Development of Assays to Study the Role of Natural Killer Cells in the Vesicular Stomatitis Virus Based *Ebolavirus* Post Exposure Therapeutic

by

Kinola Jeanette Noella Williams

A Thesis submitted to the Faculty of Graduate Studies of

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in partial fulfilment of the requirements of the degree of

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Of

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This thesis is dedicated to
the memory of my mother (Rose Jasmine Warner Williams)
and to my father (Vernon P. Williams)
who taught me patience, love, and the worth
of hard work, and to all my brothers, sisters, family, godparents
and friends who were always there and believed in me.

"A journey of a thousand miles starts with a single step."

- Mao Tse-tung

"Are you bored with life? Then throw yourself into some work you believe in with all your heart, live for it, die for it, and you will find happiness that you had thought could never be yours."

- Dale Carnegie

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LIST OF ABBRIVIATIONS

Abbreviation	Meaning
ADCC	Antibody Dependent Cellular Cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
αMEM	Alpha Modified Minimum Essential Medium Eagle
APC	Antigen Presenting Cell
APS	Ammonium Persulfate
βME	2-Mercaptoethanol
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
BSC	Biosafety Cabinet
CCR	Chemotactic Protein-1 Receptor
CD	Cluster of Differentiation
CEBP-γ	CCAAT/Enhancer-Binding Protein γ
CFDA-SE	Carboxyfluorescein Diacetate Succinimidyl Ester
CFSE	Carboxyfluorescein Succinimidyl Ester
CL	Containment Level
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CPE	Cytopathic Effect
DAPI	4',6-diamidino-2-phenylindole
DC	Dentritic Cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
DR	Death Receptor
dsRNA	Double Stranded RNA
E. coli	*Escherichia coli*
E:T	Effector : Target Ratio
EBOV	*Ebolavirus*
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FasL	Fas Ligand
FATAL	Fluorometric Assessment of T Lymphocyte Antigen Specific Lysis
FBS	Fetal Bovine Serum
Flt3-L	FMS-Like Tryosine Kinase 3 Ligand
GLB	Gel Loading Buffer
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor

G	VSV Glycoprotein gene
GP	Glycoprotein
GP_1	Glycoprotein 1
GP_2	Glycoprotein 2
HEPA	High-Efficiency Particulate Air
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HLA	Human Leukocyte Antigen
HLH	Helix-Loop-Helix
HRP	Horse Radish Peroxidase
HSC	Hematopoietic Stem Cell
ID	Inhibitor of DNA
IFA	Immunofluorescence Assay
IFN	Interferon
Ig	Immunoglobulin
Ig-SF	Immunoglobulin Superfamily
IL	Interleukin
i.m	Intramuscular
iNK	Immature NK
IRC	Leukocyte Receptor Complex
IRF	Interferon Regulatory Factor
ITAM	Immunoreceptor Tyrosine-Based Activating Motif
i.v	Intravenous
KIR	Killer cell Immunoglobulin-like Receptor
L	RNA dependent RNA polymerase
LAMP	Lysosomal-Associated Membrane Protein
LB	Luria Broth
LIR	Leukocyte Immunoglobulin-like Receptor
LMP	Low Melting Point agarose
LP-2000	Lipofectamine 2000
LPS	Liopolysaccharide
LT	Lymphotoxin
mAb	Monoclonal Antibody
MARV	*Marburgvirus*
MEF	Myeloid ELF-Like Factor
MHC I	Major Histocompatibility Complex class I
MIC	MHC I Polypeptide-Related Sequence
MIP-1	Macrophage Inflammatory Protein 1
MITF	Microphthalmia-associated Transcription Factor
mNK	Mature NK
MOI	Multiplicity of Infection
MPR	Mannose 6-Phosphate Receptor

N	Nucleoprotein
NCR	Natural Cytotoxicity Receptor
NK	Natural Killer
NKC	NK gene Complex
NKP	NK cell progenitor
NKR-P1	NK cell Receptor Protein 1
NML	National Microbiology Laboratories
OD	Optical Density
PAGE	Protein Acrylamide Gel Electrophoresis
PAMP	Pathogen Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
pBS	pBluescript II plasmid
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PFU	Plaque Forming Units
PI	Propidium Iodide
PMA	Phorbol-12-myristate-13-acetate
Pro	Promoter
PVDF	Polyvinylidene Flyoride Transfer Membrane
Rae1	Retinoic Acid Early Protein
RANTES	Regulated Activation Normal T cell-Expressed and Secreted
RFADCC	Rapid Fluorometric ADCC
RNA	Ribonucleic Acid
rNAPc2	Recombinant Nematode-Derived Anticoagulation Protein 2
RT-PCR	Reverse Transcription PCR
SCF	Stem Cell Factor
SDS	Sodium Dodecyl Sulfate
SE	Standard error
SEBOV	*Sudan ebolavirus*
SEM	Standard error of the mean
siRNA	Small Interfering RNA
ssDNA	Single Stranded DNA
ssRNA	Singe Stranded RNA
STAT	Signal Transducer and Activator of Transcription Factor
T-bet	T-box Expressed in T cells
TCR	T cell Receptor
$TCID_{50}$	50% Tissue Culture Infective Dose
TEMED	Tetramethylethylenediamine
TF	Transcription Factor
TGF-β	Transforming Growth Factor β

Th	T Helper Cell
TLR	Toll-Like Receptor
TMB	3.3',5.5'-tetramethylbenzidine substrate
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
Tris HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol Hydrochloride
ULBP	UL16 Binding Protein
USAMRIID	U.S. Army Medical Research Institute of Infectious Disease
VLP	Virus Like Particle
VP	Viral Protein
VSV	Vesicular Stomatitis Virus
WB	Western Blot
wt	Wild type
ZEBOV	*Zaire ebolavirus*

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ABSTRACT

Zaire ebolavirus (ZEBOV) causes severe haemorrhagic fever with a case fatality rate of 90% for which no therapeutic or prophylactic interventions are currently available. Our lab has created a replication-competent recombinant virus (VSVΔG/ZEBOVGP) by replacing the glycoprotein gene (G) of Vesicular Stomatitis virus (VSV) with that of ZEBOV. Post-exposure therapy studies with this vector suggest innate immune responses are important for protection. Natural Killer (NK) cells are prominent effectors in innate immune responses and, therefore, we were interested in characterizing the interaction between NK cells and VSVΔG/ZEBOV GP.

Cell lines were either incubated in media or infected with VSVΔG/ZEBOV GP at different MOIs for 24 h, then incubated with or without NK cells at different E:T ratios. At 4 h following NK cell addition, cell samples and supernatants were harvested and the percentage of target cell killing by NK cells was determined by FACS analysis. Supernatant of infected cells taken over a 24 h period were also utilized in ELISA assays to determine secretion of cytokines from either cell type.

Results suggest that infection with VSVΔG/ZEBOV GP causes target cells to express the ZEBOV GP on the cell surface and to decrease the expression of MHC class I molecule on the cell surface. In addition, it has been demonstrated here that NK cell killing is increased when the target cells are virally infected compared to uninfected target cells. Cytokine release from either cell line may not have a major role in the causing increased NK cell cytotoxicity in these assays. Collectively, these results suggest that NK cells become highly activated due to direct stimulation and/or interaction with target cells infected with the VSVΔG/ZEBOV GP therapeutic.

1. Introduction

In 1969 large granular 'non-specific' lymphocytes with the capacity of destroying cancer cells were first discovered and described on a functional basis in following years (Herberman *et al.*, 1975b; Hogan and Basten, 1988; Newman and Riley, 2007; O'Connor *et al.*, 2006). These bone marrow derived lymphocytes were also found to have the ability to lyse mutated, viral or bacterial infected cells (Newman and Riley, 2007). These cells were eventually called Natural Killer (NK) cells (Herberman *et al.*, 1975a; Herberman *et al.*, 1975b; Kiessling *et al.*, 1975a; Kiessling *et al.*, 1975b) and they were soon considered a major cell type in the first line of defense against invading microorganisms without prior sensitizing (Colucci *et al.*, 2003; Di Santo, 2006; Hogan and Basten, 1988; Janeway *et al.*, 2001; Vos *et al.*, 1998; Yokoyama *et al.*, 2004). Since NK cells have the ability to lyse without prior sensitization, they have been used as adoptive immunotherapy against solid tumors with limited effectiveness (Chiorean and Miller, 2001; Moretta and Moretta, 2004).

NK cells make up the third largest lymphocyte population, comprising 5-20% of human peripheral blood mononuclear cells (Chiorean and Miller, 2001; Santoni *et al.*, 2007; Sigal, 2003) and approximately 3% of mouse peripheral blood mononuclear cells (PBMC) (Raulet, 2003). NK cells are considered a unique population as they do not contain cell surface markers characteristic to B and T cells (Abbas and Lichtman, 2001; Herberman *et al.*, 1975a; Janeway *et al.*, 2001; Kiessling *et al.*, 1975a; Lanier *et al.*, 1986). NK cells also contain surface receptors that do not undergo rearrangement (Di Santo, 2006; Moretta and Moretta, 2004).

