a minimum of three times and a representative result is shown. Where a single peak was observed, the mean fluorescence intensity is reported. The same protocol as above was used for the rat cell line with the primary Ab being OX18 (PharMingen Inc., Torry Pines, CA). For IFNγ stimulation of class I MHC, cells were cultured 72 hr in media containing 400U/ml IFNγ with fresh media replacement after the first 48 hr

Cytotoxicity Assay

A non-radioactive colorimetric assay was used to determine specific cytotoxicity of random donor Peripheral Blood Mononuclear Cells (PBMC) towards vector transduced and control target cells. The Cytotox® 96 (Promega Inc., Madison, WI) assay is based on measuring lactate dehydrogenase (LDH) release by a reaction with a tetrazolium salt (INT) to produce a red formazan product (Korzeniewski and Callewaert, 1983). In brief, target cells are plated in a 96 well plate and effector cells are added at the appropriate ratio. After 4 hr, supernatant is collected and assayed for LDH content. Controls include maximum LDH release (detergent lysed), media background, and spontaneous LDH release from both targets and effector cells. Data presented is an average from four wells/data point. PMBC were isolated from whole blood of a random donor using a sucrose gradient (Ficoll-Paque® Plus, Amersham Biosciences, Upsala Sweden) to obtain the buffy coat. Cells were collected and expanded seven days in RPMI media plus 10% FBS, MEM sodium Pyruvate, 2-

Mercaptoethanol, HEPES, MEM non-essential amino acids, and L-Glutamine (Gibco BRL), plus 100 units/ml IL2 to stimulate $CD8^+$ cell proliferation. $CD8^+$ enriched PBMC were obtained using a human $CD4^+$ cell depletion column (Cytovax Biotechnologies Inc., Edmonton, AB, Canada) to remove both $CD4^+$ cells and large granular cells.

RESULTS

Expression of ICP47 and/or US11 decreases class I MHC levels on the cell surface of transduced cells

In order to directly compare the function of HSV ICP47 and HCMV US11, both genes were cloned into the retroviral vector LXSN (Fig. 1A). PA317 based retroviral vector producer cell lines were constructed and target cells transduced. Functional transcription of the vectors was demonstrated by Northern blot analysis (Fig. 1B) in the A375 cell lines where all three LXSN derived vectors were shown to functionally transcribe RNA of the expected sizes. To detect cell surface class I MHC expression in the A375 cell line, the anti-class I MHC antibody H58A was used. H58A is a monoclonal Ab raised against a highly conserved region of the goat class I MHC heavy chain molecule (Davis *et al.*, 1987). It has been shown to cross react with class I MHC molecules from multiple species, including all human and canine haplotypes tested. A FITC labeled anti-mouse secondary Ab was used to detect H58A binding. Characteristic cell surface staining was noted and the results were quantified by flow cytometry (Fig. 2A). A single peak was observed and the mean fluorescence of the transduced mixed cell population reported (Fig. 2B). In the A375 cell line, US11 decreased class I MHC by 94% as compared to LXSN transduced controls. ICP47 reduced cell surface class I MHC by 52%. These results remained consistent over 6 months in continuous culture (data not shown).

Down Regulation of class I MHC cell surface expression in multiple human cell lines

To compare cell line variability of function of ICP47 and US11, both vectors were introduced into several human cell lines of different tissue origin and class I MHC expression

was quantified (Fig. 3). All human cell lines transduced with the ICP47 and US11 genes showed decreased cell surface expression of class I MHC compared to the LXSN vector control. For ICP47, class I MHC cell surface levels dropped over a range of 31% in the OVCAR-3 ovarian cancer cells to 98% in the MOLT-3 lymphoma cells (Fig. 3 and 7A). For US11, the range of class I MHC cell surface decrease was 64% in VA13 fibroblasts to 96% in the MOLT-3 cells. In most cell lines, ICP47 and US11 decreased cell surface class I MHC to similar levels. However, in A375 and OVCAR-3 cells ICP47 only reduced class I MHC expression to approximately one half that observed with US11. Thus, cell specific differences in ICP47 and US11 function are likely to occur.

Down regulation of class I MHC cell surface expression in dog and rat cell lines

ICP47 has minimal function in rodent cells due to its decreased affinity for the N-terminal 45 amino acids of murine TAP (Tomazin *et al.*, 1996; Tomazin *et al.*, 1998). To determine if dogs or rats would be appropriate for *in vivo* studies canine D17 osteosarcoma cells and rat 9L gliosarcoma cells were transduced. US11 reduced class I MHC expression in both canine D17 and rat 9L cells by 71% and 64% respectively (Fig. 4). The ICP47 gene showed similar response in the canine cells to that measured in the human cells lines with a 87% reduction of class I MHC expression. As expected, ICP47 showed a much lower (24%) reduction of the cell surface class I MHC over LXSN control cells than was observed in most of the human cell lines.

Co-expression of ICP47 and US11

ICP47 and US11 work at different points in the class I MHC presentation pathway and co-expression of the two genes was examined to determine if further decreases could be induced. In order to co-express US11 and ICP47, US11 was cloned into MSCVp, a puromycin selectable alternate murine retroviral vector to allow for selection of doubly transduced cells (Fig. 5A). MSCVp has a mutated 5' LTR containing a murine stem cell virus promoter designed to maintain long term expression in embryonic stem cells (Grez *et al.*, 1990). The MSCVp vector has also been reported to function in other mammalian cells (Hawley *et al.*, 1994). When compared to the LUSN vector, US11 in MSCVp was considerably less effective at reducing cell surface class I MHC. This was demonstrated in both A375 and VA13 cells (Fig. 5B, 5C). However, in both cell lines tested, co-expression of ICP47 and US11 further decreased the cell surface class I MHC over levels observed with either gene alone.

Decreased specific lysis of transduced target cells by primed PBMC

Specific lysis of target cells was determined using primed, random donor PBMC or $CD8^+$ enriched population of primed PBMC. Cytotoxicity results using primed PBMC populations follow an expected pattern based upon the class I MHC cell surface expression experiments. Both non-transduced and LXSN vector transduced target cells are killed more efficiently than LISN or LUSN vector transduced targets (Fig. 6A). When $CD8^+$ enriched cells are employed these significant differences are more pronounced (Fig. 6B). After $CD4^+$ depletion, the $CD8^+$ enriched population was 82% $CD8^+$ (data not shown). At the 10:1 effector:target ratio, specific lysis of non-transduced cells was 59%, LXSN transduced 47%,

LISN transduced 20%, and LUSN 10%. This confirms that the down regulation of class I MHC observed in cells expressing ICP47 and US11 leads to an *in vitro* decrease of CTL lysis.

Interferon gamma restores high levels of cell surface expression of MHC I in ICP47 and US11 transduced cells

Molt-3 lymphoma and VA13 fibroblast cells were cultured in media containing 400U/ml interferon gamma (IFNγ). IFNγ is produced by NK cells and subpopulations of T cells and is known to upregulate cell surface class I MHC in many cell types. Although both ICP47 and US11 virtually eliminated (decreased by 98% and 96% respectively) cell surface class I MHC in the Molt-3 cell line, IFNγ restored expression to about 35% the amount seen in LXSN transduced cells (Fig.7). In the VA13 cell line, the IFNγ effect was even more pronounced. Although ICP47 and US11 reduced cell surface class I MHC by 73% and 64% respectively, incubation with IFNγ increased its expression by greater than 300% in both transduced and non-transduced cell lines. Only a minor reduction of cell surface class I MHC in the ICP47 and US11 transduced cell lines (4% and 7% respectively) was observed after IFNγ treatment.

DISCUSSION

Suppression of the immune response only in the transduced cells may aid in prolonging transgene survival. ICP47 and US11 decrease expression of class I MHC and the corresponding lysis by CTL which is the earliest antigen specific host response to viral infection. To assess and compare the use of ICP47 and US11 for gene therapy purposes, both genes were cloned and incorporated into otherwise identical retroviral vectors (Fig. 1A). Transcription of the genes was demonstrated by Northern blot (Fig 1B) and function of the proteins was indicated by a reduction in cell surface class I MHC in transduced cells (Fig. 2). The observed class I MHC reduction correlates with a drop in specific lysis by *ex-vivo* primed $CD8^+$ CTL as expected (Fig. 6). To directly compare function of ICP47 to US11 and to determine if tissue specific variabilities present a problem, a panel of human cell lines were transduced with both vectors (Fig. 2, 3, and 7). This study identifies and demonstrates three potential limits to the application of ICP47 and US11 for long term therapeutic immune suppression. First, the reduced function of ICP47 in two of nine cell lines indicates choice of gene used for immune suppression needs to be optimized for each application. Second, the range of class I MHC cell surface reduction across cell lines indicates that the choice of target cells will be important in determining the successful application of this immune suppression. Third, cytokine mediated upregulation of class I MHC must be circumvented to achieve long term transgene expression.

Comparing ICP47 to US11 within individual cell lines, both genes decreased class I MHC to nearly identical levels in seven of the nine human cell lines tested (Fig. 3, 7). These results are consistent with a previously published report on ICP47 and US11 expressed in primary human T cells and transformed fibroblasts where both genes decreased class I MHC

to similar levels as compared to non-transduced parental cells (Berger *et al.*, 2000). However, in the A375 melanoma and OVCAR-3 ovarian carcinoma cells, ICP47 was less than half as effective as US11 at decreasing class I MHC (Fig. 2, 3). Thus, careful testing of these genes in each specific target tissue will be necessary to ensure that adequate class I MHC decrease occurs for the given application.

The question as to what level of *in vivo* class I MHC decrease is needed to circumvent host $CD8^+$ CTL response is not easy to address. The antigenicity of specific epitopes and the amount of epitope presented are two key factors determining whether or not a CTL response occurs. As little as 1 to 200 class I MHC epitopes presented per cell are reported to be sufficient to mount a host response (Christinck *et al.*, 1991; Sykulev *et al.*, 1996). Antigenicity of specific epitopes varies greatly and with highly antigenic epitopes, less of it needs to be presented to initiate CTL mediated killing (Sette *et al.*, 1994). Therefore depending on the antigenicity of the transgene in question, it may be necessary to achieve near complete suppression of class I MHC in order to defeat the CTL response. In the seven cell lines where ICP47 and US11 functioned at comparable levels, the decrease of class I MHC ranged from 67% in the SW620 colorectal adenocarcinoma cells to 97% in the MOLT-3 lymphoblastic leukemia cells (Fig. 3, 7). Our data demonstrates further decreases of class I MHC where both genes are co-expressed (Fig. 5) in agreement with previous reports (Berger *et al.*, 2000). Thus, reducing class I MHC expression to a required level is technically feasible using vectors tailored for high expression of the best gene or genes in the given target tissue. However, the complete or near complete elimination of class I MHC presentation on the cell surface brings a potential new issue to the foreground. Cell surface decrease or loss of self class I MHC is one of the known triggers of natural killer (NK) cell

mediated killing (Timonen and Helander, 1997). Class I MHC negative cell lines are often the model of choice used to demonstrate NK mediated lysis (Yeoman and Robins, 1988). Additional strategies might be considered to avoid initiation of NK mediated cell killing such as to incorporate an inhibitor of NK mediated lysis into the vector design. HCMV UL18 is a distant class I MHC homologue recently shown to protect HCMV infected cells from NK mediated lysis (Leong *et al.*, 1998). UL18 is resistant to US11 down regulation and thus may prove particularly useful in combination with US11 (Park *et al.*, 2002).

Another potential obstacle to obtaining long term immune suppression by down regulation of class I MHC are factors that enhance class I MHC expression and presentation. Although partially inhibited by ICP47 and US11, the components of the class I MHC processing and presentation pathway are still present and able to function. Many cytokines including tumor necrosis factor alpha, IFN-alpha, IFN-beta, IFNγ, and others up regulate cell surface levels of MHC (Raval *et al.*, 1998). IFNγ in particular is known to increase cell surface class I by increasing transcription of the genes involved via activation of the Jak-STAT signal transduction pathway (Rosa *et al.*, 1983; Darnell *et al.*, 1994). IFNγ is released by several types of lymphocytes in response to immune challenge, and thus the ability of ICP47 and US11 to prevent epitope presentation may be negated during an inflammatory response. To evaluate this issue, VA13 fibroblasts and MOLT-3 lymphoblasts were cultured in media containing IFNγ. Co-expressing ICP47 and US11 in human fibroblasts can lead to a complete loss of cell surface class I MHC (Berger *et al.*, 2000). In our study of VA13 human fibroblasts, ICP47 and US11 decreased cell surface class I MHC by 73% and 64% respectively. Subsequent incubation with a relevant level of IFNγ (400 IU/ml) resulted in a greater than 3 fold increase of class I MHC levels in transduced and non-transduced cells and

minimized the ICP47 and US11 effects observed (Fig. 7)(Saito *et al.*, 1983). These data suggest a potential limitation in an *in vivo* setting. A systemic cellular immune response against transduced cells may lead to the release of IFNγ overriding the class I MHC suppression induced by ICP47 and US11 thereby revealing the transgene being protected. A less prominent upregulation effect was observed when MOLT-3 human lymphoblasts were studied (Fig. 7). These findings suggest that careful testing and evaluation will be required to apply this immune evasion technique and its application will be transgene and cell type specific. Given the wide variation in epitope immunogenicity, the effectiveness may differ greatly for specific transgenes. Although it may be possible to overcome IFNγ increased class I MHC levels by simply increasing the number of inhibitory molecules present, constitutive expression of ICP47 or US11 at a significantly higher level may be difficult to achieve. A potentially attractive approach may be to use IFNγ inducible promoters to increase ICP47 and US11 expression coincident with increase in transcription of the class I MHC genes. IFNγ inducible promoters have been characterized from several class I and II MHC genes and have been shown to function in transgene models (Eades *et al.*, 1990; Sims *et al.*, 1993).

In conclusion, ICP47 and US11 decrease class I MHC in a wide variety of cell types. This ability makes them attractive potential candidates for inducing localized immune suppression to delivered transgenes. Two obstacles to the application of ICP47 and US11 for use in suppression of host CTL response to transgene were examined. First, cell line variability in the function of ICP47 and US11 demonstrates the need to specifically tailor the vector to the target cell type being treated. Such narrowly optimized vectors would limit application where multiple tissues are involved, such as with organ transplants or with *in vivo*

transduction. Second, the up regulation of class I MHC by inflammatory cytokines needs to be further addressed to achieve the goal of long term suppression of CTL mediated lysis.

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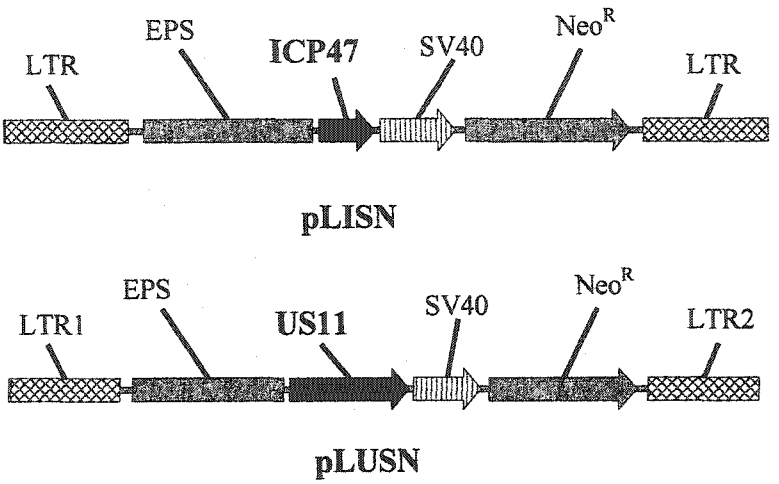
Figure 1. Schematic representation of the LISN and LUSN retroviral vectors and detection of the transgene expression in the A375 melanoma cells. (A) Both ICP47 and US11 were cloned into LXSN at the Eco RI restriction site. Abbreviations: LTR Long Terminal Repeat, EPS Extended Packaging Signal, SV40 SV40 promoter, Neo^R Neomycin Phosphotransferase, ICP47 308 nucleotide gene sequence cloned into the EcoR I site (see methods) encoding the HSV ICP47 gene, US11 689 nucleotide gene sequence cloned into the EcoR I site (see methods) encoding the hCMV US11 gene. (B) Northern analysis of RNA expression in the A375 cell line. Three separate probes detect the Neo^R, the ICP47, and the US11 RNA transcripts at their expected sizes.

