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University of Alberta

A Strategy of Local Immunomodulation:

Engineering Fas Ligand Expression by Islet Allografts to Prevent Rejection

by

Camille Hancock Friesen

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of ***Master of Science***

in

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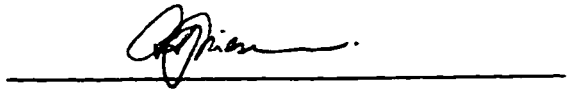


Camille Hancock Friesen
P.O. Box 160
Marwayne, Alberta
T0B 2X0

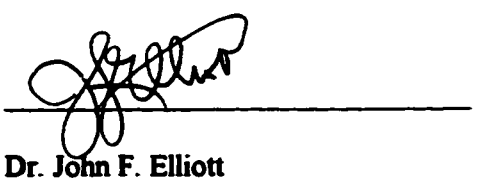
December 12, 1996

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***A Strategy of Local Immunomodulation: Engineering Fas Ligand Expression by Islet Allografts to Prevent Rejection*** submitted by ***Camille Louisa Hancock Friesen*** in partial fulfillment of the requirements for the degree of ***Master of Science*** in ***Experimental Surgery***.

Dr. Ray V. Rajotte (Supervisor)



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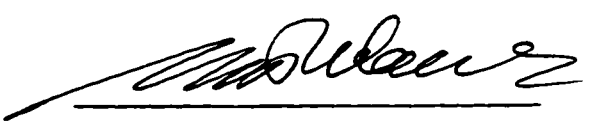

Dr. Garth L. Warnock

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ABSTRACT

Diabetes, a manifestation of islet cell destruction can be treated by islet cell allograft transplantation. Graft rejection remains the primary barrier to successful islet cell transplantation. The initiation of acute allograft rejection has been attributed to helper T lymphocytes. Fas receptor (FasR) and its ligand (FasL) are instrumental in peripheral clonal deletion of activated T cells whereby FasL causes FasR bearing cells to apoptose. We hypothesize that islet cells can be engineered to express FasL thereby protecting them from T cell mediated acute rejection while preserving their endocrine function. The cDNAs for human FasL and mouse FasL were cloned by RT-PCR. Human FasL was sequenced and then truncated constructs were derived from both wildtype FasL genes. Human FasIg, a fusion of human FasR and the Fc region of human IgG1, was designed and cloned from existing constructs as was human FasR. Bioassay of the FasL gene products present in supernatants from transfected COS cells, revealed that the wildtype constructs, but not the shortened constructs, had the expected cytotoxic effects against human FasR+ cells (causing an 80% reduction in cell viability in a species-independent manner). HFasIg was capable of blocking thirty percent of the wildtype FasL cytotoxicity in a species-independent manner. Wildtype human FasL did not have any deleterious functional effect on islets, mouse or human, demonstrated by glucose stimulation indices which were unaltered in islets exposed to human or mouse FasL. Transplantation results are too preliminary to be conclusive about the *in vivo* immunoprotective role of Human FasL. However, this strategy warrants further investigation as we move toward the goal of allowing transplantation of islet cell allografts without the need for chronic immunosuppression.

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2XYT:	Bacterial culture media composed of 1.6% (w/v) bacto-trypton, 1.0% (w/v) bacto-yeast extract and 0.5% (w/v) NaCl
BSA:	Bovine serum albumin
cDNA:	Complementary deoxyribonucleic acid; from which DNA has been copied
Con A:	Concanavalin A
CTL	Cytotoxic T lymphocytes
DMEM:	Dulbecco's Minimum Essential Media
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
dNTPs:	2'-deoxynucleotide-5'triphophates (G, A, T, C)
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
ELISA:	Enzyme-linked immunosorbent assay
FasR:	Fas Receptor designated with prefix to indicate species (M=mouse / H=human); also referred to as Apo-1 and CD95
FasL:	Fas Ligand: the binding partner for FasR designated with prefix to indicate species (M=mouse, H=human); also referred to as anti-Apo-1 and CD95 ligand
FBS:	Fetal bovine serum
FCS:	Fetal clone serum (I/II)
FITC:	Fluoroscein isothiocyanate conjugated antibody
FACS:	Fluorescence Activated Cell Sorting: a process of quantitating cell surface protein expression using fluorescently labelled antibody staining
gld:	mutant mouse strain with deficient FasL genotype
HBSS:	Hanks' balanced salt solution
HFasIg:	chimeric construct with HFasR binding specificity and human IgG1 Fc portion
IDDM:	Insulin dependent diabetes mellitus
IFN:	Interferon
IL:	Interleukin
kb:	kilobases (1000 base pairs)
kDA:	kiloDalton (1000 Daltons)
lpr:	mouse strain with FasR deficient genotype
mAb:	monoclonal antibody
MHC:	Major Histocompatibility Complex
MTX:	methotrexate
PBMC:	peripheral blood monocytes
PCR:	Polymerase chain reaction
RNA:	Ribonucleic acid
RNase:	Ribonuclease
RPMI:	Rothwell Park Memorial Institute media

RT-PCR	Reverse transcriptase PCR; process of generating cDNA from mRNA and synthesizing double stranded DNA which is amplified with PCR
TCR:	T cell receptor
T.E.:	Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA)
TNF:	Tumor necrosis factor

Chapter 1

INTRODUCTION

Diabetes mellitus is a disease of both young and old. The treatment of diabetes by insulin injection has been largely unsuccessful in preventing the metabolic effects of hyperglycemia. The progression of premature atherosclerosis with resultant renal, cardiovascular, nervous system, and ocular damage continues even in patients with "good" blood sugar control.

The recognition that physiologic function can be restored to patients with end-organ failure secondary to insulin-dependent diabetes mellitus (IDDM) by transplanting insulin-producing tissue has encouraged much clinical and basic science investigation in the recent past. The fact that insulin independence has been achieved in a number of patients by islet cell transplantation at the University of Alberta and in several other centers has been gratifying.[1] However, graft rejection remains the greatest obstacle to successful tissue transplantation. Current therapies aimed at abrogating allograft rejection target the recipient's immune system nonspecifically. While protecting the allograft from host immune attack, these immunosuppressant agents increase the patient's risk of opportunistic infection and predispose the patient to the development of various malignancies (B cell lymphoma, squamous cell carcinoma of the skin, Kaposi's sarcoma).[2]

The mechanisms of acute allograft rejection (with a key role played by activated T cells), a newly understood system of activated T cell control (the Fas system), and the potential application of Fas system to transplantation will be reviewed with the intention of developing a strategy of local immunomodulation so that islet cell allografts may be transplanted without the need for broad-spectrum, long-term immunosuppression.

1. ALLOGRAFT REJECTION

Allograft rejection is a process by which a recipient's immune system recognizes foreign antigens expressed by donor tissue resulting in both nonspecific and specific immune reactions, which uninterrupted lead to demise of the donor islet cell allograft.

Rejection is subdivided into three distinct subtypes (hyperacute, acute, and chronic) on the basis of histologic classification and clinical course.

1.1 Hyperacute rejection

Hyperacute rejection occurs rapidly (within hours of transplantation) as a result of preformed antibodies which cross-react with antigenic determinants expressed by the donor allograft endothelium. The antibody-antigen interaction triggers the complement cascade. Complement attacks the endothelium of the graft vessels resulting in thrombosis, ischemia and secondary graft malfunction.[16]

1.2 Chronic rejection

Chronic rejection is characterized by progressive fibrosis of the graft vasculature and ultimate loss of graft function. No single injurious mechanism has been identified as the cause of tissue damage in chronic graft rejection. Pathophysiologic theories include scarring secondary to healing acute rejection induced wounds, chronic ischemia, or a chronic delayed-type hypersensitivity reaction with direct cell mediated cytotoxicity or cytokine release responsible for graft damage.

1.3 Acute rejection

Acute allograft rejection occurs at a slower tempo than hyperacute rejection (days to weeks) and is thought to be the result of both humoral and cellular immune responses. Similarities between environmentally encountered antigens (bacterial, protein, or carbohydrate moieties) and donor tissue antigen are thought to allow previously formed antibodies and

previously sensitized T cells to cross react with donor tissue antigens causing acute allograft rejection.[5]

2. ALLOANTIGEN

The target antigen in acute allograft rejection has been alluded to; however, there are a host of antigens which may initiate or accelerate rejection. The major histocompatibility complex (MHC) is a tightly linked group of alleles localized to the short arm of chromosome 6 in humans which encode several unique membrane bound proteins, the recognition of which are responsible for rapid graft rejection. The MHC molecules are classified as Class I and II. Class I MHC is a 45 kD protein comprised of an α subunit with an associated β-2 microglobulin expressed by most tissues of the body and recognized by host CD8+ cells . Class II MHC is a heterodimer comprised of an α and a β chain with CD4+ restricted recognition by immune competent cells.[6] Donor MHC molecules are thought to be responsible for recipient T cell activation characteristic of acute allograft rejection.

Other non-MHC molecules responsible for allorecognition include minor histocompatibility antigens which are defined as non-MHC antigens capable of inducing cell-mediated allograft rejection. Initially these antigens were thought to cause slower paced rejection but it is now acknowledged that the rate and extent of rejection can be as marked in response to minor histocompatibility antigens as to foreign MHC antigenic stimulation.[5] The alloresponse to minor histocompatibility antigens is induced by the presentation of donor antigen peptide in the context of recipient MHC. The development of alloreactivity to minor histocompatibility antigens is attributed to recipient T cell clones responsive to self peptides with allelic variation (presented as "foreign" in the context of self MHC) which cross react with homologous donor antigens. There are a number of other non-MHC targets of allorejection

including tissue specific antigens (i.e. skin antigens, blood group antigens and endothelial glycoproteins).

The immune response to alloantigen can be divided into the afferent limb comprising both cellular (CD4+ T cell activation) and humoral responses (B cell antigen presentation) and the efferent limb comprising the development of local and systemic immune responses, organization of the inflammatory infiltrate, MHC induction in donor tissue and finally allograft destruction.[6]

3. ACUTE ALLOGRAFT REJECTION

3.1 Afferent Limb

The afferent limb of allograft rejection begins with CD4+ T lymphocytes (generally T helper cells) stimulated either by direct or indirect antigen presentation. CD4+ (the Th1 subtype) have been identified as the most significant mediators of cytotoxic insult in acute allograft rejection. Experimental evidence in support of the central role of CD4+ T lymphocytes was provided by an athymic mice mouse model (i.e. mice with no native T cells). Mice were reconstituted with only one T cell population (either CD4+ or CD8+). The mice that were reconstituted with CD4+ cells had a normal rate of graft rejection while those reconstituted with CD8+ T lymphocytes experienced a slowed rate of rejection implying that the CD8+ cell population was either insignificant in graft rejection or dependent on CD4+ cell activation.[3] Similarly when anti-CD4+ antibodies were administered to mice (thus knocking out CD4+ cell function) graft survival was improved while anti-CD8+ antibodies did not alter rate of graft rejection. These findings confirmed the role of CD4+ T lymphocytes in acute allograft rejection.[4] Recipient antigen presenting cells process foreign Class I MHC and express the antigen in the context of self-MHC, so called, "indirect" antigen presentation. Alternatively, and less efficiently, the T cells may directly recognize the donor MHC expressed constitutively on the surface of donor antigen presenting cells (so called "passenger

leukocytes"). In fact, much of the immunogenicity of an allograft is reportedly attributable to donor antigen presenting cells.[6]

T cell activation requires two signals. The first signal is provided by TCR binding of antigen in the context of self MHC (just described). The second signal (or costimulus) may be one of a number of factors which bind T cell receptors other than the TCR. The second signal may be provided by passenger leukocytes in the donor organ, by endothelial cells of the graft, or by various growth factors and cytokines. A number of different molecules on the recipient's T cell membrane have been recognized to function as receptors for costimulation, including CD28, IBB4. Once activated, CD4+ T cells begin to secrete a number of cytokines responsible for initiating the efferent limb of allorejection (IFN-α, IL-2, IL-4, IL-5, IL-6, TNF-α, TNF-β, GM-CSF).[6]

The humoral component of acute allograft rejection is mediated by alloantigen specific activated B cells. The primary role of B cells is antigen presentation (in the context of Class II recipient MHC) with antibody production and cytokine elaboration being a less important function in the acute setting.[6]

3.2 Efferent Limb

The efferent limb is mediated by alloreactive CD4+ cells which recruit and activate nonspecific inflammatory cells (including macrophage and polymorphonuclear cells) which elaborate a number of proinflammatory cytokines (including TNF-α, IFN-α, IFN-β, IL-1, IL-6, eicosanoids, platelet activating factor, oxygen free radicals) resulting in nonspecific tissue injury. Soluble cytokines may be directly toxic to graft parenchyma or may damage the graft indirectly by causing cellular infiltrate, vasodilation, capillary leak, endothelial cell necrosis, interstitial edema, and fibrin deposition resulting in secondary coagulation and tissue ischemia. More importantly, CD4+ cells cause the activation, proliferation and differentiation of CD8+ T lymphocytes (cytotoxic T lymphocytes, CTL). CD8+ cells can alternately be activated directly

by donor MHC Class I expressed by the nucleated cells of the graft parenchyma although this method of activation is less important than that mediated by CD4+ T cells. Both Class I and Class II MHC expression are upregulated on donor tissue during rejection. This effect is modulated by IFN-α and is said to be necessary but not sufficient for graft rejection. It may be that at a critical level of MHC expression antigen presentation is enhanced augmenting the already established rejection cascade.[6] The CD4+ and CD8+ cell populations clonally expand and differentiate with helper, cytotoxic and suppressor functions. The CD8+ CTL ultimately comprise the major immune effector in allograft rejection.[5] Specific cytolysis of graft parenchyma is mediated by CD8+ CTL whose effect is mediated by the release of a membrane-toxic protein, perforin.

4. AUTOIMMUNE GRAFT DESTRUCTION

Aside from acute allograft rejection, a unique issue in islet cell transplantation is the milieu of autoimmune islet cell destruction present in the recipient. Autoimmune destruction, responsible for the demise of native islet β cells, may be an ongoing source of injury to grafted endocrine pancreas cells as the recipient is "presensitized" to islet autoantigens which may cross react with transplanted islet antigens causing accelerated rejection. The occurrence of IDDM is now recognized to be MHC-restricted, and as islet allografts are MHC-typed (to avoid hyperacute rejection), the grafted islets are likely at a higher risk of spontaneous destruction because of MHC-associated autoimmunity, whereas transplanting donor / recipient MHC-mismatched islets may in fact be protective against autoimmune attack.[10] Allograft destruction by autoimmune recurrence has been witnessed even in the absence of acute allograft rejection and it is felt by some to be as important to treat or prevent the autoimmune facet of the disease as it is to treat acute rejection.[10]

5. PREVENTION OF ALLOGRAFT REJECTION

Immunosuppressive modalities can be categorized as ***nonspecific***, ***T cell specific***, or ***antigen-specific***.

5.1 Nonspecific Immunosuppression

The goal of nonspecific immunosuppressive therapy is to reduce the overall immunocompetence of the individual. Anti-metabolites such a cyclophosphamide, methotrexate, 5-fluorouracil nonspecifically interact with rapidly dividing cells (a population of cells that include alloreactive T cells) by interfering with DNA synthesis thus preventing cellular proliferation. This strategy obviously impacts all rapidly dividing cells including normal gut mucosa and bone marrow.

5.2 T cell Specific Immunosuppression

T cell specific strategies include anti-T cell antibodies from pooled gamma globulin known as antilymphocyte globulin, ALG, which targets T cells, B cells, and NK cells. OKT3 is an anti-T cell antibody which binds to the CD3 complex and prevents T cell activation by inhibiting TCR triggering. A similar strategy entails blocking the costimulatory signal on the T cell (anti-CD28/CTLA4) or on the antigen presenting cells (anti-B7) to prevent T cell activation. Drugs such as corticosteroids, cyclosporin A, and FK506, target T cell metabolic pathways and interfere with protein expression, notably IL-2, which inhibits the cascade of rejection discussed earlier. These strategies target the entire T cell pool rather than isolated alloreactive T cells.

5.3 Antigen Specific Immunosuppression

Antigen specific immunosuppression allows suppression of the immune response to donor antigen but preserves the immune response to other foreign antigens. A number of strategies are aimed at donor tissue modification and some at recipient modification. *'Donor'* strategies include: (1) deplete the amount of antigen present by culture of islets in high oxygen

concentration or by cryopreservation;[11-13] (2) modulate MHC expression by donor islet cells by low temperature culture or by cryopreservation.[14] *'Recipient'* strategies include: (1) prevent interaction of donor antigen with recipient lymphocytes by transplanting islet cells to immune privileged sites or by islet encapsulation;[15,16] (2) educate immature thymocytes by exposing them to foreign antigen in the thymus thus inducing tolerance;[11] (3) induce anergy in alloreactive T cells; (4) induce suppressor cells.[11]

With our current understanding of the pathophysiology of allograft rejection the ability to target T cells specifically will minimize the side effects of treatment while preventing graft rejection. One system that has shown some promise in the field of transplantation because of its ability to specifically target activated T cells is the Fas system. The rest of this review will be focused on the historical development of the Fas system and the current understanding of its physiologic role and some potential antirejection applications.

6. FAS RECEPTOR

The regulators of cell proliferation and differentiation are a series of membrane-bound receptors that transduce a signal when they are bound to their respective ligand. Tumor necrosis factor (TNF) was one of the first proteins (called cytokines because of their cellular influence) to be identified as having an role in inducing of cytotoxicity in various tumor cell lines among other biological effects.[17] Once the gene encoding TNF was cloned and pure preparations were available for study it became apparent that there were cell lines with high affinity cell surface receptors for TNF.[18,19] However studies of the response of these TNF-sensitive cell lines revealed that there was no correlation between the killing effect of TNF and the number of TNF receptors or the binding affinity of TNF to its receptor. Thus it was presumed that another factor was involved and responsible for the biological effects initially ascribed to TNF. A panel of monoclonal antibodies were raised against a TNF-sensitive

hybridoma formed by fusing a fibroblast cell line with the murine myeloma, NS-1.[17] The clones of this fusion were screened for the production of a monoclonal antibody with cell killing activity against various murine and human cell lines (the antibody was named anti-Fas antibody). The specificity and intensity of cell killing were identical between TNF and anti-Fas antibody but Western blotting of one of the tumor cell lines sensitive to TNF and anti-Fas antibody cytotoxicity (U397, a histiocytoma) revealed a unique protein, named Fas antigen.

Another group isolated the same anti-Fas antibody (and called it anti-Apo-1) and noted the antibody's effect in inducing tumor regression when applied to certain malignant lymphocytes (B cell lymphoma, BJAB).[20] It was concluded that Fas antigen, (also referred to as Apo-1 and CD95) must either trigger a cytotoxic message or block growth factor receptors. Cells susceptible to anti-Fas antibody toxicity were subjected to total mRNA extraction. cDNA libraries were constructed and cloned into plasmids for transfection into eukaryotic cell culture. The transfected eukaryotic cells were panned with anti-Fas antibody (IgM) and cells binding the antibody were subjected to DNA extraction. Common sequences between expressing clones were compared, sequenced and identified as the Fas receptor (FasR) gene.[21,22]

Further characterization of FasR localized it to chromosome 19 in the mouse and chromosome 10 in human. It is a type I protein with a signal sequence and an extracellular amino terminus, a hydrophobic transmembrane domain and an intracytoplasmic carboxy terminus.[23] The genomic DNA is >26 kb in length and the mature protein is 335 amino acids long. A soluble form of FasR (sFasR) has been identified which is truncated by alternate mRNA splicing thus lacking the transmembrane region. Soluble FasR has been shown to block apoptosis and is hypothesized to be responsible for the accumulation of activated T cell in a number of autoimmune diseases.[24,25]

FasR is expressed by a variety of somatic cell lines including heart, lung, liver and spleen, as well as in cells of lymphoid origin including T lymphocytes, B lymphocytes,

monocytes, and polymorphonuclear cells. There has been weak mitogen-induced expression of FasR expression by thymocytes and speculation that FasR may play a role in central negative selection of self- reactive T cells, although this remains controversial. Germ line tissues including ovary and testis also have also been found to express FasR. FasR expression is either constitutive as in the liver or inducible as in activated T cells. FasR expression is upregulated by IFN-α, actinomycin-D, and cyclohexamide.[26]

Comparison of FasR amino acid sequence with the tumor necrosis factor super family revealed significant species-independent homology in the C-terminus region (which is responsible for apoptotic signal transduction), as well as in the extracellular cysteine-rich domain of the protein.[23,24] The members of the TNF receptor super family include two forms of TNF-R, α and β, low affinity NGF, lymphocyte membrane proteins CD30, CD27, the T cell antigens OXO40 and 4-1BB as well as the B cell antigen CD40.[23]

FasR was now known as a cell surface protein that bound to an unidentified cytokine and induced cell death. The physiologic roles of FasR appeared to be peripheral regulation of immune responses by clonal deletion and a CTL effector mechanism (calcium-independent cell mediated cytotoxicity).

7. FAS LIGAND

The search for a binding partner for FasR began. Using a chimeric protein construct with FasR binding specificity combined with the Fc-portion of the heavy chain of immunoglobulin gamma-1 (MFas-Fc) a T cell hybridoma was identified that expressed FasL at high levels by fluorescent activated cell sorting (d10S, a rat-mouse T cell lymphoma hybridoma).[27,28] Expression cloning for FasL was performed whereby COS cells were transfected with a cDNA library from the high level FasL expressor cell line. The cells were then panned with MFas-Fc which positively identified those cells which expressed the FasL

protein on the cell surface. The plasmid DNA was amplified in *E. coli* and then isolated.[28] Positive clones were restriction enzyme mapped and overlapping sequences used to identify the gene which was cloned and sequenced.[27,28] The same researchers went on to purify FasL from cell membranes but also from the supernatant of cell cultures transfected with FasL indicating that FasL exists both in membrane bound and soluble forms. Both forms were shown to have equal biological activity. The purified protein caused apoptosis of cell bearing FasR but not of cells without FasR expression. The cytolytic activity of FasL on FasR bearing cells could be blocked by the receptor-decoy MFas-Fc (this soluble form of FasR binds Fas ligand preventing it from interacting with the membrane bound receptor and triggering apoptosis) but not by an unrelated soluble chimeric fusion protein TNFR-Fc.[28]

Further characterization of Fas ligand revealed that it was a type II membrane protein with the amino terminus residing within the cytoplasm and the carboxy terminus found in the extracellular region. FasL has no signal sequence and the soluble form is believed to arise by proteolytic shedding. The gene was localized to chromosome one in both human and mouse genomes. Genomic DNA is 8.0 kb long and mature cDNA is 843 base pairs long encoding the 281 amino acid peptide. HFasL has 76.7% homology with MFasL and there is species-independent binding of FasR by FasL.[29] Study of the amino acid sequence revealed significant homology with the TNF/NGF family confined to the carboxy terminus (receptor recognition site). FasL shows greatest homology with TNF-α, TNF-β (28% homology), and lymphotoxin α and less with the CD40, CD30, and CD27 ligands.[27]

The expression of Fas ligand is much more limited than that of Fas receptor. FasL is found expressed by activated lymphocytes (both T and B), by germ line cells (especially high levels in testis) and in all structures of the eye. FasL expression is either constitutive (as in the eye and testis) or induced (as in activated T cells). FasL expression can be stimulated by mitogens PMA, ionomycin, concanavalin A, and 1L-2.

8. FAS SYSTEM PHYSIOLOGY

Apoptosis is the term applied to a histologically characteristic cell death that occurs as a result of coordination of extracellular stimuli (soluble signaling molecules, cellular interactions and substrate availability) and intracellular milieu (including a number of second messenger signals and expression of modifier genes).[30] Apoptosis is the most common form of eukaryotic cell death and has a role in embryogenesis, tissue atrophy, tumor regression, and neurological development. As well, apoptosis is active in ongoing homeostatic processes in mature organisms such as peripheral clonal deletion of activated T cells.[20,24] Interaction of FasR with FasL causes cell demise of the FasR+ cell via apoptosis. This system of cell death is responsible for regulating normal immune responses, mediating immune tolerance, and conferring immune privilege to various tissues.

Apoptosis, also known as activation induced cell death (AICD) or programmed cell death (PCD) represents either a "*default death*" which ensues because of the absence of necessary growth factors, or in the case of Fas-mediated apoptosis as "*active cell death*" as a result of the presence of a negative effector cytokine. Within two to three hours of engagement of the FasR by FasL in a susceptible cell, endonucleases are activated and chromosomal DNA degradation occurs with the DNA fragmented into 180 base pair oligomers. Nuclear segmentation, cytoplasmic condensation, cytoplasmic membrane bleeding and breaking up of the cell into apoptotic bodies are the other characteristic histologic features that distinguish apoptosis from other forms of cell death.[24] Apoptosis can occur in anucleate cells indicating that the death signal is mediated intracytoplasmically with nuclear demise as a secondary event.

Single activated T cells have been isolated and found to undergo apoptosis indicating that one cell has both stimulus and effector mechanisms sufficient to induce suicide.[31] Apart

from FasR expression other intracellular influences on apoptotic signaling include the state of cellular activation. It is important to note that FasR does not always induce apoptosis in cell lines bearing the receptor. In fact, FasR has the effect of cellular activation in select circumstances when chronically activated T lymphocytes or thymocytes are copresented with FasL and immobilized monoclonal anti-T cell antibodies.[32] Naive T cells when activated upregulate FasR expression but are not sensitive to FasR mediated apoptosis. Contrariwise, previously activated or chronically activated T cells upregulate both FasR and FasL expression and become sensitive to FasR mediated apoptosis within several hours. There seems to be a threshold of FasR/FasL expression required for a population of activated T cells to undergo apoptosis (sororicide) since within hours of activation a T cell will upregulate FasR and FasL expression but population death does not occur for 72 hours.

Extracytoplasmic modulators of apoptosis are recognized to either promote or inhibit Fas-mediated apoptosis. Apoptosis promoters include p53, bax, bcl-x5, ced-3, ced-4, ICE, prICE. Apoptosis is inhibited by another group of mediators including bcl-2 family products, FAP 1, bcl-xl and ced-9.[33] Other less well elucidated but hypothesized modifiers of apoptosis include ICAM-1, LFA-1, 4-1BB, TNF, CD30, CD40, MHC Class I and II.[34]

The trigger at the cell surface for the transduction of a death signal is the cross linking of FasR by either its native ligand, FasL or agonistic antibodies (anti-Fas antibody, IgM; anti-Apo1, IgG3). Trimerization of the FasR appears necessary for activation induced cell death as evidenced by the fact that $F(ab)_2$ cannot induce apoptosis but its cytotoxicity can be restored by cross linking the antibody with secondary antibody or protein A.[26] The purported homotrimer structure of FasL parallels TNF-α which exists as a homotrimer and aggregates membrane-bound receptors in triplicate.

The intracellular details of death signaling secondary to FasR engagement remain vague. A variety of second messengers have been implicated but their exact functional role

remains poorly characterized. Evidence for the roles of tyrosine phosphorylase, cysteine protease, sphingomyelinase, interleukin-1-β converting enzyme have all been purported.[35] The proposed sequence of events is receptor aggregation by FasL or agonistic antibody binding followed by activation or recruitment of receptor-associated effectors (such as FADD and RIP) which are intimately associated with the death domains of the receptor. With trimerization of the receptor, the effectors are also brought into close proximity which may result in their activation and the enactment of cell death.[35]

Both physiologic and pathophysiologic manifestations of the Fas system have been elucidated. In normal physiology Fas system plays a role in maintaining normal immune responses, allowing immune tolerance, and conferring immune privilege

8.1 FAS SYSTEM: Maintaining Normal Immune Responses

FasL appears to be an alternate route of killing by CTL. The classically described method of T cell mediated cytotoxicity is the perforin pathway. Perforin, a 66 kDa protein, undergoes conformational changes in the presence of calcium and inserts into the lipid bilayer of a biological membrane causing pore formation, disrupting the homeostatic balance and resulting in osmotic lysis. Perforin-induced injury cannot account for all of the cytotoxic effects mediated by CTL, and it appears to act in consort with granzyme. Granzymes are a group of proteases found in the granules of CTL, which are released with the release of perforin and are theorized to be taken up by endocytosis as the cell repairs perforin-mediated damage, or directly through the perforin-induced pores.[7] However, mice with a mutation in the perforin gene retain a measure of cytotoxic ability specific to FasR+ cells implicating the Fas system in the effector mechanism of CTL.[8] As well, CTL can engage in cell mediated cytotoxicity in experiments devoid of calcium, implying again that a mechanism other than perforin-granzyme must be present.[7] This CTL effector system has been much studied the past 5 years and has

come to be known as the Fas system with a number of other physiologic functions that make it particularly useful as part of an antirejection strategy.[9]

8.2 FAS SYSTEM: Immune Tolerance and Peripheral Clonal Deletion

Fas is involved in both T and B lymphocyte homeostasis. B cells producing autoreactive antibodies in the periphery are sensitive to Fas-mediated apoptosis.[37] T cell control is less clear and the question remains whether Fas is operative in central or peripheral T cell selection. Normal T cell ontogeny involves the migration of pluripotent stem cells from the bone marrow to the thymus where the immature T cells are known as thymocytes. The thymocytes (initially CD4-CD8-) first acquire both cell surface markers (CD4+ CD8+) and subsequently they express the TCR/CD3 receptor complex each clone having a unique binding epitope. Four possible fates await the developing thymocyte. Thymocytes that interact with self-MHC plus peptide expressed on thymic epithelium are positively selected. The thymocytes that bind peptides in a non-MHC-restricted manner are allowed to die (via apoptosis). If a more mature thymocyte interacts with self-MHC plus peptide recognized on thymic epithelium clonal anergy is induced . Precursor T cells that interact too strongly with self-antigen in the context of MHC presented by antigen presenting cells are deleted (negative selection). Ultimately each T cell down regulates the expression of either CD4 or CD8 and becomes a single positive T cell (CD4+8-or CD4-8+) with unique TCR specificity. Experiments have been performed to identify the level of FasR expression of thymocytes at different stages of differentiation. The highest levels of FasR expression are in undifferentiated thymocytes (CD4-CD8-) with intermediate levels of TCR expression (this population comprises 1-3% of the total thymocyte population). CD4+/CD8+ double positive cells (i.e. more mature) and thymocytes marked by higher levels of TCR expression, exclusive expression of CD4+ or CD8+ or increased expression of CD28, CD44 CD69 (other markers of thymocyte maturity) down regulate the

expression of FasR. The conclusion that FasR plays a role in central T lymphocyte selection is based on the fact FasR expression is greater in more immature T cells.

Central thymic education is only effective in inducing tolerance to antigens that are presented as "self" by the neonatal thymus. Self-antigens that appear later in development are not introduced to T cells in this manner so a method of peripheral selection is necessary. FasR expression has been identified in the thymus by Northern blot analysis.[28] However, there is little or no FasL mRNA transcript present in the thymus, and the fact that in mice with mutant forms of FasR and FasL the thymic T cell selection is unperturbed with normal TCR repertoire and intact MHC-restricted responses implies that the role of Fas is limited to peripheral T cell selection.[26,34,36] FasR and FasL deficient mice (lpr/gld) are normal developmentally except for the accumulation of activated T cells, B cells and development of autoreactive T lymphocytes.[8] Ultimately nephritis, arthritis and autoimmune-type diseases manifest and the mice die prematurely.[37] This lack of self-tolerance evident in FasR/FasL mutants has lead to the inference that the Fas system is involved in the maintenance of self tolerance.[38-40] The specific role of Fas in the periphery is deletion of autoreactive T cell clones that have escaped thymic negative selection and elimination of activated/expanded T cell clones after antigenic stimulation.

8.3 FAS SYSTEM: Immune Privilege

Consideration of immune privileged sites and the substrate which confers immune privilege have long been debated. Immune privileged sites are defined as tissues or regions of the body that can support allogenic or xenogenic tissue without rejection. Such sites include testis and ovary, pregnant uterus and placenta as well as many somatic cell lines including cornea, retina, brain, cartilage, liver, adrenal cortex, prostate and a few others.[41,42] Immune privilege in these sites was originally ascribed to an anatomic barrier (either absence of

lymphatic drainage or presence of a blood-tissue barrier) which was felt to prevent the interaction of mature immunocompetent lymphocytes with antigen expressed by the tissue maintaining a state of "immune ignorance." However, lymphocytes are not sequestered from all immune privileged tissues so there must be another explanation for their immune privilege.[42]

Testes, which are an immune privileged tissue, are exempt from usual allogenic rejection. When transplanted across MHC incompatibilities the testis, specifically the Sertoli cells, do not succumb to allograft rejection. It was hypothesized that host lymphocytes could recognize a mismatched testicle graft as any other MHC-incompatible tissue but because Sertoli cells express FasL the activated T cells would be induced to apoptose as soon as they were activated.[15] To prove this hypothesis, testicular tissue was transplanted in a non-immune privileged site (under kidney capsule) and found to survive without evidence of any rejection. However when testicular tissue was donated from mice incapable of producing FasL (gld) the testicular transplants were rapidly rejected indicating the absence of any deterrent to cytotoxicity of the infiltrating alloreactive T cells.[15] Semi-quantitative RT-PCR was performed to identify the level of FasL mRNA expression of various tissues. Testicular tissue in general and specifically the Sertoli cell population was found to express high levels of FasL. The researchers concluded that graft associated FasL could be an effective method of interfering with T cell mediated acute allograft rejection.