The primary effector function of the NK cell is its innate ability to recognize non-self cells, mutated cells, virally/bacterially infected cells or cancer cells and rapidly kill them. The targeted cells are recognized by a repertoire of activating and inhibitory receptors found on the surface of the NK cells. The binding of the NK receptors to the target cell ligands determines the action the NK cells will take towards that cell. The killing function of the NK cell is highly regulated by a balance of these activating and inhibitory receptors (Smyth *et al.*, 2005). The signals received from the binding of these receptors to their ligand either inhibits activation, such as the binding of the major histocompatibility complex class I (MHC class I), or activates the NK cell to kill the target cells by granular exocytosis, such as the binding of the Fc receptor, CD16. NK cells also possess receptor members of the tumor necrosis factor (TNF) superfamily, such as TRAIL (TNF-related apoptosis-inducing ligand), and Fas ligand (FasL) which can cause death in target cells through the death receptor signaling pathway.

NK cells have been identified as the producers of important cytokines, such as interferon gamma (IFN-γ) and TNF alpha (TNF-α), which affects many cell types, both in the innate and adaptive immune systems and this will be discussed further shortly. The cytokines secreted by the NK cells have been seen to regulate the effects of other immune cells, such as T cells and Dendritic cells (DCs) (Zhang *et al.*, 2006).

1.1 Regulation of NK Cell Functions

NK cells detect target cells by binding to them through different receptors on their cell surface. These functional cell surface receptors (Table 1.1) on human and murine NK cells are described either as inhibitory or activating. It is the balance of signals delivered from the binding of these two types of receptors that leads the NK cell to either kill or not

kill the target cell. Inhibitory receptors, containing immunoreceptor tyrosine-based inhibitory motifs (ITIM) on their intracytoplasmic tails, bind to MHC class I molecules (Janeway *et al.*, 2001; Yokoyama and Kim, 2006). The binding of these clonally distributed receptors to the MHC class I molecule arrest the cytotoxic effects of the NK cell, such as release of cytotoxic granules and cytokines. Activating receptors bind to different ligands on the target cell, ranging from viral proteins to antibodies. These activating NK cell receptors associate with intracellular molecules that bear immunoreceptor tyrosine-based activating motif (ITAM). By binding to these ITAM bearing molecules, the NK cell can perform perforin dependent natural killing or produce and secrete proinflammatory cytokines such as IFN-γ or TNF-α. There are four families of both human and/or murine activating and inhibitory NK cell receptors: the Killer cell Immunoglobulin-like receptors (KIR), the Natural Cytotoxicity Receptors (NCR), the Immunoglobulin-like transcripts or Leukocyte immunoglobulin-like receptors (ILT or LIR) and the C-type lectins (Sigal, 2003).

1.1.1 Activating Receptors

An individual NK cell is capable of responding to multiple activation ligands because it can express a range of various receptors. In mice, there are three classes of activating receptors: the members of the Ly49 family, the members of the NKG2 family and the low affinity Fc receptor (Chiorean and Miller, 2001; Karlhofer *et al.*, 1992; Lai *et al.*, 1998; Raulet, 2006; Yokoyama *et al.*, 2004). In addition to other types of receptors, human KIR activatory receptors also include members of the NKG2 family and the low affinity Fc receptor.

Table 1.1: NK Cell Receptors

Cell Marker	Known Ligand	Function	Reference
CD16 ($F_c\gamma$III)	IgG, IgE	Activation, ADCC	(Ahmad and Ahmad, 2003; Hogan and Basten, 1988; O'Connor *et al.*, 2006; Sigal, 2003)
DNAM-1	Poliovirus receptor (CD155), Nectin-2 (CD112)	Activation	(Ahmad and Ahmad, 2003; Moretta and Moretta, 2004; O'Connor *et al.*, 2006)
NKG2C/CD94	HLA-E	Activation	(Moretta *et al.*, 2001; O'Connor *et al.*, 2006)
NKG2D	Human: MICA, MICB, UL16 Mouse: Rae1, H60, ULBPs	Activation	(Ahmad and Ahmad, 2003; Kirwan and Burshtyn, 2007; Markiewicz and Shaw, 2006; Moretta *et al.*, 2001; Moretta and Moretta, 2004; Newman and Riley, 2007; O'Connor *et al.*, 2006; Rodriguez-Rodero *et al.*, 2007; Sigal, 2003; Yokoyama, 2005)
NKp30	Human CMV pp65	Activation	(Bottino *et al.*, 2000; Moretta *et al.*, 2001; O'Connor *et al.*, 2006)
NKp44	Influenza HA	Activation	(Bottino *et al.*, 2000; Moretta *et al.*, 2001; O'Connor *et al.*, 2006)
NKp46	Influenza HA, parainfluenza HA/NA	Activation	(Ahmad and Ahmad, 2003; Bottino *et al.*, 2000; Lee *et al.*, 2007; Moretta *et al.*, 2001; O'Connor *et al.*, 2006)
KIR2DS1	HLA-C, Lys.p80	Activation	(Ahmad and Ahmad, 2003; O'Connor *et al.*, 2006)
KIR2DS2	HLA-C, Asn.p80	Activation	(Ahmad and Ahmad, 2003; O'Connor *et al.*, 2006)
KIR2DL4	HLA-G	Activation	(Ahmad and Ahmad, 2003; O'Connor *et al.*, 2006)
KIR3DS1	HLA-B	Activation	(O'Connor *et al.*, 2006)
KIR3DL1	HLA-B	Activation	(O'Connor *et al.*, 2006)
TLRs	PAMPs	Activation	(O'Connor *et al.*, 2006)
p58/KIR2DL	MHC I	Activation	(Moretta *et al.*, 2001)
p50/KIR2DS	HLA-C	Activation	(Moretta *et al.*, 2001)
2B4 (CD244)	CD48	Co-stimulation	(Ahmad and Ahmad, 2003; Kirwan and Burshtyn, 2007; Moretta *et al.*, 2001; Moretta and Moretta, 2004; Newman and Riley, 2007; O'Connor *et al.*, 2006; Sigal, 2003)
CD2	CD58, LFA-2	Co-stimulation	(Cao *et al.*, 2006; O'Connor *et al.*, 2006)
CRACC (CD2-like receptor-activating cytotoxic cells)	MHC I	Co-stimulation	(Cao *et al.*, 2006)
NKp80	Unknown	Co-stimulation	(Moretta *et al.*, 2001; O'Connor *et al.*, 2006; Sigal, 2003)
NTB-A (NK-, T- and B-cell antigen) aka Ly108	NTB-A	Co-stimulation	(Cao *et al.*, 2006; Moretta and Moretta, 2004; O'Connor *et al.*, 2006)
CD85j	Variety of MHC I	Inhibition	(Kirwan and Burshtyn, 2007)
NKG2A/CD94	HLA-E	Inhibition	(Ahmad and Ahmad, 2003; Braud *et al.*, 1998; Kirwan and Burshtyn, 2007; O'Connor *et al.*, 2006; Sigal, 2003)
KLRG1	Cadherins	Inhibition	(Kirwan and Burshtyn, 2007)
NKRP1	Ocil	Inhibition	(Ahmad and Ahmad, 2003; Kirwan and Burshtyn, 2007)
KIR2DL1	HLA-C, Lys.p80	Inhibitory	(Ahmad and Ahmad, 2003; O'Connor *et al.*, 2006)
KIR2DL2/3	HLA-C, Asn.p80	Inhibition	(Ahmad and Ahmad, 2003; O'Connor *et al.*, 2006)
KIR3DL1	HLA-B, Bw4.Iie.p80	Inhibitory	(Ahmad and Ahmad, 2003; O'Connor *et al.*, 2006)
KIR3DL2	HLA-A	Inhibition	(Ahmad and Ahmad, 2003; O'Connor *et al.*, 2006)

NOTE: Sources are cited from articles on receptor function and/or reviews for illustration purposes.

In the mouse, members of the Ly49 family are encoded by genes on the NK gene complex (NKC): of these, Ly49D and Ly49H can trigger cytotoxic function by the NK cells (Karlhofer *et al.*, 1992; Yokoyama, 2005; Yokoyama and Kim, 2006; Yokoyama *et al.*, 2004). Ly49D was the first member of the Ly49 family identified to be an activation receptor (Yokoyama, 2005). The ligands for the Ly49D receptor is the murine equivalent of the human MHC class molecule, H2-D (Raulet, 2006). Ly49H recognizes viral ligands on infected cells such as the m157 protein of the mouse cytomegalovirus (Arase *et al.*, 2002; Hamerman *et al.*, 2005; Lee *et al.*, 2007; O'Connor *et al.*, 2006; Voigt *et al.*, 2003; Webster and Johnson, 2005; Yokoyama, 2005).