A.


LTR
EPS
ICP47
SV40
Neo^R
LTR
pLISN
LTR1
EPS
US11
SV40
Neo^R
LTR2
pLUSN

B.

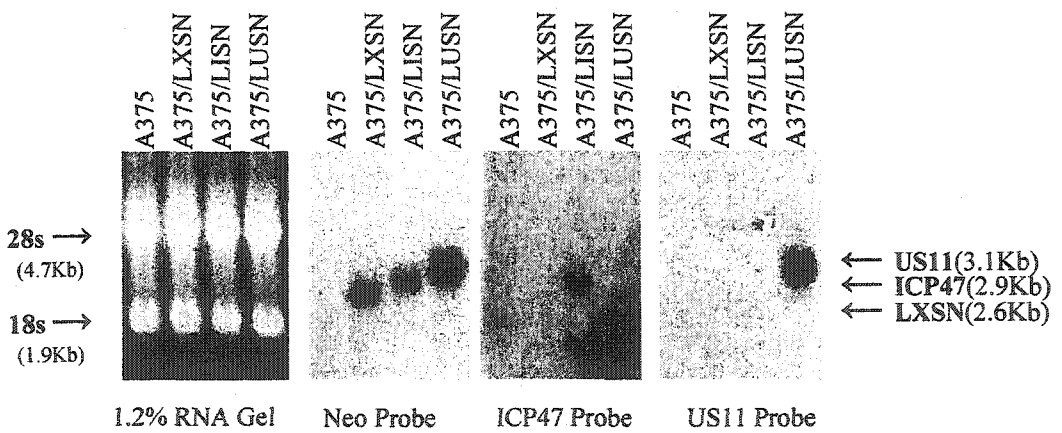

A375
A375/LXSN
A375/LISN
A375/LUSN
28s ⟶
(4.7Kb)
18s ⟶
(1.9Kb)
1.2% RNA Gel
Neo Probe
ICP47 Probe
US11 Probe
⟵ US11(3.1Kb)
⟵ ICP47(2.9Kb)
⟵ LXSN(2.6Kb)

Figure 2. A375 human melanoma cells transduced with US11 or ICP47 retroviral vectors exhibit reduced cell surface expression of MHC I. (A) NV (No Vector), LXSN, LISN, LUSN transduced A375 cells were labeled with the H58A MAb that recognizes a conserved MHC I epitope. A FITC labeled anti-mouse secondary Ab was used and the samples were analyzed by flow cytometry. The control is an untransduced A375 sample labeled with Isotype matched (IgG2a) primary MAb and the same FITC secondary Ab. (B) Where single peaks were observed, the mean fluorescence was graphed. The percent decrease in expression (↓%) was determined by subtracting the negative control from the experimental values and calculating the decrease compared to the LXSN transduced.

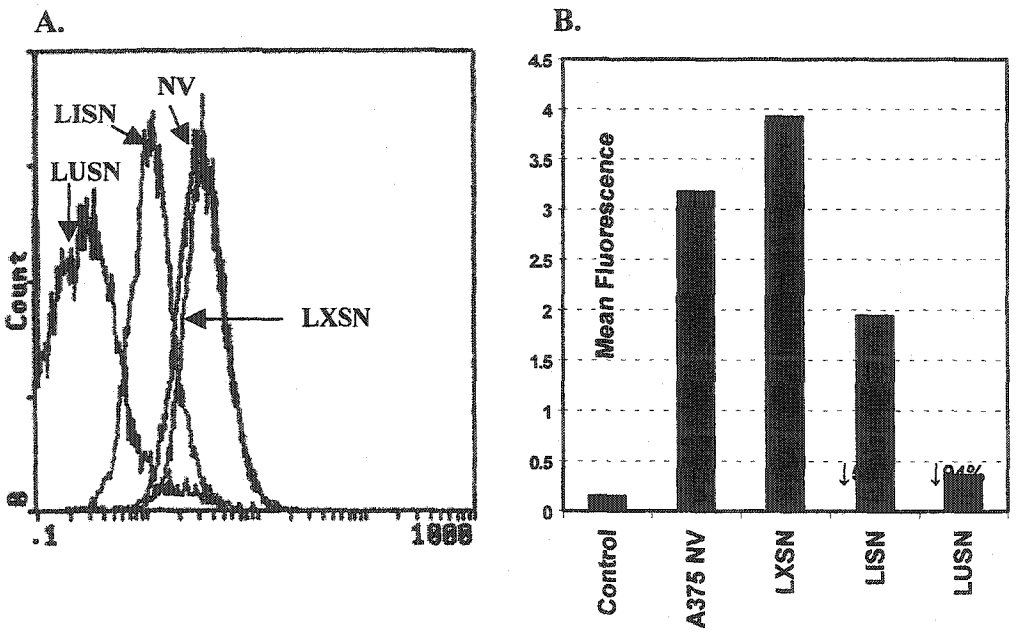

A.
NV
LISN
LUSN
LXSN
Count
0
.1
1000
B.
Mean Fluorescence
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Control
A375 NV
LXSN
LISN
LUSN

Figure 3. Multiple human cell lines transduced with ICP47 or US11 retroviral vectors exhibit reduced cell surface expression of MHC I. Transduced mixed populations of cells were labeled with anti-MHC I Ab and a FITC labeled secondary. A single peak from flow cytometry analysis is reported as the mean fluorescence intensity. Control represents isotype matched Ab plus the FITC labeled secondary Ab. The percent decrease in expression (↓%) was determined by subtracting the negative control from the experimental values and calculating the decrease in expression compared to LXSN transduced cells. (A) SW620 colorectal adenocarcinoma. (B) A549 lung carcinomma. (C) 293 transformed human kidney fibroblasts. (D) IGROV ovarian adenocarcinomma. (E) OVCAR ovarian adenocarcinomma. (F) DU145 metastatic prostate carcinoma.

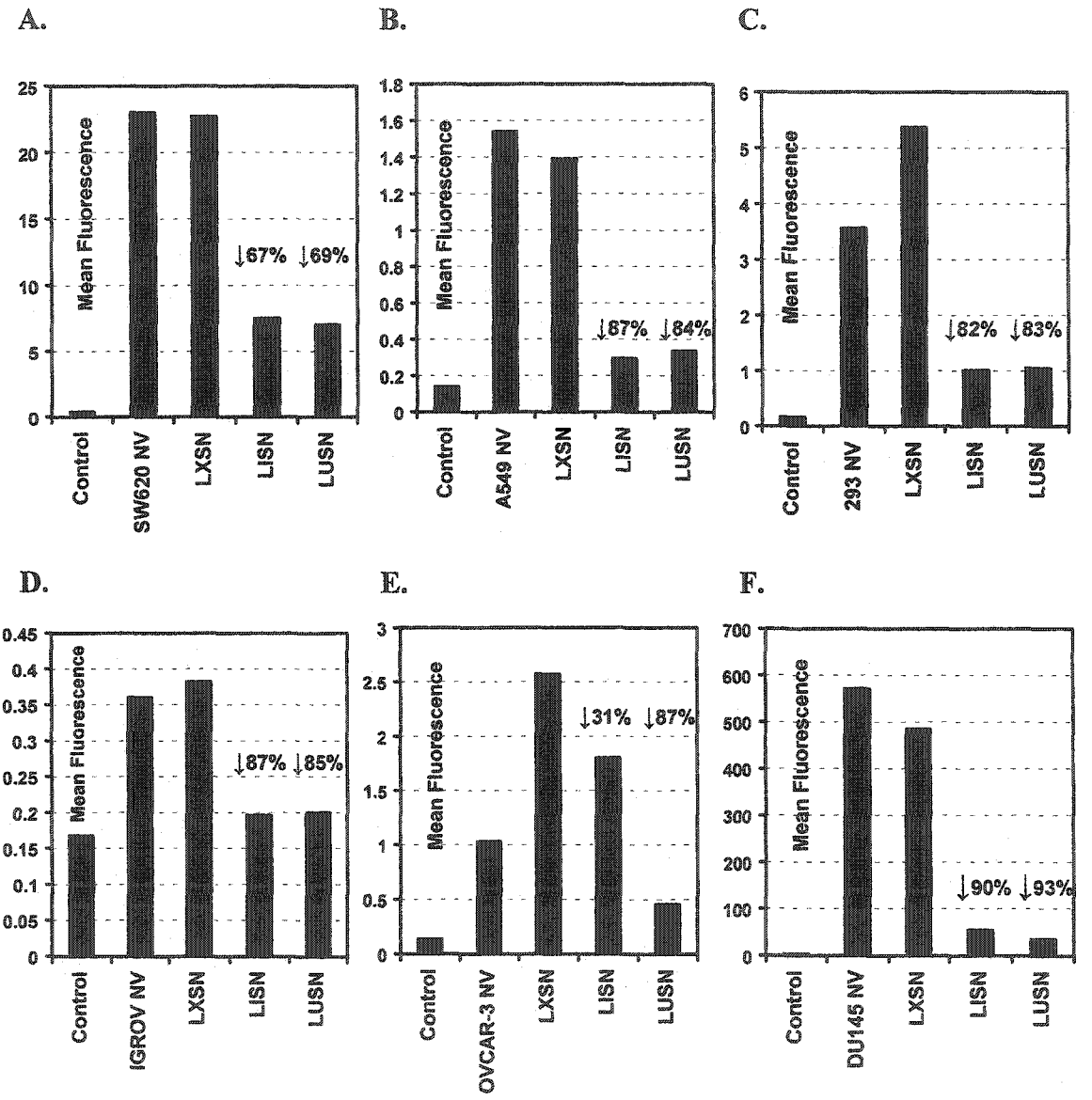
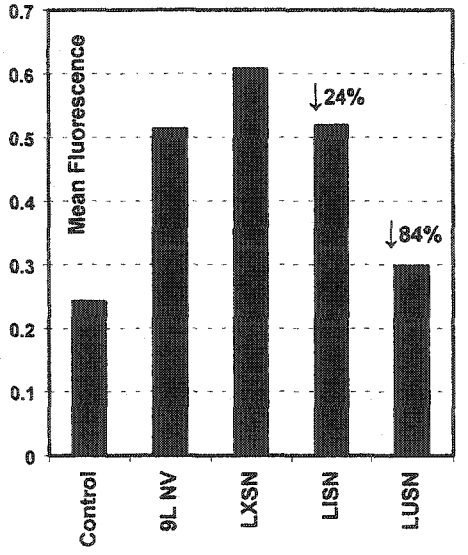

A.
B.
C.
D.
E.
F.
Mean Fluorescence
↓67% ↓69%
↓87% ↓84%
↓82% ↓83%
↓87% ↓85%
↓31% ↓87%
↓90% ↓93%
Control
SW620 NV
A549 NV
293 NV
IGROV NV
OVCAR-3 NV
DU145 NV
LXSN
LISN
LUSN

Figure 4. ICP47 and US11 function in dog and rat cells. (A) Rat brain gliosarcoma 9L, and (B) Canine osteosarcoma D17 cells were transduced with LXSN, LISN, and LUSN vectors. Transduced and non-transduced cells were assayed for cell surface class I MHC as previously described.

A.


Mean Fluorescence
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
↓24%
↓84%
Control
9L NV
LXSN
LISN
LUSN

B.

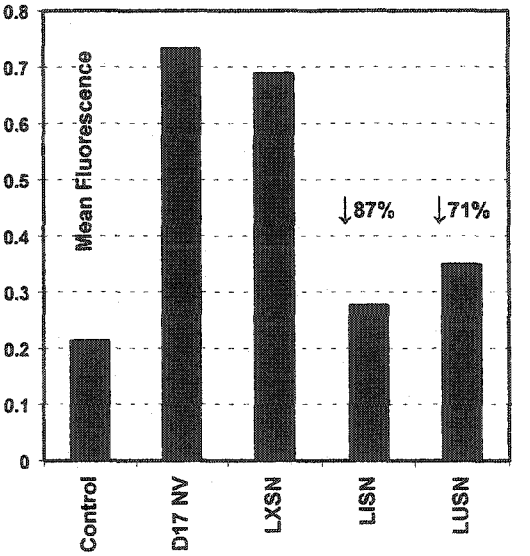
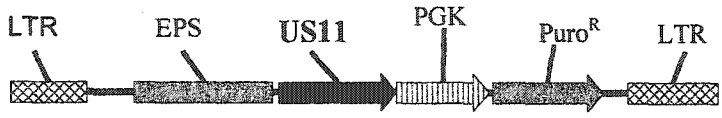

Mean Fluorescence
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
↓87%
↓71%
Control
D17 NV
LXSN
LISN
LUSN

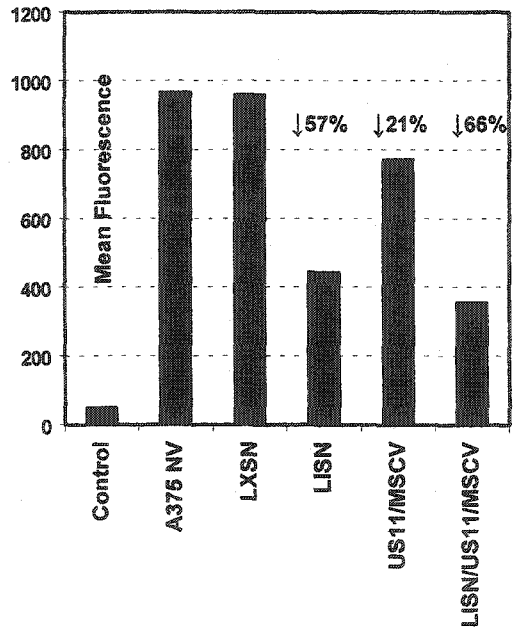
Figure 5. Expression of both ICP47 and US11 in the same cell line leads to further decrease in cell surface MHC I. (A) Schematic representation of the US11 in MSCVp vector. MSCVp is an alternate murine Molony leukemia virus based retrovirus containing a murine stem cell virus promoter and a puromycin selection marker ($Puro^R$) driven by the PGK (PhosphoGlycerate Kinase) promoter. (B) A375 and (C) VA13 cells were transduced with both vectors and assayed for class I MHC expression.

A.



US11 in MSCVpuro

B.



C.

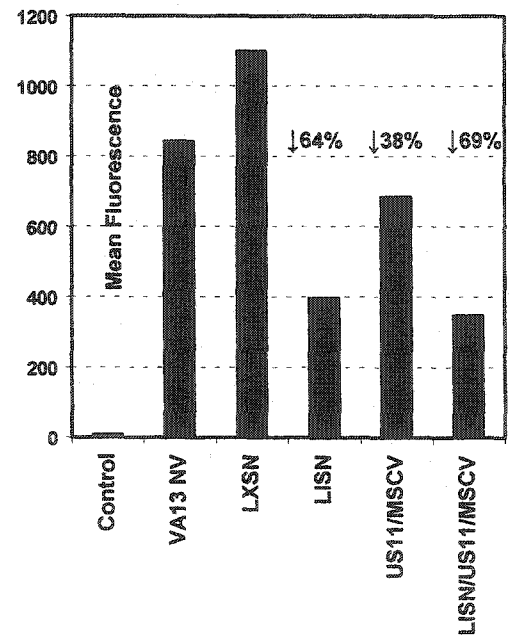


Figure 6. ICP47 and US11 protect A375 cells from lysis by anti-A375 primed effector cells. Peripheral blood mononuclear cells (PBMC) were isolated from two random donors and co-cultured with irradiated A375 cells for 14 days to generate an anti-A375 effector cell populations. Specific lysis was determined by the release LDH from dying cells (see methods) (A) PBMC (15% $CD8^+$ data not shown) isolated from random donor number 1. (B) $CD8^+$ enriched effector cell population (82% $CD8^+$ data not shown) generated using a $CD4^+$ depletion column on PMBC from donor 2.