Following this report another immune-privileged site was found to express Fas ligand. Histological assessment of cornea and anterior chamber of the eye were conducted after introducing an infectious virus into the anterior chamber. In normal mice (B6) there was a brief inflammatory response followed in 24 hours by rapid resolution of the reaction as the activated T cells committed apoptosis. In models with defective Fas ligand function there was a sustained inflammatory response and loss of the immune privilege in the eye. Gld mice irradiated and then reconstituted with B6 (wildtype) bone marrow still had the same prolonged

inflammatory reaction as the FasL deficient mice (gld) implying the protective effect was local rather than systemically derived. The eye structures were stained with an anti-FasL antibody . All structures of the eye (iris, cornea, ciliary body, retina) expressed FasL. This was the first recognition of somatic cell line FasL expression.[41]

Isolated anterior chambers of the eye were inoculated with Fas R positive and Fas R negative tumor cell lines to verify that the effect was FasR specific. In wild type (B6) anterior chambers there was apoptosis of the Fas R positive cells while in the anterior chambers of gld mice (Fas L deficient) there was no apoptosis. This indicated that the FasL responsible for the apoptosis noted in this isolated system did not come from systemic circulation but rather from the local environment of the eye. MRNA transcripts for Fas ligand were identified in Northern blots from eye.

8.4 FAS SYSTEM: Pathophysiology

Pathophysiologic manifestations have been associated with either under expression or over expression of FasL and FasR. Functional alterations induced by mutations of the FasR and FasL gene have been important to inferences about the physiologic role of Fas system in normal circumstances.

Three mice models with mutations in the Fas system genes have been identified and studied to characterize the effect of subnormal levels of FasR and FasL. Generalized lymphoproliferative disease mice (gld) are a loss of function mutation with a point mutation in the FasL gene encoding an amino acid in the C terminus of the extracytoplasmic domain of FasL protein.[8] As a result of this mutation, FasL, while produced constitutively, cannot engage FasR thus clonal deletion of activated T cells is impaired as is peripheral negative selection of autoreactive T cells. These physiologic deficits result in characteristic lymphadenopathy and autoimmune-type diseases.[37]

The MLR strain designated as classic lymphoproliferation (lpr) arises from one of two mutations. Lpr has a phenotype similar to gld induced by a mouse retrovirus which causes entransposon insertion into the FasR gene (intron 2) resulting in aberrant mRNA transcript splicing and truncation of the transcript. While this is a leaky mutant, it greatly diminishes FasR expression and thus impairs FasR mediated apoptosis.[26,39] Lpr^{cg} describes the strain of mice which are heterozygous at both the gld and lpr alleles (lpr complementing gld). These mice have a point mutation in the portion of the FasR gene encoding the cytoplasmic domain which prevents the receptor from transducing the apoptotic signal so while there is abundant mRNA encoding full length FasR the protein is nonfunctional.

Both gld and lpr phenotypes include lymphadenopathy, splenomegaly, B cell activation and hypergammaglobulinemia (IgG and IgM anti-DNA antibodies, rheumatoid factor antibody) as well as accumulation of activated T cells (CD4-/CD8-) with ultimate autoimmune manifestations and premature death.

Similar to lpr phenotype, a number of human cases of autoimmune lymphoproliferative syndrome have been identified. The mutations underlying the autoimmune lymphoproliferative syndrome phenotype are either loss of function (as in lpr) or dominant negative expressing alleles at the FasR gene.[40]

FasR expression is also aberrant in diabetic patients' peripheral blood mononuclear cells (PBMC, either T cells or B cells). In patients with overt insulin-dependent diabetes mellitus (regardless of stage of disease) and those at high risk for developing IDDM, FasR expression on PBMC is significantly down regulated compared to controls. The PBMC were also resistant to up regulation of FasR expression secondary to TCR stimulation (via anti-CD3 mAb).[43] As we know, CD4+ cells have been shown to have a role in initiating autoimmune responses to pancreatic B cells. The immunologic consequence of impaired FasR expression by PBMC is the accumulation of activated T cells (such as CD4+) and the impaired ability to

enact peripheral negative selection. FasR may serve as a useful screening method to identify those at risk of developing the disease and instituting early treatment or preventative measures as they become available. The down regulation of FasR in the PBMC of IDDM patient appears to involve all T cells (no subset has been identified as the sole target for down regulation). If all T cells are equally down regulated then both the clones responsive to autoantigens (i.e. the T cells responsible for autoimmune β cell destruction) and those responsive to alloantigens (i.e. the T cells responsible for acute allograft rejection) will be equally affected, a fact which may limit the usefulness of FasL in creating a local immune haven for islet cell allografts.

Another pathophysiologic manifestation of Fas system is over expression of either FasR or FasL. Some tumors (leukemia/lymphoma) have been found to shed large amounts of soluble FasL which reach detectable levels in patients serum.[44] Other tumors (human B cell tumors, gliomas) express FasR.[45,46] As well, detectable serum levels of soluble FasR have been identified in the serum of some patients suffering from Systemic Lupus Erythematosus (SLE, an autoimmune disorder). Soluble FasR can exert a pathophysiologic effect by acting as a decoy to bind available FasL and preventing the engagement of membrane-bound FasR thus preventing FasR+ cell apoptosis.

8.5 FAS SYSTEM: Transplantation Application

Recent studies have begun to focus on transplantation strategies employing manipulation of the Fas system to intercept T cell mediated acute rejection and thus prolong graft survival. Given the importance of CD4+ helper T cells in acute graft rejection and the likely ubiquitous cellular expression of FasR it was hypothesized that FasL expression by a recipient's activated infiltrating T cells was responsible for the destruction of FasR bearing transplanted organ tissue. An experiment was performed in which heterotopic cardiac allografts were transplanted in one of three combinations: Normal donor/normal recipient;

FasR deficient donor/normal recipient; Normal donor/FasL deficient recipient. The FasL and FasR mRNA transcripts of the cardiac grafts were analyzed post-transplant. In normal donor/normal recipient allografts, expression of FasL transcripts were up regulated during acute allograft rejection on both donor and recipient tissues. FasR was constitutively expressed in normal and syngeneic cardiac tissue but there was no appreciable increased expression in allogeneic grafts.

In FasR deficient donor/normal recipient transplants the absence of FasR on donor tissue was anticipated to prevent an apoptotic response and thus no damage would be mediated by FasL expressed by the infiltrating recipient T cells. In Normal donor/FasL deficient recipient transplants the recipient's T cells are incapable of expressing the necessary signal to induce apoptosis of the donor tissue and thus graft rejection should be ameliorated. Neither the absence of Fas R on donor cells nor the inability of recipient T cells to express functional Fas L altered the rate of allograft rejection. The conclusion was that allograft rejection can proceed in the absence of Fas mediated killing and its role in direct cytotoxicity was questioned again.[48]

Trials now need to address the effect of FasR expression by recipient lymphocytes. Normal donor/FasR deficient recipient and FasL deficient donor/normal recipient pairs could be transplanted. In Normal donor/FasR deficient recipient transplants, recipient alloreactive T cells devoid of FasR would not be subject to any FasR-mediated apoptosis so the rate of rejection would be normal or accelerated. In FasL deficient donor tissue transplanted into normal recipients experiments even though alloreactive T cells express FasR, there would be no FasL expression by the donor organ to induce T cell apoptosis thus the rate of graft rejection would be normal or accelerated.

9. HYPOTHESIS

While the evidence for the alloprotective effect of FasL is not unequivocal, and recognizing that allograft rejection is the result of a complex interconnected web of reactions we hypothesize that islet cell allografts can be engineered to express Fas ligand, thus rendering them protected from alloreactive T cell mediated acute rejection, while preserving their endocrine function. The goal of such a study is to allow transplantation of islet allografts without the need for chronic immunosuppression.

10. OUTLINE OF STUDIES

In this thesis we carried out the following series of experiments in order to create and test the necessary reagents. Our experiments were technically diverse but fell into four basic categories and for the presentation of Methods and Materials, Results and Discussion they will be discussed as they were performed chronologically. The first group of experiments comprised the **cloning** of all the constructs (HFasL, MFasL, HFasR, HFasIg). The second group of experiments were designed to test the biologic activity of each of the constructs by ***in vitro* FasR+ cell cytotoxicity** testing of each construct (HFasL, MFasL), the ability to block cytotoxicity (HFasIg), and the ability to confer FasL sensitivity to a cell line (HFasR). Subsequently HFasL was the only construct to be tested for ***in vitro* islet cytotoxicity** in the third group of experiments. A fourth and final group of experiments were the **transplantation** of a number of experimental groups with islets transfected with HFasL-1 (Table 1). This may be a useful reference to refer to during the course of reading this thesis to orient yourself both vertically to the experiments performed in a given discipline and also horizontally to follow the course of each construct.

Chapter 2

METHODS AND MATERIALS

1. DNA CLONING

1.1 Oligonucleotide Synthesis

A 0.1 μM oligonucleotide synthesis was performed using the appropriate mass of deoxynucleotide coated CPG beads (500 angstrom, Glen Research) which were aliquoted into commercially available plastic columns (Biogenex) with ABI-style Teflon filters at either end. The sequence was entered into the Applied Biosystems 391 DNA synthesizer for automatic DNA synthesis. The oligonucleotides were deprotected with 70% NH_4OH (1 mL per oligonucleotide) for 12 hours at 55°C. The deprotected oligonucleotides were dried on a speed vacuum (Savant SVC 100) and the pellet was resuspended in T.E. (10 mM Tris HCl, 1 mM EDTA) and quantified by spectroscopy on the Beckman DU-7 Spectrophotometer. The DNA concentration was calculated using the equation DNA μg/μL = optical density (260λ) x dilution x 25 μg/mL.

A number of oligonucleotides were synthesized for sequencing (HFasL-S3, -S4, -S5), repairing mutations (HFasLrp-5', HFasLrp-3'), engineering wildtype and truncated gene constructs (HFasL-5', -3', -S1, -S2), and to clone other constructs needed for our experiments (Human Fas Receptor: HFasR-3', sHFasR-5', sHFasR-3'; Human FasIg: HFasIg-5', HFasIg-3', HFasL-137: MG-CSF, MG-CSF-5', MG-CSF-3') (Table 2).

1.2 Polymerase Chain Reaction

In a total reaction volume of 50 μL, 5 μL polymerase buffer (10x, Stratagene), 2 μL dNTPs (2.5 mM, U.S. Biochemical), 1 μL DTT (0.1 M) and 2.5 μL dimethyl sulfoxide (20% DMSO) were combined with 1 μL cloned *PFU* DNA polymerase (2.5 U/μL Stratagene),

1.5 μM concentrations of 5' and 3' primers and 100 ng of template DNA. The reaction was placed in micro Eppendorfs and placed in a PTC-100 programmable Thermal Controller Polymerase Chain Reaction (PCR) machine. For HFasL-1 PCR was performed with an 80°C x 5 minutes denaturing step followed by 5 cycles of 95°C x 20 seconds denaturing step, 54°C x 45 seconds annealing step, and 70°C x 2 minutes elongation step. The elongation step was altered to 64°C for 45 seconds with the other steps remaining unaltered for an additional 25 cycles. This cycle was altered for each construct to optimize the products of various primer and enzyme combinations. While we were unable to obtain our products using *PFU* polymerase the reaction was duplicated successfully using 1 μL Thermo aquaticus polymerase (5 U/μL *Taq* polymerase, Boehringer) and its buffer (10x DNA polymerase buffer, Birch Scientific). The resulting PCR products were purified using a Centricon 100 system (Amicon Inc Beverly, MA). The reaction was placed in 2 mL of Tris HCl (5 mM pH 7.4) in a Centricon 100 column and spun at 3000 rpm x 40 minutes in a Beckman J21-12 centrifuge. Another 2 mL of Tris HCl were added to the column and spun again at 3000 rpm x 40 minutes. The column was inverted and capped with the bullet-shaped reservoir and spun 3000 rpm x 3 minutes. 50 μL of purified PCR product was recovered and used in subsequent experiments.

1.3 Restriction Endonuclease Digest

pBluescript cloning vector was prepared for cloning HFasL-1 by double digesting with restriction endonucleases XbaI/NotI. In a total reaction volume of 30 μL, 3 μL of buffer compatible with both enzymes (Buffer 2, NEB), 1 μL bovine serum albumin (BSA; 10 mg/mL, NEB), 1 μL XbaI (10,000U/mL, NEB) and 1 μL NotI (10,000U/mL, NEB) were combined in sterile milliQ water. 1 μg of vector DNA was cut. The reactions were incubated at 37°C for 18 hours. The vector digests were then run on a 10.1 cm x 6.6 cm 1% low melting point gel (0.6 g sea plaque LMP agarose in 60 mL 1x TAE buffer (0.004 Tris acetate; 0.001M EDTA)) at 70 mV for 1 hour in a BioRad electrophoresis gel box (17 cm between anode and cathode).

The portion of gel containing the appropriately cut vector was cut out of the gel with a scalpel blade, flash spun in an Eppendorf tube and diluted with a volume of sterile milliQ water equal to the approximate volume of the gel slice (60 µL). These samples were stored at -20°C.

A similar digestion was performed on the Centricon-purified PCR products of HFasL-1(XbaI/NotI), HFasL-131 (BamHI/NotI), HFasL -132 (BamHI/NotI), and HFasL-137 (MluI/NotI).

1.4 Ligation Reaction

a. Gel slice ligation: HFasL-1 was ligated into pBluescript plasmid vector in the following manner: a diluted gel slice of XbaI/NotI digested vector (pBluescript KS+) and diluted gel slice of XbaI/NotI cut insert (HFasL-1) were brought to 70°C in a water bath and then mixed thoroughly (finger flicked), cooled and reheated for 5 minutes. In a total reaction volume of 20 µL, 10 µL milliQ water were added to 2 µL of the vector gel slice and 5 µL of the insert gel slice. 2 µL T4 ligation salts (10x, NEB) and 1 µL T4 DNA ligase (400,000U/mL, NEB#202S) were added, mixed well and then incubated at 14°C overnight. Control ligations with vector DNA alone (no insert) were also performed.

b. β agarase digest/ligation: Insert DNA was digested with the appropriate enzyme combination and run on a LMP gel as was the vector. The gel slice was harvested (~35 µL) and diluted with an equal volume of sterile milliQ water. Half the volume of the diluted gel slice (~35 µL) of each vector and insert were spun down in an Eppendorf tube (15,000 rpm for 30 seconds in the Eppendorf centrifuge, 5415C) and 1/10 reaction volume of β agarase buffer (10x, NEB (10 mM Bis Tris HCl (pH 6.5) 1 mM Na_2EDTA)). The reaction was heated to 70°C for 10 minutes then vortexed, reheated to 70°C and again mixed thoroughly. The sample was allowed to equilibrate to room temperature for 5 minutes and then 1 µL β agarase was added to the reaction (1 µL per 100 µL reaction, 1000 U/mL, NEB). Incubation at 42°C for 2 hours was followed by DNA precipitation in cold 95% ethanol and 0.3 M NaOAc. The

DNA was washed with 70% ethanol and then resuspended in 18 µL of sterile milliQ water and stored at -20°C. Our gene constructs were cloned into a number of vectors (Table 3).

1.5 Bacterial Transformation

Replication competent *E.coli* (strain DH5α, lab stock) were thawed on ice (20 minutes) and resuspended gently by triturating with a P1000 pipetman. 50 µL of cells were aliquotted into each Eppendorf (cooled on ice) and then 5 µL of the ligation reaction was added. The reaction was mixed by finger-flicking and incubated on ice for 10 minutes. Heat shock at 37°C for 90 seconds was applied to the reaction after which a second incubation on ice for 10 minutes was performed. In later experiments this incubation was omitted with no apparent impairment of the reaction. 1 mL of 2XYT media (without ampicillin) was added to each Eppendorf tube and the transformation was outgrown at 37°C for 45 minutes. Following outgrowth, the transformation reaction was flash spun (Eppendorf centrifuge at 14000 rpm x 5 seconds), the supernatant decanted and the cell pellet resuspended in the remaining approximately 50 µL of media. The cells were then plated on 10 cm carbenicillin plates (agarose with carbenicillin 100 µg/mL), inverted and incubated at 37°C overnight.

1.6 Mini Preparation of DNA

Depending on the number of colonies growing on control plates (control plate contained *E. coli* transformed with ligation reaction containing only vector, no insert) a proportionate number of colonies were selected for growth in liquid media followed by DNA extraction to ensure that the correct insert was present. On average, with low background rates, 6 colonies per plate were selected to grow mini preparations from. 2.5 mL of 2XYT media plus 100 µg/mL ampicillin (Sigma #A-9518) were transferred to sterile capped glass culture tubes (Falcon #14-961-26). Using sterile forceps a P200 pipette tip was touched to a single colony and then dropped into the liquid media. The mini preps were incubated at 37°C overnight on a roller wheel.

1.7 Mini Preparation Alkaline Lysis

A total of 3 mL of mini preparation culture was spun in an Eppendorf tube (in 2 x 1.5 mL aliquots) at room temperature and 15000 rpm x 2 minutes in the Eppendorf centrifuge. The supernatant media was aspirated using at P200 pipetman to dry the cell pellet completely. The bacterial pellet was resuspended in 100 µL of Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris HCl) and vortexed until all the cells were in suspension. 200 µL of freshly made Solution II (0.2 M NaOH, 1% SDS ; Solution II has a shelf-life of 2 weeks) were added and the Eppendorfs shaken until the solution cleared then incubated at room temperature for 5 minutes. 150 µL of Solution III (60 mL 5 M KOAc, 11.5 mL glacial acetic acid, 28.5 mL water) were added to neutralize the reaction, which was vortexed and then incubated on ice for 5 minutes. The reaction was spun at 4°C and 15000 rpm x 15 minutes. The supernatant was transferred to fresh Eppendorf tubes and phenol:chloroform extracted once with 400 µL (1:1 phenol:chloroform) once with cevag 400 µL per tube, then precipitated with 2-3 volumes cold 95% ethanol and 0.3 M NaOAc and chilled at -20°C for 30 minutes. The DNA was pelleted at 4°C x 15000 rpm x 15 minutes. The DNA pellet was washed with 70% ethanol, spun again at 4°C x 15000 rpm x 5 minutes and then air dried at 37°C for 7-10 minutes. The pellet was then resuspended in 100 µL T.E. and quantitated with spectrophotometry.

1.8 Restriction Endonuclease Mapping

To ensure that the correct insert was present, alkaline lysis purified DNA was subjected to double restriction endonuclease digestion and the fragment length post-digest assessed.

1.9 Large Scale DNA Purification

a. Qiagen® plasmid maxi protocol: Mini preparation culture was used to grow up large *E. coli* culture for increased DNA yield. 250 mL of super broth media with 100 µg/mL ampicillin was inoculated with 1 mL of mini preparation culture in a 500 mL glass Erlenmeyer flask. The culture was incubated at 37°C in the air shaker for 24-48 hours. The culture was

harvested in 250 mL Oakridge tubes and spun at 20°C x 5000 rpm x 20 minutes (Beckman J21-12 centrifuge, JA14 rotor). The supernatant media was aspirated until the bacterial pellet was dry. 10 mL of cold solution P1 was used to resuspend the bacterial pellet which was then transferred to an Oakridge 40 mL tube. 10 mL of solution P2 was added and mixed well by shaking manually then incubated at room temperature for 5 minutes. 10 mL of cold P3 was added to the reaction and mixed by inverting the tube repeatedly and then incubated on ice for 20 minutes. The reaction was spun at 4°C x 15000 rpm x 30 minutes (Beckman J21-12 centrifuge JA-20 rotor). The Qiagen-tip 500 (Qiagen Inc; Chatsworth, CA) was equilibrated with 10 mL QBT and the DNA supernatant was strained through two layers of cheesecloth and applied to the column. The column was washed with 30 mL QC twice and the DNA eluted with 15 mL QF into ultracentrifuge tubes (1x3 1/2 inch Beckman #326823). The DNA was precipitated with cold isopropanol (1 volume) and cooled at -20°C x 30 minutes. The DNA was pelleted using the ultracentrifuge at 4°C x 15000 rpm x 30 minutes (Beckman , L8-80 using the SW27 rotor), washed with cold 70% ethanol and transferred to Eppendorf tubes then respun at 4°C x 15000 rpm x 10 minutes in the Eppendorf centrifuge. The pellet was allowed to air dry and then resuspended in 100 µL T.E. and quantified spectrophotometrically.

b. Cesium chloride gradient DNA purification: Large preparation DNA (250 mL cultures grown for 24 hours with 250 µL ampicillin 100 mg/mL) was harvested in 250 mL Oakridge tubes in Beckman centrifuge at 4°C spun x 6000 rpm x 15 minutes. The pellet was resuspended in 5 mL of Solution I (50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA + fresh 5 mg/mL lysozyme) and then incubated with 10 mL of freshly made Solution II (0.2 N NaOH, 1% SDS in ddH_2O) at room temperature for 5 minutes. 7.5 mL of chilled Solution III (7.5 M NH_4OAc) was added and the reaction incubated on ice for 5 minutes. The reaction was centrifuged at room temperature x 8000 rpm x 10 minutes (Beckman J21-12 centrifuge JA-14 rotor) and the supernatant transferred to clean tubes. 25 µL RNase A (10 mg/mL) was added

and the reaction incubated at 37°C for 1 hour. Water saturated phenol (1/2 volume of reaction ≈10 mL) and chloroform (1/2 volume of reaction ≈10 mL) were added and the reaction shaken vigorously for 2 minutes. The tubes were spun at room temperature x 5000 rpm x 10 minutes and the aqueous layer of the phenol extract was transferred to a clean tube then isopropanol precipitated (0.6-1.0 volume). The DNA was pelleted at 4°C x 5000 rpm x 30 minutes (Brinkmann Eppendorf centrifuge, 5403). The pellet was washed with 70% ethanol and air dried then resuspended in 1.5 mL T.E. Cesium chloride 3 mL (1.2 g/mL) was added to the DNA, mixed and transferred to Vti 65 ultracentrifuge tubes (Quickseal centrifuge tubes, 1/2"x2", Beckman #342412). 100 µL ethidium bromide (50 mg/mL) was added to each tube and dilute cesium chloride (2:1 CsCl in ddH_2O) filled the tube to the neck. The tubes were weight balanced, heat sealed and mixed, then spun at 19°C x 54000 rpm x 8 hours (Beckman ultracentrifuge L8-80). The ethidium-stained DNA band was harvested by perforating the ultracentrifuge tube with a 21 gauge needle at the lower edge of the band and aspirating with a 3 mL syringe. The DNA solution was transferred to a 15 mL conical tube (Falcon #2096) containing water saturated butanol in equal volume (≈1 mL) and the reaction mixed vigorously, then allowed to settle and the butanol extraction containing the ethidium bromide aspirated off. This step was repeated multiple times (4-8) until the butanol extraction was colorless. The DNA solution was transferred to ultracentrifuge tubes (1"x31/2" Beckman #326823) and precipitated with 95% ethanol (2-3 volumes) and 0.3 M NaOAc. The DNA was pelleted by spinning at 4°C x 15000 rpm x 30 minutes (Beckman Ultracentrifuge L8-80). The pellet was washed with 70% ethanol and air dried then resuspended in an appropriate volume of T.E. (400-1200 µL) and DNA content quantified using spectrophotometry.

1.10 Sequencing

a. Chain termination cycle sequencing: Template DNA was obtained from Qiagen large preparation purification (or cesium chloride purification) and used at 1 μg per reaction with 10 pmol of each of two primers (5' and 3'). 1 μL NaOH (1N) was added to the DNA (template and primers) and mixed then incubated at 37°C for 10 minutes. A Sequenase® Kit Version 2.0 was used according to manufacturers specifications in "Protocol for DNA Sequencing with Sequenase® Version 2.0" (US Biochemical, Cleveland, Ohio). In brief, 1 μL HCl (1 N) was added to the reaction followed by 2 μL reaction buffer (5x) and the annealing reaction incubated at 37°C for 5 minutes. 1 μL dithiothreitol (0.1 M DTT), 2 μL diluted labeling mix (1:5 dilution in distilled water) and 0.5 μL ^{35}S-dATP were mixed with 2 μL diluted sequenase (1:8 dilution in enzyme dilution buffer). The labeling reaction was incubated at room temperature for 3-4 minutes. The termination reaction was performed in a 96 well plate and 2.5 μL ddNTP of the appropriate base (one lane each of ddATP, ddGTP, ddCTP, ddTTP) was added to each well. The plate was placed in 37°C incubator to warm the reaction. 3.5 μL of the sequenase reaction mixture was added to the bottom of each well and then the plate was covered and slammed on the counter top to mix all reagents simultaneously. The reaction was briefly incubated at 37°C again (2-3 minutes). Stop solution (4 μL) was added to each well and the plate covered and slammed on the counter top to mix the reagents. The plate was then heated to 75°C briefly before loading onto a sequencing gel.

b. Single strand sequencing: The *E. coli* replication competent strain TG2 was transformed with DNA obtained by alkaline lysis. TG2 cells (lab stock) were thawed on ice and gently resuspended then 20 μL were aliquotted into an Eppendorf tube on ice. 2 μL of alkaline lysis DNA was added to the cells and finger flicked to mix. The cell/DNA mixture was incubated on ice for ten minutes then heat shocked at 37°C for 90 seconds and outgrown in 1 mL of 2XYT media without ampicillin at 37°C for 1 hour. The outgrowth was spun at 15000

rpm for 30 seconds, the supernatant decanted and the cells resuspended in the residual media. The cells were plated on carbenicillin plates and incubated for 18 hours at 37°C. Single colonies were picked from the carbenicillin plate and grown up in 2 mL of 2XYT + 100 µg/mL ampicillin. A small inoculum (0.2 mL) from mini preparation was added to fresh 2XYT media without ampicillin and outgrown for 1 hour at 37°C. The culture was then superinfected with helper phage (R408, 0.2 mL of stock $5x10^{11}$ plaque forming units / mL) and grown overnight (maximum 10 hours) at 37°C on a roller wheel. The culture was transferred to an Eppendorf tube and spun 7 minutes at 4°C x 15000 rpm. The supernatant was transferred to a new Eppendorf tube keeping well away from the cell pellet. The supernatant was spun again and transferred to a fresh tube. The phage were precipitated using 0.3 mL polyethylene glycol (20% PEG, BDH #B80016 in 2.5 M NaCl), which were mixed by inverting the tubes and incubated on ice for 15 minutes. The reaction was spun three times at 4°C at 15000 rpm for 15 minutes. A drawn out pasteur pipette was used to remove all the PEG supernatant from the phage pellet. The phage pellet was resuspended in 350 µL T.E. per tube then extracted twice with 400 µL of phenol, twice with 400 µL phenol:chloroform, and twice with chloroform. The DNA was precipitated and pelleted in the usual manner. It was washed with 70% ethanol then dried on the speed vacuum for 5 minutes. The pellet was resuspended in 50 µL sterile water and stored at -70°C.

A sequencing reaction premix was prepared with 4 µL Thermo Sequenase enzyme dilution buffer (5x), 1 µL of each dNTP DyDeoxy terminators (ddA, ddC, ddG, ddT previously diluted as per protocol), 1.0 µL of NucleixPlus terminator sequencing blend® (18 mM dITP, 6 mM dATP, 6 mM dCTP, 6 mM dTTP), and 0.5 µL TTAQ Polymerase (6 U/µL) for final volume = 5.5 µL from the sequencing kit (Thermo Sequenase dye-terminator cycle sequencing kit, Amersham Life Science). The premix was stored in the dark to prevent light exposure. 500 µg of the single stranded DNA template was added to the reaction premix

with 10 pmol of each primer (S3,S4,S5) and the reaction was brought to a final volume of 20 µL with sterile milliQ water. The reaction was aliquotted into mini-Eppendorfs and PCR performed with a denaturing step of 94°C for 30 seconds, annealing step of 50°C for 15 seconds and an elongation step of 60°C for 4 minutes repeated 25 times. The amplified samples were precipitated with 0.3 M NaOAc and 95% ethanol (2-3 volumes) at -20°C. The DNA was pelleted at 4°C x 15000 rpm x 15 minutes and the pellet was washed with ice cold 70% ethanol. The pellets were then dried in the speed vacuum. Prior to loading on the gel they were resuspended in 4 µL of EDTA/formamide (10 mM). The samples were heated to 70°C for 2-5 minutes to denature, then placed on ice and immediately loaded onto the sequencing gel.

A 50 mL denaturing 8% acrylamide gel buffered by Tris-borate-EDTA (TBE) was poured. 25 g of urea were dissolved in acrylamide (40%, Biorad). 1-2 g of mixed bead anion exchange resin (AG 501-x8, 20-50 mesh; Biorad) were added to the urea mixture and adjusted with water to bring the volume to 45 mL. The reaction was covered with styrofoam and stirred with a magnetic stir bar. Once the urea was dissolved 5 mL TBE (10x, lab stock) was added and mixed. The resin beads were filtered through a 0.22 µm filter and the filter rinsed with water several times and then the solution adjusted to 50 mL. Ammonium persulfate 1500 µL (10% w/v APS) was added. A pair of 60 cm 1/4 inch glass plates were washed with Alconox and 3% acetic acid then rinsed with 95% ethanol twice. 4 mm spacers were placed on the sides and bottom between the sheets of glass. Clamps were used to secure the two sheets of glass together the then the head of the plates elevated 15° and using a 60 mL syringe the gel was cast. A lane comb was placed in the superior border of the gel and then it was covered with aluminum foil to set for 2 hours.

The gel was mounted upright in a home-made S2 sequencing gel electrophoresis apparatus. The gel was prerun at 35 watts for 15 minutes (using an ECPS 3000/150 power

source, Pharmacia). The gel was loaded and run at 35 watts until the bromophenol blue indicator ran off the bottom of the gel ≈4 hours. The gel was soaked in a 10% acetic acid/10% methanol solution for 15 minutes to remove urea, dried at 80°C under vacuum on a slab gel drier (Hoefer Scientific Instrument, San Francisco, CA), and exposed on Kodak scientific imaging film (X-Omat AR) in an autoradiography film cassette (Fisher Biotech FBXC 1417).

1.11 Basic Tissue Culture Protocol

Cells were passaged in small tissue culture flasks (Nunclon 50 mL tissue culture flasks) in 7 mL of appropriate media (Table 4).

1.12 COS cell Transfection /Recombinant Protein Expression

COS cells were grown in DMEM supplemented with 10 % FCS II serum in a 10 cm tissue culture coated plate (150x20 mm, Sarsedt, #83.1803) to a confluence of 50-60%. DNA (5-15 μg of a Qiagen or Cesium gradient purified preparation of DNA in pSRαSD7) was mixed with 20 mL of DMEM incomplete, 0.8 mL DEAE (10 mg /mL Sigma, in phosphate buffered saline for a final concentration of 400 μg/mL; PBS [8g/l NaCl, 0.2g/l KCl, 1.44 g/l Na_2HPO_4, 0.24g/l KH_2PO_4]) and 20 μL of chloroquine (100 mM for final concentration of 100 μM, Sigma #C-6628). The reaction was mixed thoroughly and incubated at room temperature for several minutes and then was applied to the COS cell monolayer (washed with DMEM incomplete twice). After a 4 hour incubation at 37°C and 5% CO_2 the media was aspirated off the cells and a 5 mL DMSO shock (10% in PBS) was applied to the cells for 2 minutes at room temperature. The plates were then washed once with DMEM complete media (DMEM + 10 % FCS II) and then 30 mL of DMEM complete media was applied to the cells. 4-6 hours later the media was changed. The cells were incubated for 24 hours following which they were trypsinized (plates washed with 5 mL of PBS once, 5 mL of trypsin/ EDTA 1x [0.05% trypsin, 0.53 mM EDTA, Gibco BRL #25300-062] per plate incubated at 37°C for 5-10 minutes). The plates were tapped gently to loosen the cells and then triturated to break up cell clumps and the

complete population plated out in fresh media (10-15 mL complete DMEM) with 1/1000 gentamicin (50 mg/mL, Gibco BRL #15140-015). The cells were then allowed to grow until the media was exhausted (72 hours) and then supernatants were harvested (spun at 4°C x 5000 rpm x 5 minutes) and frozen at -72°C for future experimental use.