The Ig-Superfamily (Ig-SF) and Ca^{+2}-dependent lectin subfamily include both inhibitory and activating receptors. In humans, there are two types of activating receptors in the Ig-SF and Ca^{+2}-dependent lectin subfamily (Hao *et al.*, 2006). In the Ig-SF, the Natural Cytotoxicity Receptors (NCR) are found strictly on NK cells (Moretta *et al.*, 2002) and include NKp46, NKp30 and NKp44. NKp46 is a 46-kDa glycoprotein with two extracellular Ig-like domains of type C2 (Bottino *et al.*, 2000; Moretta *et al.*, 2001). They are expressed only on resting and activated NK cells and when cross-linked with monoclonal antibodies (mAb) they induce cytotoxic ability, Ca^{+2} flux and cytokine release (Bottino *et al.*, 2000; Moretta *et al.*, 2001; Moretta and Moretta, 2004). To this point, only viral ligands are known for this killer cell receptor. These ligands include the haemagglutinin of influenza virus and the haemagglutinin-neuraminidase of parainfluenza virus (Bottino *et al.*, 2000; Moretta *et al.*, 2001).

NKp30 is a 30-kDa glycoprotein with one extracellular Ig-like domain of V type. It is expressed on resting and activated NK cells and when cross linked by mAbs, it can

induce the cytotoxic mechanism, Ca^{+2} flux and cytokine production (Bottino *et al.*, 2000; Moretta *et al.*, 2001; Moretta and Moretta, 2004). NKp30 has only been found to bind to the pp65 protein of human cytomegalovirus (O'Connor *et al.*, 2006). NKp44 is a 44-kDa glycoprotein, also found with a single extracellular Ig-like V type domain (Bottino *et al.*, 2000; Moretta *et al.*, 2001). Unlike the two previous NCRs, it is expressed only on IL-2 cultured human NK cells and it is considered the first marker for the identification of activated NK cells (Bottino *et al.*, 2000; Moretta *et al.*, 2001; Moretta and Moretta, 2004; Sivori *et al.*, 2000). Currently, the only known viral ligand for NKp44 is the haemagglutinin protein of influenza virus (O'Connor *et al.*, 2006).

The last set of receptors is found both in humans and mice. NKG2D is found in the C-type lectin superfamily and is predominantly expressed on NK cells that present low NCR surface densities. NKG2D is also found on cytolytic T lymphocytes (Markiewicz and Shaw, 2006; Moretta *et al.*, 2001; Moretta and Moretta, 2004). Unlike other NKG2 family members, NKG2D is manifested as a homodimer and does not affiliate with CD94 (Moretta *et al.*, 2001). Ligands for NKG2D in humans include stress-induced molecules MHC I polypeptide-related sequence (MIC) A and B and the human cytomegalovirus UL16 binding proteins (ULBPs) (Alessandro Moretta, 2001; Geraldine M. O'Connor, 2005; Lorenzo Moretta, 2004; Moretta *et al.*, 2001; Moretta and Moretta, 2004; O'Connor *et al.*, 2006; Rodriguez-Rodero *et al.*, 2007; Sandra Rodriguez-Rodero, 2007). In the mouse, NKG2D binds to GP_I-linked molecules that share high homology with human ULBPs (Yokoyama and Kim, 2006) and a series of proteins induced by retinoic acid, retinoic acid early proteins (Rae1) and minor histocompatibility antigen (H60) (Moretta *et al.*, 2001; Sigal, 2003). These molecules are induced when the cell is

under stress. Therefore the expression of these stress molecules signals to the NK cell that the cell is to be disposed of.

The CD16 ($F_c\gamma$III) molecule within the Ig-SF family contains two Ig-like C2 type domains and is involved in antibody dependent cellular cytotoxicity (ADCC), binding with the highest affinity to IgGI and IgG3 (Abbas and Lichtman, 2001; Janeway *et al.*, 2001; Sigal, 2003). The majority of NK cells, activated monocytes and a subset of T cells express CD16. The antibodies that CD16 recognizes are bound to an invading microorganism or to an infected cell through the variable portion of the antibody. CD16 on the NK cell recognizes the Fc portion of the bound antibody. The cross linking of several CD16/antibody complexes triggers the NK cell to degranulate and destroy the target.

Toll-like receptors (TLR) are not considered a NK cell activation receptor. However, they are found on NK cells and recognize conserved pathogen associated molecular patterns (PAMP) such as lipopolysaccharide (LPS) or double stranded RNA (dsRNA). NK cells have a range of TLRs. These receptors help the cell to either release proinflammatory cytokines, such as IFN-γ or TNF-α, or stimulate degranulation of perforin to kill target cells. The TLRs on the NK cells include TLR-2, 3, 4 (on human cells), 5, 7, 8 and 9 (O'Connor *et al.*, 2006).

1.2.2 Inhibitory Receptors

The inhibitory receptors found in both human (KIRs) and mice (Ly49 family) abrogate cytotoxicity and cytokine production by binding to, but are not restricted to, the MHC class I molecules found on the surface of the target cell. In the mouse, the MHC class I molecule (H2) is recognized by a subset of Ly49 receptors (Raulet, 2006). *In vitro*

studies of the engagement of these inhibitory Ly49 members determined that ligand binding to these receptors causes an inhibitory signal cascade. The cascade induces a down regulation of any corresponding Ly49 receptors in the presence of a self-MHC class I molecule (Karlhofer *et al.*, 1994; Yokoyama *et al.*, 2004). These studies have also shown that the engagement of the Ly49 receptor with the MHC class I on stromal cell reduces further expression of additional Ly49 receptors on the cell surface that can detect the same MHC class I molecule (Sjostrom *et al.*, 2001; Yokoyama *et al.*, 2004; Zimmer *et al.*, 2001). Homologues of the Ly49 receptors do not exist in humans. Human NK cells contain killer inhibitory receptors specific for HLA-C or HLA-A (Di Santo, 2006), to which gene orthologues do not exist in mice.

NKG2A is an inhibitory receptor found in both humans and mice. It belongs to the C-type lectin family and heterodimerizes with a 30 kDa glycoprotein, CD94 (Braud *et al.*, 1998; Di Santo, 2006; Janeway *et al.*, 2001; Raulet, 2006). NKG2A is specific for HLA-G (Webster and Johnson, 2005) and HLA-E, non-classical HLA class I molecules. By binding to these non-classical HLA molecules, the NK cells are inhibited from cytotoxic activities and producing cytokines.

1.2.3 Activation or Repression of NK Cells Mechanisms

The change in balance between the binding of the activation and inhibitory receptors sends signals to the NK cell that determine its action (Figure 1.1). The binding of more activation ligands than inhibitory ligands triggers activation signals that lead the NK cell to secrete cytokines or to degranulate and lyse the target cell. The opposite is also seen when the binding of more inhibitory ligands than activatory, lead to an

inhibition of NK cell mechanisms. The manner in which the NK cell may balance out these signals was seen in studies of the Ly49 receptor.

Each NK cell expresses only up to five Ly49 molecules in order to detect potential targets that may have lost some of their MHC I molecules due to infection (Rosmaraki *et al.*, 2001; Vosshenrich *et al.*, 2005). Studies of the Ly49 gene discovered two overlapping bidirectional regulatory elements of transcription. Promoter (Pro) 1 was located upstream of Pro2. The binding strength of transcription factors to either promoter established which subset of Ly49 gene receptors would be transcribed (Saleh *et al.*, 2004). By controlling the expression of Ly49 receptors on the NK cell surface, the action perpetrated by the NK cells against the target cell was then determined by the avidity of the interaction between the NK and the target cell. Doucey *et al* also discovered that mice expressing the $H2^d$ ligand constitutively bind to NK cells *in cis*. By binding to the