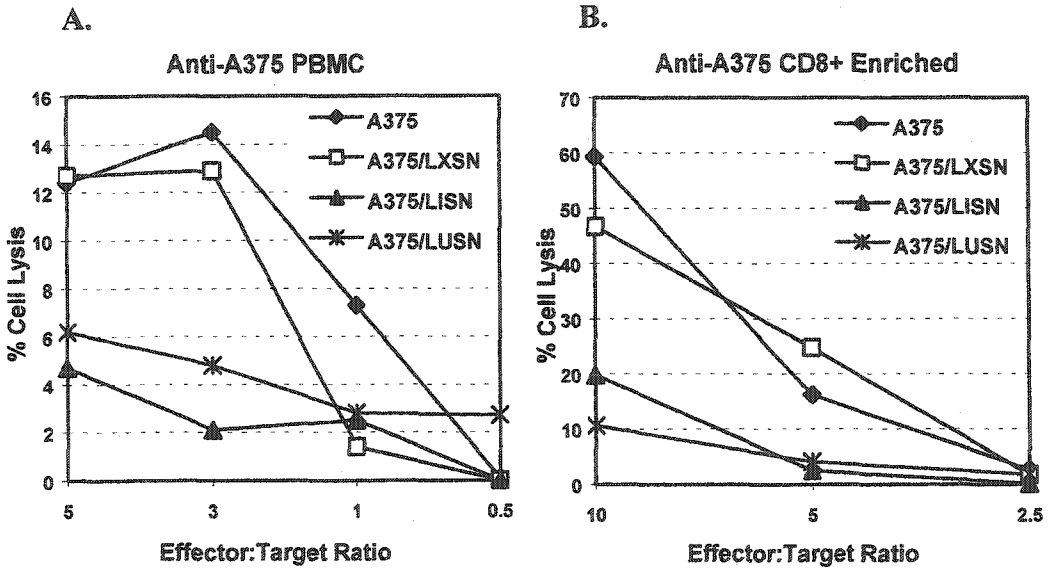
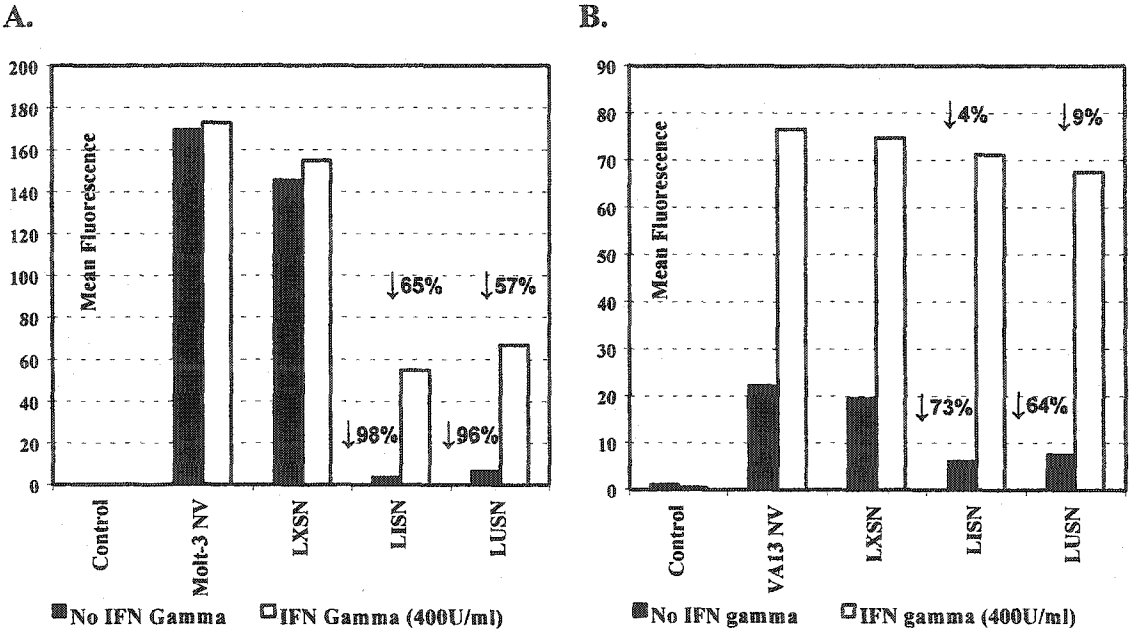

A.
Anti-A375 PBMC
% Cell Lysis
16
14
12
10
8
6
4
2
0
5
3
1
0.5
Effector:Target Ratio
A375
A375/LXSN
A375/LISN
A375/LUSN
B.
Anti-A375 CD8+ Enriched
% Cell Lysis
70
60
50
40
30
20
10
0
10
5
2.5
Effector:Target Ratio
A375
A375/LXSN
A375/LISN
A375/LUSN

Figure 7. Incubation of cells in media containing IFNγ restores cell surface MHC I expression in the VA13 and Molt-3 cell lines. IFNγ is a common cytokine known to upregulate MCH I expression. (A) Molt-3 acute lymphoblastic leukemia and (B) VA13 transformed human fibroblast cells were transduced with the LXSN, LISN, and LUSN vectors. Cells were incubated with 400 I U/ml IFNγ for 72 hr and cell surface class I MHC was determined as before.


A.
Mean Fluorescence
200
180
160
140
120
100
80
60
40
20
0
↓65%
↓57%
↓98%
↓96%
Control
Molt-3 NV
LXSN
LISN
LUSN
No IFN Gamma
IFN Gamma (400U/ml)
B.
Mean Fluorescence
90
80
70
60
50
40
30
20
10
0
↓4%
↓9%
↓73%
↓64%
Control
VA13 NV
LXSN
LISN
LUSN
No IFN gamma
IFN gamma (400U/ml)

CHAPTER 3. TALL-104 CYTOTOXICITY CORRELATES WITH THE LEVEL OF CLASS I MHC EXPRESSION IN A NONRESTRICTED MANNER

A paper submitted to the journal *Cancer Research*

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Running Title: TALL-104 cytotoxicty and class I MHC

Key Words: ICP47, US11, CD8+, immune response, TALL-104, class I MHC, TCR $\alpha\beta^+$, NK

Abbreviations:

HSV: Herpes Simplex Virus MHC: Major Histocompatibility Complex

CTL: Cytotoxic T Lymphocyte CMV: Cytomegalovirus

TAP: Transporter associated with Antigen Processing

LDH: Lactate Dehydrogenase NK: Natural Killer

TALL: T-Acute Lymphoblastic Leukemia

TCR: T Cell Receptor IL: Interleukin

LAK: Lymphokine Activated Killer β2m: β2-microglobulin

ABSTRACT

The acute T lymphoblastic leukemia cell line, TALL-104, is in clinical testing for its ability to preferentially kill tumor cells. The ability of TALL-104 cells to lyse tumor targets of multiple class I MHC haplotypes as well as targets from other species led to their designation as class I MHC nonrestricted. Our current data also demonstrated that TALL-104 cytotoxic activity correlates with expression of class I MHC on human cells in a nonrestricted manner. Two class I MHC positive cell lines A375 melanoma, VA13 fibroblasts, and two class I MHC negative cell lines, K562 leukemia, and HCT15 colon cancer, were transduced with retroviral vectors encoding two viral genes that are known to decrease class I MHC cell surface levels. The herpes simplex virus type I ICP47 and the human Cytomegalovirus US11 gene product use differing mechanisms to decrease cell surface class I MHC. Interestingly, both genes reduce TALL-104 lysis of targets as compared to cells transduced with control vectors. ICP47 transduced class I MHC positive cells decreased cell surface expression of class I MHC by 66-69% and the corresponding lysis by TALL-104 cells dropped 32-34%. US11 transduced class I MHC positive cells decreased cell surface expression of class I MHC by 73-97% and the corresponding lysis by TALL-104 cells dropped 43-59%. Class I MHC negative cells transduced with either gene showed no change in their susceptibility to TALL-104 lysis. The ability to spare normal cells while killing a wide array of tumor cells indicates a tumor specific TALL-104 activity mediated by molecules present on virtually all cell types and conserved across species. Highly conserved regions of class I MHC and class I MHC associated molecules fulfill this criterion and may explain the data presented here. In contrast to traditional class I MHC associated killing, TALL-104 cells also exhibit NK surface markers and kill class I MHC negative cells

efficiently. The presence of NK markers and the ability to kill NK sensitive cells implies that TALL-104 cells can also kill class I MHC negative tumors using a traditional NK mechanism. Understanding how TALL-104 cells recognize targets has the potential to improve the clinical application of these cells and may indicate a novel anti-tumor response.

INTRODUCTION

$CD3^+$, $CD8^+$, TCR $\alpha\beta^+$ lymphocytes typically are cytotoxic against target cells in a manner restricted by peptide presentation on class I MHC molecules. Although TALL-104 cells display these markers, they lyse a broad range of tumor cells in a class I MHC nonrestricted manner (1, 2). Their ability to lyse tumor cells crosses species and has been exploited with promising results in both dog and human clinical trials (3-5). In addition to expressing markers consistent with CTL, TALL-104 cells are CD 56^+ similar to both NK and LAK cells and secrete several cytokines including IFNγ and TNFα when stimulated through contact with tumor cells (1, 6). In accordance with their abundance of cell surface markers, TALL-104 cells kill targets using two distinct mechanisms; release of cytolytic factors and induction of apoptosis (2). The broad range of tumors lysed by TALL-104 cells includes typical NK targets lacking cell surface class I MHC and normally NK resistant class I MHC positive cells. Thus, Tall-104 cells have many characteristics of both NK and CTL cells. Understanding how these cells recognize targets may aid in improving their clinical application and provide new insight into their unique abilities to target a broad range of tumors.

Class I MHC molecules are known to trigger peptide restricted CTL mediated lysis by their presence and NK mediated lysis by their absence. To explore the potential contribution of class I MHC molecules in TALL-104 recognition and lysis of targets, two class I MHC inhibitors were employed. Infected cell protein (ICP)47 is an HSV type I protein encoded by a 267 nucleotide immediate early gene (7). ICP47 binds to the cytoplasmic side of the Transporter associated with Antigen Processing (TAP) and blocks the transport of 8-9 amino acid peptides into the Endoplasmic Reticulum (ER) (8, 9). Without

these peptides, class I MHC molecules are degraded and therefore not displayed at the cell surface (10). A second Herpes virus protein that prevents cell surface class I MHC expression is encoded by the 645 nucleotide human Cytomegalovirus (HCMV) gene US11 (11). US11 binds to mature class I MHC molecules and dislocates them back to the cytoplasm where they are rapidly degraded (12, 13). Although they work by different mechanisms, both genes have proven effective at decreasing cell surface class I MHC in a variety of cell types (14). In this study, ICP47 and US11 inhibitors of class I MHC expression are used in multiple cell lines and the effects on TALL-104 cytotoxicity are reported.

MATERIALS AND METHODS

Vector construction. Polymerase chain reaction (PCR) amplification of the ICP47 gene from purified HSV DNA (Sigma-Aldrich Corp., St. Louis, MO) and the US11 gene from HCMV DNA (Sigma-Aldrich Corp.) was performed and the products were cloned into the T/A cloning vector PCR 3.1 (Invitrogen Inc., Carlsbad, CA). ICP47 and US11 were excised from PCR3.1 using the restriction endonuclease Hind III and each sub-cloned into the murine retrovirus derived LXSN vector at the Hind III site in the multicloning region (15) (Figure 1).

Cell lines. The LISN, LUSN, and control LXSN vectors were each transfected into the GP+E86 cell line (16). Supernatants were collected, filtered, (0.2 μm) and used to transduce PA317 amphotropic vector producer cells (VPC) (17). PA317 cells were selected for in the neomycin homologue G418 (1 mg/ml) for 2 weeks and supernatents were used to transduce the following cell lines: A375 human malignant melanoma (18); VA13 SV40 transformed human fetal fibroblast (19); K562 human lymphoblast (20); HCT15 colorectal adenocarcenoma (21). TALL-104 cell cultures were maintained in the recommended medium (American Type Culture Collection, Rockville, MD).

Class I MHC Expression assay. Transduced, mixed populations of cells were briefly trypsinized and labeled with anti-class I MHC monoclonal Ab H58A (VMRD Inc., Pullman, WA) at 1.5 μg/ml/0.5×10^6 cells in serum free media at 37°C for 1 hr (22). The anti-mouse IgG2a isotype control UPC 10 Ab was used to control for nonspecific binding (Sigma-Aldrich Corp.). Cells were washed 5 min in Hank's buffer and a 1:64 dilution of goat anti-mouse FITC labeled secondary Ab (Sigma-Aldrich Corp.) in serum free media was added for 30 min at 37°C. Cells were again washed 5 min in Hank's buffer and resuspended

in Phenol red free/serum free media (OptiMEM®, Invitrogen Corp., Inc.Grand Island, NY) for fluorescent analysis. Where a single peak was observed, the mean fluorescence intensity is reported. The same protocol as above was used to detect cell surface expression of β2 microglobulin (β2m) with the primary Ab being BM-63 which recognizes a conserved region of β2m (Sigma-Aldrich Corp.). The anti-mouse IgG1 isotype control MOPC 21 Ab was used to control for nonspecific binding (Sigma-Aldrich Corp.). Data shown is a representative result from a minimum of 3 experiments. For IFNγ stimulation of class I MHC, cells were cultured 72 hr in media containing 400U/ml IFNγ with fresh media replacement after the first 48 hr.

Cytotoxicity Assay. A non-radioactive colorimetric assay was used to determine specific cytotoxicity of TALL-104 effector cells against vector transduced and control target cells. The Cytotox® 96 (Promega Inc., Madison, WI) assay measures lactate dehydrogenase (LDH) release by a reaction with a tetrazolium salt (INT) to produce a red formazan product (23). In brief, target cells are plated in a 96 well plate and effector cells are added at the appropriate ratio. After 4 hr, supernatant is collected and assayed for LDH content. Controls include maximum LDH release (detergent lysed), media background, and spontaneous LDH release from both targets and effector cells. Each data point is an average of 4 wells and data presented is a representative result from a minimum of two experiments.

RESULTS

TALL-104 lysis of class I MHC positive cells correlates with the amount of class I MHC on the cell surface. Using class I MHC inhibitors introduced by retroviral transduction allows for the generation of cell populations derived from a parental line that maintain a consistent, decreased cell surface level of class I MHC while introducing a minimal number of variables. Using two class I MHC inhibitors that function through distinct mechanisms minimizes the potential that either inhibitor has an undefined effect on TALL-104 lysis of target cells. A375 melanoma cells transduced with the LISN or LUSN vectors express a decreased level of class I MHC on the cell surface (Figure 2A). Class I MHC presentation is decreased by 66% using LISN and 97% using LUSN as compared to the LXSN transduced control population. In subsequent cell killing assays using TALL-104 cells as effectors and the transduced populations as targets, cells with less Class I MHC expression are killed less efficiently by TALL-104 cells. At an effector to target ratio of 10:1, TALL-104 specific cytotoxicity decreased by 32% and 43% against LISN and LUSN transduced populations respectively, as compared to the LXSN transduced control (Figure 2B). As these were unexpected results using a class I MHC nonrestricted effector cell, a second class I MHC positive cell line was transduced and analyzed.

In transduced VA13 fibroblast cells, class I MHC presentation is decreased by 69% using LISN and 73% using LUSN over levels measured in the LXSN transduced cells (Figure 3A). Measuring specific cytotoxicity of TALL-104 cells against VA13 targets at an effector target ratio of 5:1, LISN and LUSN decrease cytotoxicity by 34% and 59% respectively. Thus, in both the A375 and VA13 cell lines, when cell surface class I MHC is decreased by two distinct mechanisms, specific lysis by TALL-104 cells is also decreased.