1.13 β Galactosidase Staining for Transfection Efficiency

In order to assess the transfection efficiency of PA317 cells (retrovirus) and 293 cells (adenovirus), control transfections with a β-galactosidase bearing plasmids (LLLSvNeo8- βgal for retrovirus and pAC-CMV-βGal for adenovirus) were performed and expression quantitated by counting the ratio of blue cells to white cells by light microscopy.

Transfection conditions were compared and optimized. The modes of transfection compared were liposome-mediated (for which D.C.Col was used, loaned to us by Dr. L. Chang) or lipofectamine-mediated (Gibco-BRL). Ratios of DNA: D.C.Col were 3 μg, 4 μg, or 6 μg in 10 μL of D.C.Col. The same mass of DNA (i.e. 3 μg, 4 μg, 6 μg) were mixed with 7.5 μL of lipofectamine. The transfection protocol used is as follows: The appropriate volume of DNA was added to 300 μL of serum-free DME (in polyethylene tube, 5 mL, Falcon). Lipofectamine or D.C.Col in appropriate volume were added to 300 μL of serum-free DME in a separate polyethylene tube (5 mL Falcon) and mixed well. The DNA and transfectant were combined and mixed then incubated at room temperature for 30-45 minutes. The cells to be transfected (PA317 at 50-60% confluence in 60 mm plates tissue culture coated, [Falcon] or 6 well plates [CoStar]) were washed with serum-free DME media once and then all media was aspirated off with vacuum suction and a glass pasteur pipette. 2 mL of serum-free DME was added to each incubation and the DNA/transfectant/media solution was added slowly, dropwise to the exposed cells. The cells were incubated in the presence of the DNA/transfectant for 5-20 hours at 37°C and 5% CO_2. Following this 2.5 mL of DME+20% fetal bovine serum (FBS) was added to each plate and left to incubate overnight. In the morning, the media was replaced

with fresh DMEM+10% FBS. After 48 hours, the media was removed (and saved to assay infective potential) and transfected cells were incubated 48 hours at 37°C and 5% CO_2 then washed once with PBS. The cells were fixed (136 µL Formaldehyde 37%, 40 µL glutaraldehyde volume adjusted to 5 mL with PBS) and incubated for 5 minutes at room temperature. Then cells were stained (50 µL ferrous cyanide 4 mM, 50 µL ferric cyanide 4 mM, 10 µL $MgCl_2$ 1 M, 750 µL X-Gal (2 mg/mL in DMSO; a generic reagent was used with MW=408.06), and volume adjusted to 5 mL with PBS). The most efficient transfection method proved to be lipofectamine at a ratio of 3 µg DNA: 7.5 µL lipofectamine (with a transfection efficiency of 5-10%).

1.14 Methods specific to human Fas ligand cloning - HFasL

a. Single strand cDNA synthesis from mRNA: Total cellular mRNA from an activated Jurkat cell line (obtained from Dr. J. Elliott) was used as template for reverse transcription into single stranded cDNA. Candidate mRNA were selected after running aliquots of mRNA (from storage at -72°C) on a 1% agarose/formaldehyde gel (1.5 mL of 37% formaldehyde in a 60 mL gel) with 5 µL ethidium bromide (10 µg/µL) in 1x MOPS buffer (3-(N-morphoninpropanesulfonic acid)). One sample had intact mRNA (Sample # 1). This sample was precipitated in 95% ethanol and 0.3 M NaOAc to remove SDS. The mRNA was resolubilized in DEPC-treated water. AMV reverse transcriptase (10,000-20,000 U/mL, U.S. Biochemical #70041) was added to oligodT, reverse transcriptase buffer (5x buffer; 250 mM Tris HCl (pH 8.3), 40 mM $MgCl_2$, 250 mM $NaCl_2$, 5 mM DTT; NEB #76218) and dNTPs (25 mM solution of dATP, dGTP, dCTP, dTTP from 0.1 M stock (5 µL of each dNTP stock solution 100 µM mixed in 180 µL sterile milliQ water, U.S. Biochemical). The reaction was incubated at 14° for ≥1 hour.

b. Second strand DNA synthesis: To the single stranded cDNA, 3 µL *E. coli* DNA polymerase I (10,000U/mL, NEB #2095) and 2 µL *E. coli* DNA ligase (10,000U/mL NEB

#205L) were added with their substrates including 50 µL SSR buffer (10x), 5 µL dithiothreitol (1 M DTT, ICN Biomedical), 2 µL BSA (10 mg/mL purified BSA, NEB), 5 µL NAD (15 mM) and 2 µL RNase A (10 mg/mL, Boehringer #109169). The reaction was incubated at 14°C for >1 h. This double stranded DNA was phenol:chloroform extracted twice with 500 µL (1:1 phenol:chloroform solution) then cevag extracted twice with 500 µL (24 chloroform: 1 isoamylalcohol). The purified cDNA was precipitated with 2-3 volumes of cold 95% ethanol and 0.3 M NaOAc and washed with cold 70% ethanol. The DNA was resuspended in T.E. buffer (10 mM Tris HCl, 1 mM EDTA) and used as template for PCR DNA amplification.

c. Cloned into pBluescript SK-: HFasL-1 was initially cloned into pBluescript KS+ but we had difficulties obtaining and purifying DNA from *E. coli* transformations with the gene in this vector. It was hypothesized that the HFasL-1 gene product had an inhibitory effect on the growth of the cells and so it was cloned into a vector (pBluescript SK-) in which the gene would be inverted visavie promoter/enhancer and thus not transcribed. This strategy was effective. Subsequently the gene was subcloned into several other eukaryotic expression vectors (see Table 3).

d. Truncated construct design: A number of shortened constructs were designed to exclude the transmembrane binding domain and include the Human T1/Leu-1 glycoprotein signal sequence. When translated these constructs would be constitutively secreted mimicking the soluble form of HFasL-1. HFasL-131 and HFasL-132 were truncated to base pairs 391 and 394 of the wildtype gene respectively (amino acids 131 and 132) based on published speculations as to the site of transmembrane cleavage. These constructs were obtained from the wildtype full length gene by PCR with the primers HFasL-131, HFasL-132, and HFasL-3' (Table 1). An additional short construct (HFasL-137) was engineered identical to one in the literature with the signal sequence from mouse granulocyte colony stimulation factor (MG-CSF) attached to the 5' end of a truncated form of HFasL (beginning at base pair 411, amino

acid 137). These constructs were submitted to the same bioassay as HFasL-1 to see the effect of a physiologic molecule (wildtype) versus the truncated constitutively secreted forms.

e. MG-CSF oligonucleotide size selection: MG-CSF, a 116 base pair oligonucleotide, was synthetically derived using the Applied Biosystems DNA synthesizer in a 0.1 μM synthesis (Table 2). The oligonucleotide was deprotected with 1 mL NH_4OH and incubated at 55°C for 2 hours (this shorter than usual deprotection step was possible because "fast G" phosphoramidite was used in the DNA synthesis). The deprotection reaction was cooled on ice and aliquotted equally into two Eppendorfs with perforated lids. The reactions were dried on the speed vacuum for 4 hours and the contents dissolved in 100 μL of T.E. per Eppendorf, then pooled and flash spun at 15000 rpm in the microcentrifuge. The supernatant was aspirated leaving behind the CPG beads. A 10% denaturing PAG was mixed (20 mL TBE 10x, 50 mL acrylamide 19:1 40%, bis urea 84.08 g, volume adjusted to 200 mL with water). The reaction was heated to 37°C to dissolve the urea and then filtered through a 0.22 μm vacuum filter (Corning #25932-200). 1500 μL APS (10%) and 50 μL TEMEDS were added to cause gel polymerization. A 60 cm gel was poured and once set was prerun at 35 watts, 1450 volts, 150 amps for 15 minutes using an ECPS 3000/150 power source (Pharmacia). The oligonucleotide (suspended in T.E.) was loaded onto the gel and run for 7 hours until the bromophenol blue ran off the gel. The gel was unloaded onto a TLC plate. The gel was shadow-banded with a long wave UV light source. Using a blunt spatula the band was excised from the gel. The harvested gel slice was cut into 1 mm squares and loaded into the barrel of a 1 mL syringe. The gel was expelled from the syringe into a 15 mL conical tube (Falcon #2096) and the syringe was rinsed with 5 mL of crush and soak buffer (3.0 mL NH_4OAc 5 M, 300 μL MgOAc 1 M, volume adjusted to 30 mL with water). A glass stir rod was used to further macerate the gel slice in the 15 mL conical. 10 mL of crush and soak solution was added and the reaction incubated at 37°C in an end-over-end roller wheel overnight. The crushed

acrylamide and buffer solution was transferred to a 30 mL syringe and filtered through a 0.45 µm syringe-adaptable filter (CoStar, µStar LB #8112) to remove the acrylamide. The conical was rinsed with buffer and filtered.

A Sep-pak cartridge (#51900 Millipore) was charged with 100% acetonitrile (10 mL, Applied Biosystems DNA Synthesis grade #400060) and then washed with water (10 mL). The filtered oligonucleotide from the size selection protocol was loaded onto the column three times. The column was washed with water (10 mL) and eluted with 4 x 1 mL 20% acetonitrile (v/v in water) into Eppendorf tubes. Spectrophotometric analysis of the eluents was performed.

f. PCR mutagenesis: HFasL-1 was obtained by RT-PCR using *Taq* polymerase which is a nonfastidious enzyme with a high rate of proofreading errors. A single base mutation occurred at base pair 502 (A to G*) changing the amino acid from isoleucine to glycine as a result of using *Taq* DNA polymerase for the synthesis of HFasL-1. In order to repair this mutation two primers (HFasLrp-5' and HFasLrp-3') were designed with a short clamp region complementary to the sense and antisense strands of the cDNA flanking the mutation, and a nonannealing tail encoding the correct sequence for base 502. Two PCR reactions were performed, one with HFasLrp-5'/ HFasL-3' and the other with HFasLrp-3'/HFasL-5'. The two reactions were combined and a final PCR performed using the outside primers HFasL-5'/HFasL-3' to amplify the corrected product. The PCR product was cloned into pBluescript SK- and sequenced (Figure 3, 4, 5).

1.15 Methods specific to mouse Fas ligand cloning - MFasL

MFasL-1 was obtained by RT-PCR from the NOD spleen cDNA library by Dr. C. Luo. Two shortened forms were designed (starting at base pair 300 and 331) to provide a comparison between membrane bound and soluble ligand physiology.

1.16 Methods specific to human Fas receptor cloning - HFasR

Human FasR was kindly given to us by Dr. L Chang cloned into pBluescript KS+ by C. Rudnisky (EcoRI/HindIII). It was PCR amplified using primers HFas-5' and HFas-3' using *PFU* DNA polymerase and a cycle of 94°C for 20 seconds, 54°C for 45 seconds and 72°C for 60 seconds repeated 30 times. HFasR was cloned into pSRαSD7 (XbaI/NotI) and then used for the creation of stable HFasR expressing transformants. (Table 1, 2).

1.17 Methods Specific to human FasIg Cloning - HFasIg

A chimeric construct combining human FasR specificity with a human IgG1 tail was engineered to use as an immunohistochemical reagent as described in the literature. Two primers were designed to amplify a truncated form of HFasR (sHFasR-5'/sHFasR-3') which is 502 base pairs in length (genomic wildtype HFasR is 1008 base pairs) incorporating an XbaI restriction site at the 5' end of the neogene and an AatII site at the 3' end. HFasR cDNA was obtained from Dr. L.J. Chang. Similarly, primers were designed complementary to the Fc portion of human IgG1, a 706 base pair fragment, which was obtained by PCR from another construct, CTLA4Ig designed by Dr. A. Gainer. The primers HFasIg-5' and HFasIg-3' incorporated an AatII restriction site at the 5' end of the Fc portion and a NotI restriction site at its 3' end. The cloning plasmid was double cut XbaI/NotI, the HFasR fragment was double cut with XbaI/AatII and the Fc fragment was double cut with AatII/NotI. A forced three way ligation was performed successfully. The construct was assayed by dot-blot analysis, protein gel, and staining of FasL expressing cells, as well as its ability to act as a receptor decoy and block FasL mediated apoptosis.

a. Polynucleotide kinase concatamerization: Short DNA fragments often cut inefficiently when exposed to restriction endonucleases. After failed attempts at ligating our short HFasR and Fas-Fc fragments into a plasmid vector we surmised the problem was inefficient enzymatic cutting and so elected to concatamerize the DNA fragments using T4

Polynucleotide Kinase (PNK). Concatamerization forms a long chain of repeated oligomers which the restriction endonuclease can cleave efficiently. 6 µL of Tris HCl (0.5 M Tris pH 7.5, 0.1 M $MgCl_2$), 3.0 µL DTT (200 µM) and 0.6 µL ATP (100 mM) were combined. 1.2 µL of PNK (10,000U/mL, NEB#201L) was added in 0.4 µL aliquots q15 minutes over a 45 minute period. The reaction was incubated at 37°C for the 45 minutes during addition of PNK then cooled on ice for 3 minutes. 1.2 µL dNTPs (2.5 mM), 0.6 µL rATP (100 mM) 1.0 µL T4 DNA Ligase (400,000 U/mL, NEB#202S) and 1.0 µL Klenow (DNA Polymerase I Large Fragment 5000 U/mL, NEB #210L) were added for a final reaction volume of 65 µL. The reaction was incubated at 14°C for 24 hours. The concatamers were diluted with 140 µL T.E. and 10 µL SDS (10%) then phenol:chloroform extracted, precipitated and resuspended in 35 µL water. The concatamers were digested (sHFas with XbaI /AatII and FasFc with AatII/ NotI). Briefly Buffer 4 (6 µL/reaction, NEB), the appropriate enzyme combination (1 µL of each enzyme per reaction, NEB), 35 µL of the concatamerized template (sHFas PNK or FasFc PNK) and milliQ water to make total reaction volume 60 µL were combined and incubated at 37°C for 16 hours. The enzyme was subsequently inactivated by phenol:chloroform extraction.

b. Three way ligation reaction: Using KS+ vector cleaved XbaI/NotI (5 µL of digest approximately 0.5 µg DNA), sHFas cut XbaI/AatII (10 µL) and FasFc cut AatII/NotI (10 µL) mixed with 4 µL ligation salts (10x NEB) 2 µL T4 DNA ligase (400,000 U/mL, NEB#202S) and milliQ water to bring reaction volume to 40 µL. The reaction was incubated at 14°C overnight

c. Dot blot: To semiquantitate the amount of FasIg protein produced by COS cell transfections of HFasIg in pSRαSD7 an alkaline phosphatase conjugated antibody dot-blot was performed. 1 µL and 3 µL aliquots of transfected COS supernatants were dotted on nitrocellulose filter paper (BA85, Schleicher and Schuell) and the filter was washed manually in phosphate buffered saline with 10% Tween (PBS with Tween 20 (1 ng/mL, diluted 1/1000),

in sufficient volume to cover filter paper) for 2-3 minutes. Excess PBST was shaken from the filter and alkaline phosphatase conjugated Fc specific anti-human antibody was diluted 1/10,000 in PBST and applied to the filter. This reaction was incubated for 20 minutes at room temperature on an automated shaker. The filter was washed four times with 25 mL of PBST for 5 minutes each wash. 6 mL of alkaline phosphatase buffer (Tris 100 mM pH 9.5 and $MgCl_2$ 10 mM) was applied to the filter and then substrate solutions (20 µL each of 5% nitroblue tetrazolium [NBT, 50 mg dissolved in 70% formamide, BioRad #170-6532] and 5% 5-bromo-4-chloro-3-indoxile phosphate [BCIP, 50 mg dissolved in 100% formamide, BioRad #170-6539] were added to the buffer. The incubation was allowed to proceed at room temperature for 40 minutes. The intensity of the blue staining protein was compared between study and control supernatants.

d. Protein A column concentration: A column was prepared by modifying a 0.3 mL insulin syringe. The needle was pulled off and the tip cut to half its original length. A small amount of glass wool was tamped into the barrel with forceps until it settled against the outlet shoulder of the syringe. Protein A (Sigma) was spooned into an Eppendorf to a volume of approximately 200 µL. 300 µL of PBS was added to the Protein A coated beads and then was mixed by vortexing. The column was mounted in a clamp stand in the 4°C cold room running into an Eppendorf reservoir. The solubilized Protein A was loaded into the barrel of the syringe and the FasIg transfected COS supernatant (15 mL) was dripped into the top of the column slowly. 10 µL Tris HCl (pH 9.0) was aliquoted into each of the elution collecting Eppendorfs. Elution buffer (0.1 M glycine HCl, 0.5 M NaOH pH 3.0) was used to elute the FasIg from the column and the effluent was collected in 6x 50 µL aliquots. A Bradford assay for protein was run on each of the eluents and a protein gel was run of each positive sample.

e. ELISA: 96 well plates were coated with capture antibody anti-FasR or anti-FasL (50 µL per well at 1 µg/mL in PBS, Santa Cruz Biotechnology, Inc: Fas[N-18[#sc-714,

FasL[C-20] #sc-957). The plates were incubated at 37°C for 3 hours then removed to room temperature overnight (wrapped in parafilm).The plates were rinsed with ddH_2O three times and then flicked dry. The wells were blocked with PBS and 20% FCS II (100 µL/well) and incubated 10 minutes at 37°C. Plates were washed with PBST twice (100 µL/well PBS with 1% Tween) and flicked dry. Transfected COS supernatants bearing FasIg were applied to the well in serial 1:2 dilutions (50 µL diluted in RPMI 1640/ 8% FCS II). The reaction was incubated at 37°C for 30 minutes. The plates were washed with PBST twice. A secondary alkaline phosphatase conjugated antibody (goat anti-human; anti-IgG, IgM, Fluoroscein Isothiocyanate Conjugated antibody, FITC; Jackson Immunoresearch) was added to the reaction (50 µL/well diluted 1:2500 in PBST). The plate was incubated at 37°C for 30 minutes and was again washed with PBST four times. 1% BSA in PBST was used (75 µL/well) and incubated 37°C for 30 minutes and the plates were washed with ddH_2O once. Substrate buffer (50 µL/well of 1 mg/mL PNPP, 10 mM Tris HCl (pH 9.5) 2 mM $MgCl_2$ in water) was added to each well. The reaction was incubated for 30 minutes at room temperature. 50 µL/well EDTA (0.1 M) was added to each well to stop the reaction and the plates were read on an ELISA plate reader at wavelengths of 405-490 nm. A similar assay was conducted for HFasL and HFasR with the only variation being that after addition of the protein bearing supernatant to the 96 well plates, incubation and washing, 50 µL of undiluted FasIg supernatant was added to each well, incubated at 37°C for 30 minutes followed by two PBST washes. The basic protocol was resumed with the addition of a secondary alkaline phosphatase conjugated goat anti-human antibody.

2. *IN VITRO* FAS LIGAND CYTOTOXICITY

2.1 Recombinant Protein Expression

Once all the gene products had been cloned into pBluescript and sequenced the various constructs (HFasL-1, -131, -132, -137; MFasL-1, -300, -331; FasIg) were cloned into pSRαSD7 (Table 3) and then were used to transfect COS cells for the purpose of recovering recombinant protein from the culture supernatants for *in vitro* assays of the killing capacity (FasL constructs) or protective ability (FasIg). The transfected COS cells were grown in 15 mL of DMEM+10% FCS II (HyClone) for 72 hours post-transfection then supernatants were harvested and spun at 1000 rpm for 10 minutes and stored at -72°C.

We did not resequence HFasR as it was obtained by PCR from a sequenced version using *PFU* DNA polymerase. We did not resequence the shortened HFasL constructs as they were also obtained by PCR from a sequenced version of HFasL-1 using *PFU* polymerase.

2.2 MTT Assay

Supernatants harvested from COS cells transfected with HFasL-1 and its various constructs were serially diluted 1:2 in MTT media (RPMI, Gibco-BRL) with 10% FBS (Gibco-BRL) 2-mercaptoethanol 50 μM (lab stock) and 1/1000 gentamicin (50 mg/mL, Gibco-BRL). Undiluted test supernatants were dispensed into the first row (Row A) of a 96 well plate in triplicate. 50 μL of MTT media were dispensed to each of the other wells using a Costar multichannel pipetter. The multichannel pipetter was used to transfer 48 μL from each well in Row A to Row B, triturated 10-15 times to mix the supernatant with the media and continue serial 1:2 dilutions of 48 μL until the last well, from which 48 μL were disposed. Cultures of cell lines to be tested were counted using the hemocytometer (cell count per mL =count in one quadrant x10^4). The volume of culture needed to provide 50,000 cells per well was calculated aliquotted into an appropriate tube and spun at 1000 rpm and room temperature for 3 minutes, the supernatant aspirated and the cells resuspended in an appropriate volume of MTT media to

provide 50 µL/well. The cells were resuspended in media by finger flicking, aliquotted into a sterile reservoir and dispensed at 50 µL/well to each well (multichannel pipetter set at 55 µL and no-touch technique used). The reaction was incubated 24 or 48 hours at 37°C and 5% CO_2 after which 50 µL of MTT (1 mg/mL in PBS; thiazolyl blue 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma #M2128) was added to each reaction (using the multichannel pipetter set at 55 µL and no-touch technique employed) and incubated 3 hours at 37°C. The reaction was stopped with 150 µL isopropanol per well and the plates were read on an ELISA plate reader at 650-570 nm wavelengths using Delta-Soft 2.1 software® (Dr. Mosmann's lab).

2.3 Quantitative Apoptosis Staining

$0.5x10^6$ -$3x10^6$ cells were pelleted at 1700 rpm for 10 minutes and washed once in PBS. The cells were resuspended in 50 µL of formaldehyde (3% in PBS) and incubated at room temperature for 10 minutes. The fixative was removed and the cells washed once in PBS and then resuspended in 15 µL of PBS containing 32 µg/mL bis-benzimide (stock 160 µg/mL, Hoechst 33258, Sigma). After another room temperature incubation for 15 minutes 10 µL were aliquotted onto a glass slide and a standard number of cells scored for apoptotic changes.

2.4 Stable Transformants

400 µg of DNA (for each of HFasIg, HFasR and HFasL-1 in pSRαSD7) were linearized with 40 µL of Sal I (2 U per µg DNA; 20 U/µL, NEB) in 40 µL Sal I buffer (10x, NEB), 4 µL purified BSA (10 mg/mL, NEB), with water to bring the final volume to 400 µL (1 µL per µg of DNA). The digest was incubated for >12 hours and then precipitated in 95% ethanol (2-3 volumes) and 0.3 M NaOAc. Likewise 20 µg of the plasmid bearing dihydrofolate reductase gene (DHFR, provided by Dr. J. Elliott, discussion with Dr. D. Denney) and 10 µg of hypoxanthine phosphoribosyl transferase gene (HPRT provided by Dr. J. Elliott) were linearized with Sal I and independently precipitated. Each DNA and plasmid sample was

resuspended in 100 μL T.E. BW5147 cells were counted and aliquotted to provide $1x10^7$ cells per electroporation, then washed twice in 50 mL of 1x HBS(ep) (25 mL of 2x HBS[ep], 25 mL H_2O). The cells were resuspended in 400 μL 1xHBS(ep). The DNA and plasmid DNA were combined (final volume=200 μL) in 200 μL of 2x Hepes-buffered saline electroporation buffer (2x HBS[ep]= 40 mM Hepes [pH 7.0], 1.5 mM Na_2HPO_4/NaH_2PO_4 [pH 7.0], 16.1g/l NaCl, 0.75 g/l KCl, 2 g/l dextrose). The combined DNA/buffer was placed in the electroporation cuvette. The BW5147 cells (volume 400 μL) were added on top of the DNA mixture and the reaction mixed by trituration. The cuvette gap was set a 0.4 cm and electroporation performed using the Biorad Gene Pulser (with capacitance of 960 μF and voltage of 0.23 kV). The time constant for each electroporation was recorded and ranged from 11-18 seconds. The DNA/cell mixture was inoculated into 30 mL of RPMI with 10% FCS and penicillin/streptomycin (1/100 dilution, Gibco BRL). 24-36 hours later each flask was counted, all the cells harvested and resuspended in selective media (100 μM hypoxanthine, azaserine 1 μg/mL) and plated in 48 well plates (CoStar) at $1x10^5$ cells/mL (0.5 mL per well of $2x10^5$ cells/mL) and $2x10^4$ cells/mL (0.5 mL per well of $4x10^4$ cells/mL) in 48 well plates. 7 days later each well was fed with an additional 0.5 mL of selective media. As colonies began to grow in the selective media (14 days) they were weaned off azaserine media into hypoxanthine media alone. The cells were passaged twice in hypoxanthine media (100 μM) and then weaned to regular media (RPMI + 10% dialyzed FBS). As later developing clones became apparent they were also picked to and weaned from the selective media. The selected clones were then assayed for expression (FasIg tested by ELISA for expression of soluble product, HFasR and HFasL both assayed by FACS analysis for membrane-bound protein expression). Of the original 100 colonies approximately 3-12 were identified as exhibiting high levels of expression and were selected for amplification.

2.5 Stable Transformant Amplification

Methotrexate was added to standard growth media (RPMI +10% dialyzed FBS) to provide concentrations of 50, 70, 90 mM. Methotrexate was prepared from 10 M lab stock. Of the chosen expressing clones, $2x10^5$ cells per well (48 well plate) were plated out in the amplification media (0.5 mL) and continued incubation at 37°C / 5% CO_2. The cells were fed 0.5 mL fresh amplification media at 7 days and observed daily for colony growth. Those clones which grew in low level methotrextate (MTX) were then screened for protein expression and the high level expressors were inoculated into higher concentrations of MTX (120,150,180 mM) in a similar manner.

2.6 Cell Staining: Immunohistochemistry and Fluorescent Activated Cell Sorting

Stably transformed cells were harvested in 5 mL polyethylene tubes (Sarstedt 57.477) and pelleted at 4°C x 1000 rpm x 3 minutes. The cell pellet was washed in 1 mL of staining media (Hanks' balanced salt solution, HBSS; Gibco-BRL), 5% FBS (Sigma) and 10 mM N-2-Hydroxyethylpiperazine-N-2-Ethanesulphonic acid, (Hepes, pH 7.0, BDH #44 285-4V) and respun. The cells were resuspended in 100 µL of primary antibody (1:200 dilution in staining media), vortexed and incubated on ice for 20 minutes. The cells were then resuspended and an additional 1 mL of staining media was added to the solution. A glass pasteur pipette was used to underlay the cells with 1 mL of fetal calf serum and the reaction was centrifuged at 4°C x 1000 rpm x 3 minutes. The serum and media were aspirated and the cell pellet resuspended in 200 µL of staining media. 100 µL of the secondary FITC antibody (1:200 dilution in staining media, goat anti-human; anti-IgG, anti-IgM; Fluoroscein Isothiocyanate Conjugated Antibody, Jackson Immunoresearch) was added. The reaction was incubated in the dark on ice for another 20-30 minutes and again underlain with 1 mL of fetal calf serum and spun. The supernatant was aspirated and the cell pellet resuspended in 200 µL of staining media. The cells were covered and placed on ice and taken for FACS analysis.

Positive controls for both the HFasR and HFasL transformants were stimulated Jurkat cells. Negative controls for these FACS analyses were wildtype BW5147.

A Hewlitt-Packard FACS machine with IBM compatible software (Becton-Dickson) was used for FACS analysis. The FACS program was entered (Lysis II) and Aquire mode activated. 10,000 cells were counted and the forward and side scatter adjusted by SSC, FL1 and FSC-H, SSC-H to center the cell population in the dot histogram. HFasR and HFasL and HFasIg stably transformed BW5147 were assayed for gene product expression by FACS analysis.

2.7 ***Methods specific to HFasL***

a. MTT Assay: HFasL-1, HFasL-131, HFasL-132, HFasL-137 were all assayed in the MTT assay. The negative control for these experiments was pSRαSD7 (empty vector) transfected COS supernatants versus Jurkat cells. There was no positive control. HFasL-1 Stable transformants (BW5147) were also assayed in MTT.

b. Stable Transformants: We elected to transform BW5147 with only the wildtype version of HFasL (rather than with the shorter constructs which exhibited less efficient killing). Thus HFasL-1 in pSRαSD7 was prepared and used to produce stable transformants. Of the 60 clones that grew in selective media only three stained significantly on FACS analysis so these were selected for amplification (clones 47, 162, 171). Staining was performed using anti-FasL antibody (diluted 1:200 , Rabbit polyclonal antibody, C-20 Santa Cruz Biotechnology, Inc. Cat # sc-957) and FasIg COS transfected supernatants (used undiluted) as primary antibodies and anti-IgG goat anti-rabbit FITC antibody (1:200 dilution) or anti-IgG goat anti-human FITC antibody (at a 1:20 dilution, Sigma #F5512). Negative control was provided by non-transformed wildtype BW5147 cells and positive control was provided by stimulated Jurkat cells.

2.8 Methods specific to MFasL

a. MTT assay: MFasL-1, MFasL-300 and MFasL-331 were all assayed by MTT for cell killing.

2.9 Methods specific to HFasR

a. MTT Assay: The HFasR stable transformants were assayed by MTT and the cytotoxicity of standard FasL COS transfected supernatants versus Jurkat were compared to the stable transformants.

b. Stable Transformants: HFasR was used to create a stably transformed BW 5147 cell line. Cell staining was performed using anti-FasR antibody as primary antibody (rabbit polyclonal 1:50 dilution of N-18, Santa Cruz Biotechnology, Inc. Cat#sc-714) and anti-IgG FITC goat anti-rabbit 1:200 (FITC anti-IgG, anti-IgM, Jackson Immunoresearch) as secondary antibody. Six clones that grew in selective media were found to have significant expression of the HFasR by FACS analysis (clones 11, 26, 33, 40, 62, 114) and these were selected for methotrexate amplification.

c. Jurkat stimulation: Jurkat E6-1 cells were stimulated one of three ways to induce FasR and/or FasL expression for use as a positive control in FACS analysis of HFasL and HFasR stable transformants. $2x10^6$ cells per condition were plated into a six well plate containing 5 mL of the media described below. The cells were incubated for 24 hours and then stained for either HFasL or HFasR and FACS analyzed.

Condition I. PMA 10 ng/mL and Ionomycin 1.0 μM: 5 μL of Phorbol 12-Myristate 13-Acetate (PMA stock solution 10 μg/mL in DMSO, Sigma #P8139) was diluted in 5 mL of RPMI + 10% FCS I to make a final concentration of 10 ng/mL. 50 μL of Ionomycin stock (100 μM in DMSO, Sigma #I.0634) was added to the solution for a final concentration of 1 μM.

Condition II. PMA 10 ng/mL and Ionomycin 500 ng/mL: 5 µL of PMA (stock solution 10 µg/mL in DMSO) in 5 mL of RPMI + 10% FCS I was mixed with 3.3 µL of Ionomycin (stock 100 µM in DMSO).

Condition III. PMA 40 ng/mL and Concanavalin A: 40 µL of PMA (stock solution 10 µg/mL in DMSO) was mixed with 500 µL of Concanavalin A (Con A 66 µg/mL Sigma # C-5275) in 5 mL of RPMI + 10% FCS I.

Condition III caused the largest shift in the fluorescent staining of Jurkat cells and so this was used in subsequent experiments to provide a positive staining control.

d. Methotrexate amplification: Each HFasR clone was expanded to T150 flasks (30 mL media) grown for 48 hours then counted using the hemocytometer. An appropriate volume of cells was calculated to provide $3x10^4$ cells per well in a 48 well plate. The cells were spun at 4°C and 1000 rpm for 3 minutes, washed once with RPMI (plain), respun and then resuspended in 25 mL of MTX (either 50 mM, 70 mM, or 90 mM). They were plated at 0.5 mL per well. After 7 days incubation at 37°C / 5% CO_2 the plates were examined for clonal growth and each well was fed with an additional 0.5 mL of original concentration MTX media. The plates were followed until several clones had grown (on average 2-3 per 48 well plate) and then they were assayed for protein expression by FACS scan as described previously.

2.10 Methods specific to HFasIg

a. MTT FasIg blocking assay: To assess the specificity of FasL induced cell death and the functional ability of our chimeric construct to block its effect, an MTT was set up with varying concentrations of HFasL and HFasIg versus Jurkat cells. In 96 well plates the following combinations of HFasL and HFasIg were mixed: Row A. HFasL serial 1:2 dilutions; Row B. HFasIg serial 1:2 dilutions; Row C. HFasL + HFasIg both serially diluted 1:2; Row D. HFasL 1:4 and HFasIg serial 1:2 dilutions; Row E. HFasIg 1:4 and HFasL serial 1:2 dilutions.

Cells (50,000 cells/ 50 μL) were aliquotted into each well with the multichannel pipette. The reaction was incubated at 37°C / 5% CO_2 for 24 hours and developed with MTT and isopropanol using the previously described protocol.

b. Stable transformants: BW5147 were stably transformed with HFasIg by electroporation. The supernatants of colonies growing in selective media (hypoxanthine plus azaserine) were assayed by ELISA and FACS.