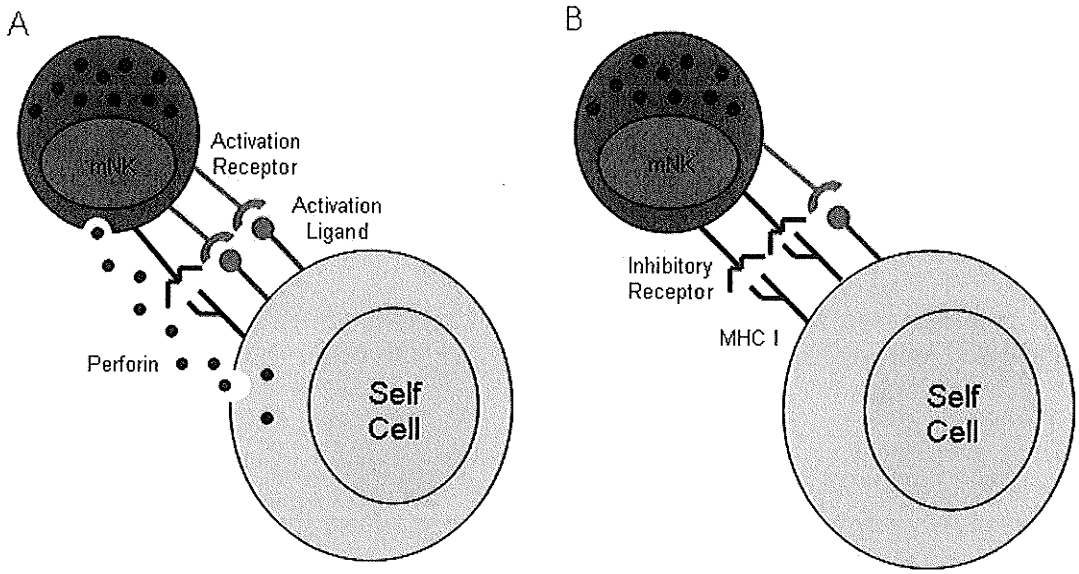

Figure 1.1: The activation or repression of NK cell function.
(A) When the target cell expresses more activation ligands than inhibitory ligands (ex. MHC I), the NK cell is sent a signal to degranulate and lyse the target cell. (B) When the target cell expresses more inhibitory ligands than activation ligands, the NK cell is sent a signal not to kill the target cell.

NK cells constitutively, this reduces the number of available Ly49A receptors on the NK cells that can bind to surrounding cells *in trans*, thus lowering the threshold necessary to overcome mechanistic inhibition (Doucey *et al.*, 2004). These methods of receptor control enable the NK cell to determine the sensitivity of the receptor/ligand complex and thus how the NK cell will react when bound to both inhibitory and activating receptors. However, with the ability to kill target cells, the NK cell still must learn how to distinguish between self-cells and "foreign" cells.

1.2 Cytokine Production

When stimulated by cytokines from other cells, such as IL-12, or by receptor binding, mature NK cells produce and secrete numerous cytokines and chemokines. Some cytokines that NK cells secrete are IFN-γ, TNF-α, Granulocyte-macrophage colony-stimulating factor (GM-CSF), transforming growth factor (TGF) - β1, interleukin (IL) – 1, 5, 8, and 10 (Anegon *et al.*, 1988; Arase *et al.*, 1996; Bancroft, 1993; Chiorean and Miller, 2001; Colucci *et al.*, 2003; Gosselin *et al.*, 1999; Ortaldo and Young, 2003; Sinkovics and Horvath, 2005; Yokoyama *et al.*, 2004). The chemokines the NK cell can secrete include macrophage inflammatory protein 1β (MIP-1β, also known as CCL-3), CCL-18 and Regulated Activation Normal T cell-Expressed and Secreted (RANTES) (Chiorean and Miller, 2001; Orange and Biron, 1996; Sinkovics and Horvath, 2005; Yokoyama *et al.*, 2004). During an infection, IFN-γ is one of the first cytokines secreted by NK cells in response to the infection and will be discussed in more detail.

During a viral infection, NK cells secrete IFN-γ in response to IL-12 secreted by the host macrophages. NK cells have been identified as the first cellular source of IFN-γ

in the draining lymph nodes following a bacterial infection (Bancroft, 1993; Newman and Riley, 2007). It activates macrophages within the first 24 to 48 hours of infection before any antigen specific T cell response can occur (Bancroft, 1993). IL-12 stimulates NK cells to produce IFN-γ through the STAT-4 pathway (Chiorean and Miller, 2001). Stimulating NK cells with IL-2 can also cause them to secrete IFN-γ. This is caused by an increase of IL-12 receptor chains on the surface and an upregulation of STAT-4 molecules (Chiorean and Miller, 2001). This leads to improved response to IL-12 and thus an increased production of IFN-γ.

IFN-γ can also induce a Th1 like adaptive immunity by inhibiting Th2 cell proliferation (Bancroft, 1993), thereby aiding the differentiation of T cells in the secondary lymphoid organs (Di Santo, 2006; Sinkovics and Horvath, 2005). The secretion of IL-10 from $CD4^+$ T cells among other cell types can abrogate IFN-γ secretion following IL-2 stimulation (Bancroft, 1993). This is caused by the inhibition of macrophage derived cytokines, such as TNF-α and IL-12 (Bancroft, 1993). IL-10 may also have a direct effect on NK cell production of IFN-γ owing to IL-2 stimulation (Bancroft, 1993).

1.3 Natural Killer Cell Mediated Cytotoxic Mechanisms

NK cells require different conditions than cytotoxic T cells in order to destroy target cells. First, NK cells kill in minutes without prior sensitization (Chiorean and Miller, 2001; Smyth *et al.*, 2005; Yokoyama and Kim, 2006). Cytotoxic T cells can take several hours before they begin to lyse a target cell (Sanderson, 1981). Second, NK cells does not require the target cell to express a MHC molecule (Chiorean and Miller, 2001).

In contrast, cytotoxic T cells require binding of their T cell receptor (TCR) to the MHC class I on the surface of a host cell offering an antigen before the cytotoxic T cell can become stimulated to kill a target cell. Third, NK cells react to various receptors and do not involve unique antigen-specific receptors (Chiorean and Miller, 2001). Cytotoxic T cells need the TCR-MHC/antigen complex along with binding of co-receptors in order to recognize a target cell. However, beside these differences, cytotoxic T cells and NK cells still lyse cells by cytotoxic granule release, which will be discussed here.

1.3.1 Cytotoxic Mechanisms

The main pathway for NK cell killing is dependent on perforin and granzymes (Figure 1.2-A). These molecules are found in cytotoxic granules that are released into a synapse formed between the NK cell and the target cell after receptor binding. Perforin is a membrane-disrupting molecule detected in NK cells during the last stage of development after the cells have expressed CD94 (Freud and Caligiuri, 2006; Smyth *et al.*, 2005). This observation was found when $CD94^-$ cells were seen to have no killing activity against K562 or Jurkat target cells. However $CD94^+$ cells displayed greater than 30% lysis response against K562 cells and more than 90% against Jurkat cells (Freud and Caligiuri, 2006). This was also observed in immature NK (iNK) cells that were unable to lyse target cells via perforin action, but was able to kill through TRAIL pathways (Bennett *et al.*, 1996; Colucci *et al.*, 2003; Zamai *et al.*, 1998). NK cells use perforin's membrane-disrupting abilities to punch holes into the surface of the target cell. Once membrane disruption has occurred, other molecules such as granzymes can enter the target cell to begin apoptosis pathways (van Dommelen *et al.*, 2006).

Once the target cell membrane has been penetrated, the NK cell secretes a series of proteases into the target in order to kill it. Granzymes are a family of serine proteases mainly found in the granules of neutrophils, but are also found in cytotoxic

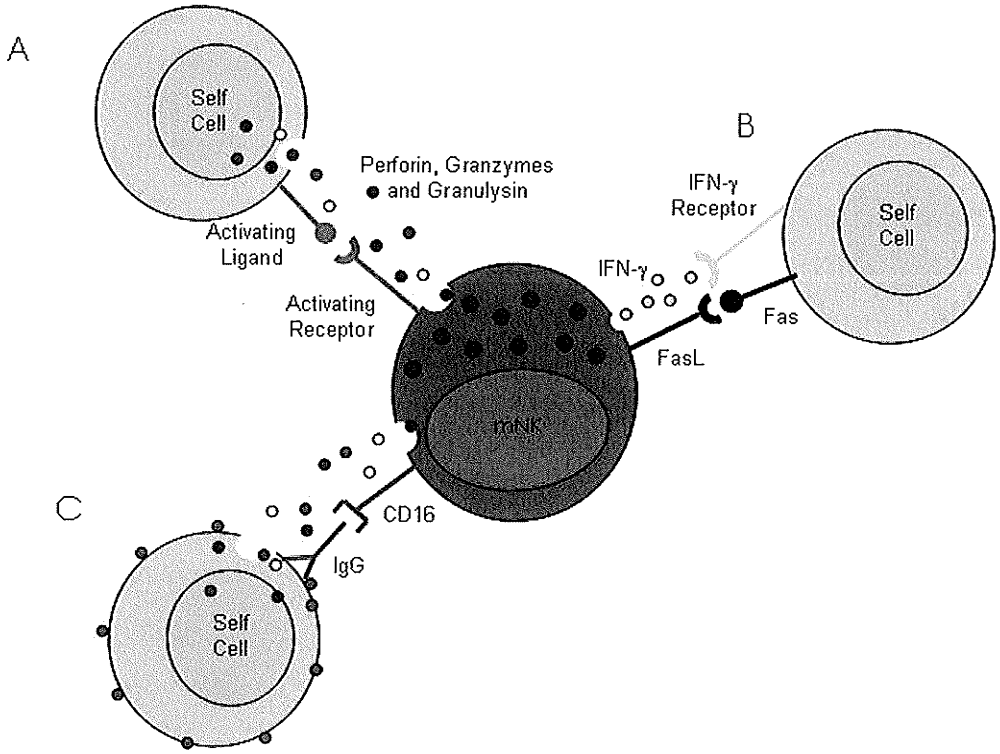

Figure 1.2: Methods of NK cell cytotoxicity
NK cells kill target cells by the release of (A) perforin, granzymes and granulysin. These cells can also cause death of the target cell by stimulating the target to upregulate (B) death receptors like Fas or TRAIL on their surface with the aid of IFN-γ. (C) CD16 on the NK cell binds to the Fc portion of the Ig on target cells, causing the NK cell to become stimulated to release perforin, granzymes and granulysin.