TALL-104 lysis of K562 cells. K562 lymphoblast cells are often used as a class I MHC negative cell line to identify and quantitate NK cell killing activity. K562 cells are one of several class I MHC negative cell lines that are efficiently killed by TALL-104 cells (2). The ability of TALL-104 cells to kill class I MHC negative and positive cells along with their being TCR $\alpha\beta^+$ led to their designation as class I MHC nonrestricted. TALL-104 killing of class I MHC negative cells is not completely unexpected as they also express cell determinant markers consistent with both NK and LAK cells (1). If however TALL-104 cells also use class I MHC levels to select targets, neither ICP47 nor US11 should have an effect on TALL-104 lysis of K562 cells. A complicating factor when using K562 cells as targets is their expression of class I MHC epitopes when grown in media containing IFNγ. The amount of class I MHC increase has been shown to inhibit their susceptibility to NK mediated killing (24-26). Upon contact with target cells, TALL-104 cells upregulate transcription of the IFNγ genes with a maximum level reached within 2 hr (6). Appearance of cell surface class I MHC on K562 cells could have complicated the interpretation of cytotoxicity results. To investigate this possibility, transduced and NV control K562 cells were grown in media with and without IFNγ (400IU/ml) and class I MHC levels were assayed (Figure 4A). Growth in media containing IFNγ caused all the K562 cell lines to express cell surface class I MHC. LISN and LUSN transduced lines displayed 20% and 71% less class I MHC on the cell surface as measured by flow cytometry compared to the LXSN transduced control. In subsequent cytotoxicity assays, there was minimal difference between the specific cytotoxicity of TALL-104 cells against K562 cell lines grown with or without IFNγ. The potential involvement of two distinct killing mechanisms demonstrates a significant drawback to using K562 cells as a class I MHC negative control when effector

cells release IFNγ. An improved control for this experiment would require a cell line unable to express class I MHC regardless of cytokine involvement.

Lack of class I MHC expression in HCT15 cell lines and corresponding TALL-104 lysis patterns. The HCT15 cell line was transduced with the LXSN, LISN, and LUSN vectors. With the NV parental control cells, these four cell lines were grown in media with and without IFNγ. All four cell lines were then analyzed for cell surface expression of class I MHC and the associated β2m proteins (Figure 5A). Flow cytometry analysis detected no expression of class I MHC or β2m with or without IFNγ addition to the media. The complete lack of class I MHC and β2m makes HCT15 cells a better negative control than K562 to determine the effect of class I MHC on TALL-104 killing. TALL-104 cytotoxicity against the HCT15 cell lines with or without IFNγ shows no differences (Figure 5B). Thus, neither ICP47 nor US11 have an effect on TALL-104 killing of HCT15 cells lacking both class I MHC and β2m.

Direct comparison of TALL-104 killing in class I MHC positive and negative cell lines. To determine whether class I MHC positive or negative target cells are more efficiently killed by TALL-104 effector cells, K562, HCT15, VA13, and A375 cells were tested as targets at a target:effector ratio of 10:1 (Figure 6). The two class I MHC negative cell lines presented the best targets for TALL-104 killing with HCT15 and K562 cells being killed 93% and 58% respectively. The class I MHC positive cells VA13 and A375 were killed 45% and 28% respectively. These data suggest that clinical use of TALL-104 cells may be more effective when used to treat class I MHC negative tumors.

DISCUSSION

TALL-104 cells have shown promise when used to treat cancer in both dog and human clinical trials (3, 5). Their ability to lyse tumor cells while bypassing normal tissue may be a side effect of their transformation or may represent a novel anti-tumor immune mechanism. Whatever the identified mechanism by which TALL-104 cells recognize and kill tumor targets, the data may aid the design of further clinical trials of this cell therapy. Their ability to lyse class I MHC negative cells even though they are $CD8^+$ and $TCR\alpha\beta^+$ led to their designation as class I MHC nonrestricted (27). Class I MHC is known to play a key role in both NK and CTL mediated targeting and lysis. To investigate whether class I MHC functions in the TALL-104 recognition of tumors, two distinct class I MHC inhibitors were employed. By decreasing class I MHC expression in A375 melanoma and VA13 fibroblast cells using retroviral vectors encoding inhibitory proteins, the effect on TALL-104 lysis was evaluated while introducing a limited number of variables. In both the A375 and VA13 cell lines, when the level of cell surface class I MHC was decreased, the specific lysis by TALL-104 cells also decreased (Figures 2 and 3). Using two distinct class I MHC inhibitors minimized the possibility that either molecule might affect TALL-104 cytotoxicity in a manner independent of their described specific class I MHC inhibition mechanisms. These data establish evidence that class I MHC or an associated molecule is involved in TALL-104 targeting of these cell types.

TALL-104 cells also express the NK marker CD56 suggesting that they may be killing class I MHC negative cells using a traditional NK killing method. In class I MHC negative K562 and HCT15 cells, neither ICP47 nor US11 had any significant effect on TALL-104 lysis. However, an exception to this occurs in K562 cells grown in media

containing IFNγ that induces cell surface expression of class I MHC. Interestingly when encountering target cells, TALL-104 cells secrete IFNγ with maximum levels of RNA detected within 2 hr (6). Although maximum upregulation of class I MHC by IFNγ has been reported to take up to 48 hr, class I MHC is known to accumulate at the cell surface at sites of cell-cell contact (28, 29). Thus, during a 4 hr cytotoxicity assay, the stimulation and secretion of IFNγ followed by upregulation of class I MHC may play a role in TALL-104 mediated lysis of K562 cells. In general, the IFNγ upregulation of class I MHC led to only slightly increased TALL-104 lysis of K562 cells. However, since two possible mechanisms may be occurring simultaneously further experiments were conducted with a cell line that does not upregulate class I MHC in response to IFNγ (Figure 4).

One such cell line is the HCT15 colon cancer cell line. HCT15 cells do not express either class I MHC or β2m when grown in the presence or absence of IFNγ (Figure 5A). TALL-104 lysis of HCT15 cells is unaffected by growing the cells in IFNγ or by transduction with the LISN or LUSN vectors (Figure 5B). The data in HCT15 cells demonstrates clearly that neither ICP47 nor US11 alter TALL-104 mediated lysis. These combined results with A375, VA13, and HCT15 cells, indicate that class I MHC apparently plays an important role in TALL-104 recognition of class I MHC positive tumor cells, but no role in the lysis of class I MHC negative cells. The presence of tumor associated antigens within cancer cells may increase the overall epitope presentation by class I MHC. This increased epitope presentation perhaps could contribute to the selective killing of cancer cells by TALL-104 lysis. Together these two apparently separate methods of killing by TALL-104 cells may account for the sensitivity of a wide array of tumor cell targets. However, the *in vitro* data when directly compared, suggests that the class I MHC negative cell lines K562 and HCT15

are better targets for TALL-104 lysis (Figure 6). These results suggest the importance of further study of TALL-104 cells to optimize their clinical utility. The two current trials of TALL-104 are for bone marrow purging and direct transplant and may have an improved opportunity for success against class I MHC negative tumors.

The ability of TALL-104 cells to lyse such a broad range of targets implies the mode of recognition is present on many if not most cell types. If TALL-104 cells are using their T-Cell Receptor (TCR) to interact with class I MHC, it suggests the possibility that MHC dominant binding is occurring at a conserved or nonpolymorphic site. This would account for TALL-104 recognition of tumor targets regardless of class I MHC haplotype. The class Ia (classical) MHC molecules (HLA-A, -B, -C) are expressed on nearly all cell types, have highly conserved regions in addition to their polymorphic regions, and thus are potential candidates as TALL-104 ligands. Of the class Ib (nonclassical) MHC molecules (HLA-E, -F, -G, -H, MICA, MICB, and CD1-a, -b, -c, -d) only HLA-E is constitutively expressed on most cell types (reviewed in (30)). In addition to their expression in very few cell types, HLA-G has been shown to be US11 resistant (31, 32) and CD1D functions independent of the TAP transporter indicating neither could be responsible for the class I MHC response demonstrated in the current study (33). In contrast, HLA-E is relatively nonpolymorphic, is expressed on virtually all cell types, and has been shown to play a key role in NK recognition and lysis (34-36). Ongoing experiments are now focusing on identifying the specific class I MHC molecule involved in the TALL-104 interaction.

Recent publications support the concept that MHC dominant binding may not be an aberrant byproduct of tumorigenesis, but rather represent a mechanism of normal anti-tumor immune surveillance. The K14 cell line is a $CD8^+$ $TCR\alpha\beta^+$ clone expressing the NK marker

NKG2C that has been shown to recognize an HLA-E molecule complexed with several different peptides derived from both class I MHC leader and viral sequences (37). This MHC dominant binding resembles in principle the nonspecific class I MHC interaction seen in TALL-104 cells. Additionally, populations of clonally expanded human $CD8^+$ $TCR\alpha\beta^+$ cells have been isolated from peripheral blood of healthy donors that show a specific response to HLA-E/β2m that can be blocked by various monoclonal antibodies (38). This population of cells, called NK-CTL, also display NK inhibitors and appears to have function similar to TALL-104 cells with the distinction that they require target cell expression of β2m for lysis to occur (39). TALL-104 lysis of the β2m negative cell line HCT15 may be explained by an additional NK mediated lysis mechanism. Like TALL-104 cells, the K14 clone and NK-CTL cells are $CD8^+$, $TCR\alpha\beta^+$, display NK markers, and lyse a broad range of tumor targets regardless of class I MHC haplotype (40). Unlike TALL-104 cells which have a identified mutation associated with a tumor genotype (1), both the K14 clone and the NK-CTL populations were isolated from healthy donors implying their normal function may be tumor surveillance and eradication. Murine cells displaying similar cell surface markers and having cytolytic activity against a broad range of tumor targets have also been reported (41). As more examples of this nonspecific anti-tumor activity are identified, their roles in tumor immunity will be better understood.

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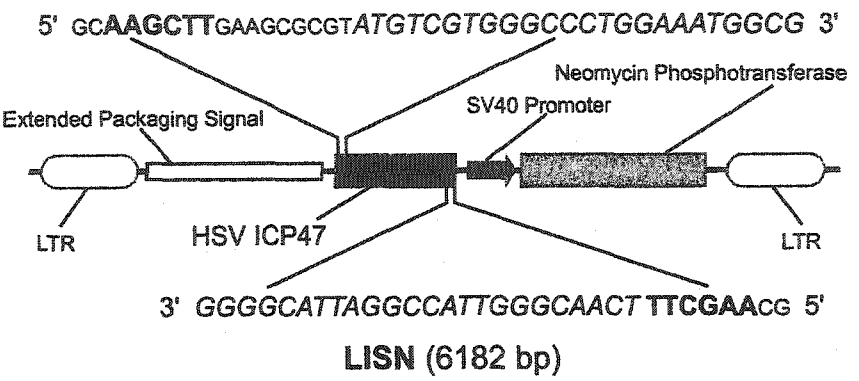
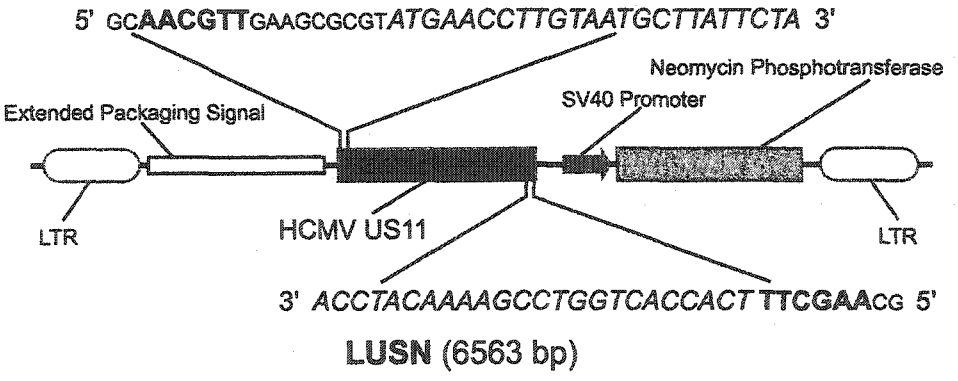
Figure 1. Schematic representation of the LISN and LUSN vectors used. The LISN and LUSN vectors were constructed from the LXSN murine retroviral vector. The 5' LTR (long terminal repeat) contains a promoter that drives transcription of the gene inserted in the multi cloning site. PCR primers shown designate orientation 5'-3', coding sequence (*italicized*), and the restriction endonuclease HindIII (**bold**).

A.



LISN (6182 bp)

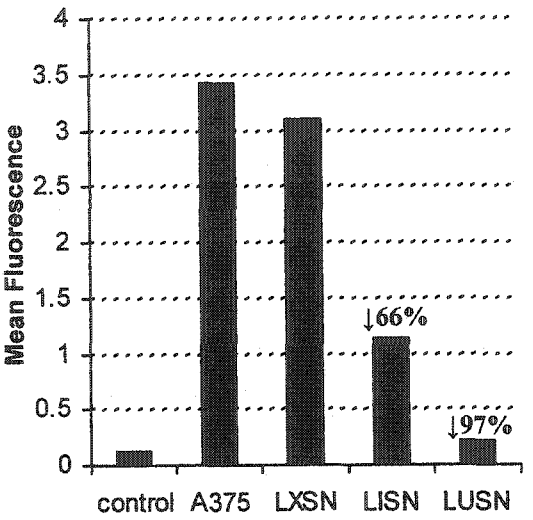
B.



LUSN (6563 bp)

Figure 2. Class I MHC expression and TALL-104 lysis of A375 cell lines. A375 cell lines including NV, LISN transduced, and LUSN transduced. A) Flow cytometry analysis of populations to determine inhibition of class I MHC cell surface expression. Single peaks from histograms are reported as mean fluorescence. B) Percent specific cytotoxicity of TALL-104 cells against the A375 cell lines at effector to target ratios of 10:1 to 1.25:1 in a 5 hr LDH release assay.

A.


Mean Fluorescence
4
3.5
3
2.5
2
1.5
1
0.5
0
↓66%
↓97%
control
A375
LXSN
LISN
LUSN

B.

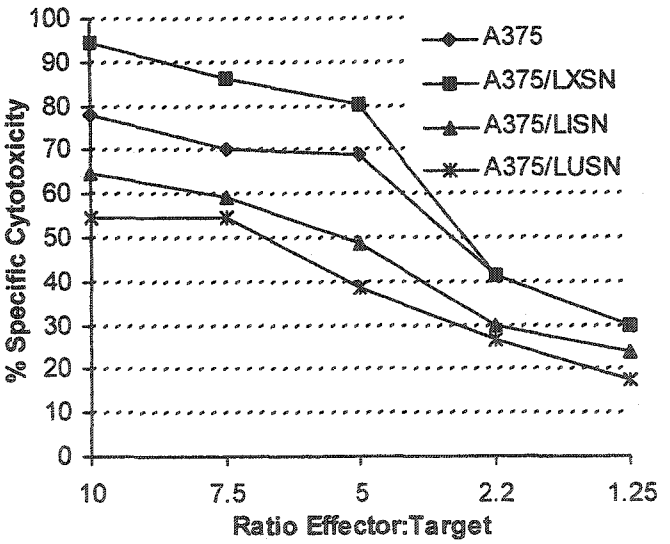
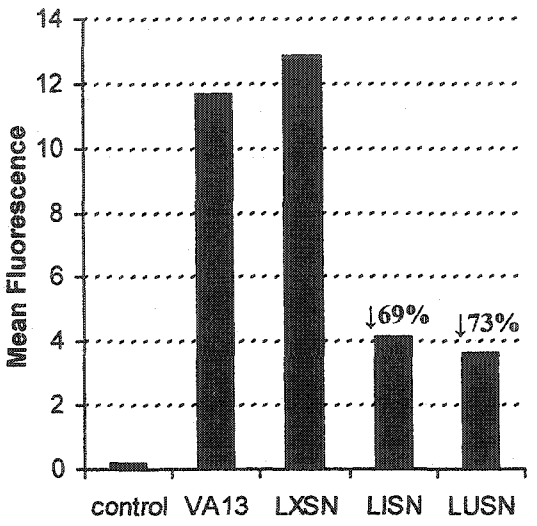

% Specific Cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
A375
A375/LXSN
A375/LISN
A375/LUSN
10
7.5
5
2.2
1.25
Ratio Effector:Target

Figure 3. Class I MHC expression and TALL-104 lysis of VA13 cell lines. VA13 cell lines including NV, LISN transduced and LUSN transduced. A) Flow cytometry analysis of class I MHC inhibition. B) Percent specific cytotoxicity of TALL-104 cells against the VA13 cell lines at effector to target ratios of 5:1 to 1.25:1 in a 5 hr LDH release assay.

A.


14
12
10
8
6
4
2
0
Mean Fluorescence
↓69%
↓73%
control
VA13
LXSN
LISN
LUSN

B.