3. *IN VITRO* ISLET ASSAYS

3.1 Effect of HFasL-1 on Islet Function

a. Islet Isolation:

i. Mouse Islet Isolation: CBA/J mice were purchased from Jackson Laboratory (Bar Harbor, Maine)and Balb/c mice were purchased from Health Sciences Lab Animal Services (University of Alberta) and housed and cared for in the Surgical Medical Research Institute animal facility (Dentistry/Pharmacy building, University of Alberta).

Islets were isolated using collagenase digestion, mechanical dissociation, and Ficoll purification. Sixteen donor mice were anaesthetized with Avertine (2,2,2 Tribromoethanol 0.4 mL of 2 g in 100 mL water and 2 mL tert-amyl alcohol; intraperitoneal injection). A midline abdominal incision was made from xiphisternum to symphysis pubis. The distal common bile duct was identified and clamped and using a 27 gauge needle 2.5 mL of 4°C collagenase (2.0 mg/mL in HBSS, with 2.0 mg/mL dextrose, 225 U/mL penicillin, 225 mg/mL streptomycin) was introduced to distend the parenchyma and begin digestion of the exocrine pancreas. The pancreas was dissected from the retroperitoneum and placed in chilled HBSS in a 50 mL tissue culture flask to which was added an additional 5.0 mL of collagenase solution. The digestion was incubated in a 37°C water bath for 32-33 minutes in an automated shaker and at the end of the incubation cold HBSS was added to inactivated the collagenase. The reaction was vortexed

for 10 seconds at speed 3 then centrifuged at room temperature x 1500 rpm x 10 seconds. The supernatant was decanted and the tissue pellet washed with HBSS three times. The tissue was resuspended in HBSS and filtered through a 400 μm screen to remove gross exocrine contamination. Post-filtration the remaining tissue was spun at 1500 rpm. The pellet was then resuspended in 10 mL of 25% Ficoll. 6.0 mL of each concentration of Ficoll (23%, 21.5%, 11%) were layered into a conical tube (Falcon) and the islets added to the upper layer of the density gradient. The gradient was spun at 2000 rpm x 10 minutes and the islets aggregated at the 21.5-11% interface and were harvested with a 10 mL syringe/14 gauge intracatheter. The islets were rewashed with HBSS three times, spun at 1500 rpm x 10 seconds between washes and then resuspended in 10 mL of 25% Ficoll and reloaded on the density gradient. The islets were then hand-picked and aliquotted into groups of 500 islets and cultured in standard Ham's media at 37°C / 95% air / 5% CO_2 for 24 hours before further processing.[13, 53]

ii. Human Islet Isolation: Human islet isolation was performed as described by Dr. Warnock et. al.[55]

b. Glucose Stimulation Assay: Groups of 500 islets (in duplicate) were counted, washed in 15 mL of low glucose DMEM (2.8 mM glucose, with 3.7 g/l $NaHCO_3$ and 2 mM glutamine; titrated pH to 7.4 with HCl, Gibco BRL) and resuspended in 25 mL standard Ham's media. 1 mL aliquots were set aside for duplicate DNA and insulin quantification assays. 2 mL per condition were then aliquotted into Falcon 2054 tubes and spun at room temperature at 1000 rpm for 90 seconds. The media was aspirated, HFasL-1 or control supernatant was added to the islets and the islets were plated out in 60 mm petri dishes (Falcon). These reactions were incubated at 37°C for 24 or 48 hours. The cultured islets were harvested (spun at room temperature 1000 rpm x 90 seconds in Falcon 2054 tubes) and washed twice with g50/RPMI media (in RPMI) and then resuspended in 1700 μL of g50 (glucose concentration 2.8 mM) or g360 (glucose concentration 28 mM in RPMI) media.

After 2 hours incubation at 37°C 700 µL were aspirated from each static incubation well and the insulin content of the supernatants assayed by ELISA (Boehringer Mannheim). 4 x 300 µL aliquots were used for the static incubation and 200 µL used for insulin content. Briefly, insulin content was measured after islets were extracted in 2 mM acetic acid containing 0.25% BSA. Samples were sonicated in acetic acid, centrifuged (800g x 15 min). Supernatants were collected and stored at -20°C until assayed for insulin content by ELISA (Boehringer Mannheim). The final 200 µL of the sample used for DNA content analysis. For assaying DNA content, aliquots were washed in citrate buffer (150 mM NaCl, 15 mM citrate, 3 mM EDTA pH 7.4) and stored as cell pellets at -20 °C. Before assay, cell pellets were placed in 450 µL of lysis buffer (10 mM Tris, 1 mM EDTA, 0.5% Triton X-100, 4° C, pH 7.5), sonicated, supplemented with 25 µL of Proteinase K solution (8 mg/mL) vortexed and incubated at 65 °C and 70°C for 45 and 10 minutes respectively. Lysates were supplemented with 25 µL RNase A (10 mg/mL), vortexed and incubated for 1 hour at 37°C. Aliquots of 25 and 50 µL were assayed in duplicate by diluting them in 1 mL of DNA buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and measuring fluorescence at 490/515 nm after the addition of 1 mL of Pico Green reagent (1/200 dilution with DNA buffer). Samples were run in parallel with and diluted in proportion to a seven point standard curve (0-400 mg/mL) which was generated using calf thymus DNA.[56]

3.2 Islet Transfection with HFasL-1

A number of different islet transfection methods were considered including biolistics, infection with adenovirus or retrovirus. Biolistics is a system whereby fine gold particles are coated with the DNA of interest, then accelerated by a high pressure helium shock wave causing them to physically impact on the islets below where approximately fifty percent will be damaged fatally by the "blast" and of the remaining viable islets approximately 3-10% will be transfected.[52] We elected to use the biolistic system because the technology was available to us

and had already been used successfully in the lab. The process of HFasL-1 biolistic transfection of islets included preparing the gold particles, isolating an adequate number of islets, and performing the biolistic transfection. A number of control experiments were done concurrent with transplantation to characterize the biolistic transfection; these experiments will be described in this section.

a. Gold particle preparation: HFasL-1 was cloned into the plasmid pCSD7 which has the human CMV Immediate Early promoter/ enhancer (Table 3). DNA was precipitated onto gold particles in the following manner: Gold particles were prepared by weighing out 30 mg of the desired size of gold particle (1.0-2.5 μm), washing once with 95% ethanol, and twice with ddH_2O and then resuspending in 500 μL of sterile water (final concentration of 60 mg/mL). 50 μL of gold particles (60 mg/mL in water) were combined with 20 μg of DNA and mixed well by finger flicking. 2.5 M $CaCl_2$ (50 μL + DNA volume) and 1 M spermidine (1/5 volume of $CaCl_2$) were added in rapid succession followed by vigorous mixing to prevent the DNA precipitate from clumping the gold particles in large aggregates. The reaction was incubated at room temperature for 10 minutes. The gold/DNA suspension was flash spun (1000 rpm x 4 seconds) and supernatant aspirated. The pellet was resuspended in approximately 500 μL of 70% ethanol, repelleted then washed again in 95% ethanol, repelleted and resuspended in 50 μL of 95% ethanol. Kapton discs were prepared by soaking in 95% ethanol, then rinsed three times with ethanol and allowed to air dry in the tissue culture hood. They were then secured in the macrocarrier ring support for blasting. The gold with precipitated DNA was finger flicked to mix and 6 μL per disc plated out on the macrocarrier Kapton discs (3 μg DNA per disc).

b. Islet isolation: Refer to pages 50 and 51 for experimental details of mouse and human islet isolation.

c. Blasting: 1000 islets cultured for 24 hours at 37°C / 95% air / 5% CO_2 were harvested from 60 mm petri dish to 15 mL conical tubes and allowed to settle. A sterile screen (40 μm cell strainer, Falcon #2340) was placed in the biolistic base and the islets were dispensed on the screen as a 0.1 mL central droplet such that the distance between the stopping screen and the cell droplet was 25 mm (Biorad Hand-Held Prototype biolistic particle delivery system). Two rupture discs (400 psi per disc) were mounted at the bottom of the helium chamber. The blast was performed in a vacuum of 15 inches of mercury at a pressure of 800 psi. Spontaneous rupture of the discs took place 4 seconds after the vacuum was applied and the helium source opened. Immediately post-blast the islets were placed in Ham's F10 media supplemented with 14.3 mM $NaHCO_3$, 10 mM glucose, 2 mM L-glutamine, 10 mM nicotinamide, 0.5% BSA, 50 mM isobutyl-methyl-xanthine 100 U/mL penicillin and 100 mg/mL streptomycin and were cultured at 37°C / 95% air/5% CO_2.

d. Luciferase assay: To assess the reproducibility of the blast technique, islets and COS cells were blasted with a pBR22-derived plasmid bearing the firefly luciferase gene driven by the human CMV Immediate Early promoter/enhancer (Dan Drucker CMV-Luc construct). The cells were blasted, incubated for 48 hours and assayed for luciferase activity. Cells were harvested and washed with PBS three times and resuspended in 100 μL of cell lysis buffer (0.1 M Tris HCl pH 7.8, 0.5 % NP40, 1.0 mM DTT added just prior to reaction). The cells were disrupted by intermittent vortex and ice incubation for several minutes. The reaction was them spun at 4°C x 15000 rpm x10 minutes. 100 μL per sample of assay buffer was prepared by combining 0.1 M luciferin (added just prior to reading) in 0.5 M Tricine, 0.1 M $MgCO_3$, 0.3 M $MgSO_4$, 0.05 M EDTA, 1.0 M DTT, 0.1 M ATP (Sigma 2383), 10 mM CoEnzyme A (Sigma 3144), 10 ng/mL BSA and water to bring the reaction volume to 100 μL. The assay buffer was placed in port II of the Lumat luminometer LB 9501 (Berthold) and the machine was washed five times (3 injection volumes per wash). Duplicate measurements of

luminescence (20 µL per measurement) were performed for each sample. Negative controls in each assay was provided by 20 µL of lysis buffer alone. A positive control (that never worked) was 20 µL of assay buffer with <1 µL of luciferase.

i. COS cells: 12 x 60 mm petri dishes at 100% confluent growth were blasted with CMV-Luc coating 1.6 µm gold particles. Post-blast they were cultured at 37°C / 95% air / 5% CO_2 for 48 hours in standard media (DMEM +10% FCS II). The cells were harvested and washed with PBS three times. They were resuspended in lysis buffer, disrupted by vortexing and then 20 µL aliquots assayed in duplicate on the luminometer.

ii. Islets: Aliquots of 500 islets were blasted with CMV-Luc coating various sized gold particles (1.0, 1.6, 2.2 µm). They were incubated at 22°C /95% air / 5% CO_2 for 48 hours then harvested, washed in PBS three times and assayed as for COS cells.

4. TRANSPLANTATION

4.1 Transplantation

CBA/J or Balb/c recipient mice (chemically induced diabetics by dorsal vein penile injection of alloxan 80 mg/kg under halothane anesthetic, 5% induction, 1.25% maintenance in 1 lpm oxygen) were made diabetic 3-7 days pretransplant and monitored daily (Medisense Glucometer and blood glucose sensor electrodes (Waltham, MA)) to ensure the animals became and sustained hyperglycemia. The recipients received no immunosuppression.

500 blasted CBA/J islets were cultured for 48 hours in standard Ham's media at 37°C / 95% air / 5% CO_2. The donor islets were hand counted and transferred to sterile Eppendorfs in 1 mL of media using a mouth-suction pasteur pipette. The nonimmunosuppressed alloxan-induced diabetic donors were anaesthetized with halothane and the left flank was shaved, then prepped with Betadine surgical scrub solution with the mouse in a prone position. An oblique flank incision was made through the latissimus dorsi into the retroperitoneum. The left kidney

was delivered through the incision and maintained in position with a cotton tip applicator. A renal subcapsular pocket was formed by making a nick in the renal capsule with a 23 gauge needle and then insertion of a fine blunt tipped glass probe along the dorsal surface of the kidney used to bluntly dissect the space between the renal capsule and underlying cortex.

A micromanipulator syringe (produced by SMRI, University of Alberta) attached to 30 cm of polyethylene tubing (PE-50, Fisher) by a 23 gauge needle was used to aspirate the islets with minimal media into the tubing which was secured at its distal end with a Ligaclip (Ethicon, Somerville, NJ). The syringe/tubing was spun using a centrifugal motor at 350 rpm for 15-20 seconds to pellet the islets in the distal tubing. The tubing was cut proximal to the Ligaclip and introduced into the renal subcapsular space. The islets were expelled from the tubing into the renal capsule, the pouch aperture was sealed with cautery and the kidney returned to its usual retroperitoneal location. The muscle layer was reapproximated with 5.0 Dexon II suture (Davis and Geck, Montreal, PQ) with a running continuous suture and skin edges were reapproximated with skin clips (Clay Adams). Blood glucose was monitored three times weekly using a Medisense glucometer and blood glucose sensor electrodes (Waltham, MA). Grafts were deemed to be functioning if blood glucose levels were maintained <11.0 mM. Technical failures were deigned as animal deaths or failure to achieve normoglycemia after transplantation. Graft failure was defined as two consecutive blood glucose readings >13.0 mM.

4.2 Histology

At an arbitrary time (depending on whether or not there was clinical evidence of graft failure) a nephrectomy was performed to remove the graft. The kidney was preserved in Bouin's solution and once fixed was embedded in paraffin and then sectioned in 5 μm thick slices. Consecutive sections were stained with hematoxylin and eosin (for general morphologic

assessment), aldehyde fuchsin (to stain islet cells) and immunohistochemical staining (for β cell insulin granules).

The immunohistochemistry was performed using a DAB peroxidase method. The tissue sections were stained with a primary anti-mouse insulin antibody and secondarily stained with a guinea pig anti-mouse antibody conjugated to the chromagen peroxidase.

Chapter 3

RESULTS AND DISCUSSION

1. DNA CLONING

1.1 Cloning of Human Fas Ligand: HFasL-1

a. Rationale: Wildtype HFasL was cloned for the purpose of transfecting islets to protect them from T cell mediated rejection. It was also used as a template for generating truncated HFasL constructs and for creating HFasL-1 producer cell lines.

b. Results: HFasL-1 was obtained by RT-PCR from total cellular mRNA of the activated Jurkat cell line (provided by Dr. J. Elliott) which are known to express HFasL.[49] Oligonucleotide primers were designed to clone the gene into plasmid vectors with an XbaI site at the 5' end of the gene and a NotI site at the 3' end. PCR using *PFU* DNA polymerase failed, but *Taq* polymerase and a cycle of denaturation at 94°C for 20 seconds, an annealing step at 55°C for 45 seconds and an elongation step of 72°C for 2 minutes succeeded in generating the wildtype gene.

The wildtype gene was first cloned into pBluescript KS+ (XbaI/NotI, Table 3). We were repeatedly unable to amplify the DNA with *E. coli* transformation. The gene was then cloned into SK- (the same vector backbone with the polylinker in reverse orientation). With the gene inverted visavie the promotor-enhancer no FasL protein was translated and large quantities of plasmid DNA were obtained. Our conclusion was that the functional HFasL-1 gene product was toxic to *E. coli* (Figure 1). A number of HFasL-1 clones which mapped correctly (Figure 2) were sequenced. HFasL-1 Clone #1 had a single transition mutation at base #502 with guanine substituted for adenine changing the coding sequence from ATT (ile)

to GTT (val). While this mutation represented a conservative amino acid change we chose to repair it.

A PCR strategy was used to repair the mutation. Two primers were generated complementary to the sequence flanking either side of the mutation and coding for the correct base at position 502 (HFasL-rp5', HFasL-rp3', Table 2). Two separate PCR reactions were performed using the mutated DNA as template (HFasL-1 Clone #1). The first reaction employed the primers HFasL-rp5' and HFasL-3' to generate the corrected 5' species from the front end of the gene to seven bases distal to the mutation (bases 1-509) (Figure 3). The second reaction used primers HFasL-rp3' and HFasL-5' to generate the corrected 3' species from nine bases proximal to the mutation through the end of the gene (bases 493-846)(Figure 4). The PCR cycle used in both these reactions was denaturing step of 94°C for 20 seconds, elongation step of 72°C and annealing step of 50°C for 45 seconds for three cycles. The annealing step was then modified to 52°C for 45 seconds with all other steps unchanged for the remaining 27 cycles. The products of these two PCR reactions were combined to provide DNA template for a third PCR reaction. In the PCR denaturing step the two species of DNA (the 5' and 3') separate from their complementary strand and either would reanneal with the original second strand, or in a few cases the overlapping complementary fragments of the two different DNA species (5' and 3' ends of the gene) would anneal and DNA polymerase would synthesize both full length strands which could then provide template in the PCR reaction for the full length repaired wild-type gene (Figure 5). The product of the third PCR reaction was cloned into pBluescript SK- (XbaI/NotI) and resequenced, confirming the repair of the mutation (Figure 1). The repaired HFasL-1 construct was then cloned into the eukaryotic expression vector, pSRαSD7 (XbaI/NotI). HFasL-1 was also used as a template for synthesizing truncated constructs HFasL-131, -132 (Figure 8).

1.2 Cloning of HFasL-131, HFasL-132

a. Rationale: HFasL-1 is known to exist both as a membrane-bound and a soluble molecule.[50] The mechanisms, kinetics and purpose of soluble FasL remain speculative. Tanaka and colleagues engineered truncated versions of HFasL (S2 beginning at amino acid 137 of wildtype HFasL making the gene fragment 437 bases long; and S1 which begins at amino acid 103 making the construct 537 bases long). The shorter construct (S2) had more killing effect when applied to FasR+ cell lines than did wildtype (HFasL-1=1200 U/mL, S1=800 U/mL, S2=3800 U/mL; 1 killing unit is defined as the concentration of FasL that gave half-maximal cytotoxicity versus $7.5x10^4$ W4 cells in 100 μL).[50]

The exact cleavage site responsible for converting FasL from membrane-bound to soluble form is not known. However, mouse FasL and human TNF have cleavage sites which bear homology to HFasL-1 in its extracytoplasmic region. Given that there is no stereotypic recognizable proteolytic cleavage site in the transmembrane domain of HFasL, planning a shortened version that will reliably be solubilized but yet not interfere with tertiary conformation and binding functions is speculative. To minimize the potential effects of construct design on protein function we chose to start the truncated sequence proximal to amino acid 137 (which is the starting base of Tanaka's construct), because we felt the proline residues in the region proximal to amino acid 137 may be important conformationally. Our shortened constructs were designed to exclude the purported cytoplasmic and transmembrane regions of the HFasL-1 cDNA. HFasL-131 was designed to begin at amino acid 131 in wildtype cDNA sequence (base 393 for final length of 453 bases, Figure 8). A slightly shorter construct, HFasL-132 was designed starting at amino acid 132 (base 396 with total length 450 bases, Figure 8).

Our constructs also differed from the published version of synthetic soluble FasL in signal sequence. Tanaka et. al. employed a mouse G-CSF signal sequence. As there was no

apparent physiologic reason for this choice we elected to use the signal sequence currently available to us in the laboratory, Human T1/Leu-1 glycoprotein.[51]

b. Results: HFasL-131 and -132 were PCR amplified from the repaired, sequence-verified HFasL-1 construct. *PFU* DNA polymerase was used as described in the Methods and Materials with the primer combinations HFasL 131-5'/HFasL 3' and HFasL 132-5'/HFasL 3' which were designed to add the restriction sites BamHI to the 5' end of the gene and NotI to the 3' end of the gene (Table 2). The PCR cycle employed was 94°C for 20 seconds; 50°C for 45 seconds; 72°C for 90 seconds repeated three times, then the annealing temperature was increased to 52°C for 45 seconds for the remaining 27 cycles.

The resulting clones were mapped but not resequenced because of the high transcription fidelity of *PFU* DNA polymerase and the short length of the genes (*PFU* transcription error rate is 1/50,000 bases). Both truncated products were then cloned into pBluescript SK- (BamHI/NotI) and then into the eukaryotic expression vector pSRαSD7 (Figure 6, Table 3).

1.3 Cloning of HFasL-137

a. Rationale: We assayed HFasL-131 and -132 transfected COS cell supernatants for cytotoxicity against FasR+ cells but these constructs produced negligible effect and given the high levels of cytotoxicity reported by Tanaka and colleagues we elected to generate an identical short construct to their published S2 construct.

b. Results: Mouse G-CSF signal sequence, a 116 base pair oligomer was synthesized and size-selected on a polyacrylamide gel as described in Materials and Methods. We performed PCR on the acetonitrile eluents from Sep-pack purification columns (which were expected to contain the synthesized 116 base pair oligomer) using MG-CSF 5' and 3' primers which incorporated MluI and XbaI sites at the 5' and 3' ends of the product respectively (Table 2). A cycle of 94°C for 30 seconds, 50°C for 45 seconds, 72°C for 60 seconds for four

cycles was followed by an increase in annealing temperature to 54°C for 45 seconds and the cycle was repeated 26 times. The PCR product was purified using the Centricon-100 system and then harvested from a LMP gel.

HFasL-137 cDNA was obtained by PCR from the corrected HFasL-1 clone using primers HFasL 137-5' and HFasL- 3' which incorporated the XbaI restriction site at the 5' end of the truncated gene and NotI at the 3' end of the truncated gene. A forced three-way ligation was performed with the signal sequence and the HFasL-137 gene fragment into the MluI/NotI cut pBSK- vector. The clones were mapped and one sequenced using the double stranded dideoxy terminator method.

A mutation was present at base 120 with deletion of one adenine base. This was due to an error in primer generation (HFasL 137-5') which was remade. PCR of the HFasL-137 portion of the construct was performed, followed by repeat ligation and sequencing with perfect sequence obtained.

1.4 Cloning Human Fas Receptor: HFasR

a. Rationale: We elected to clone HFasR for the purpose of generating cell lines which stably expressed HFasR as a control to the FasL-mediated killing of native FasR+ cell lines (i.e. Jurkat E6-1).

b. Results: HFasR protein (generated by transfected COS cells) was a reagent used for FACS staining for expression of FasL transfected cell lines.

1.5 Cloning Human FasIg Fusion Construct: HFasIg

a. Rationale: A chimeric product was designed based on previous work in which a construct comprising the 5' 502 bases of HFasR with the 3' 706 bases of the heavy chain coding region of IgG1 (starting at base 469 of wildtype IgG1 which includes the hinge (H) region and the constant heavy chain regions CH2 and CH3, Figure 9).[28]

b. Results: The Fas portion of the fusion construct was PCR amplified from wildtype HFasR using *PFU* polymerase and the primers sHFas-5', sHFas-3' incorporating XbaI sites at the 5' end of the gene fragment and AatII site at the 3' end of the gene fragment. The IgG1 fragment comprising bases 469-1175 of IgG1 was PCR amplified from CTLA4Ig construct (designed and kindly given to us by Dr. A. Gainer), including an AatII recognition site at the 5' end of this fragment and a NotI site at the 3' end of the gene in the primers FasFc-5' and FasFc-3' (Table 2). The PCR cycle used for each of these reactions was 94°C for 20 seconds denaturation step followed by 53°C for 45 seconds annealing step and 72°C for 90 seconds elongation step. This series was repeated for a total of 30 cycles.

2. *IN VITRO* FAS LIGAND CYTOTOXICITY

2.1 In vitro Cytotoxicity of HFasL, MFasL and shortened constructs

a. Rationale: After we had cloned and sequenced our constructs (and thus knew that we had the correct genes), we had to prove that the genes were translated into biologically active molecules. To answer this question recombinant proteins (harvested from COS cells transfected with the various forms of FasL including mouse and human, wildtype and truncated versions) were applied to FasR+ cells and the cytotoxic effect quantitated by the biological assay (MTT) as described in Methods and Materials.

b. Results:

i. HFasL-1 had the expected in vitro cytotoxic effect against Jurkat cells: To ensure that we had biologically active gene products HFasL-1 transfected COS supernatants were applied to FasR+ cells (Jurkat E6-1 and Yac-1 and L1210+). A negative control was provided by the supernatants of COS cells transfected with the empty pSRαSD7 vector. These experiments were conducted in triplicate unless otherwise specified.

HFasL-1 at a 1:2 dilution applied to 50,000 unstimulated Jurkat E6-1 cells caused a 79% ±3% reduction in mean optical density (reflecting a reduction in cell viability) versus control. The mean effects of HFasL-1, HFasL-131, and HFasL-132 versus Jurkat E6-1 at a 1:2 dilution were statistically significant (ANOVA $p<0.0001$; t-test with Bon Feronni adjustment at $\alpha=0.05$, $p<0.01$). While numerically an effect was still apparent at a 1:8 dilution the difference in means failed to achieve significance (ANOVA $p>0.07$) (Figure 10).

HFasL-1 applied to other FasR+ cells caused little or no discernible cytotoxicity. When a 1:2 dilution of HFasL-1 bearing COS supernatant was applied to Yac-1 cells the mean optical density of HFasL-1 treated wells was consistently and statistically significantly higher than those treated with control supernatants (t-test $p<0.04$). Likewise HFasL-1 caused no discernible difference in mean optical density compared with control when applied at a 1:2 dilution to L1210+ cells (data not shown).

ii. HFasL-1 had the expected absence of effect versus FasR- cell lines: Throughout all dilutions HFasL-1 treated L1210- wells displayed more viable cells than did control treated wells. This is represented by a negative difference in mean optical densities (implying a protective effect) compared to control. At a 1:2 dilution HFasL-1 caused a -44±0.5% reduction in mean optical density versus control, and at a 1:32 dilution a -48±6% reduction. This "protective effect" was statistically significant ($p<0.02$, Student's t test) and the curves remained parallel throughout the dilutions (data not shown).

BW5147 another FasR- cell line, was resistant to any HFasL-1 cytotoxicity (data not shown).

iii. HFasL-131 and -132 had no cytotoxic effect against FasR+ cells: HFasL-131 caused a mean reduction in optical density of -25%±8% versus control supernatant at a 1:2 dilution when applied to Jurkat E6-1 cells. HFasL-132 likewise had an absence of cytotoxic effect at a 1:2 dilution with a mean reduction in cell viability of -14%±28% (Figure 10). This

effect failed to achieve statistical significance (t-test with Bon Feronni correction with α=0.05, p>0.1). At a 1:16 dilution HFasL-131 and 132 had a -14%±16% and -16%±11% reduction in mean optical density respectively versus control supernatant. Given the absence of cytotoxicity evidenced by these constructs they were abandoned and attention turned to generating a shortened HFasL construct (HFasL-137) identical to that published by Tanaka et. al. (Figure 8).[50]

iv. HFasL-137 has weak cytolytic effect versus FasR+ cells: The recombinant protein derived from COS cells transfected with HFasL-137 was analyzed for cytotoxicity in an MTT assay against Jurkat E6-1 cells. HFasL-137 caused a mean reduction in optical density of 18%±3% at a 1:2 dilution versus control supernatant. This effect failed to achieve statistical significance (t-test p<0.1)The effect rapidly tapered to a 1:16 dilution where a 0%±12% effect was observed (Figure 11).

Compared to control, HFasL-137 had no discernible effect on Yac-I in duplicate experiments (data not shown).

As predicted, HFasL-137 had no cytotoxic effect against FasR- cell line L1210- (data not shown).

v. MFasL-1 had a greater cytotoxic effect than HFasL-1 on FasR+ cells:

Compared with control supernatant at a 1:2 dilution, MFasL-1 caused a mean reduction in optical density of 82.8%±4%. This effect was 55.3%±2% at 1:4 dilution and 26%±10% at a 1:16 dilution. MFasL-1 had a 12.8%±3% greater effect at 1:2 dilution than HFasL-1 (and the effect was sustained to a 1:64 dilution at which point no discernible effect was present). However, these mean differences were not statistically significant (t test with Bon Feronni adjustment at α=0.05, p>0.1). This result is different to the pattern of HFasL-1 cytotoxicity with its cytotoxic effect extinguished by a 1:16 dilution (Figure 12).

vi. MFasL-1 had no cytotoxic effect versus FasR- cells: Like HFasL-1, MFasL-1 had no significant cytotoxic effect when applied to L1210- or BW5147 cells compared to control (data not shown).

vii. MFasL-300 and -331 had an absence of any detectable cytotoxicity against FasR+ cells: MFasL-300 caused a mean reduction in optical density of 5%±23% versus control at 1:2 dilution when applied to Jurkat E6-1 cells. At a 1:16 dilution a -10%±23% reduction in optical density was observed. Likewise, MFasL-331 caused a mean reduction in optical density of -21%±5% at a 1:2 dilution and at a 1:16 dilution -25%±14% versus control. In a comparison between the effects of MFasL-1, MFasL-300 and MFasL-331 versus Jurkat E6-1 only MFasL-1 was found to be significantly different from control and from both MFasL-300 and MFasL-331 (statistically significant to a 1:16 dilution by ANOVA $p<0.01$; t test with Bon Feronni adjustment $\alpha=0.05$, $p<0.05$) (Figure 13). Similar to our results with truncated forms of HFasL, the shortened forms of MFasL had insignificant killing effect against FasR+ cells. Tanaka et. al. were also unable to demonstrate any killing effect by a soluble form of MFasL.[50]

c. Discussion:

i. The effect we witnessed of HFasL-1 (wildtype) versus Jurkat E6-1 was reproducible and consistent: The same killing curve was reproduced in numerous repeated experiments with 80% reduction in cell viability at a 1:2 dilution and a rapid taper of effect with dilution so that no effect was appreciable at a 1:16 dilution. A similar magnitude of effect was seen against some of the highest HFasR expressing BW5147 stable transformant cell lines.

The effect of HFasL-1 against other FasR+ cell lines including Yac-I and L1210+ likely reflects the level of FasR expression by these cell populations. FasR+ cells populations are heterogenous with respect to level of FasR expression. Some researchers have needed to

FACS their L1210+ cell populations repeatedly to assemble a FasL-sensitive colony (verbal report; D Green).[41] The apparent protective effect (i.e. negative reduction in mean optical density when comparing HFasL versus control) most likely represents a technical error in aliquotting cells during the experiment, allotting more cells to one group of wells than another. Repeated experiments have in fact shown overlapping lines with near identical optical densities between control and HFasL-1 treated wells. If there were truly a protective effect provided by HFasL-1 one would expect that as the dilution increased, the protective effect would decrease. In the reported experiments the curves remained parallel indicating that initial disparity in allotment of Jurkat E6-1 cells is the most logical explanation for these findings.

ii. The largest cytotoxic effect of our constructs against FasR+ cells was several orders of magnitude lower than the effect reported by Tanaka et. al.: Tanaka et. al. reported a 100% reduction in cell viability at 1:80 dilution of HFasL-1 supernatant, a 50% reduction in cell viability a 1:100 dilution and 15% reduction in cell viability at 1:150 dilution.[50] Our highest reduction in mean optical density was 80% at a 1:2 dilution and the effect was insignificant by a 1:16 dilution.