T lymphocytes and NK cells (Ganz, 2002; Smyth *et al.*, 2005). The rapid granzyme uptake is either mediated via perforin or the mannose 6-phosphate receptor (MPR) by receptor-mediated endocytosis (Smyth *et al.*, 2005; van Dommelen *et al.*, 2006).

There have been many different granzyme proteins identified in humans and mice. Granzymes A, B, H, K and M have been described in humans and granzymes A

through K, M and N in mice (Smyth *et al.*, 2005). Here, only the major granzymes will be discussed. Granzyme A is a trypsin protease that cleaves at arginine and lysine residues on target proteins (Smyth *et al.*, 2005). It can trigger nuclear damage when in the presence of perforin and can induce caspase-independent cell death by generating single stranded DNA (ssDNA) nicks (Smyth *et al.*, 2005). Granzyme B is an aspase that cleaves after aspartic acid residues (Smyth *et al.*, 2005). It can trigger apoptosis directly or by activating caspases. Like granzyme A, granzyme B can trigger nuclear damage when in the company of perforin (Smyth *et al.*, 2005).

To list a few other proteases, granzyme K is a trypsin that cleaves at arginine and lysine residues. Granzyme H is a chymase that cleaves after hydrophobic residues such as phenylalanine. Granzyme C through G belongs to the chymase family. Finally, granzyme M uniquely cleaves after methionine, leucine or isoleucine (Smyth *et al.*, 2005).

Granulysin is another protease found in NK cell cytotoxic granules. It is a small human protease (9 and 15 kDa forms) that NK cells use to kill target cells (Ganz, 2002). It belongs to the saposin-like protein family that includes NK lysin (Smyth *et al.*, 2005). Granulysin is also present in granules of cytolytic T cells (Smyth *et al.*, 2005).

NK cells can utilize these cytotoxic granules to kill target cells by ADCC using CD16 (FcRγIIIα) (Figure 1.2-C). CD16 is expressed on the NK cell when it has been exposed to IL-21 (Sivori *et al.*, 2003). This cytokine induces the production of cytokines and lytic ability in mature NK cells in both humans and mice (Colucci *et al.*, 2003). By binding CD16 to the Fc portion of an immunoglobulin G (IgG), the NK cell can kill the target through the release of perforin and granzymes (Chiorean and Miller, 2001).

1.3.2 Receptor Mediated Cytotoxicity

NK cells can also kill by pathways other than cytotoxic granules. Specifically, by receptor mediated cytotoxicity by FasL (CD178) and TRAIL (Figure 1.2-B). FasL, combined with stimulation from IFN-γ, induces Fas expression on the target cell (Smyth *et al.*, 2005). TRAIL stands for TNF-related apoptosis-inducing ligand. Both FasL and TRAIL receptors are transmembrane proteins that belong to the TNF superfamily (Smyth *et al.*, 2005). Binding of these NK cell death receptors induces programmed cell death or "apoptosis" in the target cell. There are five receptors of TRAIL identified in humans. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) transduce apoptotic signals in the target cell (Smyth *et al.*, 2005). The three other receptors, lack death receptors and are thought to be decoys regulating TRAIL-mediated death (Smyth *et al.*, 2005). In mice, two apoptotic TRAIL receptors have been identified and two decoy receptors (Smyth *et al.*, 2005).

1.3.3 Cytotoxic Mechanisms During a Viral Infection

During a viral infection, the NK cell searches for cells that have become infected. The virus binds to the targeted cells and infects it. Inside the cell, the virus begins to replicate causing the infected cell to undergo changes such as an increase or decrease in MHC class I molecule on the cells surface and/or secrete cytokines or chemokines. The infected cells can also express viral proteins on its surface that can trigger NK cell functions. Once the infected cell is found and recognized, the NK cell binds to the target cell and signals are sent to the NK cell. The decision to lyse the cell causes the NK cell to release its cytotoxic granules within the synapse formed between the target and the NK cell. The granule releases perforin which punches holes into the membrane of the target cell to allow in granzyme proteases. The granzymes bind to and cleave caspases that

degrade DNA, inducing the apoptosis pathway. The infection can also cause a cell surface expression of the death ligands TRAIL and FasL on the NK cell to bind to the death receptors on the target cells. Ligand binding to the death receptors, DR4/5 or Fas respectively, also leads to a caspase cascade that induces apoptosis in the target cell.

Antigen specific antibodies found in the body, secreted by B cells, can bind to infected target cells. The antibodies bind, exposing their Fc portion. The $F_c\gamma$III receptor, CD16, then binds to the antibodies and sends signals to the NK cell to release cytotoxic granules. The perforin released from the granules causes holes in the target cell membrane allowing in granzyme proteases. The proteases then cleave caspases, inducing apoptosis pathways. As the NK cell binds to and kills the infected cell, the NK cell also interacts with other immune cells during a viral infection. This interaction will be discussed next.

1.4 NK Immune Interactions

During an infection, one of the first cells to become activated are the dendritic cells (DCs). The immature DCs (iDCs) are found in the skin and at mucosal surfaces. They bind to invading microorganisms and internalize them for antigen processing. Upon internalization of the microbe, the iDCs become activated, undergo maturation and begin to secrete Interleukin 12 (IL-12) as they migrate to the lymphoid organs for antigen presentation to T cells (Wilson *et al.*, 1999). DCs secrete IL-12 through their 'stimulatory synapses,' which activates NK cells to produce IFN-γ and enhances NK cytotoxicity (Andoniou *et al.*, 2006; Hamerman *et al.*, 2005; Moretta, 2005; Moretta *et al.*, 2008; Moretta *et al.*, 2006). The activated NK cells secrete other cytokines such as TNF-α and GM-CSF that boost antigen processing abilities and maturation of more DCs (Moretta,

2005). Interaction between NK cells and DCs involves the binding of the NKG2D activatory receptor on the NK cell to an unknown ligand on the DCs (Moretta, 2005; Moretta *et al.*, 2006; O'Connor *et al.*, 2006).

NK cells maximize the efficiency of antigen presentation of the DCs by secreting IFN-γ that enables the DCs to enhance its antigen processing by promoting the upregulation of MHC class I and II receptors on the DCs. Another effect that the NK cells have on DCs, is to induce apoptosis in the iDC by the TRAIL pathway if the iDC is unresponsive to viral antigen or does not become mature (Hayakawa *et al.*, 2004; Moretta, 2005; Sivori *et al.*, 2004). It is known that both mature and immature DCs have TRAIL receptors on their surface. However, iDCs have low levels of MHC class I on their surface and mature DCs have upregulated MHC class I molecules and are resistant to NK cell induced apoptosis (Moretta *et al.*, 2008; O'Connor *et al.*, 2006). This "clean up" of unresponsive iDCs allows the host to maximize the efficiency of antigen presentation by having more DCs that can process and present antigen to T cells (Zhang *et al.*, 2006).

The IL-12 activated NK cells begin to express molecules such as L-selectins and CCR7 on their cell surface. This allows the NK cells to migrate to regional lymph nodes (Lucas *et al.*, 2007). In the lymph nodes, human NK cells predominately have a $CD56^{high}CD16^{low}$ receptor profile (Moretta, 2005; Moretta *et al.*, 2008; Zhang *et al.*, 2006). The high levels of cytokines produced by this subset of NK cells push the immune response towards a Th1 profile by increasing antigen presentation by antigen presenting cells (APCs) such as $CD11c^{high}$ DCs (Lucas *et al.*, 2007; Moretta, 2005; Moretta *et al.*, 2008).