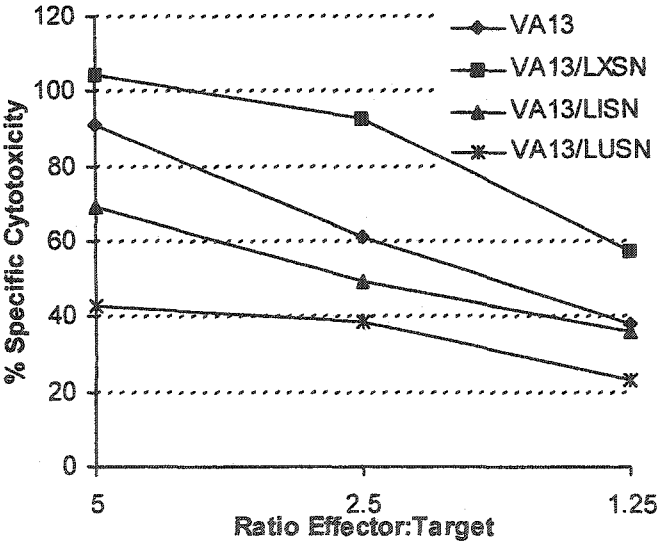
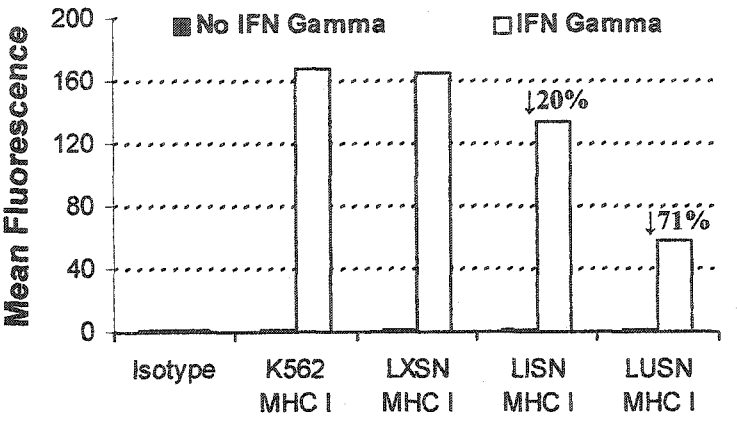

120
100
80
60
40
20
0
% Specific Cytotoxicity
VA13
VA13/LXSN
VA13/LISN
VA13/LUSN
5
2.5
1.25
Ratio Effector:Target

Figure 4. IFNγ effect on class I MHC presentation and TALL-104 lysis of K562 cell lines. K562 cell lines including NV, LISN transduced, and LUSN transduced. A) Flow cytometry analysis of class I MHC expression both with and without IFNγ stimulation. B) Percent specific cytotoxicity of TALL-104 cells against the K562 cell lines with and without IFNγ stimulation at effector to target ratios of 5:1 to 1.25:1 in a 5 hour LDH release assay.

A.


200
160
120
80
40
0
Mean Fluorescence
No IFN Gamma
IFN Gamma
↓20%
↓71%
Isotype
K562 MHC I
LXSN MHC I
LISN MHC I
LUSN MHC I

B.

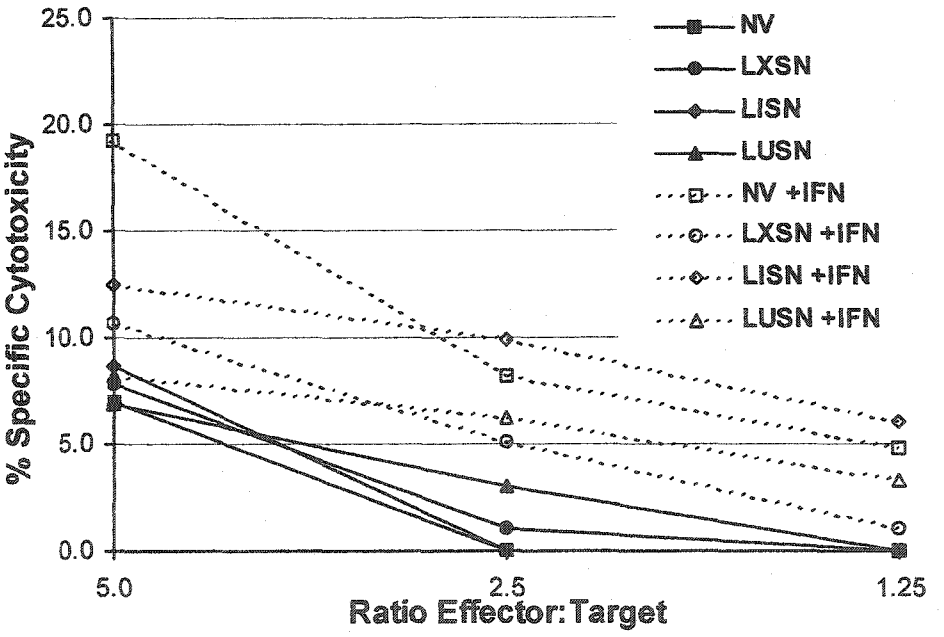
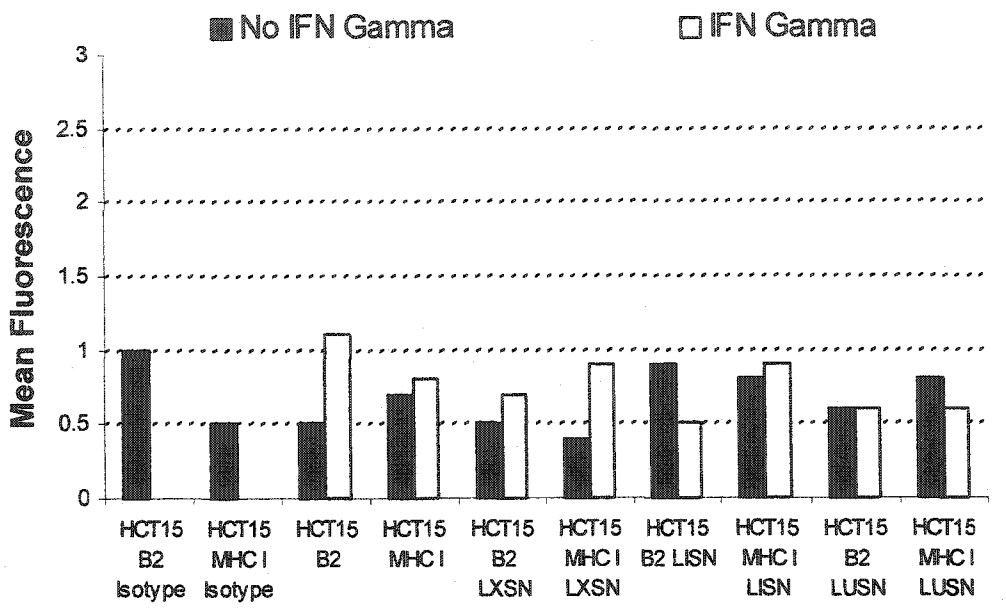

25.0
20.0
15.0
10.0
5.0
0.0
% Specific Cytotoxicity
NV
LXSN
LISN
LUSN
NV +IFN
LXSN +IFN
LISN +IFN
LUSN +IFN
5.0
2.5
1.25
Ratio Effector:Target

Figure 5. TALL-104 lysis of the class I MHC negative cell line HCT15. HCT15 cell lines including NV, LISN transduced, and LUSN transduced. A) HCT15 cells lack cell surface expression of both class I MHC and β2m regardless of the presence of IFNγ in the growth medium. B) Flow cytometry analysis of TALL-104 cytotoxicity against the class I MHC negative HCT15 cell lines in a 4 hr LDH release assay.

A.


No IFN Gamma
IFN Gamma
Mean Fluorescence
3
2.5
2
1.5
1
0.5
0
HCT15 B2 Isotype
HCT15 MHC I Isotype
HCT15 B2
HCT15 MHC I
HCT15 B2 LXSN
HCT15 MHC I LXSN
HCT15 B2 LISN
HCT15 MHC I LISN
HCT15 B2 LUSN
HCT15 MHC I LUSN

B.

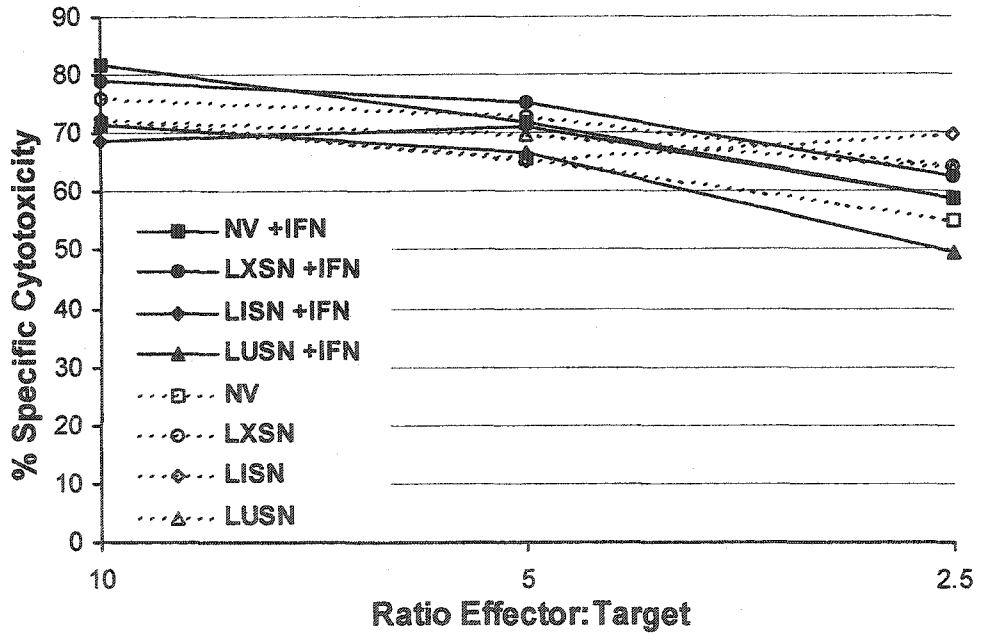
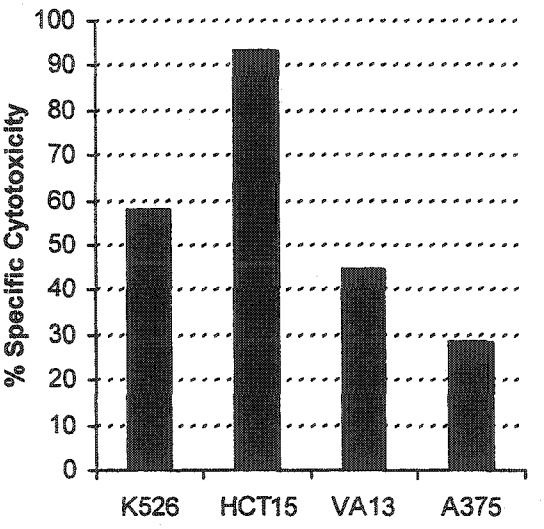

% Specific Cytotoxicity
90
80
70
60
50
40
30
20
10
0
NV +IFN
LXSN +IFN
LISN +IFN
LUSN +IFN
NV
LXSN
LISN
LUSN
10
5
2.5
Ratio Effector:Target

Figure 6. Direct comparison of TALL-104 lysis of class I negative and positive cell lines. Flow cytometry analysis of TALL-104 specific cytotoxicity against the four cell lines used in this study at an effector:target ratio of 15:1 in a 3 hr LDH release assay.


% Specific Cytotoxicity
100
90
80
70
60
50
40
30
20
10
0
K526
HCT15
VA13
A375

CHAPTER 4. APPLICATION OF CLASS I MHC IMMUNE SUPPRESSION FOR USE IN ALLOGENEIC TRANSPLANT AND TUMOR MODEL CELL EVASION OF HOST RESPONSE.

ABSTRACT

Class I MHC inhibition by ICP47 and US11 has been achieved in a variety human, canine, and rodent cell lines. Although this represents down regulation of only one aspect of host immune response, it may be possible to use this inhibition to achieve specific short-term immune suppression goals. In this application, the immune suppression delivered serves as the therapeutic treatment. Allogeneic transplant of anti-tumor cells is one treatment that could benefit from short-term suppression of host CTL response. There are at least two cell lines that are currently under investigation for their potential to treat various tumors. The $CD8^+$, $TCR\alpha\beta^+$, $CD56^+$, T Acute Lymphocytic Leukemia (TALL)-104 cell line has demonstrated an ability to kill tumor cells while sparing normal tissue. This cell line has been used in clinical trials to treat cancer in both dogs and humans and has shown promising results. In humans, vigorous CTL responses have been documented to the TALL-104 cells used in the treatment. If class I MHC expression were reduced or eliminated from TALL-104 cells, their duration in patient may increase and improve the effectiveness of the treatment. NK-92, a natural killer (NK) cell line with similar function to TALL-104 has also been characterized and is under investigation for use in treating cancer. Both of these cell lines were transduced with retroviral vectors expressing ICP47 and US11 and the resulting class I decrease is reported herein. A third application of short-term class I MHC aims to prolong the survival of reintroduced tumor cells generated from outbred mice for use as a tumor model. After isolating and establishing primary tumor cell lines, it is often difficult to

achieve tumor growth when the cells are reintroduced back into mice. With outbred mice, host immune responses are likely candidates for the inability to sustain tumor growth. By suppressing class I MHC expression, tumor cells may be able to establish *in vivo* for a long enough period of time to provide a useful model system. Preliminary results for these three applications of class I MHC suppression are presented and their significance discussed.

INTRODUCTION

One form of cancer treatment now in clinical trials involves transplanting allogeneic lymphocytes that preferentially kill tumor cells into patients with the hope that these lymphocytes will locate and kill tumor cells *in vivo*. In an alternate form of this approach, cancer cells can be purged from a patient's bone marrow *ex vivo* while the patient undergoes aggressive cancer treatment. After treatment, the purged bone marrow with its resident population of pluripotent stem cells is reintroduced to repopulate the patient's blood system. TALL-104 and NK-92 demonstrate an ability to kill tumor cells preferentially and are currently undergoing clinical trials to treat a number of different types of cancers. Because these are both cancer cell lines, they are irradiated to a point where they maintain their cytotoxic functions while losing the ability to divide before they are administered to patients.

The TALL-104 cell line has demonstrated a remarkable ability to kill tumor cells from a number of species while sparing normal tissue [1-3]. To exploit this ability, three clinical trials to treat cancer have been performed [4-6]. The TALL-104 cells are irradiated to avoid transferring leukemia to the patients and injected in escalating doses throughout the treatment. Two trials treating both spontaneous and introduced tumors in dogs provided promising results. A third Phase I clinical trial to treat refractory metastatic breast cancer in humans demonstrated mixed results. Over the course of the treatment, 7 of 15 patients developed specific CTL responses to TALL-104 cells which likely limited the time TALL-104 cells were available to attack tumors [6]. In a previous clinical trial, anti HIV $CD8^+$ cells transduced with a retroviral vector encoding a marker gene were administered to HIV immunocompromized patients [7]. Initial injections of marked lymphocyte were detectable by polymerase chain reaction (PCR) assay at decreasing levels for the first seven days. With

subsequent injections into these now primed patients, labeled cells were often undetectable even one day post injection. Thus, with haplotype mismatched TALL-104 cells presenting unknown numbers of non-self antigens, the prospect of effective cancer killing cells remaining in the patients any significant time seems minimal. By decreasing the presentation of class I MCH antigens on TALL-104 cells, we hope to prolong their persistence in patients and improve their effectiveness at treating cancers.

NK-92 is a NK cell line that has been shown to preferentially kill tumor cells similar to TALL-104 cells [8-10]. Clinical trials using NK-92 are now under way [11, 12]. How NK-92 cells function to recognize and kill tumor cells is not yet understood, but much like TALL-104 cells it involves a class I MHC non restricted process [13]. NK-92 cells appear to be particularly effective when used for *ex-vivo* bone marrow purging [14, 15]. During this bone marrow purging process, NK-92 cells are exposed to host CTL and thus would likely benefit by having less cell surface class I MHC presenting targets. By decreasing the presentation of class I MCH antigens on NK-92 cells, we hope to improve their utility for both bone marrow purging and allogeneic transplant.