Possible explanations for the disparity in effect seen by this group and ourselves include the fact that the HFasL-1 gene sequence used by Tanaka et. al. was not published; a mutation in this construct could account for the increased cytotoxicity apparent in their reports. Even without invoking a theory or mutation, gene polymorphisms of HFasR may exist physiologically and cause increased or decreased sensitivity to FasL mediated apoptosis in different cell lines. Simple differences in cell line sensitivity to Fas-mediated apoptosis may account for the differences in cytotoxicity observed. W4, Tanaka's own stable transformant expresses MFasR whereas we were relying on the natively expressing HFasR+ cells, Jurkat for the highest degree of sensitivity to FasL mediated cytotoxicity. The level of FasR expression by a given population (i.e. the proportion of cells in the population expressing FasR) may also vary

between cultures. Cultures that have been stressed may down regulate or select against FasR expression as evidenced by the need to repeatedly FACS a population of L1210+ cells to obtain a Fas ligand-sensitive group of cells.[41] The definition of one unit of killing activity also differs between Tanaka and ourselves. Tanaka et. al. report their data as killing units per mL (versus ourselves who have observed the killing effect per 100 μL of supernatant) which accounts for ten-fold difference in our results leaving a remaining ten-fold difference to explain.

iii. Our truncated constructs have less activity than literature reports: Tanaka et. al. found that their shortened constructs (S1 truncated at amino acid 103, S2 truncated at amino acid 137 of wildtype HFasL) exhibited marked cytotoxicity against the FasR+ cell line they were using (W4, a MFasR expressing cell line from parental WR19L cell line which is a virus transformed tumor cell line). In fact the shortest construct (S2) had more killing effect (3800 U/mL) than wildtype HFasL (1200 U/mL) or a longer truncated construct, S1, which had 800 U/mL killing activity.[50] There are a number of possible explanations for the lack of effect incurred by our truncated constructs. Our short constructs were never sequenced after being PCR amplified from the sequence perfect wildtype HFasL-1. It is possible that these constructs bear a mutation that impairs the delivery of a cytotoxic insult. As well, different FasR+ cell lines were used (W4 in Tanaka's experiments which express MFasR and Jurkat E6-1 used in our cytotoxicity assays which express HFasR). These cell lines may have different sensitivity to FasL-mediated apoptosis which is species-dependent. By making longer constructs than those engineered by Tanaka et. al. we may have interfered with tertiary conformational changes necessary for trimerization and binding to FasR. HFasL-131 is 16 base pairs longer, and HFasL-132 is 13 base pairs longer than S2. This hypothesis is consistent with the findings of Tanaka et. al. who found the shorter of the truncated constructs (S2 which is 102 base pairs shorter than S1) had more than three times the cytotoxic effect that the longer (S1) construct. A final hypothesis to explain the disparity of our results with Tanaka's is that

the FasL constructs which we engineered were poorly or not at all soluble, that is by conserving more of the transmembrane domain we inadvertently caused the protein to be constitutively membrane bound. This issue could be addressed by a cell-to-cell MTT whereby an HFasL stably transformed cell line would be exposed to low dose irradiation and then added to FasR+ cells in an MTT assay. As the transformants are irradiated they would be unable to process the MTT and the assay would reflect the effect of the transformant cell surface molecules on FasR+ cells. Additionally the cells could be FACS analyzed to determine the level of HFasL expression. If, however, as for HFasL-137, the issue is a lower level of cytotoxicity rather than a complete absence of cytotoxicity , some of the protein is being solubilized thus the necessary enzymes and substrate must be present, perhaps just quantitatively less than on more sensitive cells.

iv. HFasL-137 truncated construct had less than expected in vitro cytotoxicity: Our objective in designing and cloning HFasL-137 was several fold. First, we wanted to have an identical reagent to that published by Tanaka et. al. to be able to assess the sensitivity of our HFasR+ cell lines compared to their MFasR+ stable transformant (W4). Secondly, we wanted to develop a very sensitive assay for FasL production which would be necessary for assaying FasL expression by transfected islets for the purpose of developing a dose-response curve for transplantation success. However, our S2 analogue, HFasL-137, had meager cytotoxic effect versus the FasR+ cell line we were using in the MTT assay for cytotoxicity (20% reduction in Jurkat E6-1 viability) and much less than HFasL-1 (80% reduction in cell viability), which is contrary to the findings of Tanaka et al. who showed their shortest construct (S2) had more killing effect than wildtype (HFasL-1) or a longer construct (S1). Similar factors likely account for the reduced cytotoxicity of our HFasL-137 as for our other truncated constructs, HFasL-131, HFasL-132. Tanaka's transfection protocol was obtained to see if this could account for our differences in cytotoxicity, but it is very similar to ours. The key differences consist of:

Tanaka's use of glycerol shock versus ours of DMSO shock. Tanaka transfects 5 μg of DNA, we have used 5-15 μg. Tanaka grows COS cells post-transfection in 10 mL of media for 72 hours. We trypsinized the transfected COS cells after 24 hours incubation (wasting the initial 24 hours worth of protein and exposed the transfected cells to trypsin which may digest newly produced protein) and then incubated them an additional 72 hours in 15-20 mL of media. The transfection method does not seem to account for even a 10-fold difference between our results. We have already raised the possibility of variable cell line sensitivity to FasL mediated apoptosis to account for the ten-fold difference in cytotoxicity incurred by our constructs versus Tanaka's. Tanaka's group designed a MFasR stable transformant cell line from parental WR19L, a murine tumor cell line induced by Abelson leukemia virus. These cells are known to produce Moloney leukemia virus. It may be that this secreted virus has a confounding cytotoxic effect unrelated to FasL-mediated apoptosis. WR19L are not susceptible to Fas mediated apoptosis whereas the MFasR transformant W4 is exquisitely sensitive with 100% killing effected caused by the application of HFasL-1 transfected COS supernatants until a 1:80 dilution when the effect begins to diminish. It is unlikely that one cell line natively insensitive to Fas mediated apoptosis rendered sensitive by FasR transfection (i.e. W4) would be more sensitive than a native FasR+ cell (i.e. Jurkat E6-1) or another stably FasR transformed cell line (our stable HFasR transformants). To address this question we generated our own HFasR stable transformants and also obtained the W4 cell line from Tanaka et. al. against which we could assay our own proteins.

v. Our MFasL-1 construct has greater cytotoxicity than literature reports: Tanaka et. al. report that their MFasL-1 construct had a greatly limited cytotoxic effect (<20 U/mL) compared to the HFasL constructs (1200-3800 U/mL) against a MFasR+ cell line. Our results are opposite Tanaka's with our MFasL-1 construct having a greater cytotoxic effect against a HFasR+ cell line than our HFasL-1 construct.

There are no other published accounts of the cytotoxicity of truncated MFasL constructs. Our findings are internally consistent between MFasL and HFasL with wildtype constructs incurring considerable cytotoxicity (80%) and shortened constructs having little or no effect. We have yet to prove that there is a dose-response curve in the cytotoxicity induced by FasL although it has been reported.[54] Concentrating the MFasL-1 bearing supernatants (by Centricon or lyophylization) and proving that more concentrated FasL protein is associated with higher levels of cytotoxicity would indirectly argue that the killing effects seen were solely due to the effects of MFasL and not due to a confounding factor.

2.2 HFasIg Blocks Fas Ligand Mediated Cytotoxicity

a. Rationale: In order to prove the specificity of FasL mediated cytotoxicity FasIg was added to MTT assays in which FasL was applied to FasR+ cells. In this experiment FasIg functioned as a 'receptor decoy', binding FasL and preventing the membrane-bound form of FasR+ from engaging FasL and initiating apoptosis.

b. Results:

i. HFasIg is able to block some HFasL-1 mediated cell death: When HFasIg (1:4 dilution) is added to HFasL-1 (1:4 dilution) the optical density was increased from 0.199 to 0.38, a 48% increase in mean optical density, which represents a 31% reduction in cytotoxicity by the addition of HFasIg. Compared to the mean optical density of an MTT with maximally dilute HFasL-1 (=0.85 depending on the actual number of cells at the beginning of the assay) the addition of HFasIg to the reaction achieved 31% reduction in the effect of HFasL-1. As HFasIg was diluted (and HFasL remained at a constant 1:4 dilution) the protective effect gradually diminished from 15% reduction in cytotoxicity at 1:32 dilution, to 5% at 1:128 dilution. The protective effect was entirely absent by a 1:256 dilution (Figure 14).

ii. HFasIg alone has no inherent cytotoxicity: Using the MTT assay of cytotoxicity, HFasIg added to Jurkat E6-1 cells did not cause any loss of cell viability compared to maximally diluted HFasL supernatants (1:4096, data not shown).

iii. HFasIg was able to block MFasL-1 induced cytotoxicity: When HFasIg (1:4) was added to MFasL-1 (1:4) and the supernatants assayed on Jurkat cells in a standard MTT, the optical density rose from 0.29 (the mean optical density for MFasL alone versus Jurkat) to 0.638, representing a 55% inhibition of MFasL induced cytotoxicity. This protective effect tapered with serial 1:2 dilutions of FasIg (and consistent MFasL 1:4) but never quite reached baseline levels of MFasL alone (at a FasIg 1:4096 dilution there was still a 13% reduction in cytotoxicity compared to 1:4 MFasL alone, data not shown).

c. Discussion:

i. HFasIg blocks one-third of FasL-1 cytotoxicity: HFasIg at a 1:4 dilution was only able to reduce by 31% the maximal cytotoxic effect exerted by FasL-1 (either HFasL-1 or MFasL-1). However this effect was species independent as both MFasL-1 and HFasL-1 were inhibited to a similar extent. As the protein concentration of the supernatants employed (both HFasIg and FasL) were not known and the binding constants of FasL to FasR is not known, this assay is a crude measure of the specificity of the cytotoxic effect seen in our assays and attributed to FasL. We surmise that the COS supernatant concentration of HFasIg was half that of HFasL-1 accounting for the limited (31%) inhibition of FasL cytotoxicity.

Other groups have reported the ability to block FasL mediated cytotoxicity with a fusion protein such as FasIg in a species independent manner.[28,50] Tanaka et. al. found that a chimeric protein (MFasIg) at a concentration of 100 μg/mL was able to block the cytotoxic effect of 1.25-fold concentrated FasL bearing supernatants of activated human peripheral blood lymphocytes by 80%. After 100-fold dilution (i.e. 1 μg/mL) the blocking effect was reduced to

5%.[44] The same group reported that the neutralizing activity of monoclonal anti-FasL antibodies are 100-fold that of HFasIg constructs. The difficulty in interpreting our data in light of these studies are several-fold: First, we have no system for quantitating our FasIg thus it is difficult to interpret our results comparatively either between batches of transfected COS supernatants or between researchers. Secondly, the literature has not quantitated FasL protein so a 1.25x concentrated supernatant from activated human peripheral blood lymphocytes has no quantitative value.

While our experiment was internally controlled for the effect of FasIg versus FasL, a positive control (Jurkat) and negative control (pSRαSD7 transfected COS supernatants) should be included in future experiments. Another control would be a nonspecific fusion protein such as CTLA4Ig which would not be expected to have any protective effect against FasL cytotoxicity.

2.3 Stable Transformants

a. Rationale: In the interest of developing a more sensitive bioassay, for comparison with literature regarding stable transformant sensitivity to FasL mediated killing, and to have a continuous source of FasL and FasIg, we elected to generate stably expressing HFasL, HFasR and HFasIg cell lines. After the BW5147 cell line had been shown by FACS analysis to not express FasR in wildtype form (Figure 17) and by MTT to be insensitive to FasL-mediated cytotoxicity and (data not shown), BW5147 cells were transformed with the gene of interest (cloned into pSRαSD7) by electroporation.

After the weak cytotoxic results of our HFasL constructs against the native FasR+ cell line Jurkat E6-1, we elected to engineer our own HFasR expressing cell line in hopes of developing a more sensitive bioassay. The purpose of developing a highly sensitive bioassay was to assay the amount of FasL expressed by FasL transfected islets and allow us to generate a dose-response curve correlating the amount of FasL expressed with transplantation success.

All our FasL constructs had less cytotoxicity than we had anticipated based on Tanaka et al. reports. However, we had a different FasR+ cell line than they did and so we hypothesized that some of the difference observed between our results was that they had selected for a more highly FasL sensitive cell line than what we had. To assess the theory of the impact of FasR+ cell sensitivity to FasL-mediated cytotoxicity we obtained the W4 MFasR stable transformant cell line from Suda et al.[28] An MTT was performed using the same COS supernatants used on our FasR+ cell lines (Jurkat and stable transformants).

b. Results:

i. HFasL stable transformants express surface-bound HFasL detected by FACS analysis: Only three of 90 clones which grew in selective media expressed FasL judged by a shift in fluorescent staining. HFasL1-47 had a small peak shift of its primary population (negative population peak intensity at $4x10^0$ and transformed population peak moved to $1x10^1$). HFasL1-162 had minimal shift of its primary population with a larger second population demonstrating a full log shift in fluorescence to $6.5x10^1$. HFasL1-171 was a polyclonal population with one prominent population displaying no shift and a smaller population with peak fluorescence at 6.5×10^1. Both HFasL1-162 and -171 appear to be multiclonal in origin and each of the second populations has an identical fluorescent character indicating that these likely are transformed similarly (Figure 15).

ii. HFasL stable transformants do not possess any cytotoxic ability against FasR+ cells: Three BW5147 HFasL stable transformant clones were picked to use in an MTT (HFasL-47, -162, -171). The clones were grown in RPMI+10% FCS II for 48 hours and the supernatants harvested for recombinant protein. The supernatants were applied to Jurkat E6-1 cells in serial 1:2 dilutions. None of the three clones had any significant killing effect compared to control supernatant. HFasL1-47 caused only a 14% reduction in mean optical density at 1:2 dilution, HFasL1-162 caused a 19% reduction and HFasL1-171 caused a 5% reduction

whereas COS transfected supernatants expressing HFasL-1 and MFasL-1 had 87% and 91% mean reduction in optical density respectively at 1:2 dilution (Table 5).

iii. HFasR stable transformants are equally sensitive to HFasL mediated cytotoxicity as the native FasR+ cell line, Jurkat: Six clones from the electroporation which grew in selective media were picked for analysis of levels of HFasR expression (HFasR-11, -26, -33, -40, -62, -114). These clones represented four early (HFasR-11, -26, -33, -114) and two late-appearing clones (HFasR-40,-162). A mixture of early and late clones were chosen because anecdotal evidence suggests that late-appearing clones often are higher level expressors than early clones. Three of the six HFasR transformant clones were exquisitely sensitive to HFasL-1 killing with all three clones experiencing a 94% reduction in cell viability at 1:2 dilution versus control (HFasR-26, -62, -114). At a 1:8 dilution the HFasR-26 cells were reduced by 37%, HFasR-62 by 54% and HFasR-114 by 79% versus control. By a 1:16 dilution the HFasL-1 cytotoxic effect was no longer apparent against any of the clones, very similar to the effect of HFasL-1 versus Jurkat E6-1 (Figure 16). Nontransformed BW5147 cells provided a negative control.

iv. The level of HFasR expression by stable transformants assessed by FACS analysis did predict the level of sensitivity to FasL mediated cytotoxicity: The three clones most sensitive to HFasL-1 mediated cytotoxicity did have the largest shift in fluorescence by FACS analysis of any of the clones growing in selective media (HFasR-26 with a $1.5x10^3$ shift in peak intensity; HFasR-62 with a $7x10^2$ shift, and HFasR-114 with a $1x10^3$ shift) (Figure 17). The less sensitive clones (-11, -33, -40) had slightly less fluorescent shift on FACS analysis (HFasR-11 $4.5x10^2$; HFasR-33 $6x10^2$; HFasR-40 $6x10^2$, data not shown).

v. HFasR stable transformants are not as sensitive to MFasL cytotoxicity as native FasR expressing cells (Jurkat): While there clearly was some cytotoxicity induced by MFasL-1 versus HFasR stable transformants compared to control this was a much weaker effect than seen when MFasL-1 is applied to natively expressing HFasR+ cells (Jurkat E6-1), or HFasL-1 is applied to the sensitive transformants (Table 6).

vi. W4 stable transformants: The effect of HFasL and MFasL on W4 is similar to the cytotoxicity of these constructs against native FasR+ cell lines: An MTT was performed with HFasL-1 and MFasL-1 COS transfected supernatants applied to 50,000 W4 cells imported from Japan (courtesy Dr. Nagata et. al.). Compared to the effect of control supernatant at a 1:2 dilution HFasL-1 COS supernatants applied to W4 caused a 33% reduction in mean optical density and MFasL-1 caused a 59% reduction in optical density (these experiments performed in duplicate and results are reported as averages). However, like the cytotoxic effect seen against the native FasR+ cell line, Jurkat, the effect tapered rapidly and by a 1:8 dilution HFasL-1 caused a mere 4% reduction and MFasL-1 a 7% reduction in optical density compared to control (Figure 18). A failed control in this experiment makes results difficult to interpret. Due to time constraints there experiments were not repeated.

vii. Jurkat E6-1 and W4 have disparate sensitivities to HFasL-1 mediated cytotoxicity: At higher concentrations of HFasL-1 Jurkat was more sensitive to HFasL-1 cytotoxicity. At dilutions of 1:16 and greater, W4 response plateaued whereas Jurkat continued to show diminishing cytotoxic effect with each dilution until plateauing at 1:64 dilution (Table 7).

viii. W4 showed similar response to MFasL-1 and HFasL-1: At higher concentrations of MFasL W4 responded with an identical curve to that of Jurkat but plateaued at 1:16 dilution whereas Jurkat continued in a dose-responsive manner until a 1:64 dilution was reached (data not shown).

c. Discussion: HFasL-1 stable transformants do not express a soluble form of FasL. Our finding that HFasL-1 stable transformants do not produce killing activity in supernatants has recently been corroborated.[44] BW5147 may not express the necessary enzyme(s) for proteolytic cleavage/shedding of soluble FasL whereas COS cells do. Recent literature indicates that the cleaving enzyme is likely a member of the matrix metalloproteinase enzymes analogous to those responsible for cleaving TNF from its membrane-bound to soluble form.[44] Overgrowth of a non-expressor: As mentioned, in the FACS analysis in two of the three clones (HFasL-47, -162, 171) there was indication of at least two cell populations present. It may be that the FasL- cells have a survival advantage and thus overgrow the FasL+ cells quickly in culture. This hypothesis could be tested by limited-dilution cloning or single-cell FACS sorting to develop single-cell origin populations and repeating the MTT bioassay to see if the FasL expressing population exhibits cytotoxicity against FasR+ cells. A further possible explanation for our weakly cytotoxic stable transformants is the proline rich sequence of the cytoplasmic domain of the wildtype FasL which has been hypothesized to destabilize FasL on cell surface.[44] A truncated construct of HFasL missing amino acids 8-69 (i.e. cytoplasmic proline-rich region) was designed and the supernatants of stably transformed cells expressing this construct had significant cytolytic activity against FasR+ cells (W4). The conclusion from these studies was the cytoplasmic domain does bear on the behavior of the membrane-bound protein thus truncated constructs may have reduced activity.

HFasR Stable Transformants exhibit similar sensitivity to FasL-1 killing as native FasR+ cells. The performance of our HFasR stable transformants in the MTT are consistent with the findings of our MTT versus natively expressing FasR+ cells (Jurkat E6-1) and remain inconsistent with Tanaka's data regarding a very sensitive transformant cell line. Did they amplify expression? We tried to amplify the HFasR stable transformants unsuccessfully, likely because FasR is membrane-bound and it is difficult to amplify membrane-bound molecules because of stearic considerations for membrane-bound proteins.

W4 were no more sensitive to FasL mediated apoptosis than Jurkat cells. One of the most likely explanations for the modest sensitivity to FasL-1 mediated apoptosis is that a metabolically stressed population of FasR+ cells (like the W4 cells we received from Japan, after a long trip, exhausted media and suboptimal temperature) down regulates FasR+ expression or some part of the intracellular signaling cascade. We may have found if we had FACS these cells that there were only a small group of FasR+ cells remaining.

3. *IN VITRO* ISLET ASSAYS

3.1 In Vitro Effect of HFasL-1 on Islets

a. Rationale: After we had determined by *in vitro* cytotoxicity assays that our HFasL-1 gene product was biologically active against FasR+ cells the next step was to determine the effect, if any, of FasL on islets. FasR expression status of islets was unknown at the time of our experiments. Given that many somatic tissues express FasR it would not be surprising if islets were FasR+. As we know from review of Fas system physiology, FasR expression by a cell line may not be sufficient to render a cell sensitive to Fas-mediated apoptosis thus rather than histologic assessment of the presence or distribution of FasR, we chose to conduct a functional assay that could reflect the effect of FasL on islet cell survival and function.[34] If islets were destroyed by HFasL-1 (as are hepatocytes) then further experiments in using FasL as an

immune protectant would be nonsensical. However, if islets were to prove insensitive to Fas-mediated cytotoxicity, the second goal of *in vitro* islet assays was to determine and characterize an efficient method of islet cell transfection.

b. Results: In order to assess the effect of FasL on islet function we isolated human and mouse islets as described in Materials and Methods. Groups of 500 islets were incubated in the presence of control (pSRαSD7 'empty vector' transfected COS supernatants) or HFasL-1 bearing supernatants at a 1:4 dilution for 48 hours. We chose this dilution because in FasR+ cells (Jurkat and W4) a 1:4 dilution of FasL was associated with approximately 50% reduction in cell viability.

The islets were then exposed to either low glucose (2.8 mM) or high glucose (28 mM) and insulin production was measured after 2 hour incubation at 37°C / 95% air / 5% CO_2. Stimulation indices were calculated by dividing the insulin production at high glucose exposure by the insulin production at low glucose exposure.

c. Discussion:

Both human and mouse islets retained the ability to increase insulin production in response to glucose challenge. The stimulation index of human islets incubated in the presence of HFasL-1 (8.6±0.8) did not differ significantly from that of control islets (8.9±0.6) by paired Students' t test analysis ($p>0.69$). Likewise, the stimulation index of mouse islets did not differ significantly between HFasL-1 exposed (4.5±0.3) and control (4.9±0.04 $p>0.19$) (Table 8).

3.2 In Vitro Islet Transfection with HFasL

a. Rationale: Once we were satisfied that HFasL-1 had no deleterious effect on *in vitro* islet function we moved to transfection strategies. A number of alternative routes of introducing FasL to islets were considered. We elected to use the Biolistic Particle Delivery System because of its previous success in islet transfection (with a reported 50% survival of blasted islets and 3-10% transfection rate in surviving islets) (Figure 19).[52]

Our blast protocol comprised HFasL-1 gene cloned into a CMV-promotor bearing vector (pCSD7) coating 1.6 μm gold particles accelerated by a spontaneous rupture (two 400 psi rupture discs thus rupture occurs at 800 psi). Concurrent with the initiation of transplants we began to characterize the reproducibility of the blast process. The variables of any blast include: the DNA/ vector employed, the gold particle size, the homogeneity of macrocarrier disc coating, the method of rupture disc release (lance perforation or spontaneous rupture), and the characteristics of islets (i.e. degree of exocrine contamination, length of time in culture etc.). For each blast the nature of these variables was recorded and the outcome reviewed to see if it correlated with these factors.

b. Results:

i. Islet blast: In vitro luciferase assay: An initial assay of the effect of gold particles size on islet transfection was performed. A reporter construct comprising the firefly luciferase gene in a pBR22-derived plasmid with a human CMV Immediate Early Promotor/Enhancer (CMV-Luc, kindly provided by Dr. Drucker, University of Toronto) was precipitated onto gold particles (1.0, 1.6 or 2.2 μm, Biorad) as described in Materials and Methods. Spontaneous rupture blasts at 800 psi (2 rupture discs per blast each calibrated to spontaneously rupture at 400 psi) were performed on groups of 500 CBA/J islets isolated by standard collagenase digestion and Ficoll purification. Blast characteristics, islet appearance and 48 hour islet recovery were recorded. After 48 hours incubation in standard Ham's F10 media at 37°C and 5% CO_2 the islets were harvested and a luciferase assay performed (Figure 20). While some of the lowest luciferase counts are difficult to appreciate graphically, there was a consistent 100-fold difference between peak and trough levels of luciferase expression regardless of the size of gold particle used as microcarrier.

ii. Blast visualization assay: Following the Luciferase assay which showed a great deal of blast-to-blast variation in the degree of luciferase expression by islet cells, a simple

visualization experiment was performed with 1.6 μm gold particles coated with the same CMV-Luc DNA as in the islet blast experiment. Nine blasts were performed using spontaneous rupture technique at 800 psi and the gold particles were trapped on tape which was placed on the islet screen. The resultant 'tape-captures' illustrated the scatter of gold particles at the usual islet location (Plate 1).

It was apparent visually that there is at least a 10-fold difference in gold particle density between the tape bearing the greatest and least amount of gold. It was also apparent that the distribution of gold is highly variable and none of the recorded subjective data about the blast (including the nature/symmetry of macrocarrier disc DNA/gold load, the length of time to spontaneous rupture, the appearance of the rupture discs after spontaneous rupture, the appearance of the macrocarrier disc after impact on the stopping screen) could predict which blast would result in central maximal gold delivery.

iii. COS luciferase assay: Quantification of the findings from tape blasts were performed by blasting confluent plates of COS cells with CMV-Luc coated 1.6 μm gold particles using the spontaneous 800 psi rupture technique (Figure 21).

Again a great deal of blast-to-blast variability was noted with a 1600-fold difference in luciferase expression between peak and trough. Five of twelve blasts resulted in luciferase values that were greater than the mean.

c. Discussion:

i. Biolistic transformation can result in variably efficient Transfection of islets with a reporter gene. Our results of *in vitro* biolistic characterization show that a blast can cause high levels of expression of a reporter gene construct. Conversely, it can also be associated with poor levels of transfection. There is a great deal of blast-to-blast variability with peak and trough levels of expression varying 100-fold regardless of particle size. The level of expression expected from each blast could not be predicted by any observable blast variables (i.e.

macrocarrier disc load characteristics, rupture disc appearance, macrocarrier impact appearance, islet appearance post-blast).

ii. Alternate methods of Transfection have been attempted: Because of the high degree of variability between blasts and the inability to determine which islets received a "good blast" we have sought other methods of transfecting islets including a second generation biolistic device and adenoviral transfection.

Numerous strategies of adenoviral transfection were undertaken and while the details of these experiments are beyond the scope of this discussion eight different experimental arms were initiated to achieve a recombinant adenoviral vector. Following lengthy incubation we were disappointed to not have produced lytic virus in any of the conditions. However, discussion with other groups indicates that a number of other groups have failed to generate a recombinant adenovirus expressing FasL (verbal, Bluestone). The reason FasL cDNA would prevent cells from the recombination event required to generate and infectious virus is unclear at this time. There was no evidence of cell demise from visualizing the transfected plates through the course of a 1 month incubation.

iii. Our bioassay is not sufficiently sensitive to measure levels of FasL production by transfected islets: In fact, our bioassay may not be sensitive enough to detect the true cytotoxic abilities of our various FasL constructs. What we need is a sensitive enough assay to be able to detect the low levels of protein production in the supernatants of blast-transfected islets (we predict this will be in the order of nanograms). A capture ELISA was attempted with anti-FasL polyclonal antibody coating the plate, HFasIg binding to the trapped FasL and a secondary alkaline-phosphatase conjugated goat anti-human antibody. No readout was obtained from this experiment (data not presented). However, there are monoclonal antibodies on the verge of being commercially available and a highly sensitive ELISA has been described.[44] Once a sensitive assay exists, even if the blast is variably efficient in transfecting islets, a dose-response

curve can be generated indicating the threshold level of FasL expression necessary to protect islet cell allografts from rejection.

4. TRANSPLANTATION

During the course of characterizing the biolistic particle delivery system we performed some preliminary transplants. While we did not have an expectation of graft outcome given that we had no method of quantitating the HFasL-1 production of a given group of islets, they did perform an important extension of our studies of the *in vitro* effect of HFasL-1 on islets. The experimental groups were:

i) Allogeneic HFasL-1 blasted study group (n=8)

ii) Allogeneic HFasL-1 blasted inverted-gene control group (n=6)

iii) Allogeneic Luciferase blasted control group (n=6)

iv) Syngeneic Luciferase blasted control group (n=3))

v) Diabetic controls (n=6)

4.1 Group 1. Allogeneic HFasL-1 blasted (n=8)

a. Rationale: To answer our hypothesis that islet cell allografts could be engineered to express HFasL-1 and be protected from acute graft rejection while preserving their endocrine function we transfected islets with HFasL-1 and transplanted them into diabetic mice. This allowed us to follow the endocrine function by blood glucose measurements and to correlate the endocrine results with histologic state of the grafts.

b. Results: Of the initial eight transplanted recipients two died within 3 days of transplantation prior to achieving euglycemia. Of the remaining six recipients, four normalized (day 3 or 4) and two of these four experienced graft failure in the usual time course of allograft rejection (day 14,17). The remaining two had longer term evidence of graft function. On day 53 post-transplant both of the long term survivors underwent nephrectomy removing the graft-

bearing kidney. Both mice remained euglycemic indicating that these animals had experienced spontaneous reversion after alloxan-induced diabetes (a phenomenon which occurred in 13-16% of our study and control animals) (Table 9).

The fact that our long term survivors were spontaneous reversions was reinforced by histologic assessment of the kidney graft sites of each of the long term survivors. There was an absence of demonstrable graft, lymphocyte infiltrate or insulin staining in these tissue sections assessed by H&E stains, aldehyde fuchsin and immunohistochemical staining for insulin (Plate 2).

4.2 Group 2. Allogeneic HFasL-1 inverted blasted controls(n=6)

a. Rationale: Our negative control for HFasL-1 transfected islets was the wildtype gene but cloned into the vector inverted such that it is not translated (the vector used was pCSD7 which is the same as pCSD8 with the polylinker in reverse orientation). This DNA was used to coat 1.6 μm gold particles as for the other blasts.

b. Results: Only two of six control transplants normalized (day 5, 7) and they both experienced evidence of early graft failure (day 22, 15). Six days after graft failure (evidenced by two consecutive blood glucose readings >13.0 mM) the mice were nephrectomized and the grafts subjected to histologic assessment with H&E, aldehyde fuchsin, and immunohistochemical staining for insulin. Control animal 1.2 had been transplanted with 500 islets, normalized on day 7 post transplant, evidenced graft failure on day 15, and had nephrectomy on day 22. This graft shows marked lymphocytic infiltrate with a few isolated nests of β cells on aldehyde fuchsin staining and a small amount of β cell insulin present. Control animal 2.3 received 670 control blasted islets and normalized on day 5 post transplant with graft failure evident on days 22 and nephrectomy on day 28. On thin section histology we see mild cellular infiltrate, a thickened capsule at the previous site of graft but not β cells present and no insulin staining (Plate 3, 4).

4.3 Group 3. Allogeneic Luciferase blasted controls (n=6)

a. Rationale: An alternate control group was transplanted with islets blasted with the CMV-luciferase gene that was used for *in vitro* characterization of blast reproducibility.

b. Results: Of the six control animals in this group four normalized (day 2, 3, 14, 14). On day 27 post transplant, the normoglycemic animals were nephrectomized. One animal remained euglycemic representing a spontaneous reversion. However, three became hyperglycemic after nephrectomy indicating that it was on the basis of a functioning islet cell allograft that they had been maintaining euglycemia. Histologic assessment of these nephrectomies revealed large well preserved grafts in all three cases with marked focal lymphocytic infiltration in one of the grafts but no infiltrate in the others. All three stained unequivocally for insulin in the grafted β cells (Plate 5, 6).

4.4 Group 4. Syngeneic Luciferase blasted controls (n=3)

a. Rationale: Another control group is syngeneic transplantation. It is expected that syngeneic transplants will survive indefinitely as there are no alloantigens present on the allograft to stimulate acute graft rejection. If the syngeneic transplants experience rejection then there must be some unexplained antigenic determinant present.

b. Results: Of the three syngeneic transplants two achieved normoglycemia, one which proved subsequent to nephrectomy (day 34) to be a spontaneous reversion, the other which became hyperglycemic post nephrectomy (day 34) and had histologic evidence of a functioning islet allograft (as would be expected in a syngeneic graft).

4.5 Group 5. Diabetic controls (n=6)

a. Rationale: The point of including data on diabetic mice not treated with an islet cell allograft is to control for the possible failure of chemically induced diabetes to persist.

b. Results: 1/6 (16.7%) of our diabetic control animals exhibited spontaneous reversion following Alloxan-induced diabetes whereas 3/23 (13%) of our study (control or experimental) animals spontaneously reverted.

c. Discussion:

i. There was no long term graft survival in our HFasL-1 transfected study group:

The effect of FasL in providing local immune protection to allografts is inconclusive. Half of the technically successful study animals (2/4) had evidence of early graft failure (day 14, 17) and it was felt that these may represent the variability inherent in the blast process. This may be so, but we had no long term graft survival to compare outcomes to thus we can make no conclusions about this point currently.

ii. There was a high rate of primary nonfunction of transplanted islets:

Primary islet nonfunction was witnessed in 50% of Group 1 transplants, 66% of Group 2, 33% or Group 3, and 33% of Group 4. Of all the allogeneic unblasted controls (n=5) none were technically successful (defined as the return to normoglycemia following islet allograft, data not shown). This experimental arm reflects the initial *in vivo* function of islets (i.e. their functional state after isolation and incubation) and indicates the expected duration of allograft survival without any immunomodulation (the usual time to allograft rejection without immunosuppression is 10-14 days).

Generally 250 islets are sufficient endocrine mass to reverse diabetes in mice and maintain euglycemia. As a rule, however, 500 islets are transplanted to surpass the requirements and allow for some islet attrition during transplantation and re-establishment of vascular supply. One possible explanation for the 100% technical failure rate seen in our unblasted allogeneic transplant controls is that insufficient tissue was transplanted (number of islets transplanted = 500, 300, 490, 250, 270; mean=362). All the animals in our transplant studies (control or experimental) received a minimum of 500 islets. Another possible

explanation for the high rate of islet graft primary nonfunction is that the islets were all quite unhealthy prior to transplantation. This theory is contradicted by the fact that 6/14 mice receiving blasted (control or study) islet allografts did normalize indicating that islet function was adequate.

iii. Luciferase blasted islets enjoyed prolonged islet cell allograft survival: Islet cell function was clinically evident at 35 days in 3/4 technically successful grafts. Histologic assessment confirmed the presence of granulated β cells in the subcapsular space. The reason for allograft protection by Luciferase is not immediately apparent. Luciferase is an intracellular molecule that should not have any modulating effect on cell surface molecule expression. If Luciferase did alter antigenicity it might be expected to have an negative effect on syngeneic allograft survival yet 1/3 syngeneic transplants blasted with Luciferase had long term graft function. In one of the graft histology slides there was a marked *focal* infiltration of lymphocytes indicating that there were lymphocytes present in the system and that there was a clear focus of inflammation quite separate from the rest of the graft which is immediately adjacent (histology not shown). Other potential causes of islet graft primary nonfunction include decreased purity of the graft (i.e. increased contaminating exocrine tissue; the amount of contamination can be quantitated by an amylase content assay), passenger leukocytes (which can be depleted in vitro by special incubation conditions) and MHC modulation (also can be manipulated by incubation conditions). It is unlikely that any of these factors played a significant role as the islets were purified identically for each experiment, and both control and study animals were transplanted with each experiment, thus receiving essentially "identical" islet aliquots. While a conclusion regarding the source of this immune protection is elusive, the take home message is clear: the choice of transfection control is significant in these *in vivo* experiments.