In the lymph nodes, NK cells interact with $CD4^+$ T cells in order to directly participate in the adaptive immune response. It has been shown by a few groups that active NK cells express MHC class II molecules and present antigen to $CD4^+$ T cells. The binding of activating receptors on NK cells can cause them to gain the ability to process pathogen-derived antigen by multiple independent and unique pathways (Hanna *et al.*, 2004; Zhang *et al.*, 2006). Hanna *et al* demonstrated through proteomic analysis that activated human NK cells not only upregulate MHC class II receptors, but also multiple ligands for TCR co-stimulation. Binding of the activation markers, along with IL-12 and IL-15 secretion from mature DCs, causes the NK cells to express CD86, a co-stimulation marker that binds to CD28 on T cells, and OX40L, another co-stimulation marker that binds to OX40 on $CD4^+$ T cells (Zhang *et al.*, 2006). By activating $CD4^+$ T cells, NK cells are able to regulate a Th1 type adaptive immune response towards the invading virus.

NK cells travel from the lymph nodes to the peripheral blood and inflamed tissues. Here, human NK cells predominately have a $CD56^{low}CD16^{high}$ receptor profile and produce low amounts of cytokines, but have potent cytotoxic abilities (Moretta *et al.*, 2008). In the periphery, NK cells lyse compromised cells through granule exocytosis, Fas/FasL, TRAIL or TNF-α pathways (Raulet, 2003). The NK cells will not only kill abnormal cells, but will also secrete IFN-γ if they are still in contact or in the vicinity of activated mature DCs that are secreting IL-12. The IFN-γ cytokine will continue to perform the functions already discussed, but will also act on surrounding host cells, causing them to enter an anti-viral state to inhibit infections within them.

1.5 Ebolavirus

Ebolavirus is one of the two genera of the *Filoviridae* family, that can cause lethal epidemics of haemorrhagic fever with high mortality rates (50-90%) in humans (Bosio *et al.*, 2004; Bradfute *et al.*, 2007; Bray and Geisbert, 2005; Leroy *et al.*, 2001; Simmons *et al.*, 2002). *Ebolavirus* was identified simultaneously in Zaire (now known as the Democratic Republic of Congo) and Sudan, Africa in 1976. Since then, two new species of the *Ebolavirus* have been discovered, Reston (1989) found in Cynomolgus monkeys imported from the Philippines Islands, Côte d'Ivoire (1994) in a laboratory worker who came into contact with chimpanzees. Although it is believed to be zoonotic in nature, following the primary human case, person-to-person transmission by intimate contact is the main route of infection in human outbreaks (Feldmann *et al.*, 1997). The natural host is currently believed to be different species of fruit bats, such as *Hypsignathus monstrosus, Epomops franqueti,* and *Myonycteris torquata* (Biek *et al.*, 2006; Gonzalez *et al.*, 2007; Leroy *et al.*, 2005; van der Poel *et al.*, 2006).

Ebola is a negative sense, single stranded RNA virus. Its 19kb genome encodes seven structural proteins: a nucleoprotein (N), viral proteins (VP35, VP40, VP30 and VP24), a glycoprotein (GP) and a RNA dependent RNA polymerase (L) (Figure 1.3) (Feldmann *et al.*, 1997; Mahanty and Bray, 2004; Peters, 1996). The glycoprotein gene is transcribed into the only structural spike protein on the surface of the viron ($GP_{1,2}$) and two secreted nonstructural glycoproteins, sGP and ssGP (Feldmann *et al.*, 1997; Feldmann *et al.*, 1999; Peters, 1996).

Severe illness from this virus begins with non-specific symptoms such as fever, headache, malaise and myalgia following an incubation period of approximately 2 - 21

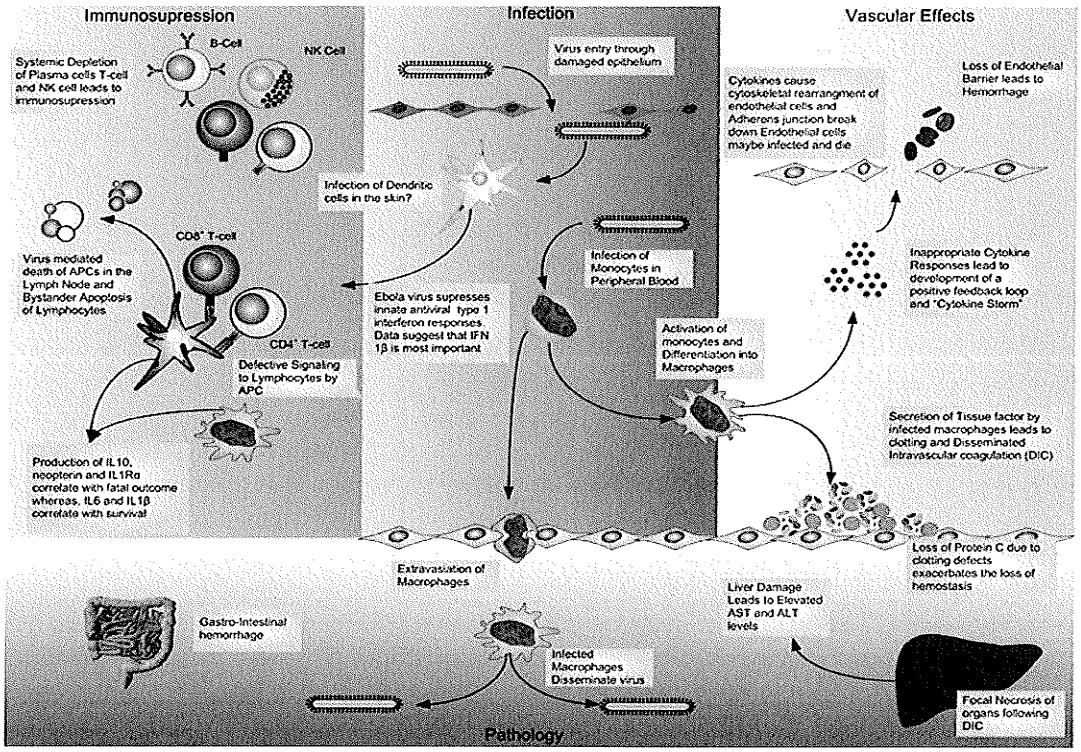

Figure 1.3 : Ebola Pathogenesis
Ebolavirus initially infects DCs and monocytes. Infection of these two cell types causes immunosuppression of some cell types such as T cells and NK cells, and improper cytokine production from other cells causing a "cytokine storm" that can lead to the breakdown of the endothelial barrier. The virus is disseminated through the body via infected cell, leading to tissue damage and hemorrhage and death of the individual (modified from Feldmann *et al.*, 2005).

days (Feldmann *et al.*, 1997; Geisbert and Jahrling, 2003). Patients rapidly deteriorate over the next few days noted by pharyngitis, severe nausea and vomiting that progresses to hematemeses and melena (Peters, 2005). Death by shock usually follows 6 – 9 days after clinical onset of disease.

During the course of infection in humans and nonhuman primates, the first cell types to be infected by *Ebolavirus* are monocyte/macrophages and DCs (Figure 1.3) (Bray and Geisbert, 2005; Feldmann *et al.*, 2005; Geisbert *et al.*, 2003; Mahanty and

Bray, 2004; Reed *et al.*, 2004). Infection of these cells allows the virus to circulate and replicate throughout the body as virus was observed in infected lymph nodes (Mahanty and Bray, 2004). The infection and death of these APCs blocks their ability to secrete proinflammatory cytokines or to upregulate co-stimulatory molecules that are needed for T cell stimulation (Geisbert *et al.*, 2003). The infection also causes a decrease in the expression of MHC class I and II proteins thereby decreasing the antigen presentation capacity (Simmons *et al.*, 2002; Sullivan *et al.*, 2005) and possibly the release of cytokines that activate other cells in the immune system, including NK cells.

Early in the infection, although not infected by the *Ebolavirus*, severe lymphopenia and lymphocyte destruction is observed. This is possibly due to a lack of co-stimulatory molecules or the engagement of death receptors such as Fas and TRAIL (Baize *et al.*, 1999; Bray and Geisbert, 2005; Geisbert *et al.*, 2003; Ignatiev *et al.*, 2000; Mahanty and Bray, 2004; Reed *et al.*, 2004; Sanchez *et al.*, 2004). This situation is observed in many non-human primate studies where the numbers of $CD4^+$ and $CD8^+$ T lymphocytes and possibly B cells decrease in the blood, spleen, lymph nodes and bone marrow (Baize *et al.*, 1999; Bray and Geisbert, 2005; Feldmann *et al.*, 2007; Geisbert *et al.*, 2003; Ignatiev *et al.*, 2000; Mahanty and Bray, 2004; Reed *et al.*, 2004; Sanchez *et al.*, 2004). The loss of lymphocytes is a hallmark feature of an Ebola infection (Reed *et al.*, 2004). The loss of $CD4^+$ and $CD8^+$ T cells and possibly NK cells, may prevent activation of macrophages and other inflammatory cells that may assist in constraining viral replication (Bray and Geisbert, 2005) because of the loss of cytokines these cells secrete.