A third application of class I MHC suppression addressed herein involves a need to prolong the survival of chemically induced tumors when re-introduced back into mice in order to establish a cell line suitable for use as an *in vivo* tumor model. Cell lines established from tumors may have difficulty growing in their host organism for a variety of reasons. Inability to establish a necessary blood supply, introduction to a new tissue site, and a variety of immunological barriers are some of the challenges these tumor cells need to overcome. By decreasing class I MHC presentation on tumor cells, they may be able to avoid CTL recognition and lysis long enough to establish a tumor. The specific tumor model chosen

involves an in-house generated squamous cell carcinoma of the dermis, SQ172 [16]. This tumor was generated using a chemical carcinogenesis regimen on a α (1,3) galactosyltransferase (α-gal) knockout outbred mouse. SQ172 was determined to be H-$2^{b/b}$ haplotype however re-introduction into H-$2^{b/b}$ matched α-gal knockout mice at varying doses resulted in regression of tumors beginning at 7-10 days post injection. Seven days is the average time for peak CTL response indicating the possibility that CTL recognition of minor antigens was responsible for the failure to establish tumors. To investigate this further, the SQ172 cell line was transduced with the LUSN vector to determine the effect on class I MHC expression and *in vivo* growth properties.

MATERIALS AND METHODS

Vectors and cell lines. The LEL vector consists of an LXSN vector with the SV40 promoter and Neo^R gene deleted and replaced with the eGFP gene. The LESN vector consists of an LXSN vector with the eGFP gene cloned into the multicloning site. PA317 retrovirus packaging cell lines were developed for the LEL, LESN, LXSN, LISN, and LUSN vectors to obtain the supernatants necessary for transduction. Eight serial transductions of TALL-104 and NK-92 cells were done using freshly prepared viral supernatants. The following cell lines were used as targets in cytotoxicity assays: A375 human malignant melanoma [17]; VA13 SV40 transformed human fetal fibroblast [18]; K562 human lymphoblast [19]; HCT15 colorectal adenocarcenoma [20]; SQ172 squamous cell carcinoma of the dermis. TALL-104 and NK-92 cell cultures were maintained in the recommended medium (American Type Culture Collection, Rockville, MD).

Class I MHC Expression assay. Transduced, mixed populations of cells were briefly trypsinized and labeled with anti-class I MHC monoclonal Ab H58A (VMRD Inc., Pullman, WA) at 1.5 μg/ml/0.5×10^6 cells in serum free media at 37° for 1 hr [21]. The anti-mouse IgG2a isotype control UPC 10 Ab was used to control for nonspecific binding (Sigma-Aldrich Corp.). Cells were washed 5 min in Hank's buffer and a 1:64 dilution of goat anti-mouse FITC labeled secondary Ab (Sigma-Aldrich Corp.) or for 2 color analysis a 1:20 dilution of anti-mouse R-Phycoerithrin labeled secondary Ab (Sigma-Aldrich Corp.) were added for 30 min at 37°. Cells were again washed 5 min in Hank's buffer and resuspended in Phenol red free/serum free media (OptiMEM®, Invitrogen Corp., Inc.Grand Island, NY) for fluorescent analysis. Where a single peak was observed, the mean

fluorescence intensity is reported. Data shown is a representative result from a minimum of 2 experiments.

Cytotoxicity Assay. A non-radioactive colorimetric assay was used to determine specific cytotoxicity of NK-92 effector cells against vector transduced and control target cells. The Cytotox® 96 (Promega Inc., Madison, WI) assay measures lactate dehydrogenase (LDH) release by a reaction with a tetrazolium salt (INT) to produce a red formazan product [22]. In brief, target cells are plated in a 96 well plate and effector cells are added at the ratio of 15:1. After 5 hrs, supernatant is collected and assayed for LDH content. Controls include maximum LDH release (detergent lysed), media background, and spontaneous LDH release from both targets and effector cells. Each data point is an average of 4 wells and data presented is a representative result from a minimum of two experiments.

Mouse Injections. SQ172 cells were seeded, grown 24 hrs, counted and resuspended to $1x10^6$ or $5x10^6$/100ul in HBSS. Female, 8-14 week old α-gal knockout mice were injected subcutaneously in the belly and tumor measurements were taken 2-3 times weekly. Tumors were measured length x width x height and animals with ulcerated tumors or tumors larger than 1 cm^3 were euthanized.

RESULTS

Retroviral eGFP transduction into TALL-104 and NK-92 cells. Efficient MoMLV transduction into lymphocytes can be difficult to achieve [23-25]. To determine if TALL-104 or NK-92 cells could be transduced, two vectors encoding humanized, red-shifted green fluorescent protein (eGFP) were used [26]. LEL encodes eGFP and has had the SV40 promoter and Neo^R gene deleted. LESN is a LXSN vector with the eGFP gene cloned into the multicloning site. After eight consecutive transductions, fluorescent cells were observed in TALL-104 and NK-92 populations transduced with either vector. 4-5% of NK-92 cells were eGFP positive in either the LEL or the LESN populations indicating that for each of the eight transductions approximately 0.5% of the NK-92 cells were transduced (data not shown). Viral supernatant was prepared fresh for each transduction and viral titers were not measured. A375 melanoma cells used as a transduction control were routinely 30-50% transduced from each transduction. After eight transductions of TALL-104 cells, populations transduced with both vectors were between 0.8 and 0.9% fluorescent (data not shown). Therefore, approximately 0.1% of the cells were transduced in each attempt. TALL-104 is a slow growing cell line requiring a minimum of $9x10^5$ cells/ml to grow and $1.5x10^7$ cells/ml to cryopreserve. Low transduction rates coupled with slow growth has limited the ability to select fluorescent TALL-104 cells for further analysis. NK-92 LEL eGFP positive cells have been selected using cell sorting and NK-92 LESN cells have been selected in media containing 1 mg/ml G418 (Figure1B).

ICP47 and US11 function in TALL-104 and NK-92 cells. LXSN, LISN, and LUSN vectors were transduced into the TALL-104 and NK-92 cell lines as noted above and transduced populations were selected in media containing 1mg/ml G418. Both genes

decreased class I MHC cell surface expression in both cell lines. In TALL-104 cells, LISN decreased class I MHC expression by 71% and LUSN decreased class I MHC expression by 52% over non transduced control cells (Figure 2E). LXSN transduced TALL-104 cells became contaminated during their expansion and were not able to be tested. It is clear that in the LISN transduced population, there are cells that are negative for cell surface class I MHC expression as evidenced by the broad dual peak histogram (Figure 2C). In NK-92 cells similar result were observed. LISN transduced NK-92 cells had 52% and LUSN had 32% less expression of cell surface class I MHC as compared to LXSN transduced controls (Figure 3F). Again, the histogram for the LISN transduced NK-92 analysis indicates there is an identifiable population of class I MHC negative cells (Figure 3D). Both LISN and LUSN function to decrease class I MHC cell surface expression in the TALL-104 and NK-92 lymphocyte cell lines.

NK-92 LEL cells transduced with LXSN, LISN, and LUSN vectors. To investigate the possibility of transducing NK-92 cells with two retroviral vectors, NK-92 LEL cells were transduced with the LXSN, LISN, and LUSN vectors. After selection in media containing 1mg/ml G418 for 2 weeks, the populations were analyzed by flow cytometry for both eGFP and class I MHC expression. G418 selection did not appear to adversely affect eGFP expression (Figure 4). LISN and LUSN selected cells had 53% and 83% less class I MHC cell surface expression as compared to LXSN transduced. Thus, transduction using two vectors with two different selection markers is a viable approach for introducing multiple genes into the NK-92 cell line.

Anti-tumor cytotoxicity is maintained in transduced NK-92 cell lines. To determine if decreasing the class I MHC presentation on NK-92 cells negatively impacted

their ability to attack tumor cells, the specific cytotoxicity of the transduced NK-92 populations was tested against a panel of tumor cell targets. The class I MHC positive A375 melanoma and VA13 fibroblast and the class I MHC negative K562 lymphoblast and HCT15 adenocarcenoma were chosen as targets. At an effector:target ratio of 15:1, in a five hour cytotoxicity assay, all the transduced NK-92 populations killed the tumor cells at efficiencies similar to non transduced NK-92 cells (Figure 5). Therefore, the transduction process, class I MHC suppression, Neo^R expression, and eGFP expression did not prevent NK-92 cells from efficiently killing tumor cells.

Decreased class I MHC expression in LUSN transduced SQ172 cell line. In a separate application of US11 class I MHC suppression, the LUSN vector was used to decrease class I MHC expression in the SQ172 cell line. Due to the lack of function of ICP47 in murine cell lines, the LISN vector was not used for these experiments [27]. SQ172 cells were transduced with the LUSN vector and class I MHC cell surface expression was determined (Figure 6). Although LUSN transduced SQ172 cells expressed 52% less than LXSN transduced, they expressed just 16% less cell surface class I MHC as compared to non-transduced. In order to see if these levels of class I MHC reduction had any effect on tumor longevity *in vivo*, cells were injected into haplotype matched outbred mice from the same α-gal knockout colony which originally generated the tumor.

Decreased mouse survival when LUSN transduced SQ172 carcinoma cells are injected. Four groups of eight $H\text{-}2^{b/b}$ haplotype matched 8-12 week old α-gal knockout mice were selected. Groups 1 and 2 were injected with $5x10^6$ and $1x10^6$ SQ172 no vector (NV) cells (Figure 7). Groups 3 and 4 received $1x10^6$ SQ172 LXSN and SQ172 LUSN cells respectively. In both the SQ172 NV control groups and the SQ172 LXSN transduced group,

at least 75% of the tumors regressed and the mice survived. In the SQ172 LUSN group, 75% of the animals grew tumors large enough where the animals had to be sacrificed according to protocol.

DISCUSSION

CTL lysis mediated by class I MHC presented antigens is one of many ways our immune system protects us from our potentially dangerous environment. Several factors must be considered when designing a useful application for the suppression of this one aspect of the immune response. First, the target cells must use class I MHC processing and presentation of antigens. In most cases class I MHC presents short peptides generated by proteasome degradation of cytoplasmic proteins. Genetic mutations producing altered proteins, haplotype mismatched cells, and viral infected cells are all capable of presenting antigens to CTL. Second, by decreasing only the class I MHC mediated immune response, the immune suppression is likely to be effective only until Ab mediated and other immune responses intervene. Cancer treatments involving allogeneic transplant of anti-tumor cells require injecting a haplotype mismatched lymphocyte into a patient [6]. Minimizing the patient's immune response to these therapeutic lymphocytes would prolong their effective duration in the patient. Cancer treatments often benefit by stimulating or augmenting a patient's immune response to cancer cells and thus general immune suppression is not an option. Therapies utilizing TALL-104 and NK-92 cell lines would likely benefit if these cell lines were able to avoid the patient's immune detection.

Lymphocytes are efficient antigen presenting cells that interact with many cells of the immune system to stimulate cytokine secretion and induce recruitment and proliferation of antigen responsive cells. Although retroviral transduction of lymphocytes is often difficult, the ability to select and propagate NK-92 and TALL-104 cells *in vitro* minimizes this obstacle (Figure 1). Both ICP47 and US11 decreased class I MHC expression in NK-92 and TALL-104 cells (Figure 2, 3) and the decrease lasted over six months in continuous culture

(data not shown). Transduction and selection of NK-92 cells did not decrease the anti-tumor activity against a panel of 4 cell lines (Figure 5). Continuing work is underway and focuses on sorting populations of class I MHC negative NK-92 and determining the extent of their ability to evade host detection *in vitro*.

To further improve NK-92 cells, it will be necessary to introduce additional therapeutic genes. To demonstrate 2 separate vector transductions into the same NK-92 cells, NK-92 LEL cells were transduced with the LXSN, LISN, and LUSN vectors (Figure 4). The cells were then selected for in media containing G418 and analyzed for both eGFP and class I MHC expression. All the populations maintained their high level of eGFP expression while the LISN and LUSN transduced populations displayed decreased cell surface class I MHC. The function of both genes indicates transduction of both vectors was successful. Incorporating suicide genes such as HSV thymidine kinase may remove the necessity to irradiate these cells prior to use in either bone marrow purging or allogeneic transplant [28, 29]. Such modified cells could be allowed to expand *in vivo* during the treatment and eliminated as needed once they are finished or if complications arise. ICP47 and US11 transduction into the NK-92 cell line already encoding IL2 is another possible improvement [30]. Such further refinements of both TALL-104 and NK-92 cells are very promising.

Using US11 to prolong tumor survival in mouse cancer models is another application where short-term suppression of cell surface class I MHC expression may prove useful. One way some tumors avoid detection and elimination by host immune response is by decreasing or eliminating class I MHC expression [31, 32]. Thus, class I MHC down regulation may aid in establishing tumor cell lines that grow *in vivo* for use as tumor models. The vast majority of tumor models are developed in rodents and the lack of function of ICP47 in rodent cells

makes US11 the choice for use in this application [33]. SQ172 carcinoma cells injected into haplotype matched mice establish tumors infrequently. After injection, growth initiates until approximately 7-10 days when the tumors begin to regress [16]. To see if decreasing the expression of cell surface antigens on class I MHC would prolong the survival of these tumors, SQ172 cells were transduced with LXSN and LUSN retrovirus (Figure 6). LXSN transduced SQ172 carcinoma cells show increased cell surface class I MHC expression that is often observed and may result from the addition of vector proteins being processed for presentation [34]. Compared to SQ172 LXSN cells, SQ172 LUSN cells display 52% less cell surface class I MHC however they are display only 16% less class I MHC than SQ172 NV control cells. In results from preliminary tumor growth experiments, these differences corresponded to an increase in tumor growth and mouse mortality (Figure 7). Although these results are promising, the many potential factors affecting *in vivo* tumor development require further testing to directly link the observed tumor growth to inhibition of host CTL response. Further experiments on SQ172 LUSN and other tumor cell lines generated from α-gal knockout mice for use as tumor models have been postponed while an inbred colony of H-$2^{b/b}$ haplotype mice is being developed.

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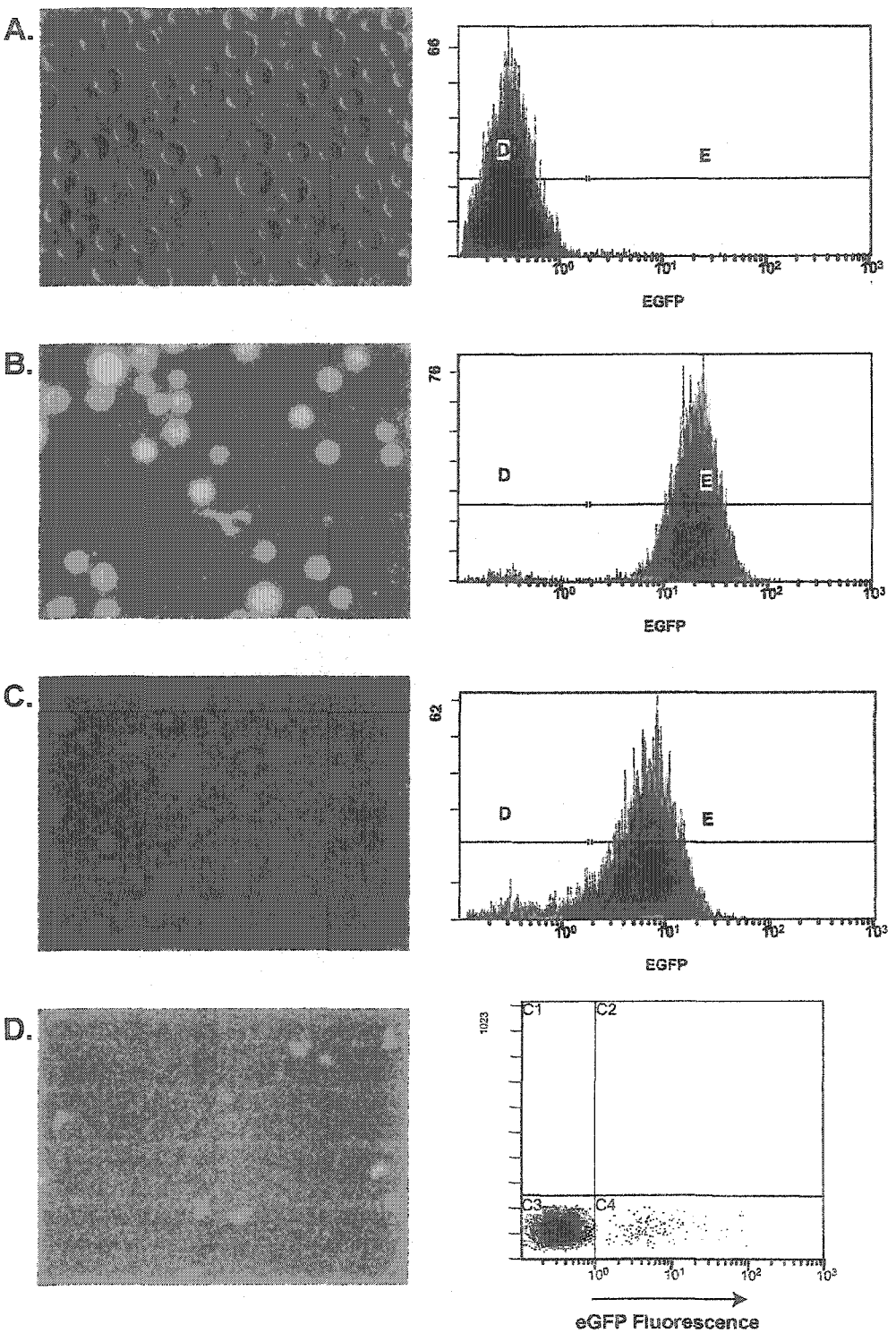
Figure 1. eGFP fluorescence in NK-92 and TALL-104 cells. TALL-104 and NK-92 were transduced with supernatants containing LEL and LESN vectors. NK-92 LEL transduced cells were selected using a flow cytometer. NK-92 LESN transduced cells were selected for by growth in media containing 1mg/ml G418. Fluorescence microscopy and flow cytometry results are presented for each sample. A) NK-92 control cells. B) NK-92 cells transduced with LEL C) NK-92 cells transduced with LESN. D) TALL-104 cells transduced with LEL.