Chapter 4

CONCLUSIONS

1. DNA CLONING

HFasL-1 was cloned, a mutation was repaired and the final sequence verified. HFasL-131, -132, -137 were all obtained by PCR from the wildtype cDNA template using a fastidious enzyme so these clones were not sequenced. HFasR was PCRd after it had been sequenced, and was subcloned into several vectors for use in our laboratory. FasIg was engineered and cloned. The construct was used as the primary antibody for immunohistochemical staining.

2. *IN VITRO* FAS LIGAND CYTOTOXICITY

2.1 HFasL

A ten-fold difference exists between the cytotoxicity of our COS transfected supernatants and those reported by Tanaka and colleagues. This effect has been standard between different batches of transfected COS cells. Several reasons have been offered for the different in degree of cytotoxicity witnessed. Transfection method has been eliminated as a factor as we have received Tanaka's transfection protocol and it does not differ significantly from our protocol. Supernatant processing (such as concentrating by centrifugation or lyophylization) have not been reported by Tanaka. The sensitivity of the FasR+ cell line has been examined as a factor. FasR expressing cell populations appear to be heterogenous with regard to the level of FasR expression by the individual cells in the population. Certain groups have reported needing to FACS their FasR+ cell lines to obtain clones that are highly sensitive to FasL-mediated apoptosis. Because of the low levels of cytotoxicity induced by our FasL constructs against L1210+ cells we obtained an aliquot of L1210+ cells from another lab to

rule out the possibility that the reason they did not respond to FasL-mediated apoptosis was that they had been contaminated or switched with L1210- cells during tissue culture handling. However, the 'new' L1210+ had the same lack of response to FasL as our original population. We also obtained and assayed our supernatants against W4, Tanaka's stable MFasR transformed cell line, and have observed a similar magnitude of cytotoxicity by our supernatants against this cell line as against Jurkat or our own HFasR stable transformant cell lines. The stress of transport of the W4 cells may have caused a nonexpressing population to overgrow the FasR+ cells in the culture.

Another variable that has been raised to account for differences in cytotoxicity reported between ourselves and Tanaka's group is the method of assaying cell viability. Again this does not appear to be a variable as Tanaka et al. report using an MTT kit, like the system we use so assay sensitivity does not account for our results although the reported results are in units which would cause our results to differ by 10-fold (they express killing in units per mL and we express ours as units per 100 μL).

One final issue that has not yet been examined is the use of dissimilar plasmid vectors by Tanaka and ourselves and the possible effect this may have on eukaryotic cell expression of the cloned gene.

2.2 MFasL

Our MFasL-1 results are also at variance with published results which suggest that MFasL has a fraction of the effect of HFasL-1 against W4 cells. We have two interesting results in this regard. First, MFasL-1 has the same magnitude of effect as HFasL-1 against the Jurkat cells that we tested. We also found that MFasL-1 truncated forms had as little effect as the shortened HFasL-1 constructs. From these two findings we infer that HFasL-1 and MFasL-1 do act in a similar and species independent manner. The fact that both human and mouse shortened constructs failed to cause any significant *in vitro* cytotoxicity in implies that the

molecules have similar cleavage and binding sites which were altered by the site at which we chose to truncate them.

A final conundrum of MFasL-1 was the finding that it had little effect against BW5147 stable transformants expressing HFasR whereas HFasL-1 had a significant effect against these cell lines. This is the first result that is discrepant between the two species. First this experiment needs to be repeated and the finding verified. If it is a real result then it may indicate polymorphism in the MFasL-1 (if the result were due to a polymorphism in the HFasR then I would anticipate that HFasL-1 would have an altered effect which it did not) although the supernatants used were the same ones which had been cytotoxic to other FasR+ cells.

3. *IN VITRO* ISLET ASSAYS

HFasL-1, at concentrations high enough to cause a 50% reduction of cell viability in sensitive FasR+ cells, did not have a toxic effect on islet insulin production *in vitro*. Several variables in the experiment conducted need further characterization. First, the DNA content of the islets pre- and post-assay should be assayed to quantitate islet survival. Second, the duration of HFasL-1 exposure was 48 hours. If islets are engineered to secrete FasL they will be exposed to high levels for a long period of time, thus to further characterize the FasL effect on islets a longer incubation should be performed, following which the islets should be allografted to assess their *in vitro* function after FasL exposure. Thirdly, MFasL-1 should be assayed for effect against human and mouse islets. If there is a species difference in effect it may have a bearing on future transplantation experiments (i.e. when transplanting mouse islet allografts it may be preferable to transfect them with MFasL-1)

4. TRANSPLANTATION

In the allografted HFasL-1 transfected islets 2/4 animals receiving functioning islets appeared to have long term graft function. However, when these animals were nephrectomized they remained normoglycemic, thus they represented failure of Alloxan-induced diabetes. Histologic assessment of the removed kidneys revealed no evidence of graft tissue. Luciferase blasted controls however had a surprising outcome. Four of six transplanted animals were considered technically successful (blood glucose normalized after islet transplantation). Three of the four successful grafts had clinical evidence of graft function at the time nephrectomies were performed and histologic assessment confirmed that each of these animals had well preserved functioning islet cell tissue. The reason for a protective effect of the luciferase gene on islet cell transplants is unclear. Systemically administered gold is an immune-suppressant used therapeutically for the autoimmune disease, rheumatoid arthritis, and some thought has been given to the blasted gold particles causing local immunosuppression. However, this should be an effect witnessed for all blasted control islets, but our inverted gene controls (HFasL-1 in pCSD8) experienced graft rejection at the expected rate and showed histologic evidence of allograft rejection.

POSTSCRIPT

Throughout this project we have been cognizant that acute allograft rejection results from an exceedingly complex web of reactions. We have been aware and even fearful that the Fas system is too simple a solution for such a complex problem. This however has been the elegance and excitement of this project. At different times throughout the year our confidence in our approach has been bolstered as the monthly journal articles have appeared with more developments in the Fas story. In October a Nature paper showed that Sertoli cells could be transplanted against allogeneic barriers and survive without immunosuppression, presumed secondary to the immune protection conferred by FasL expressed by the Sertoli cells of the graft. In November Science a second paper described the role of FasL in conferring immune privilege to all the structures of the eye.[15,41]

The fruit of our labor this year is the accrual of a number of useful reagents, not the least of which are the FasL wildtype and truncated genes (both mouse and human), human FasR, the human FasIg construct and stable producer cells lines (HFasL-1, HFasR, HFasIg). Further characterization of these constructs since my departure from the lab has revealed that one of our HFasR stable transformants that had been identified as a high level expressor (Clone 114) is exquisitely sensitive to HFasL-137 killing but has little sensitivity to MFasL-1. These results are consistent with those of Tanaka et.al. and imply that the FasR+ cell lines I was employing in my bioassays (Jurkat E6-1) were not sufficiently sensitive to detect this effect.[50] Recloning our native FasR+ cell lines (Yac-1, L1210+ or Jurkat E6-1) may have provided us with a sensitive assay without the need for creating stable transformant cell lines.

While our transplantation efforts were confounded by the technical problems of biolistic transfection, other strategies were attempted including the coencapsulation of stably transformed HFasL-1 cells lines with islet cells (which were unsuccessful as the transformed BW5147 survived <24 hours once encapsulated), and the construction of a recombinant

adenovirus (which to date this has not been accomplished by any of the labs attempting it). Ongoing work with coencapsulation of FasL-expressing Sertoli cells with islet cells and the development of a sensitive FasL ELISA assay for the purpose of establishing a dose-response curve in transplantation experiments were technically not feasible earlier because the reagents or the techniques were not yet available.

Our intention to use FasL as a local immunomodulator has been further validated by a recent study published in Science.[54] In this work, islet allograft rejection has been prevented by cotransplantation of islets with syngeneic myoblasts which are stably transformed with FasL. This effect has been proven both site-specific and antigen-specific (in that third party donor tissues are rejected), and a dose-response has been noted in the mean survivals of the transplanted animals.

While the goal of this research, which was to allow transplantation of islet cell allografts without the need for chronic immunosuppression, remains elusive, our approach has been, and continues to be, valid and worthy of continued attention.

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Table 1. Outline of Studies Performed

Construct	I. Cloning	II. Cytotoxicity *In vitro*	III a. Islet Cytotoxicity *In vitro*	III b. Islet Transfection *In vitro*	IV. Islet Transplantation
HFasL-1	-Cloned into vectors pBluescript SK-, pSRaSD7, pCSD7, pCA14/Not1 -Sequenced	-Applied to FasR+ cells and had expected effect against Jurkat, our own stable HFasR transformed cell lines (HFasR-26, 62, 114) and W4 -Generated HFasL stable transformants	-No cytotoxic effect was caused by applying HFasL-1 to islet cells as measured by islet responsiveness to a glucose challenge	-Biolistics used for transfection; no assay sensitive enough to quantitate the amount of FasL expressed by transfected islets. -Failed attempt to create a recombinant adenoviral construct	-Preliminary transplant experiments were performed using HFasL-1 as the sole immuno-protectant
HFasL -131	-Cloned into pBluescript SK-pSRaSD7	-No significant cytotoxic effect against FasR+ cell lines therefore abandoned	-Not carried on because minimal cytotoxicity *in vitro*	-Not done	-Not done
HFasL -132	-Cloned into pBluescript SK-pSRaSD7	-No significant cytotoxic effect against FasR+ cell lines therefore abandoned	-Not carried on because minimal cytotoxicity *in vitro*	-Not done	-Not done
HFasL -137	-Cloned into pBluescript KS+, pBSK-, pSRaSD7, pCSD7, pCA14/Not1	-No significant cytotoxic effect against FasR+ cell lines therefore abandoned	-Not carried on because minimal cytotoxicity *in vitro*	-Not done	-Not done
MFasL-1	-Cloned by Dr. C. Luo and E. Chan -recloned into pBluescript KS+, pSRaSD7, pCSD7, pCA14/Not1	-Similar cytotoxic effect against FasR+ cells as HFasL-1	-Only HFasL-1 assayed because of time constraints	-Not done	-Not done
MFasL -300	-Cloned by Dr. C. Luo and E. Chan	-No significant cytotoxic effect against FasR+ cell lines therefore abandoned	-Not carried on because minimal cytotoxicity *in vitro*	-Not done	-Not done
MFasL -331	-Cloned by Dr. C. Luo and E. Chan	-No significant cytotoxic effect against FasR+ cell lines therefore abandoned	-Not carried on because minimal cytotoxicity *in vitro*	-Not done	-Not done
HFasR	-Cloned into pBluescript KS+, SK-pSRaSD7, pCSD7	-Used to generate stable transformants (BW5147/ HFasR)	-Not done	-Not done	-Not done
HFasIg	-Cloned into pBluescript KS+, SK- pSRaSD7, pCSD7	-Used as reagent to block FasL mediated cytotoxicity -Generated Stable transformants (BW5147/HFasIg) -Also used to stain FasL expressing cells for FACS analysis	-Not done	-Not done	-Not done

Table 2. Oligonucleotide Reference

Oligo name	cDNA Binding Region	Oligo Length	Restriction Site	Sequence
HFasL-5'	1-20	37	XbaI	ctctcttctaga*ccaccATGCAGCAGCCCT TCAATTA
HFasL-3'	822-846	39	NotI	ctctctgcggccgcTTAGAGCTTATATAA GCCGAAA
HFasL 131-5'	391-408	30	BamHI	ctctctggatccATAGGCCACCCCAGTCC A
HFasL 132-5'	394-409	28	BamHI	ctctctggatccGGCCACCCCAGTCCAC
HFasL 137-5'	409-428	32	XbaI	ctctcttctagaCCCCCTGAAAAAAAGGA GC
HFasLm-5'	400-416	41	-	gctagcgcatctgcctcagctagcCCCAGTCCA CCCCCTG
HFasLm-3'	393-369	41	-	gctagctgaggcagatgcgctagcTGTGTGCAT CTGGCTGG
HFasL-S1	231-249	19	-	CAGCACAGGCCTGTGTCTC
HFasL-S2	423-443	20	-	AGGAAAGTGGCCCATTTAAC
HFasL-S3	603-621	19	-	CAGGGGCAGGTTGTTGCAA
HFasL-S4	405-424	20	-	CCTTTTTTTCAGGGGGTGGA
HFasL S5	221-239	19	-	CCTGTGCTGTGGTTCCCTC
HFasLrp-5'	493-523	31	-	acctatggaaTTGTCCTGCTTTCTGGAG TGA
HFasLrp-3'	480-509	30	-	aggacaatTCCATAGGTGTCTTCCCAT TCC
sHFasR-5'	1-18	35	XbaI	ctctcttctagaccaccATGCTGGGCATCTG GACC
sHFasR-3'	482-502	32	AatII	ctctctgacgtcCTCTTTGCACTTGGTGTT GC
HFasR-3'	988-1008	34	NotI	ctctctgcggccgcCTAGACCAAGCTTTG GATTTC
HFasIg-5'	469-488	32	AatII	ctctctgacgtcGAGCCCAAATCTTCTGA CAA
HFasIg-3'	1158-1175	24	NotI	agagagGCGGCCGCTCATTTACCC
MG-CSF	1-116	116	-	ATGGCTCAACTTTCTGCCCAGAGG CGCATGAACCTAATGGCCCTGCAG CTGCTGCTGTGGCAAAGTGCACTA TGGTCAGGACGAGAGGCCGTTCCC CTGGTCACTGTCAGCGCTCTagaa**
MG-CSF-5'	1-18	37	MluI	ctctctacgcgtccaccATGGCTCAACTTTC TGCCCA
MG-CSF-3'	98-116	29	XbaI	agagagttctAGA**GCGCTGACAGTGA CCA

*Restriction enzyme recognition sites underlined in each entry

**The oligonucleotide ends at base 116 but the final four bases in lower case are proved by the MG-CSF-3' primer and with the terminal cytosine and thymidine residues comprise the final two codons (Leu, Glu) of the MG-CSF protein.

Table 3: Cloning Reference

Insert	Vector pBluescript KS+	pBluescript SK-	pBSK-*	pSRaSD7	pCSD7	pCA14/NotI
HFasL-1	(XbaI/NotI)	XbaI/NotI	-	EcoRI/NotI**	XbaI/NotI	XbaI/NotI
HFasL-131	(BamHI/NotI)	BamHI/NotI	-	EcoRI/NotI**	-	-
HFasL-132	(BamHI/NotI)	BamHI/NotI	-	EcoRI/NotI**	-	-
HFasL-137	MluI/NotI	Mlu/NotI	Mlu/NotI	Mlu/NotI	Mlu/NotI	XhoI/NotI
MFasL-1	XbaI/NotI	-	-	XbaI/NotI	XbaI/NotI	XbaI/NotI
HFasR	-	XbaI/NotI	-	XbaI/NotI	XbaI/NotI	-
HFasIg	XbaI/NotI	XbaI/NotI	-	XbaI/NotI	XbaI/NotI	-

* pBSK- is pBluescript SK- with a modified polylinker (MluI substituted for EcoRV, see Figure 7).
**pSRαSD7 has two BamHI sites thus unable to clone the truncated constructs as engineered (BamHI/NotI) so cloned EcoRI/NotI
( ) constructs which were never recloned into this vector after the initial mutation in HFasL-1 was repaired

Table 4. Tissue Culture Reference

Cell Line	Origin	Growth Media	Characteristics
COS	-Lab stock	DMEM + 10% FCSII	-Green monkey kidney cells
L1210+	-Lab stock (ATCC CCL219) -Bleakley lab	RPMI + 10% FCSI	-Murine lymphocytic leukemia (FasR+)
L1210-	-Lab stock	RPMI + 10% FCSI	-Murine lymphocytic leukemia (FasR-)
Jurkat E6-1	-Ordered (ATCC E6-1)	RPMI + 10% FCSI	-Human acute T cell leukemia (FasR+) -FasR expression induced with mitogens (PMA, Ionomycin, Concanavalin A)
Yac-1	-Bleakley lab (ATCC TIB160)	RPMI + 10% FCSI	-Murine lymphoma (FasR+) -Moloney leukemic virus induced lymphoma in newborn mice
BW5147 .G.1.4.OUAR.1	-Lab stock (ATCC CRL 1588)	RPMI + 10% FCSI	-Murine T cell lymphoma (FasR-)
EL4-IL2	-Ordered (ATCC TIB 181	DMEM + 10% FCSI	-Murine lymphoma cell line
WR19L	-Ordered (ATCC TIB 52)	DMEM + 10% FCSI	-Murine tumor induced in Balb/c mouse after innoculation with virus originiating from Abelson leukemia virus -W4 is the WR19L cell line stably transformed with mouse FasR
293	-Lab stock (ATCC CRL 1573)	DMEM + 10% FBS	-Human transformed primary embryonal kidney tissue
PA317	-Chang lab	DMEM + 10% FBS	-Retroviral packaging cell line -Amphotrophic

DMEM: Dulbecco's Minimum Essential Media (Gibco)
RPMI: Rothwell Park Memorial Institute (Gibco)
FCSI: Fetal Clone Serum I (Gibco)
FCSII: Fetal Clone Serum II (Gibco)
FBS: Fetal Bovine Serum (Gibco/Hyclone; Hyclone preferred)

Dilution	Control Mean O.D.*	Clone 47 Mean O.D.	Clone 47 Reduction vs control (%)	Clone 162 Mean O.D.	Clone 162 Reduction vs control (%)	Clone 171 Mean O.D.	Clone 171 Reduction vs control (%)
1:2	0.57	0.4915	0.14	0.4595	0.19	0.5395	0.05
1:8	0.5435	0.512	0.06	0.48	0.12	0.52	0.04

*O.D.= optical density

Table 5. HFasL-1 Stable Transformants versus FasR+ cells BW5147 cells transformed with HFasL-1 were selected for HFasL-1 expression by FACS. The supernatants from the high level expressors Clones 47, 162 and 171 were applied to Jurkat E6-1 cells and an MTT performed to quantitate their apoptotic effect. All the supernatants had minimal effect. Clone 162 had the greatest reduction in cell viability at 19% with 1:2 dilution, and Clone 171 had the least effect with 5% reduction in cell viability (compared to the effect of control supernatant from nontransformed BW5147 cells) implying that either the bioassay is insufficiently sensitive or these cells do not have the enzymes necessary to cleave FasL to its soluble form.

Dilution	Control Mean O.D.*	Clone 26 Mean O.D.	Clone 26 Reduction vs control (%)	Clone 62 Mean O.D.	Clone 62 Reduction vs control (%)	Clone 114 Mean O.D.	Clone 114 Reduction vs control (%)
1:2	.944	.575	39%	.729	23%	.725	23%
1:8	.819	.79	4%	.952	-16%	.907	-11%

*O.D.= optical density

Table 6. HFasR Transformant Sensitivity to MFasL-1 Killing BW5147 cells transformed stably with HFasR were selected for HFasR expression by FACS. The highest level expressors (Clones 26, 62, 114) were exposed to MFasL-1 or control supernatant (ie. supernantant from wildtype BW5147 cells) in an MTT assay to assess their sensitivity to FasL-1 killing compared to other FasR+ cell lines (ie. Jurkat E6-1). Clone 26 was the most sensitive with a 39% reduction in mean optical density at a 1:2 dilution. A similar but two-fold greater effect was seen with HFasL-1 applied to these stable HFasR transformants (Fig 15). The effect tapered rapidly and was insignificant by a 1:8 dilution.

Dilution	W4 Mean O.D.*	Jurkat Mean O.D.*
1:2	0.228	0.142
1:4	.575	.489
1:8	.801	.878
1:16	.862	1.027
1:32	.902	1.029
1:64	.886	1.1
1:128	.874	1.111
1:256	.814	1.166

*O.D. = optical density

Table 7. HFasL-1 Effect Against Jurkat E6-1 and W4 HFasL-1 supernatant was applied to both W4 and Jurkat E6-1 cells (50,000 cells per well, 100 µl of supernatant incubated for 24 hours in a standard MTT assay). As indicated by the mean optical density readout from the MTT the cell lines were similarly sensitive to HFasL-1 cytotoxicity although W4 cells were less sensitive than Jurkat at the highest dilution (1:2, mean optical density W4 was 0.228 versus Jurkat at 0.142) and the effect plateaued in the W4 at a lower dilution than in the Jurkat.

Incubation Conditions		Human Islets		Mouse Islets	
1:4 Dilution	[Glucose]	Insulin Production ng/islet/h Mean±SD	Stimulation Index	Insulin Production ng/islet/h Mean±SD	Stimulation Index
hFasL n=4	2.8 mM	1.2±0.12	8.6±0.8	2.3±0.3	4.5±0.3
	28 mM	10.1±0.6		10.2±0.6	
Control n=4	2.8 mM	0.88±0.1	8.9±0.6	2.1±0.2	4.9±0.04
	28 mM	7.8±0.4		10.1±1.0	

Table 8. Islet Function in the Presence of HFasL-1. Groups of 500 islets(human and mouse) were incubated with HFasL-1 for 48 hours. The Stimulation Index (ratio of insulin production in the presence of high versus low glucose) was the same whether islets had been exposed to HFasL-1 or a control supernatant.

Table 9. Preliminary Transplantation Results

Study Group	Animal No.	No. Islets Transplanted	Graft Success	Days to Graft Failure/ Nephrectomy	Spontaneous Reversion	Histology
Group 1: Allogeneic HFasL-1 Blasted	6.2	500	-	-	-	-N/A
	6.3	500	+	Failure day 14	-	-N/A
	6.4	500	-	-	-	-N/A
	6.5	500	+	Failure day 17	-	-N/A
	7.2	500	+	Nephrectomy day 50	+	-no graft -no infiltrate
	7.3	500	-	-	-	-N/A
	7.4	500	-	-	-	-N/A
	7.5	500	+	Nephrectomy day 50	+	-no graft -no infiltrate
Group 2: Allogeneic HFasL-1 Inverted Gene Blasted Controls	1.1	500	-	-	-	-N/A
	1.2	500	+	Failure day 15 Nephrectomy day 22	-	*graft infiltrate *few isolated nests of B cells *small amount insulin staining
	1.3	500	-	-	-	-N/A
	1.4	500	-	-	-	-N/A
	1.5	500	-	-	-	-N/A
	2.3	670	+	Failure day 22 Nephrectomy day 28	-	*lymphocyte infiltrate -no insulin staining
Group 3: Allogeneic Luciferase Blasted Control	2.1	500	+	Nephrectomy day 42	+	+mild cellular infiltrate -no insulin present
	4.2	500	-	-	-	-N/A
	4.3	500	+	Nephrectomy day 27	-	*small graft with insulin staining
	4.4	500	+	Nephrectomy day 27	-	*large graft with insulin staining
	4.5	500	-	-	-	-N/A
	4.6	400	+	Nephrectomy day 27	-	*cellular infiltrate in focal portion of graft **insulin
Group 4: Syngeneic Luciferase Blasted Controls	3.1	450	+	Nephrectomy day 33	+	-N/A
	3.2	500	-	-	-	-N/A
	3.3	450	+	Nephrectomy day 34	-	*graft with insulin staining
Group 5: Diabetic Controls (no transplant)	1.6	-	-	Normal day 11	-	-N/A
	2.6	-	-	Sacrificed day 42	-	-N/A
	3.4	-	-	Deceased day 8	-	-N/A
	4.7	-	-	Sacrificed day 33	-	-N/A
	6.6	-	-	Sacrificed day 19	-	-N/A
	7.6	-	-	Sacrificed day 52	-	-N/A

```
1/1
ATG CAG CAG CCC TTC AAT TAC CCA TAT CCC CAG ATC TAC TGG GTG GAC AGC AGT GCC AGC
Met gln gln pro phe asn tyr pro tyr pro gln ile tyr trp val asp ser ser ala ser
61/21
TCT CCC TGG GCC CCT CCA GGC ACA GTT CTT CCC TGT CCA ACC TCT GTG CCC AGA AGG CCT
ser pro trp ala pro pro gly thr val leu pro cys pro thr ser val pro arg arg pro
121/41
GGT CAA AGG AGG CCA CCA CCA CCG CCA GCG CCA CCA CTA CCA CCT CCG CCG CCG CCG
gly gln arg arg pro pro pro pro pro pro pro pro pro leu pro pro pro pro pro pro
181/61
CCA CCA CTG CCT CCA CTA CCG CTG CCA CCC CTG AAG AAG AGA GGG AAC CAC AGC ACA GGC
pro pro leu pro pro leu pro leu pro pro leu lys lys arg gly asn his ser thr gly
241/81
CTG TGT CTC CTT GTG ATG TTT TTC ATG GTT CTG GTT GCC TTG GTA GGA TTG GGC CTG GGG
leu cys leu leu val met phe phe met val leu val ala leu val gly leu gly leu gly
301/101
ATG TTT CAG CTC TTC CAC CTA CAG AAG GAG CTG GCA GAA CTC CGA GAG TCT ACC AGC CAG
met phe gln leu phe his leu gln lys glu leu ala glu leu arg glu ser thr ser gln
361/121                                    ↓  ▼                    ▽
ATG CAC ACA GCA TCA TCT TTG GAG AAG CAA ATA GGC CAC CCC AGT CCA CCC CCT GAA AAA
met his thr ala ser ser leu glu lys gln ile gly his pro ser pro pro pro glu lys
421/141
AAG GAG CTG AGG AAA GTG GCC CAT TTA ACA GGC AAG TCC AAC TCA AGG TCC ATG CCT CTG
lys glu leu arg lys val ala his leu thr gly lys ser asn ser arg ser met pro leu
481/161
GAA TGG GAA GAC ACC TAT GGA (A)T GTC CTG CTT TCT GGA GTG AAG TAT AAG AAG GGT GGC
glu trp glu asp thr tyr gly ile val leu leu ser gly val lys tyr lys lys gly gly
541/181
CTT GTG ATC AAT GAA ACT GGG CTG TAC TTT GTA TAT TCC AAA GTA TAC TTC CGG GGT CAA
leu val ile asn glu thr gly leu tyr phe val tyr ser lys val tyr phe arg gly gln
601/201
TCT TGC AAC AAC CTG CCC CTG AGC CAC AAG GTC TAC ATG AGG AAC TCT AAG TAT CCC CAG
ser cys asn asn leu pro leu ser his lys val tyr met arg asn ser lys tyr pro gln
661/221
GAT CTG GTG ATG ATG GAG GGG AAG ATG ATG AGC TAC TGC ACT ACT GGG CAG ATG TGG GCC
asp leu val met met glu gly lys met met ser tyr cys thr thr gly gln met trp ala
721/241
CGC AGC AGC TAC CTG GGG GCA GTG TTC AAT CTT ACC AGT GCT GAT CAT TTA TAT GTC AAC
arg ser ser tyr leu gly ala val phe asn leu thr ser ala asp his leu tyr val asn
781/261
GTA TCT GAG CTC TCT CTG GTC AAT TTT GAG GAA TCT CAG ACG TTT TTC GGC TTA TAT AAG
val ser glu leu ser leu val asn phe glu glu ser gln thr phe phe gly leu tyr lys
841/281
CTC TAA
leu OCH
```

Figure 1. HFasL-1 cDNA Sequence. A PCR error was identified at base 502 in wildtype Clone 1 (circled). This was repaired using a PCR strategy and verified sequence perfect in at least one strand throughout the length of the gene. Truncated constructs HFasL-131 ↓ HFasL-132 ▼ and HFasL-137 ▽ were generated from sequence perfect wildtype.

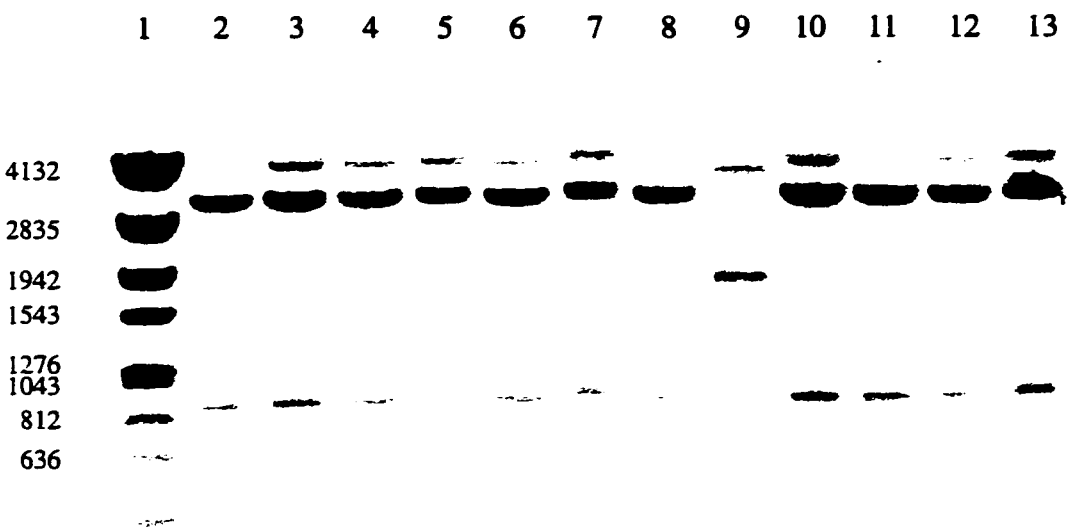


Figure 2. Restriction Enzyme Analysis of HFasL-1 Mini Preps. Digested pCDM8 (lane 1) provides size markers. Clone 1 (lanes 2-4), Clone 2 (lanes 5-7), Clone 6 (lanes 11-13) all mapped appropriately with XbaI/NotI restriction enzyme analysis. Clone 4 (lanes 8-10) had two of three colonies map appropriately. Subsequently Clone 1 and Clone 6 were sequenced. Clone 6 had multiple mutations and was abandoned. Clone 1 had a single transition mutation which was repaired.

Wildtype HFasL-1

GAA GAC ACC TAT GGA A*TT GTC CTG CTT TCT GGA

Glu Asp Thr Tyr Gly Ile* Val Leu Leu Ser Gly

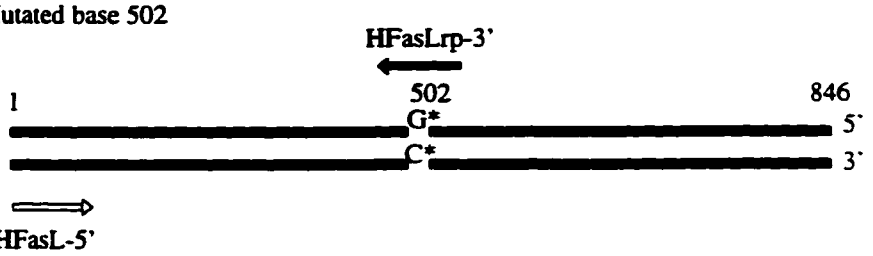
HFasL-1 Mutated base 502

GAA GAC ACC TAT GGA G*TT GTC CTG CTT TCT GGA

Glu Asp Thr Tyr Gly Val * Val Leu Leu Ser Gly

PCR Reaction I.

Template: HFasL-1 Mutated base 502



Primers: HFasLrp-3' Identical to lower strand except encodes correct base (T) at base 502
Anneals to upper strand bases 480-509

HFasL-5' Identical to upper strand HFasL-1 cDNA
Anneals to lower strand bases 1-20

Product: 5' Species

Figure 3. Polymerase Chain Rection Mutation Repair Strategy I. The mutated gene HFasL-1 Clone 1 was used as template for a several step repair process. Primers were designed to correct the base mutation and in the first reaction generate the 5' end of the gene up to and including the repaired mutation.