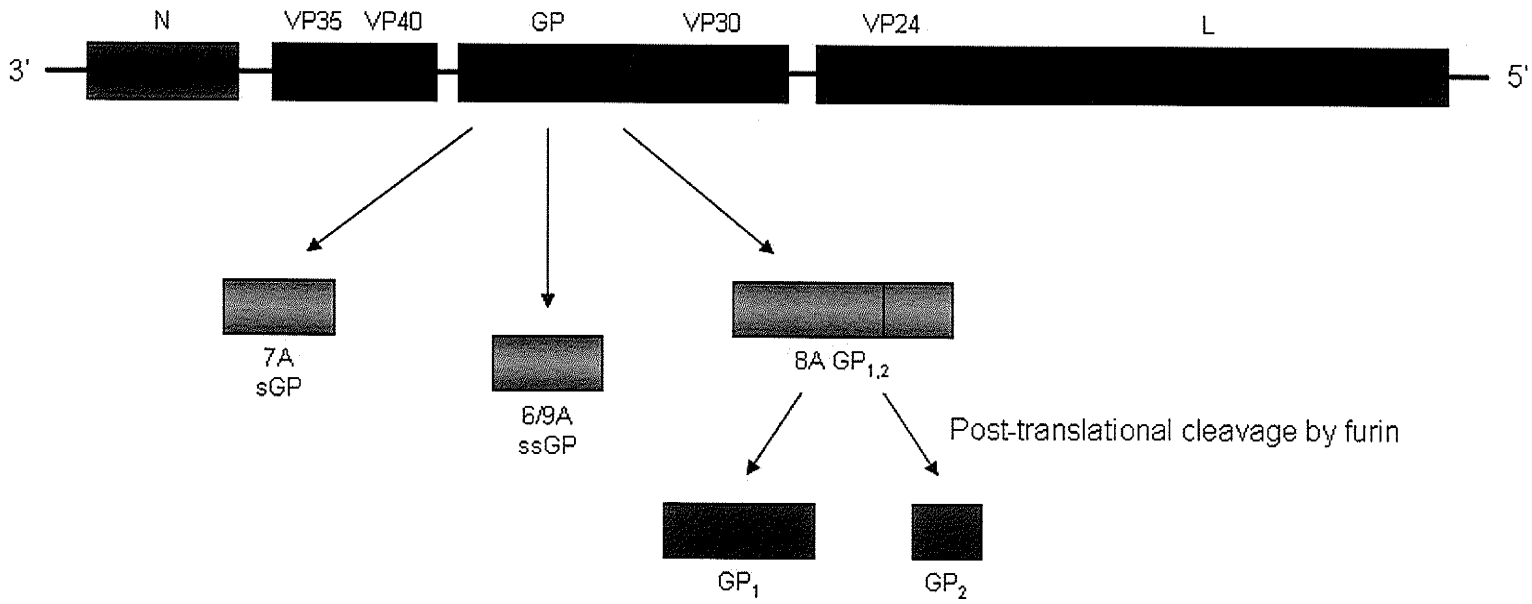

Figure 1.4: The genome of *Ebolavirus*.
The glycoprotein gene of the *Ebolavirus* produces multiple gene products: sGP, ssGP and $GP_{1,2}$. The $GP_{1,2}$ gene is translated and later cleaved into two proteins GP_1 and GP_2.

During the course of the disease, *Ebolavirus* can inhibit the immune response. An example of the innate immune response is the suppression of induction of type 1 interferons by the VP35 protein and the blockage of interferon action by the VP24 protein (Mohamadzadeh *et al.*, 2007; Peters, 2005). Interferons, again, are important in a viral infection. They inhibit viral growth; activate immune cells (eg. NK cells, macrophages and dendritic cells) and augment the adaptive immune response.

1.6 NK Cells and *Ebolavirus*

Not much is known about the fate of NK cells during an *Ebolavirus* infection. However, numerous sources have tried to determine what may occur to them. A non-human primate study was performed by Ignatiev *et al* on *Papio hamadryas* baboons. These NHPs were injected subcutaneously with 100 PFU/animal of guinea pig-adapted *Zaire ebolavirus*. The animals began displaying signs of infection by day 3 PI and succumbed to death between days 10 and 11 while demonstrating acute hemorrhagic syndrome (Ignatiev *et al.*, 2000). This experiment found that NK cell numbers actually increase in the blood during the course of infection. Despite of the increase in numbers, the killing activity of the NK cells found in the blood was decreased significantly (Ignatiev *et al.*, 2000). The killing activity, determined against K562 target cells using a radioactive 3H-uridine precursor, showed an increase in cell activity on day 8 post infection, but a significant decrease of activity near the end of the disease course on day 10 (Ignatiev *et al.*, 2000). This decrease in cytotoxic activity could be due to the lack of stimulus from other immune cells such as DCs which have become infected and do not function fully. Conversely, as the cytolytic activity of the NK cells decreased, the production of IFN-γ and TNF-α continued to increase until the disease had run its course

(Ignatiev *et al.*, 2000). This cytokine data corresponds to levels of proinflammatory cytokines found in the bloodstream of human victims of Ebola towards the end of the disease. This contrasts with human survivors who have high levels of these cytokines early in the course of infection (Mahanty and Bray, 2004).

This NK cell data is also supported by the analysis of PBMCs of human patients who have survived or succumbed to an outbreak of *Sudan ebolavirus* in 2000 that demonstrated a 50% mortality rate (Sanchez *et al.*, 2004). As seen in the non-human primate models, severe leukopenia developed throughout the human infection (Sanchez *et al.*, 2004). However, unlike the non-human primates, there was no significant difference in the percentage of NK cells in either fatality or survival cases (Sanchez *et al.*, 2004).

In an experiment performed by Geisbert *et al*, *Macaca fascicularis* macaques were inoculated with a lethal dose of 1000 PFU of ZEBOV (Geisbert *et al.*, 2003). Fever was observed in a few of these macaques by day 3 PI. The remaining animals began showing signs of infection by day 4 PI, including macular cutaneous rashes, fever and anorexia (Geisbert *et al.*, 2003). As with other lymphocytes, it was found that murine NK cells are not infected by *Ebolavirus* (Warfield *et al.*, 2004). However, in the Cynomolgus macaques infected in this experiment, flow cytometric analysis showed that the number of NK cells (determined as $CD8^{+}CD4^{lo}$ cells) dramatically declined by day 4 of an Ebola infection (Geisbert *et al.*, 2003). NK cell levels dropped quickly within a matter of days to nearly 25% of the original population of NK cells seen on day 1 (Geisbert *et al.*, 2003). Furthermore, when transmission electron microscopy was done on

the blood of these macaques on day 3, large granular cells that seemed morphologically identical to NK cells, were seen to have gone through apoptosis (Geisbert *et al.*, 2003).

Further study of the blood of these *Ebolavirus* infected Cynomolgus macaques showed a 60 – 70% decrease in the $CD8^+$ lymphocytes by day 4 (Reed *et al.*, 2004). A closer look at the $CD8^+$ cells, specifically the $CD8^{lo}CD16^+$ cells, deemed NK cells, showed the most significant decrease in the absolute number of NK cells (Reed *et al.*, 2004). Observations of the cells showed an increased expression of Fas, which suggested that the death of these $CD8^{lo}CD16^+$ cells could be due to the Fas/FasL pathway (Reed *et al.*, 2004). Additional data in this paper suggested to the investigators that the lymphocytes were dying owing to the lack of stimulation by DCs which had already undergone apoptosis early in the infection (Reed *et al.*, 2004).

The wild type *Ebolavirus* does not cause disease in mice; however a mouse-adapted strain is lethal in mice. Bradfute *et al* performed experiments in Balb.c mice by injecting them intraperitoneally with 1000 PFU of mouse-adapted ZEBOV (Bradfute *et al.*, 2007). This studies performed showed a significant decrease in number of NK cells found in the blood and spleen in the first few days of the Ebola infection (Bradfute *et al.*, 2007). However, lymphocyte death was observed as classical apoptosis and lymphocytolysis, a broad definition of different types of cell death, whereas in non-human primate models only classical apoptosis was seen (Bradfute *et al.*, 2007).

There may be several reasons why there was a difference seen in the fate of NK cells during these experiments. One reason may be due to the different viruses used to infect the different animal species. The work done by Ignatiev *et al* used a guinea pig adapted virus to infect baboons. By adapting the virus to guinea pigs, it may have

changed the properties of the virus compared to the wild type *Ebolavirus*, given that wild type *Ebolavirus* cannot kill adult mice and must be adapted by serial passage in order to achieve this end result (Bray, 2001; Bray *et al.*, 1998). Thus, a change in the host immune response to this new virus could be explained. Furthermore, giving a guinea pig adapted virus to baboons may cause the animals' immune system to behave differently than what would be observed when giving the animals a virus normally adaptive to that host, as seen in the paper by Sanchez *et al.* A different species of *Ebolavirus* was given to the Cynomolgus macaques in the two papers by Geisbert *et al* and Reed *et al.* The four different species of the *Ebolavirus* show a high degree of sequence divergence at both the nucleotide and amino acid levels (Sanchez and Rollin, 2005; Sanchez *et al.*, 1996). This divergence may explain why there is a discrepancy in data from *Zaire ebolavirus* and *Sudan ebolavirus*.