A.
66
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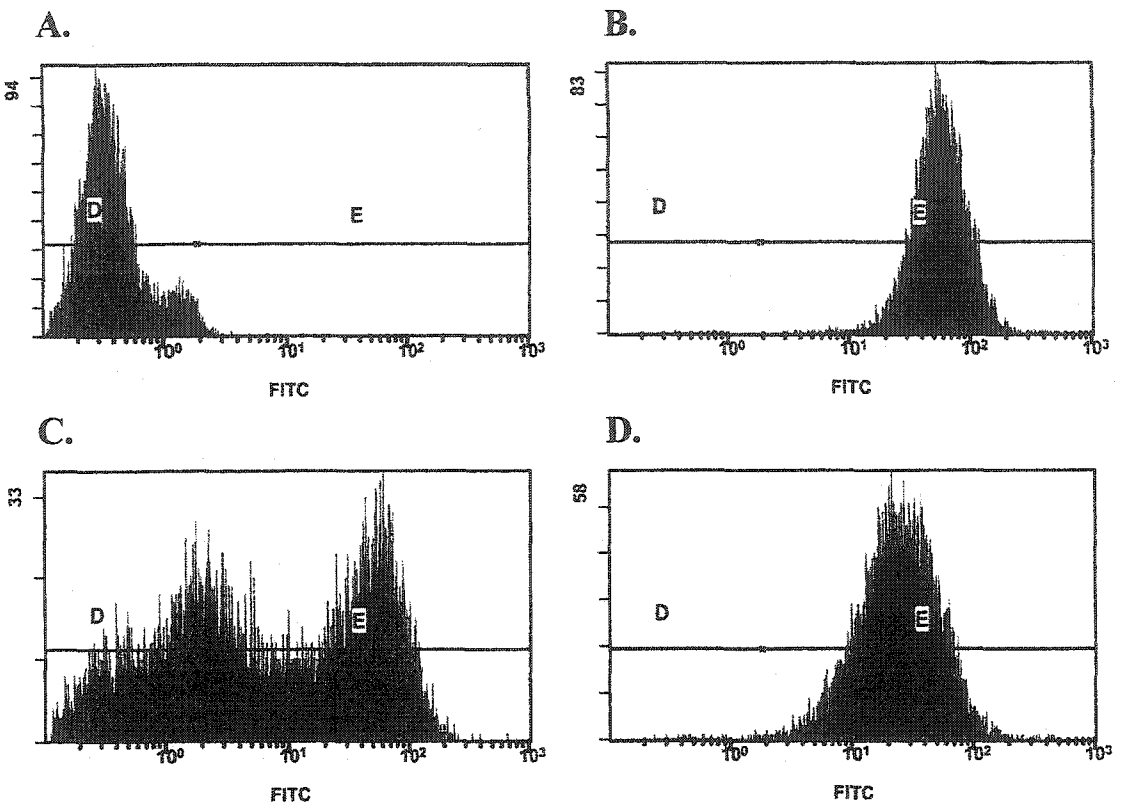
EGFP
B.
76
D
E
EGFP
C.
62
D
E
EGFP
D.
1023
C1
C2
C3
C4
eGFP Fluorescence

Figure 2. Class I MHC down regulation in LISN and LUSN transduced TALL-104 cells. TALL-104 cells were transduced with the LISN and LUSN vectors, selected in media containing G418, and assayed for cell surface class I MHC expression. A) TALL-104 no vector (NV) cells labeled with isotype control and a FITC secondary Ab. B) TALL-104 NV cells labeled with the H58A anti-class I MHC Ab. C) TALL-104 LISN cells labeled with anti-class I MHC Ab. D) TALL-104 LUSN cells labeled with anti-class I MHC Ab. E) Average class I MHC expression of the 4 cell lines. Although the average level of class I MHC expression in TALL-104 LISN cells is 52% as compared to LXSN control, there are clearly class I MHC negative cells in the population.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B.
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58
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10^3

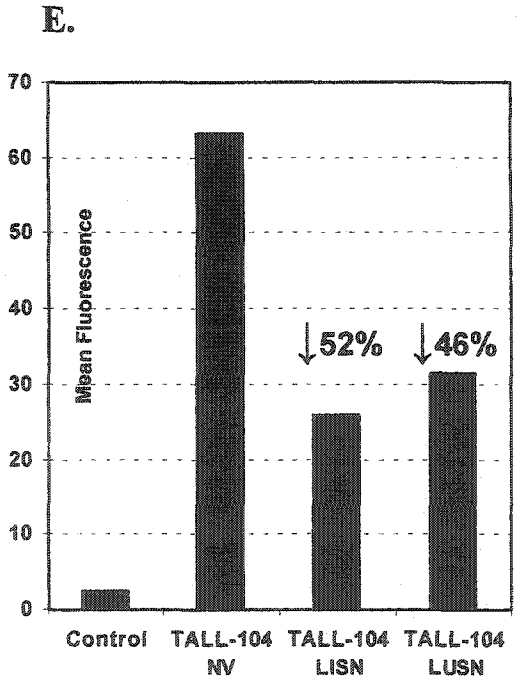
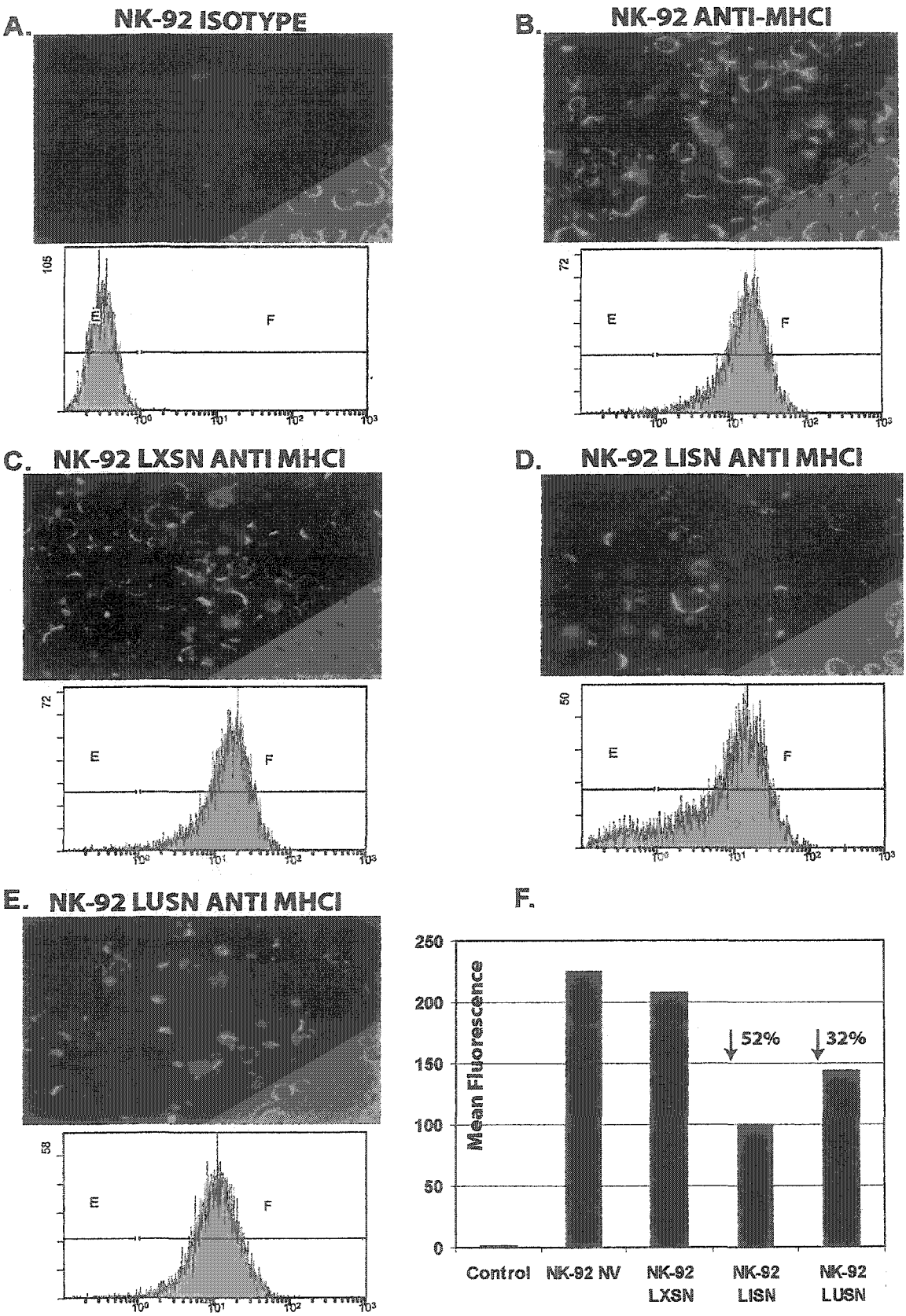

E.
Mean Fluorescence
70
60
50
40
30
20
10
0
↓52%
↓46%
Control
TALL-104 NV
TALL-104 LISN
TALL-104 LUSN

Figure 3. Class I MHC down regulation in LISN and LUSN transduced NK-92 cells. NK-92 cells were transduced with the LISN and LUSN vectors, selected in media containing G418, and assayed for cell surface class I MHC expression. Cell pictures were taken at 40X magnification using a GFP fluorescence microscope. A) NK-92 no vector (NV) cells labeled with isotype control and a FITC secondary Ab. B) NK-92 NV cells labeled with the H58A anti-class I MHC Ab. C) NK-92 LXSN cells labeled with anti-class I MHC Ab. D) NK-92 LISN cells labeled with anti-class I MHC Ab. E) NK-92 LUSN cells labeled with anti-class I MHC Ab. F) Average class I MHC expression of the 5 cell lines. Although the average level of class I MHC expression in NK-92 LISN cells is 52% as compared to LXSN control, there are clearly class I MHC negative cells in the population.


A.
NK-92 ISOTYPE
105
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B.
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72
E
F
C.
NK-92 LXSN ANTI MHCI
72
E
F
D.
NK-92 LISN ANTI MHCI
50
E
F
E.
NK-92 LUSN ANTI MHCI
58
E
F
F.
Mean Fluorescence
250
200
150
100
50
0
52%
32%
Control
NK-92 NV
NK-92 LXSN
NK-92 LISN
NK-92 LUSN

Figure 4. NK-92 LEL/LISN and NK-92 LEL/LUSN dual expression in NK-92 cells. NK-92 LEL cells were transduced with supernatants containing LISN and LUSN. Populations were selected for in media containing G418 and cell surface expression of class I MHC was determined. A) Two color flow cytometry of NK-92 LEL cells labeled with isotype control primary Ab and R-Phycoerithrin secondary Ab. Increasing class I MHC is indicated on the Y axis and increasing eGFP fluorescence is indicated on the X axis. B) Two color flow cytometry of NK-92 LEL cells labeled with anti MHC I Ab. C) Two color flow cytometry of NK-92 LEL/LISN cells labeled with anti MHC I Ab. D) Two color flow cytometry of NK-92 LEL/LUSN cells labeled with anti MHC I Ab. E) Average fluorescence of the 4 labeled populations. While eGFP fluorescence remains high, LISN and LUSN populations show 53% and 83% less cell surface class I MHC.

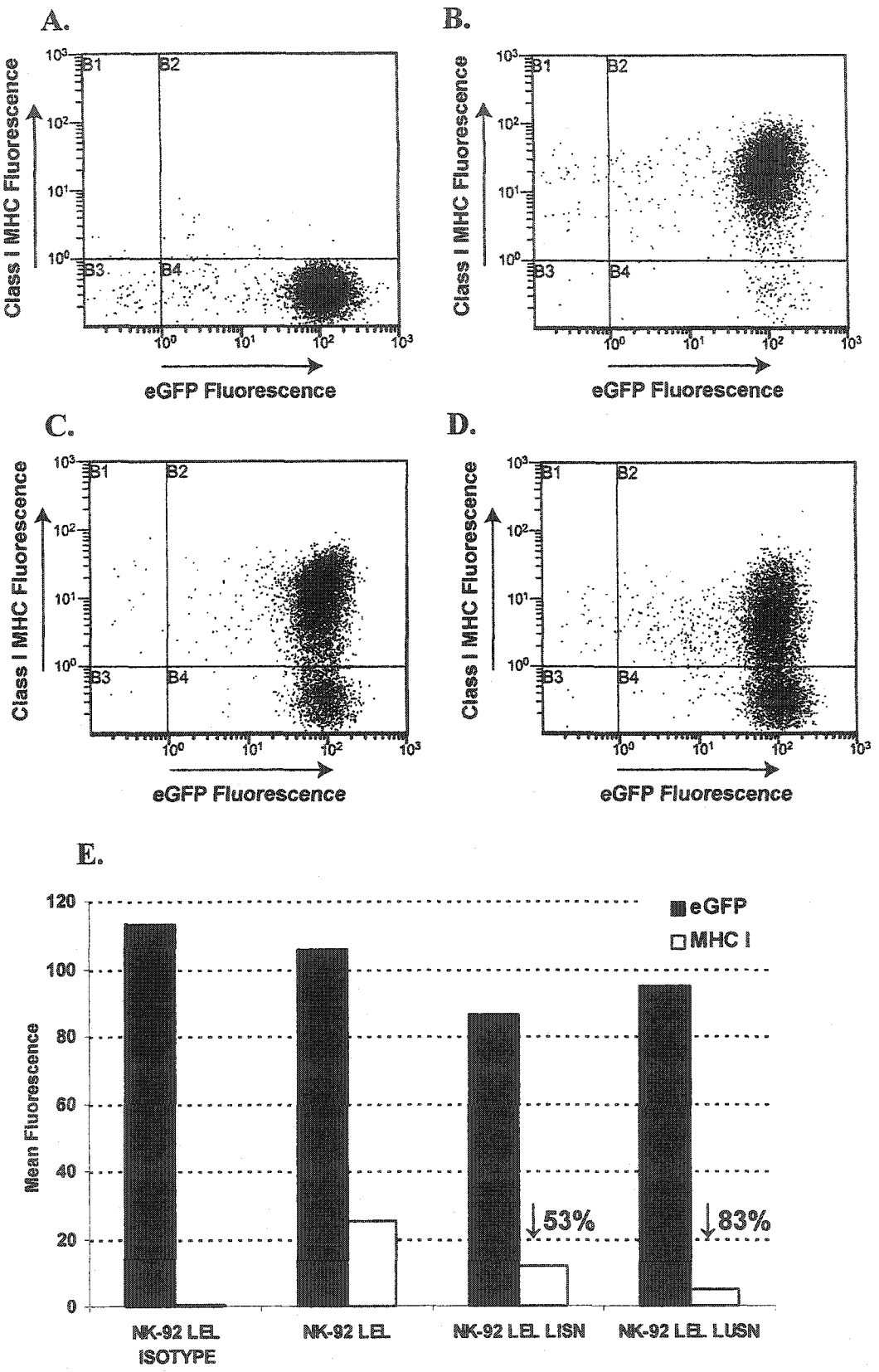

A.
B.
C.
D.
E.
Class I MHC Fluorescence
eGFP Fluorescence
B1
B2
B3
B4
Mean Fluorescence
eGFP
MHC I
53%
83%
NK-92 LEL ISOTYPE
NK-92 LEL
NK-92 LEL LISN
NK-92 LEL LUSN

Figure 5. Anti-tumor cytotoxicity maintained in NK-92 transduced cell lines. At a 15:1 effector:target ratio in a 5 hour cytotoxicity assay, NK-92 cells maintained between 25-40% specific cytotoxicity was maintained against a panel of 4 cell lines. A375 melanoma and VA13 fibroblast cells are class I MHC positive while K562 and HCT15 cells are class I MHC negative.