PCR Reaction II

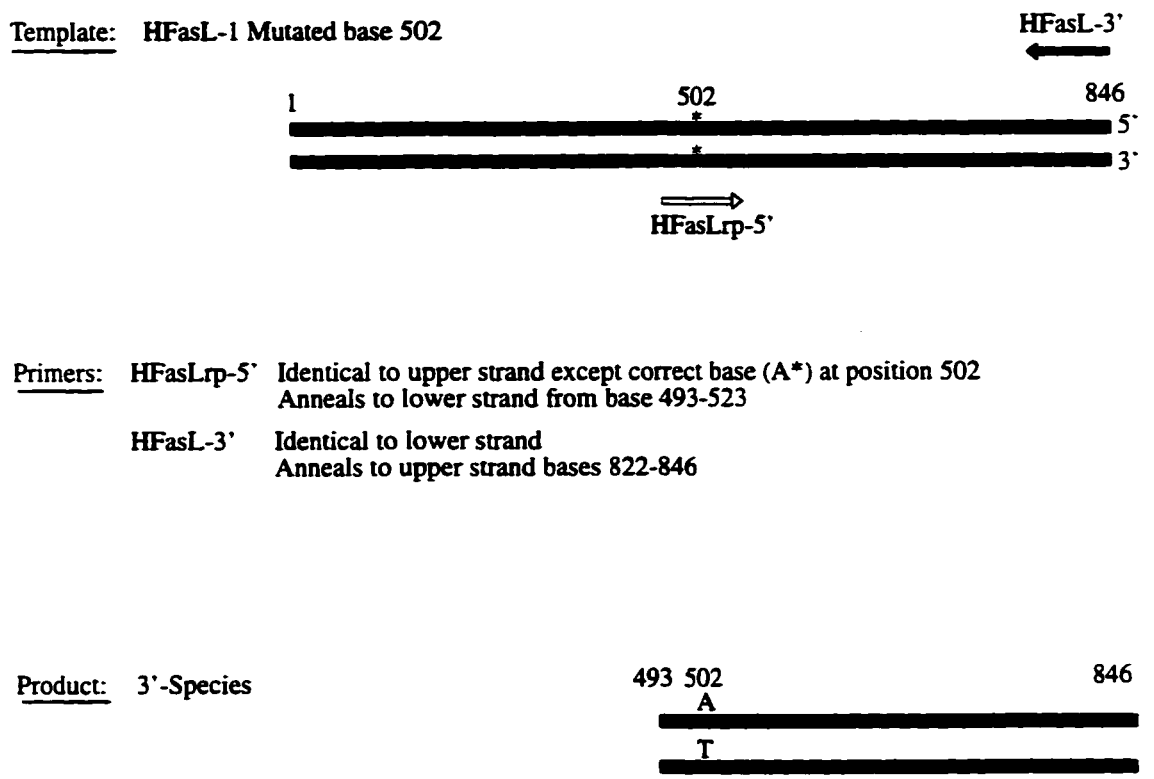
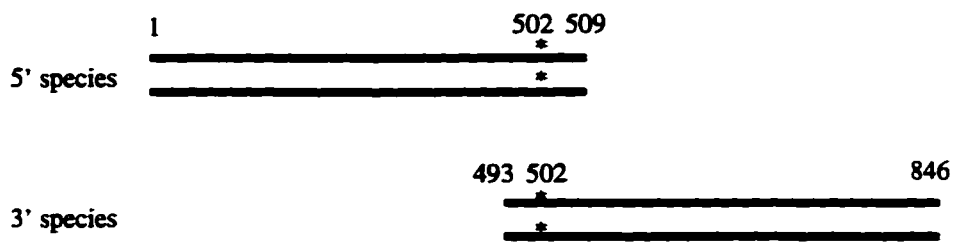


Figure 4. Polymerase Chain Rection Mutation Repair Strategy II. In the second step of mutation repair another set of primers were desgined to repair and amplify the 3' end of the gene starting just proximal to the mutation (enough to allow a 16 base pair overlap between the 5' and 3' species).

PCR Reaction III

Template:



Primer: HFasL-5'

HFasL-3'

Product:

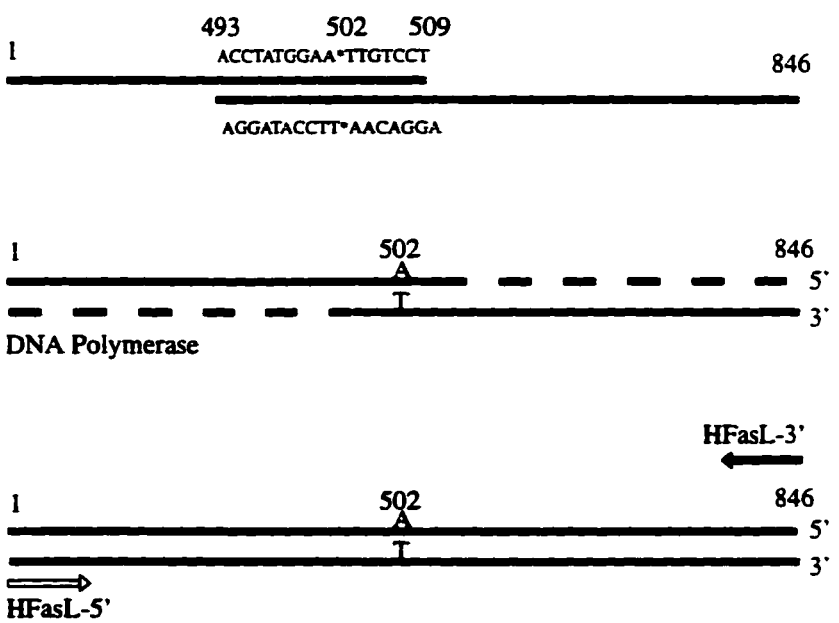


Figure 5. Polymerase Chain Rection Mutation Repair Strategy III. The repaired 5' and 3' portions of the HFasL-1gene provided the template DNA for the final PCR reaction. During the denaturing step the double-stranded DNA dissociates from its complementary strand. Some of these dissociated strands will reanneal with their complementary strands but some will anneal with the opposite DNA species because of the 16 base complementary overhang. DNA polymerase fills in the bases to generate a full length repaired HFasl-1 gene which is amplified by the PCR reaction.

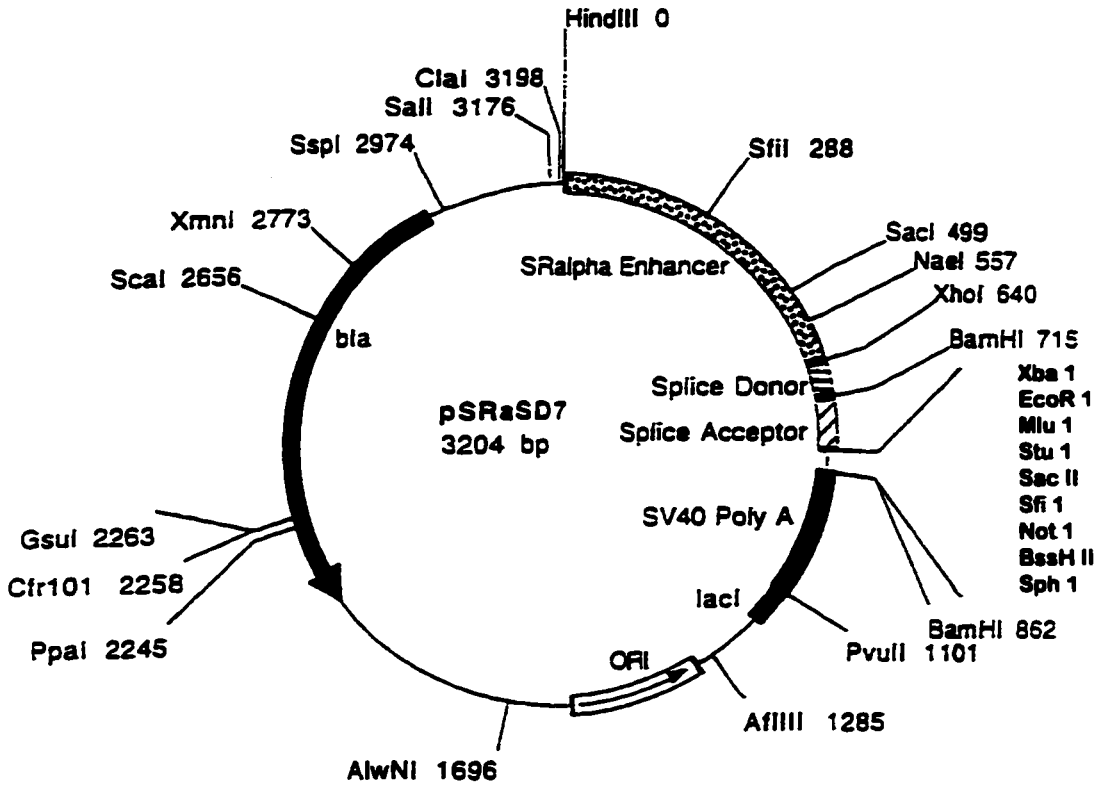


Figure 6. pSRαSD7 Eukaryotic Expression Vector.

This vector constructed by Dr. Dan Denney was used for eukaryotic transfection and transformation.

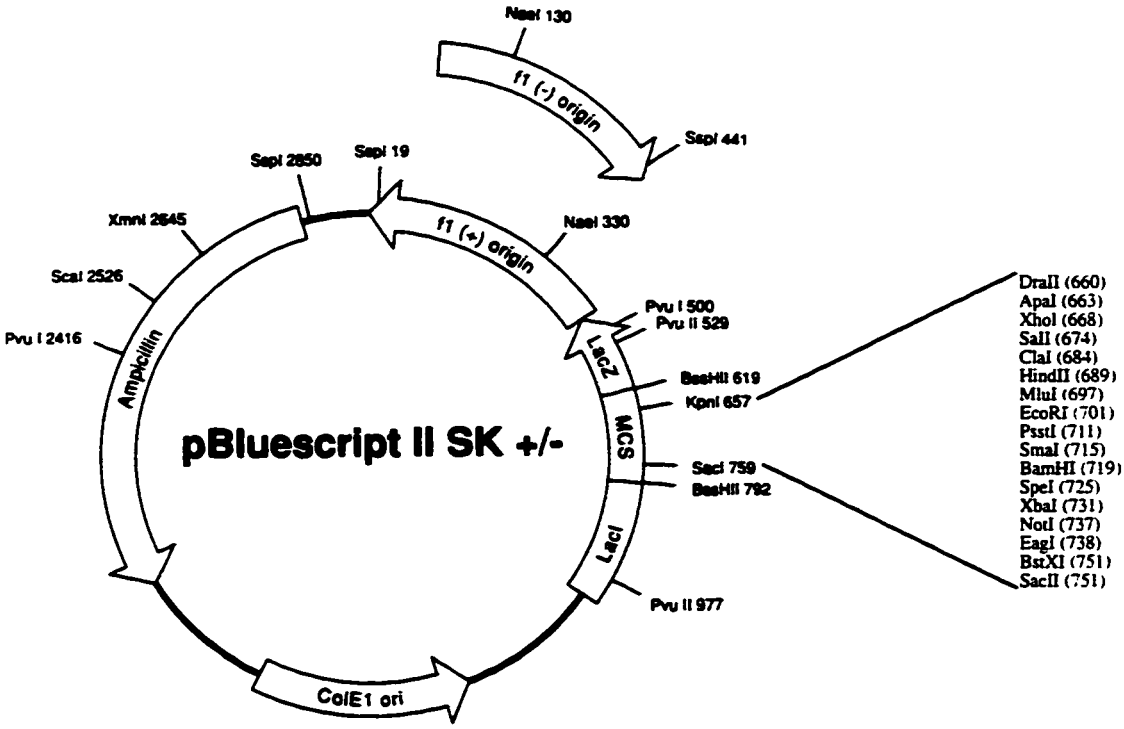


Figure 7. pBSK- Polylinker. This polyliner originally from pBluescript SK- designed by Lisa Purdy has an MluI site which replaces the EcoRV site and was used for cloning HFasL-137 (MluI/NotI).

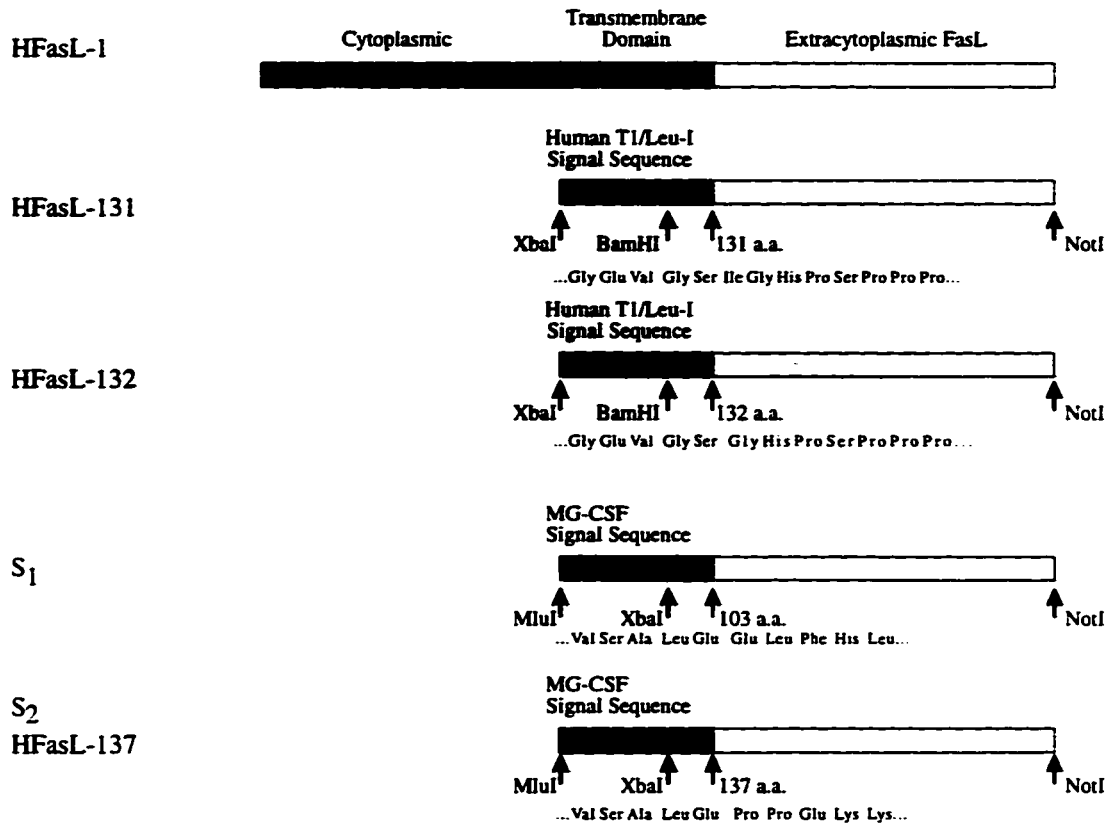


Figure 8 (a). HFasL Constructs. Wildtype HFasL is depicted schematically with cytoplasmic, membrane-spanning and extracytoplasmic regions of the protein. In the shortened constructs the cytoplasmic and transmembrane domains are excluded and replaced with a signal sequence. HFasL-131. and HFasL-132 were engineered with the Human T1/Leu-1 signal sequence (see box inset). HFasL-137 was engineered identical to published literature (S2, Tanaka et al.) with mouse G-CSF signal sequence. S1 was not generated but is included for comparison.

Human T1/Leu-I Signal Sequence

XbaI — Kozak Consensus

```
tct aga   cc acc ATG CCC ATG GGG TCT CTG CAA CCG CTG GCC ACC TTG
                  Met Pro Met Gly Ser Leu Gln Pro Leu Ala Thr Leu

                                                        BamHI
TAC CTG CTG GGG ATG CTG GTC GCT TTC TGC CTC GGA gaa gtc gaa tcc
Tyr Leu Leu Gly Met Leu Val Ala Ser Cys Leu Gly Glu Val Gly Ser
```