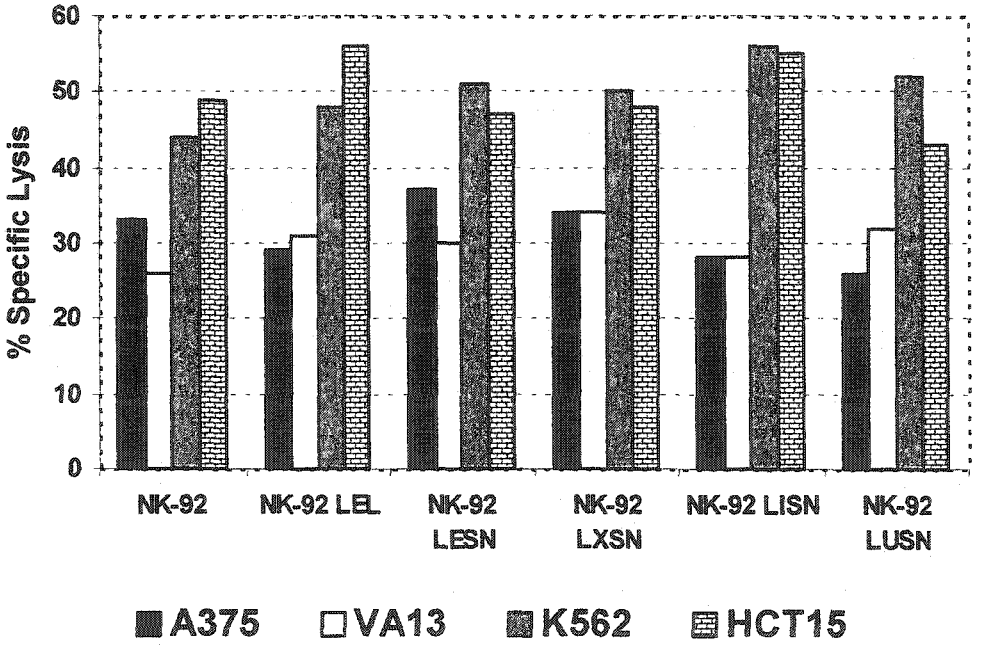

60
50
40
30
20
10
0
% Specific Lysis
NK-92
NK-92 LEL
NK-92 LESN
NK-92 LXSN
NK-92 LISN
NK-92 LUSN
A375
VA13
K562
HCT15

Figure 6. Decreased class I MHC expression in LUSN transduced SQ172 cell line. SQ172 cells were transduced with the LXSN and LUSN vectors. Class I MHC expression was determined using an anti-class I MHC Ab, fluorescent secondary, and flow cytometry. An isotype matched Ab was used to control for non specific labeling and mean fluorescence is reported for each population.

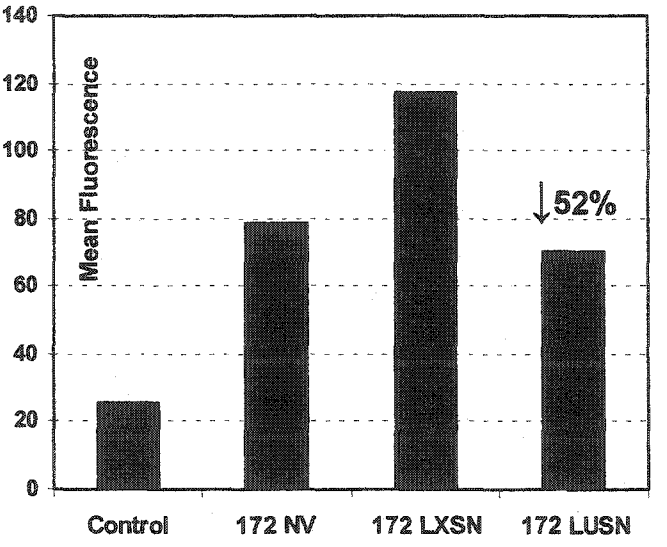
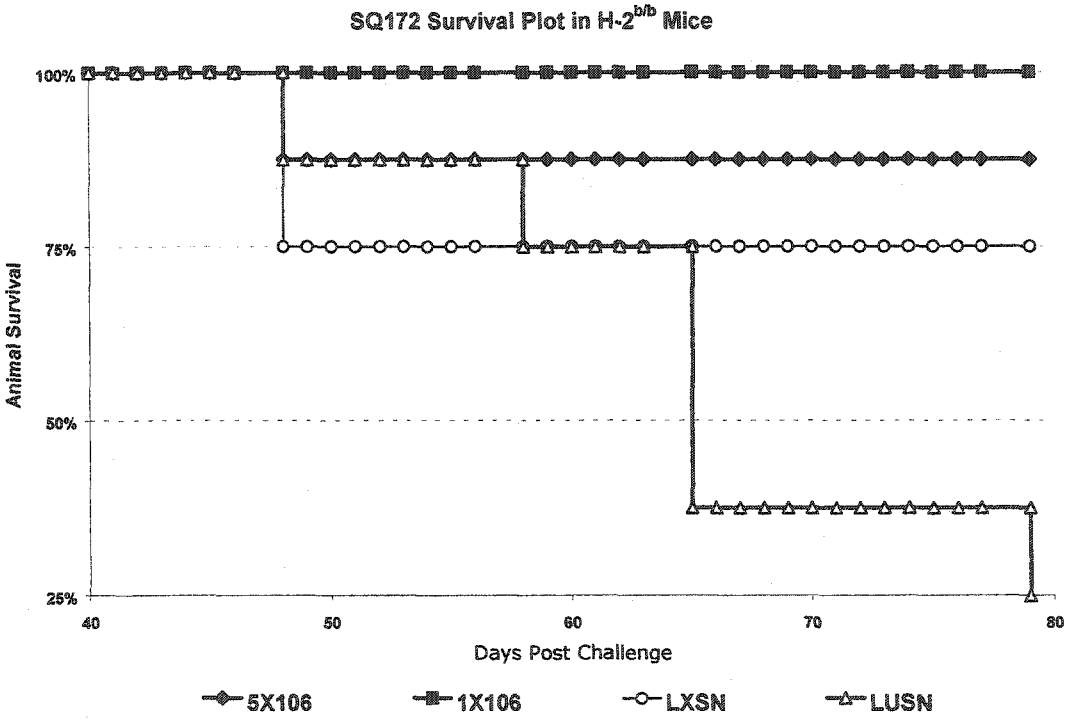

140
120
100
80
60
40
20
0
Mean Fluorescence
52%
Control
172 NV
172 LXSN
172 LUSN

Figure 7. Decreased mouse survival when challenged with SQ172. SQ172, SQ172 LXSN, and SQ172 LUSN cells were injected subcutaneously at the doses noted and tumor growth was monitored. Animals were euthanized if tumors ulcerated or grew beyond 1 cm^3.


SQ172 Survival Plot in H-2b/b Mice
100%
75%
50%
25%
Animal Survival
40
50
60
70
80
Days Post Challenge
5X106
1X106
LXSN
LUSN

CHAPTER 5. GENERAL CONCLUSIONS

GENERAL CONCLUSIONS

Therapeutic genes administered to patients often encode proteins with epitopes foreign to the patient's immune system. These epitopes are processed and presented at the cell surface on class I MHC molecules. Retrovirus mediated ICP47 and US11 expression in cells leads to a decrease in cell surface presentation of class I MHC proteins. The decrease in cell surface class I MHC expression results in an inhibition of the CTL activity as measured by an *in vitro* cytotoxicity assay. Although both genes function in all the human cell lines tested, variability in function between the two genes was noted. Variability of ICP47 function across the panel of cell lines was also noted. Growth of cells in media containing IFNγ lessened or negated the class I MHC suppression. Both variable function of ICP47 and US11 and cytokine mediated upregualation of class I MHC present obstacles to the use of these genes for promoting long-term transgene survival.

As two independent inhibitors of class I MHC presentation, ICP47 and US11 are outstanding tools to study the mechanisms involved with specific CTL/target interactions. The class I MHC nonrestricted TALL-104 cell line kills tumor cells preferentially regardless of their haplotype. Target cells transduced with ICP47 and US11 present reduced levels of class I MHC and were killed less efficiently by TALL-104 cells. Transduction of the genes into class I MHC negative cells had no effect on the ability of TALL-104 cells to kill them. Thus, TALL-104 cells can distinguish target cells based on the amount of their cell surface class I MHC expression in a nonrestricted fashion. Understanding the mechanism of TALL-104 cell targeting will aid in designing clinical applications for these cells.

TALL-104 and NK-92 are two cytotoxic cell lines that preferentially kill tumor cells and are currently in clinical trials for use as an allogeneic transplant to treat cancer. ICP47 and US11 decrease class I MHC expression in both of these cell lines. The transduction and selection process does not decrease the ability of NK-92 cells to kill tumor cells. By decreasing class I MHC expression in these cells, allogeneic transplants may persist longer in patients improving the outcome of this therapy.

Establishing tumor model systems from primary tumor cells is often difficult. Immunological barriers can prevent tumor growth when cells are reintroduced into mice. The SQ172 cell line failed to establish tumors when injected into mice from the same outbred colony where it was generated. US11 expression decreased cell surface class I MHC expression in this cell line. SQ172 cells transduced with the LUSN vector formed tumors more often than control vector transduced SQ172 cells. Although further analysis is needed to demonstrate an evasion of CTL response, US11 reduction of class I MHC expression may prove useful in establishing murine tumor models.

RECCOMMENDATIONS FOR FUTURE RESEARCH

ICP47 and US11 have proven useful for improving our understanding of the presentation and processing of class I MHC antigens and may improve a variety of *in vivo* therapeutic treatments that are subject to CTL mediated host immune response. To further these advances, several lines of investigation look promising.

Commandeering interferon gamma (IFNγ). Genes encoding class I and II MHC proteins are regulated by gamma interferon activated sites (GAS) that respond to IFNγ [1, 2]. The signal transduction occurs through the Janus kinase (JAK) signal transducer and activator of transcription (STAT) pathway (Figure 1). Many immune responder cells secrete IFNγ as an initial response when encountering stressed, damaged, or antigen presenting cells. IFNγ then up regulates class I and II MHC gene expression in the surrounding cells enabling recruited lymphocytes to sample intracellular and extracellular antigens present [3, 4]. With appropriate antigen stimulation, cell proliferation followed by removal of the target cells occurs. Proteins encoded by therapeutic transgenes are also subject to this process and physiological levels of IFNγ can negate ICP47 and US11 suppression (Chapter 2 figure 6, 7). One approach that may solve this dilemma is to construct gene transfer vectors with GAS sequences regulating the expression of ICP47 and US11. In response to IFNγ, the up regulation of ICP47 and US11 may be able to counteract the up regulation of class I MHC genes.

Characterizing non-classical (class Ib) class I MHC molecules. The class I MHC is polygenic, polymorphic, and variants inherited from both parents are expressed co-dominant. The resulting array of class I MHC molecules are categorized into haplotype groups based on function and structure. ICP47 and US11 inhibitors of class I MHC

presentation are useful tools to clarify the functions of these proteins. CD1D and HLA-E are two class Ib MHC molecules with unique functions that require further study. CD1D presents glycolipid antigens in a pathway distinct from the classical class I MHC [5, 6]. HLA-E presents leader sequences clipped from other class I MHC proteins and knowledge of its roles in both transplant rejection and NK regulation continues to expand [7]. Determining the function of ICP47 and US11 with respect to both of these class I MHC proteins could answer questions about TAP involvement, peptide requirement, and sub-cellular localization.

Quantification of CTL responses. Down regulation of class I MHC presentation by ICP47 and US11 in TALL 104, NK-92, and SQ172 cells was demonstrated. It will be useful confirm a decrease in CTL activity against these target cells. For TALL-104 and NK-92 cells, this will require *in vitro* priming of random donor lymphocyte populations followed by an ^{51}Cr release assay to measure specific cytotoxicity [8]. A ^{51}Cr release is necessary for mixed lymphocyte reactions such as this when both cell lines can act as effector or target.

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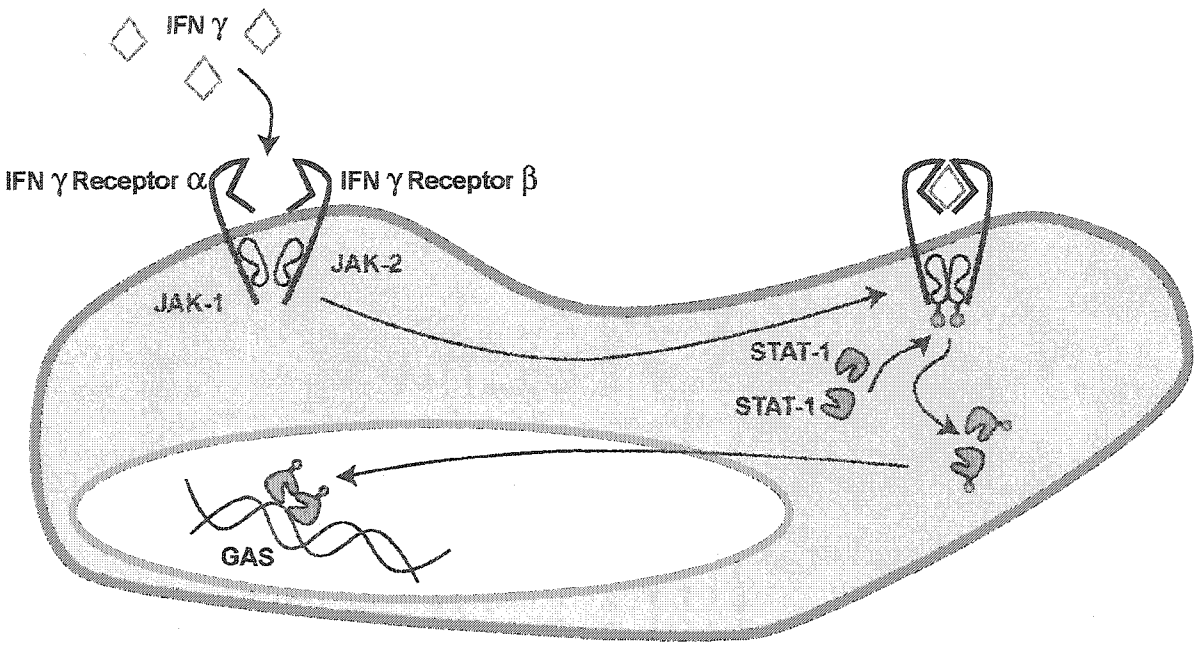
Figure 1. Interferon gamma signal transduction through the JAK STAT signal transduction pathway. IFNγ binds to cell surface receptors forming dimers. The conformational change leads to the phosphorylation of the Janus kinase (JAK) 1 and 2. These activated kinases then phosphorylate the signal transducer and activator of transcription (STAT) proteins. Phosphorylated STAT proteins are transported into the nucleus where they bind to gamma interferon activated sites (GAS) and initiate gene transcription.


IFN γ
IFN γ Receptor α
IFN γ Receptor β
JAK-1
JAK-2
STAT-1
STAT-1
GAS