MG-CSF Signal Sequence:

```
ctc tct acg cgt ccacc ATG GCT CAA CTT TCT GCC CAG AGG CGC ATG AAC CTA ATG GCC
      MluI  Kozak      Met Ala Gln Leu Ser Ala Gln Arg Arg Met Lys Leu Met Ala
            Consensus
CTG CAG CTG CTG CTG TGG CAA AGT GCA CTA TGG TCA GGA CGA GAG GCC GTT
Leu Gln Leu Leu Leu Trp Glu Ser Ala Leu Trp Ser Gly Arg Glu Ala Val

                                  XbaI
CCC CTG GTC ACT GTC AGC GCT CTa gaa ctc tct
Pro Leu Val Thr Val Ser Ala Leu Glu
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Figure 8 (b). Signal Sequences.

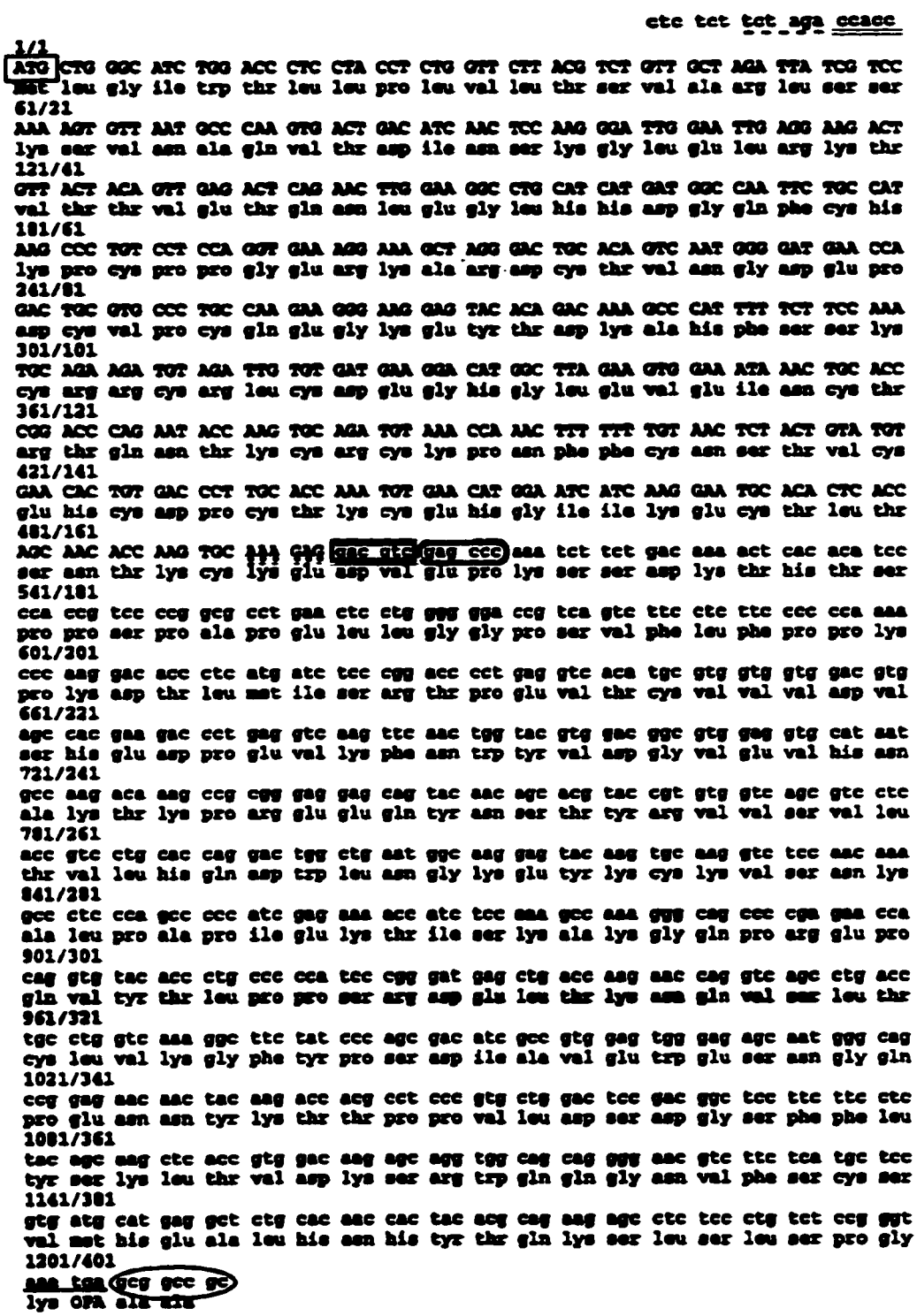

Figure 9. HFasIg Construct cDNA Sequence. A chimera was designed with HFasR binding specificity and a human IgG1 tail (the Fc portion of the immunoglobulin). It is comprised of the first 502 bases of HFasR joined at an AatII restriction cleavage site to the terminal 706 bases of IgG1. The following sites are coded by the gene sequence. XbaI cleavage site - - - - -; Kozak concensus sequence ═══, HFasR wildtype start codon ▭, HFasR (bases 496-502) ... ..., AatII cleavage site ▭, IgG1 (bases 469-475) ⬭, IgG1 (bases 1170-1175) ———, and NotI cleavage site ⬭.

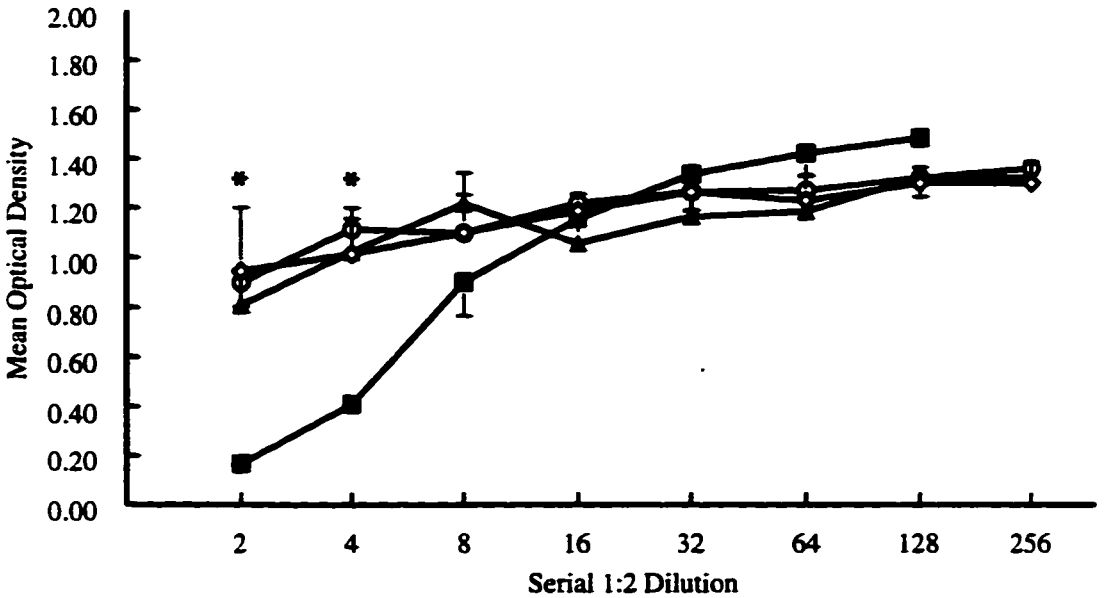

Figure 10. HFasL Constructs versus Jurkat E6-1 Cells. The cytotoxic effect of HFasL transfected COS supernatants versus FasR+ human T cell line, Jurkat E6-1. A significant difference between the means of this experiment performed in triplicate persisted to a 1:8 dilution (*ANOVA $p < 0.005$). The only construct to have statistically significant difference in mean optical density compared to control —▲— was HFasL-1 —■— (t-test $p<0.01$ using Bon Feronni adjustment with $\alpha = 0.05$) and this effect extinguished by 1:8 dilution. None of the other constructs HFasL-131 —◇—or HFasL-132 —○— displayed any significant cytotoxicity.

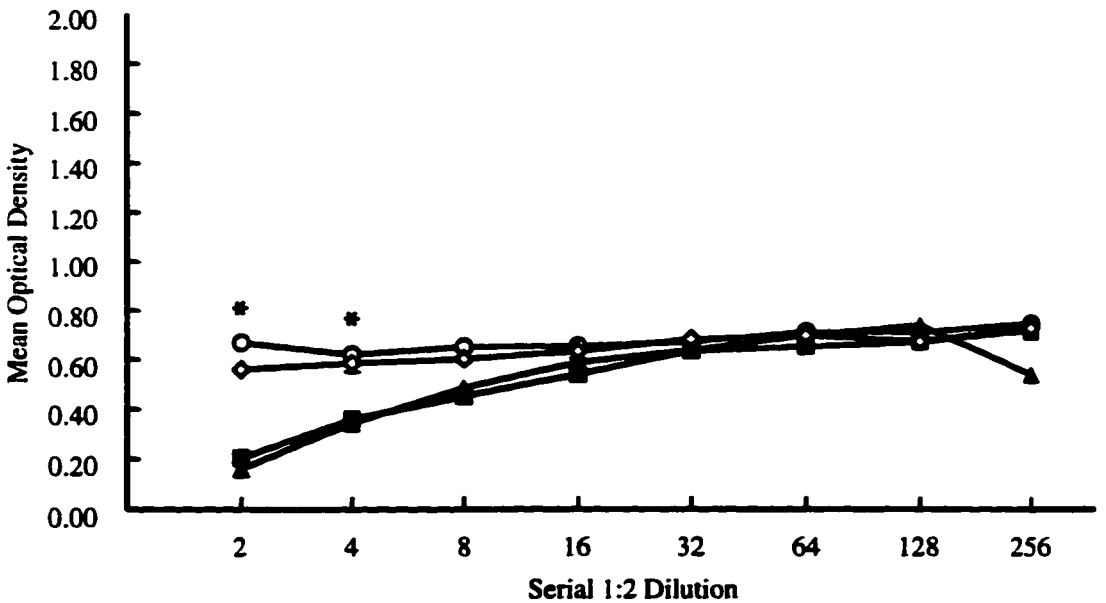

Figure 11. FasL Constructs versus FasR+ Cells. HFasL-1 —■— and MFasL-1 —▲— had similar cytotoxic effects versus the FasR+ human T cell line Jurkat E6-1 with a 79±0.3% and 82.8±4% reduction in cell viability at 1:2 dilution respectively. The differences in mean optical density compared to control —○— was significant (*ANOVA $p < 0.01$) to a 1:4 dilution (t-test $p \leq 0.05$ using Bon Feronni adjustment with $\alpha = 0.05$. The truncated construct HFasL-137 —◇— caused only a 18±3% reduction in mean optical density at the highest concentration (1:2) compared to control and the effect was not statistically significant.

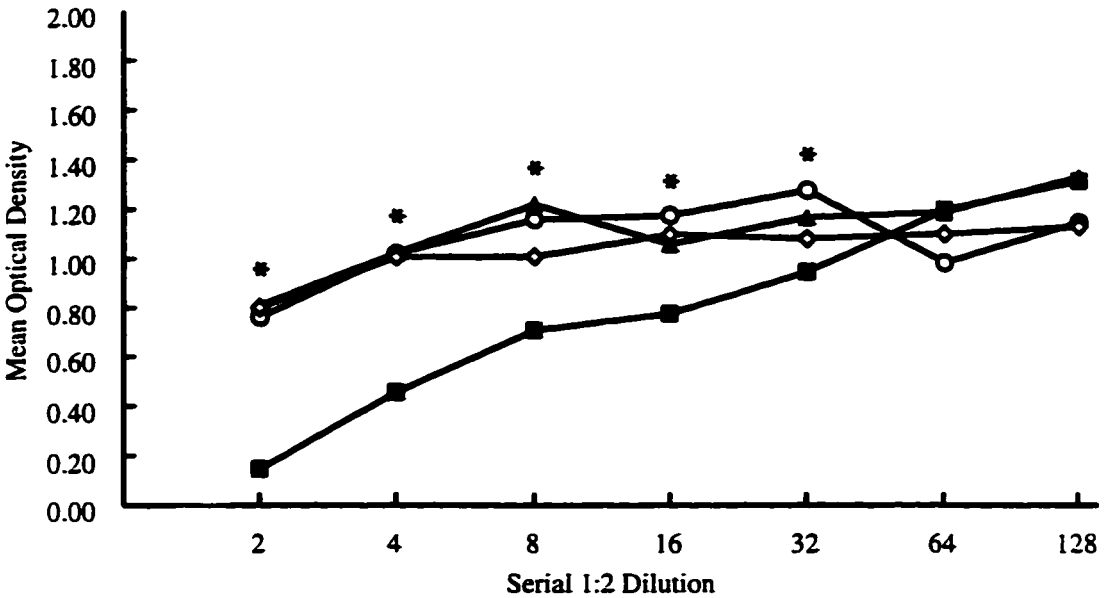

Figure 12. MFasL-1 versus FasR+ Cells. The cytotoxic effect of MFasL-1 transfected COS supernatants versus FasR+ cell lines, Jurkat E6-1 —■— , Yac-1 —◇—, L1210+ —○—. Statistically significant differences between mean optical densities were present throughout all dilutions (*ANOVA $p<0.003$). MFasL-1 caused 82±4% reduction in Jurkat E6-1 viability at a 1:2 dilution compared to control —▲—. This cytotoxic effect was significant by t- test ($p<0.05$ using Bon Feronni adjustment with $\alpha = 0.05$). MFasL-1 had no significant cytotoxicity against other FasR+ cell lines (Yac-1 or L1210+). Plotted results represent the mean values of experiments performed in duplicate.

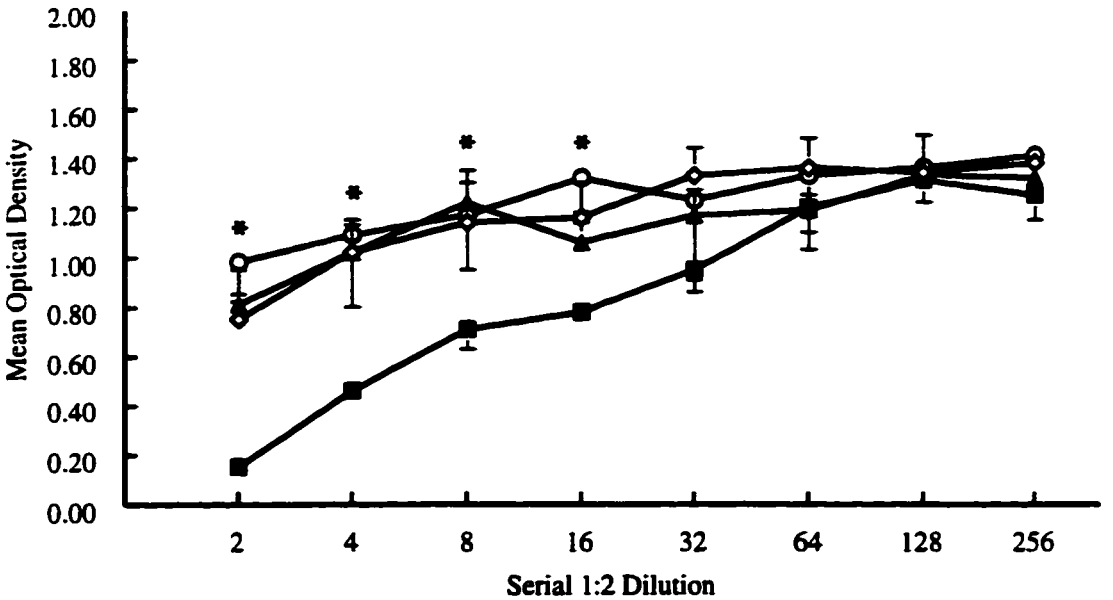

Figure 13. MFasL Constructs versus FasR+ Cells. The cytotoxic effect of MFasL-1 —■— MFasL-300 —◇— , MFasL-331 —○— transfected COS supernatants versus FasR+ cell line. Jurkat E6-1. Significant differences im mean optical densities were witnessed to a 1:16 dilution (*ANOVA $p<0.01$). MFasL-1 reduced cell viability significantly compared to control —▲— up to a 1:8 dilution ($p<0.05$ using t-test Bon Feronni adjustment with $\alpha =$ 0.05). It also caused significantly more cytotoxicity than either MFasL-300 or MFasL-331 (which did not differ significantly from control) to a 1:16 dilution.

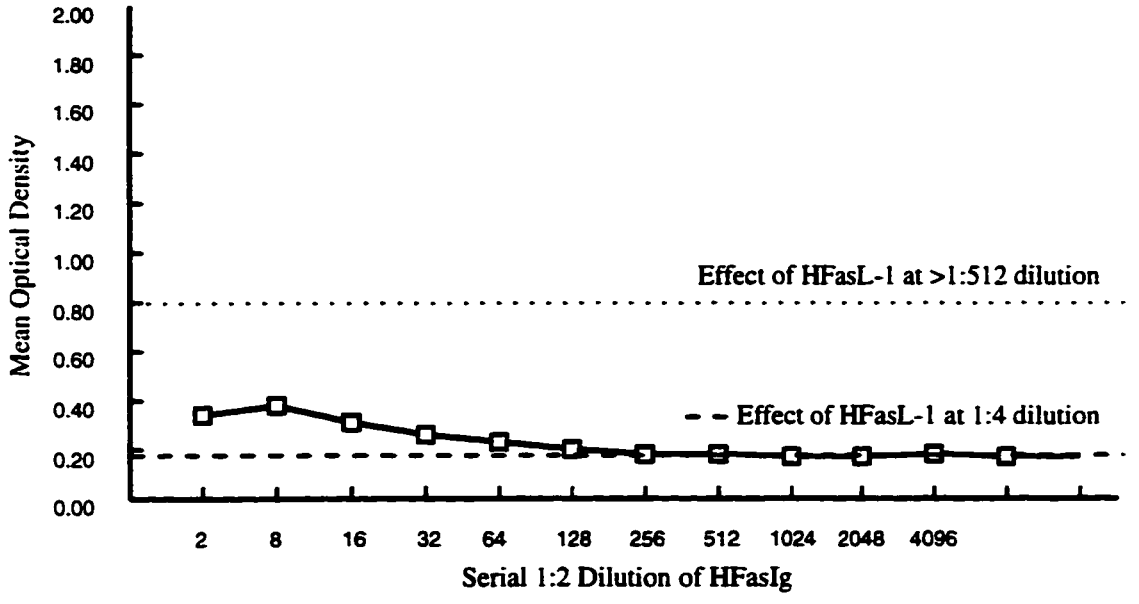

Figure 14. HFasIg Blocks HFasL-1 Cytotoxic Effect on Jurkat cells. HFasIg was added in serial 1:2 dilutions to a standard 1:4 dilution of HFasL-1. At its highest concentration (1/4), HFasIg was able to block only 31% of the maximal cytotoxic activity of HFasL-1 —□— . The effect tapered to 14.5% reduction at 1:32 and 0% by 1:256. The line – – – represents the maximum killing effect of HFasL-1 at a 1:4 dilution. The line · · · · represents the least effect of HFasL-1. This is the expected curve if HFasIg were capable of blocking all HFasL-1 cytotoxicity.

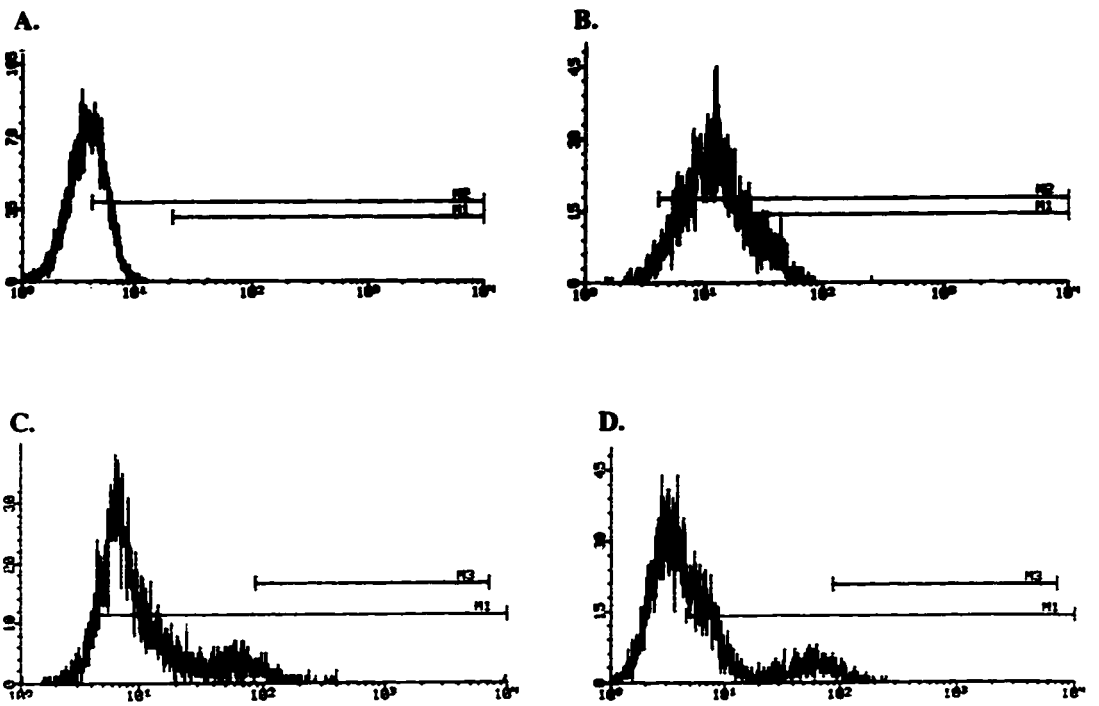

Figure 15. HFasL Stable Transformants FACS Analysis. BW5147 cells were transformed with HFasL- 1, DHFR, and HPRT genes simultaneously. Those cells able to grow in selective media were then analyzed for HFasL-1 expression by staining with antiFasL antibody and secondarily with FITC conjugated goat anti-human antibody then FACS analyzed. BW5147 wildtype cells were used as negative control with a peak fluorescence of $4x10^0$ (A). Three colonies that grew in selective media gave evidence of increased HFasL-1 expression judged by a shift in peak fluorescence. HFasL1-47 was a single population with a moderate shift in peak fluorescence (from $4x10^0$ to $1x10^1$) (B). HFasL1-162 had two populations, one with minimal shift and a smaller population with a full log increase in fluorescence ($6.5x10^1$) (C). HFasL1-171 was polyclonal with two peaks, one at $7x10^0$ and a smaller population shifted significantly to $6.5x10^1$ (D).

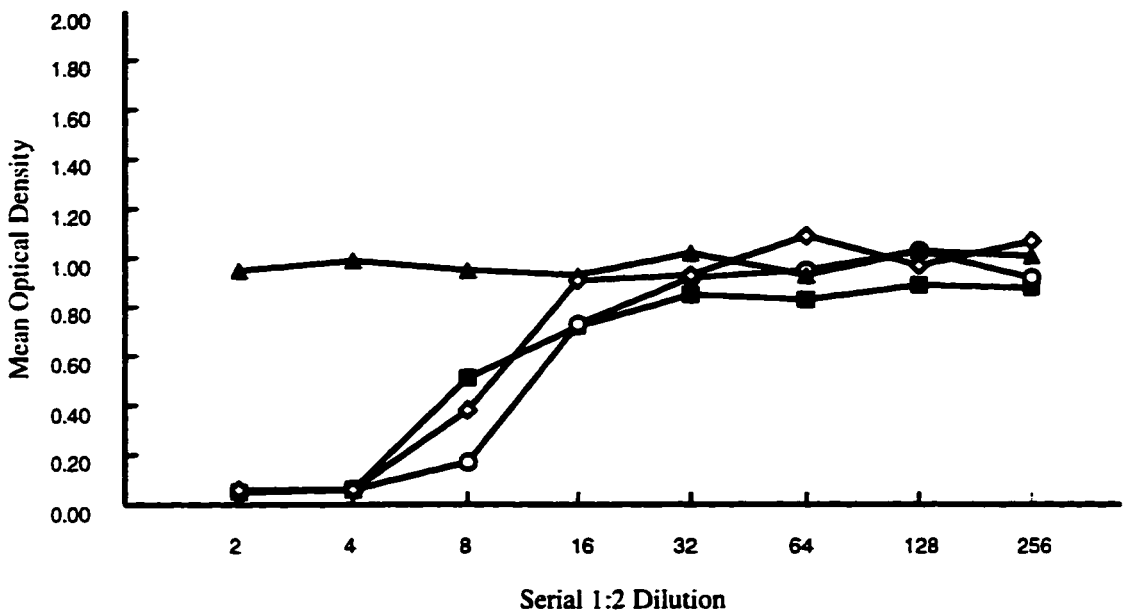

Figure 16. HFasL-1 versus HFasR Stable Transformants. Three stable transformant HFasR clones chosen by FACS analysis for high levels of FasR expression were exquisitely sensitive to HFasL-1 killing, HFasR 26 —■—, HFasR 62 —◇— HFasR 114 —○— with 94% reduction in cell viability at a 1:2 dilution compared to control —▲— . Two clones had intermediate sensitivity with a 70% reduction in cell viability and a 72% reduction in cell viability (data not shown). One insensitive clone had only a 7% decrease in cell viability compared to control (data not shown). The results plotted are means of duplicate experiments.

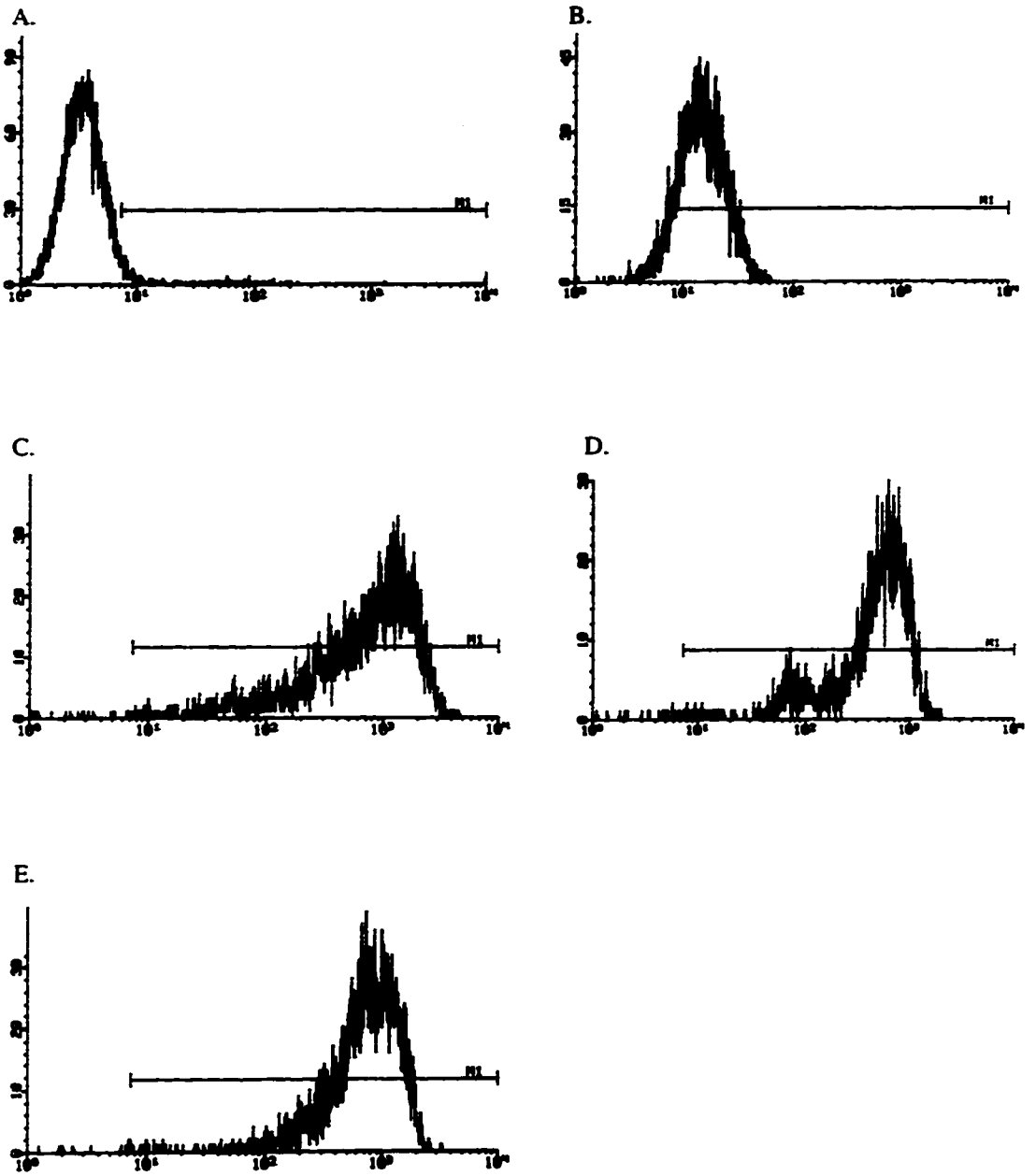

Figure 17. HFasR Stable Transformants FACS Analysis. BW5147 cells were stably transformed with HFasR. Negative control for FACS analysis was provided by nontransformed BW5147 cells (A) and positive control was the native FasR+ cell line Jurkat E6-1 (B). The highest level expressors of those colonies growing in selective media were Clone 26 with a shift of peak fluorescence to $1.5x10^3$ (C), Clone 62 with a shift to $7x10^2$ and a second smaller population at $8x10^1$(D) and Clone 114 with $1x10^3$ shift in fluorescence (E).

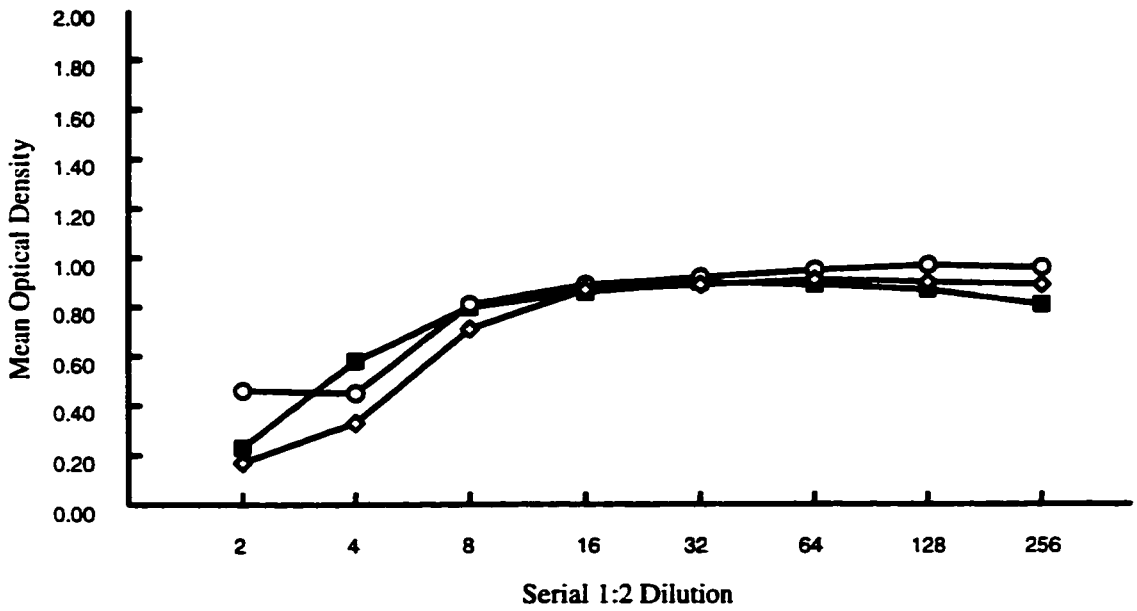

Figure 18. Wildtype FasL Constructs versus W4. The cytotoxic effect of FasL transfected COS supernatants versus the imported MFasR+ cell line W4. HFasL-1 and MFasL-1 caused a significant reduction in optical density at 1:2 dilution compared to control (33% and 59% reduction respectively). The control in this experiment was as in the other MTT assays, a measure of the effect of supernantants from COS cells transfected with the empty PSRαSD7 vector applied to the study cell line (W4). In this experiment W4 appreared more sensitive to control supernantants than other cell lines. Due to time constraints, these experiments were not repeated. At higher dilutions the FasL-1 supernatnats had no effect compared to control. The results plotted represent means of duplicate experiments.

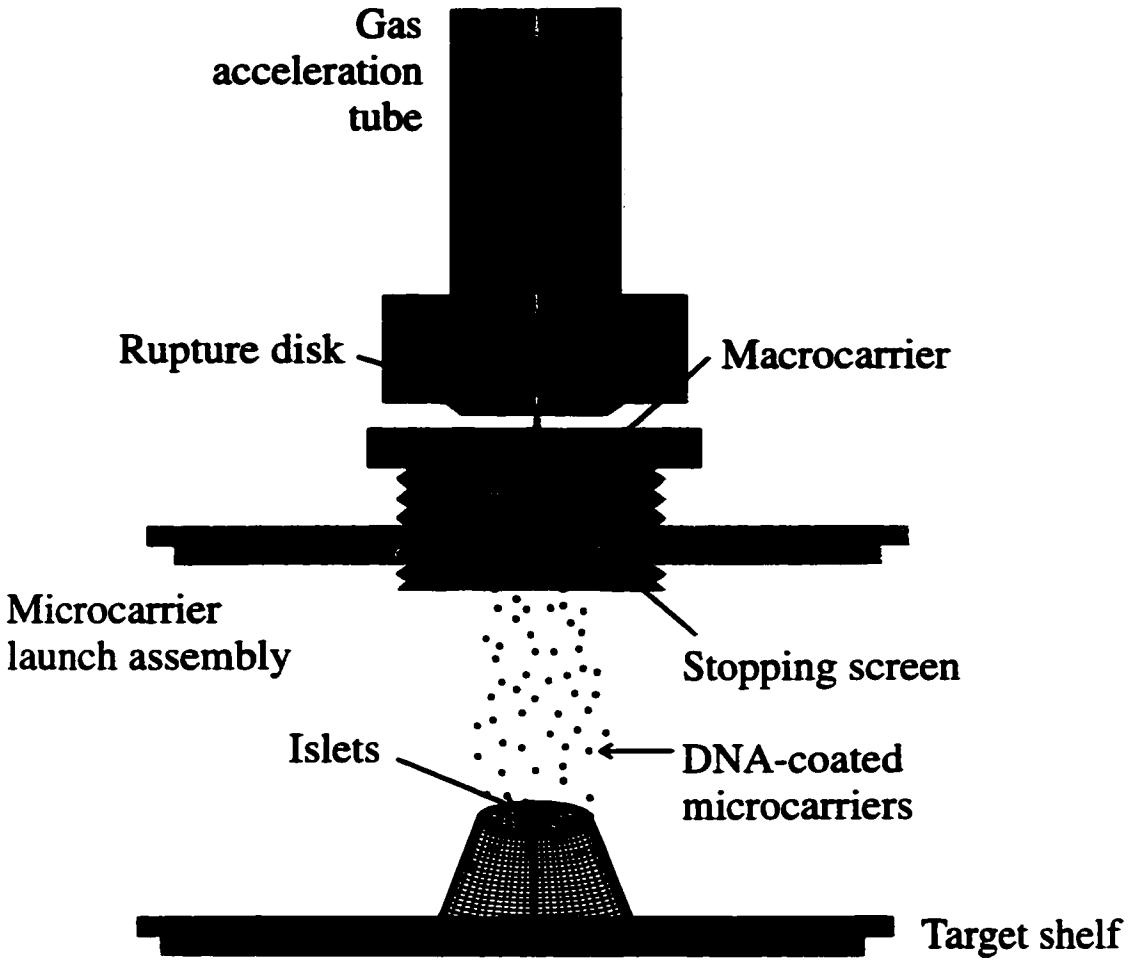

Figure 19. Biolistic Particle Delivery System Schematic. The biolistic particle delivery system or gene gun uses a helium shock wave to accelerate the macrocarrier on which rests DNA-coated gold particles (microcarriers). The microcarriers physically impact islets which rest on a screen. Of the fifty percent of islets which survive this physical impact, 3- 10% will be transformed.

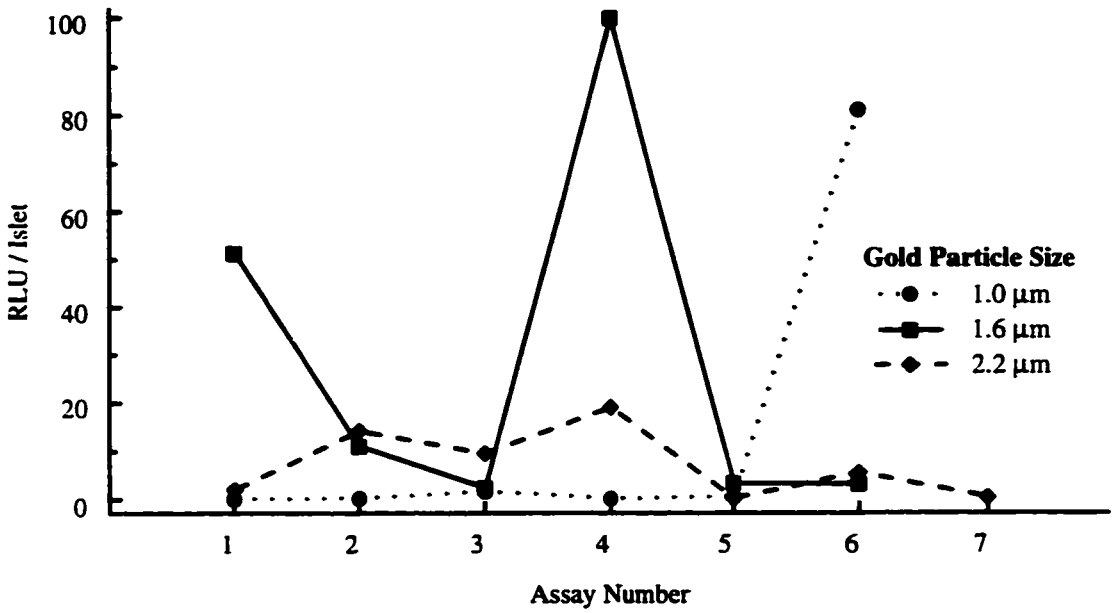

Figure 20. Luciferase Assay on Biolistically Transfected Islets. Three gold particle sizes were used to blast islets with a reporter gene, firefly luciferase. This assay quantitates transfected DNA. There was a high level of variability in peak and trough levels of expresssion of the reporter gene (>100 fold) irrespective of the size of gold particle employed.

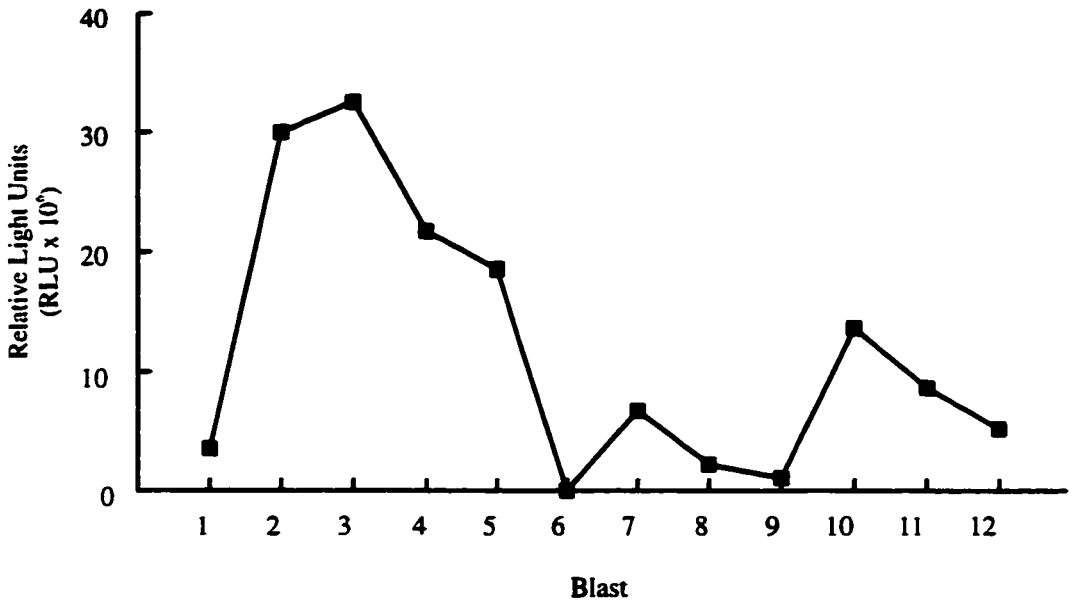

Figure 21. Luciferase Assay on Biolistically Transfected COS cells. Uniform COS cell monolayers were blasted with the reporter gene firefly luciferase coating 1.6 μm particles. The cells were harvested and assayed for luciferase activity as a quantitation of the DNA transfection. Like the islet blast experiment, there was a high degree of blast-to-blast variability (1600-fold difference between peak and trough levels of expression) although five of twelve blasts caused a greater than mean level of luciferase expresion.

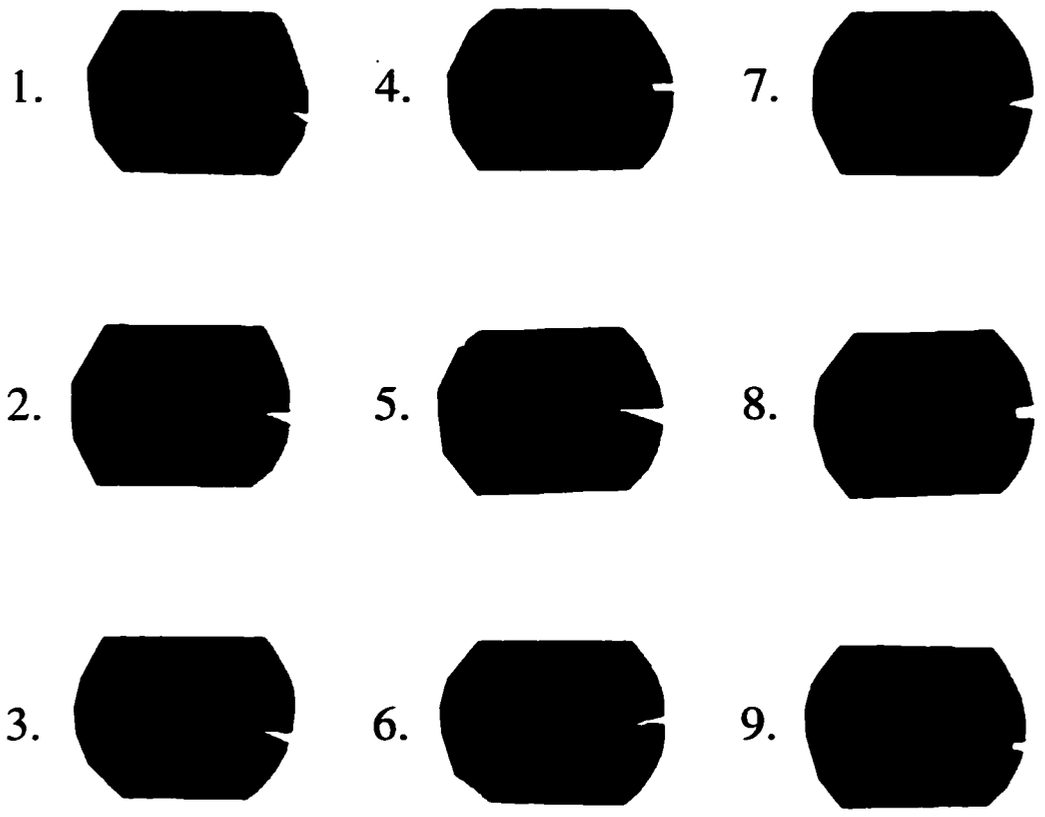

Plate 1. Blast Visualization Assay. Nine consecutive blasts were performed and the gold particles trapped on tape that was positioned at the usual site of islets. It was apparent visually that there was a great deal of variability between the blasts that was not predicted by discernible blast variables.

Plate 2. HFasL-1 Transfected Islet Allograft Histology. This graft was removed from a recipient that was normoglycemic post transplant to day 50. The recipient had spontaneously reverted after chemically induced diabetes as it remained normoglycemic post-nephrectomy. The graft histology reveals renal capsular thickening with no active cellular infiltrate and an absence of any staining islet graft tissue. All the HFasL-1 transfected allografts failed clinically and revealed an absence of graft by histological assessment (Aldehyde fuchsin, magnification 250x).

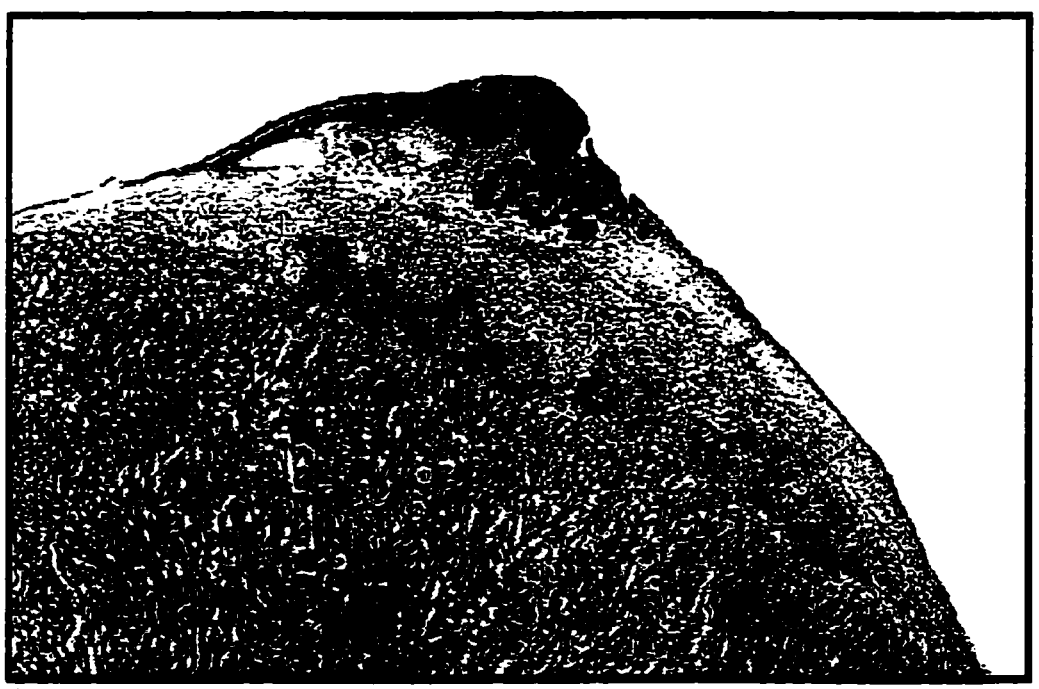

Plate 3. Inverted HFasL-1 Control Islet Allograft Histology. This graft was removed from a control recipient at day 22 after experiencing clinical graft failure at day 15. The histology of a cross section through the kidney subcapsular islet allograft shows marked cellular infiltrate at the interface betwen the graft and renal cortex with residual islet cell clusters present (Hematoxylin and eosin, magnification 125x).

Plate 4. Inverted HFasL-1 Control Islet Allograft Histology. The same graft from Plate 3 stained for insulin reveals a small number of insulin granules present in the β cells of the graft. This confirms that the initial normoglycemia was due to fuctioning islet allograft (immunohistochemical anti-insulin staining, magnification 250x).

Plate 5. Luciferase Control Islet Allograft Histology. This graft was removed after the recipient had been normoglycemic for 35 days post allograft transplant. Post-nephrectomy the recipient became hyperglycemic. Histologic assessment confirmed that there was a large subcapsular islet cell graft present (Aldehyde fuchsin, magnification 250x).

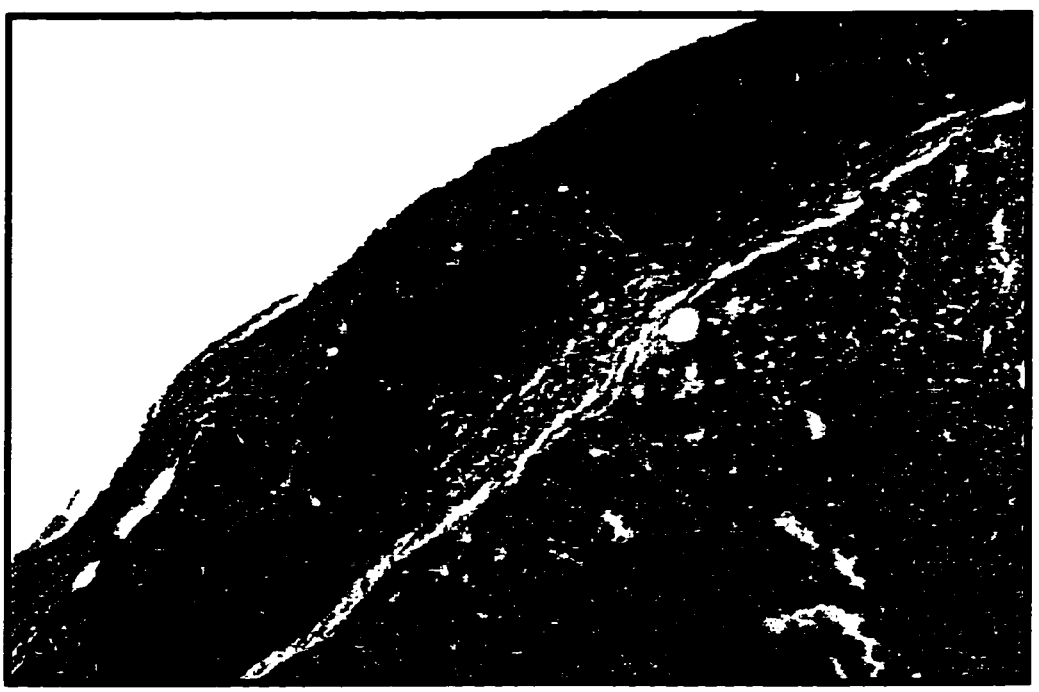

Plate 6. Luciferase Control Islet Allograft Histology. The same graft from Plate 5 was analyzed for insulin expression. The graft's β cells were found to have high levels of insulin expression indicative of a healthy functioning graft (immunohistochemical anti-insulin staining, magnification 250x).