Another difference in the experimental designs of these papers is how they determine what a NK cell is. In the paper by Ignative *et al*, the authors performed an E- and EAC- rosette-formation test to determine the numbers of circulating T and B cells. The NK cells were considered to be "null" lymphocytes, i.e. not T cells and not B cells. Hence the test for NK cells truly determined that lymphocytes in the PBMCs that did not bind to make a rosette would be an NK cell. The papers by Sanchez *et al*, Geisbert *et al* and Reed *et al*, used a more modern approach to determine the number of NK cells in the blood. By using flow cytometry, Sanchez *et al* correctly determined the NK cells to be the $CD56^{+}CD16^{+}$ cells within a $CD45^{+}/CD3^{+}$ gate. Geisbert *et al* and Reed *et al* determined the NK cell population to be $CD8^{+}CD4^{lo}$ cells. The Reed paper went further to look at the $CD8^{lo}CD16^{+}$ cells. CD16 is an Fcγ receptor found on all NK cells and

should be used, especially in non-human primate experiments, to determine what an NK cell is. However, it might have been better for these groups to look at the $CD3^-$ (not T cells) $CD16^+$ population and called these NK cells.

The data from these groups all show evidence of what may be occurring to NK cells during an Ebola infection. However, all the data stems from experiments done on PBMCs. Since known data about NK cells during the Ebola infection is from one compartment of the body, it is unclear if the NK cells might be migration from compartment to compartment. No data has been generated that determines whether there really is an increase or decrease of NK cell numbers during infection. If a migration does occur, then activities of the NK cells in these other compartments may be different than what is seen in the PBMCs. Nonetheless, since the data from Sanchez *et al* is based upon an authentic human viral infection, this information may be more reliable than the other publications about what transpires with NK cells in the blood during an Ebola viral infection.

1.7 NK Cells and Vaccines Against the *Ebolavirus*

Many studies have used the Ebola VP40 and structural glycoprotein to make virus like particles (VLPs) as a vaccine against *Ebolavirus* (Bosio *et al.*, 2004; Wahl-Jensen *et al.*, 2005; Warfield *et al.*, 2004; Warfield *et al.*, 2005; Warfield *et al.*, 2007; Ye *et al.*, 2006). VLPs are particles that look like virus, but do not contain the viral RNA necessary for replication. The use of these non-infectious VP40/GP VLPs indicated that NK cells are needed to survive an Ebola infection. Human DCs become mature and are activated when stimulated with these VLPs (Bosio *et al.*, 2004). This causes them to secrete cytokines that can lead to the activation of NK cells. Since there is a decrease in

Table 1.2: Examples of Filovirus Vaccine Candidates

Animal Model	Vaccine Vector	Challenge Virus	Survival	Reference
Rhesus macaques (*Macaca mulatta*)	VSVΔG/ZEBOV GP (Mayinga)	ZEBOV (Kikwit)	4/6	(Geisbert *et al.*, 2008)
Balb/c mice	VSVΔG/ZEBOV GP (Mayinga)	[b]Mouse adapted-ZEBOV	5/5	(Feldmann *et al.*, 2007)
Guinea pigs (strain Hartley)	VSVΔG/ZEBOV GP (Mayinga)	[c]Guinea pig adapted-ZEBOV	4/6 24h prior 5/6 1h post 3/6 24h post	
Rhesus macaques (*Macaca mulatta*)	VSVΔG/ZEBOV GP (Mayinga)	ZEBOV (Kikwit)	4/8	
Rhesus macaques (*Macaca mulatta*)	[a]HPIV3/EboGP	ZEBOV (Mayinga)	Expt 1: 2/2 Expt 2: 2/3 (1 dose), 3/3 (2 doses)	(Bukreyev *et al.*, 2007)
	HPIV3/EboGP+NP		1/1	
	HPIV3/EboGP+GM-CSF		2/2	
Balb/c mice	DNA vaccine (GP) +/or glycosylation deletions (not included)	Mouse adapted-ZEBOV	24/27	(Dowling *et al.*, 2007)
Cynomolgus macaques (*Macaca fascicularis*)	[d]Ad/GP + NP	ZEBOV (Kikwit)	4/4	(Sullivan *et al.*, 2006)
	Ad/GPΔTM + NP		2/3	
	AdGPΔTM		0/3	
Balb/c mice	[e]AdC7-ZGP	Mouse adapted-ZEBOV	5/5	(Kobinger *et al.*, 2006)
Guinea pigs (strain Hartley)		Guinea pig adapted-ZEBOV	6/6	
Guinea pigs (strain Hartley)	HPIV3/EboGP	Guinea pig adapted-ZEBOV	100%	(Bukreyev *et al.*, 2006)
	HPIV3/EboGP + NP		100%	
Rhesus macaques (*Macaca mulatta*)	VSVΔG/MARV GP (Musoke)	MARV (Musoke)	5/5	(Daddario-DiCaprio *et al.*, 2006b)
Cynomolgus macaques (*Macaca fascicularis*)	VSVΔG/MARV GP (Musoke)	MARV (Ravn)	3/3	(Daddario-DiCaprio *et al.*, 2006a)
		MARV (Angola)	3/3	
		MARV (Musoke)	1/1	
Guinea pigs (strain 13)	ZEBOV (Kikwit) VP40 + GP VLP	Guinea pig adapted-ZEBOV (Kikwit)	10/10	(Swenson *et al.*, 2005)
		Guinea pig adapted-MARV	2/8	
	MARV (Musoke) VP40 + GP VLP	Guinea pig adapted-MARV (Musoke)	10/10	
		Guinea pig adapted-ZEBOV	0/10	
Cynomolgus macaques (*Macaca fascicularis*)	VSVΔG/ZEBOV GP (Mayinga)	ZEBOV (Mayinga)	4/4	(Jones *et al.*, 2005)
	VSVΔG/MARV GP (Musoke)	MARV (Musoke)	4/4	

Balb/c mice	VSVΔG/ZEBOV GP (Mayinga)	Mouse adapted-ZEBOV	5/5	(Garbutt *et al.*, 2004)
Cynomolgus macaques (*Macaca fascicularis*)	ADV-GP ADV-NP	ZEBOV (Kikwit)	4/4 4/4	(Sullivan *et al.*, 2003)
Balb/c mice	[f]L(EV) [g]EV	Mouse adapted-ZEBOV	i.v: 64/64 i.m: 33/43 i.v: 11/20 i.m: 9/20	(Rao *et al.*, 2002)
Cynomolgus macaques (*Macaca fascicularis*)	L(EV)	ZEBOV (Kikwit)	0/3	
Balb/c mice	EBOV VP24 EBOV VP30 EBOV VP35 EBOV VP40	Mouse adapted-ZEBOV	(3 dose) 18/20 (2 dose) 19/20 (3 dose) 17/20 (2 dose) 11/20 (3 dose) 5/19 (2 dose) 4/20 (3 dose) 14/20 (2 dose) 17/20	(Wilson *et al.*, 2001b)
C57Bl/6 mice	EBOV VP24 EBOV VP30 EBOV VP35 EBOV VP40		(3 dose) 0/20 (3 dose) 2/20 (3 dose) 14/20 (3 dose) 1/20	
Cynomolgus macaques (*Macaca fascicularis*)	DNA expressing Zaire GP, Ivory Coast GP, Sudan GP, Zaire NP and boosted with ADV-ZGP	Zaire (Mayinga)	4/4	(Sullivan *et al.*, 2000)

[a]HPIV3 = Human parainfluenza virus type 3
[b]Mouse adapted ZEBOV = strain Mayinga
[c]Guinea pig adapted ZEBOV = strain Mayinga unless otherwise noted
[d]AdGP = E1/E3-deleted Adenovirus GP
[e]AdC7 = Chimpanzee adenovirus pan 7
[f]L(EV) = Liposome-encapsulated irradiated ZEBOV (Kikwit)
[g]EV = Nonencapulated irradiated ZEBOV (Kikwit)

NK activity during an Ebola infection, activation of the NK cells by the DCs via the VLPs can cause them to release soluble molecules to counteract viral infections. Human NK cells produce cytokines when directly stimulated with Ebola VLPs and activate DCs to mature, produce their own cytokines and upregulate activating ligands, such as NKp30 and MHC class I and II molecules (Fuller *et al.*, 2007). By activating NK cells directly or

indirectly, there are more activated immune cells able to assist in the fight against the disease, thus causing the individual to survive infection.

VLP studies in mice have demonstrated that mice possessing NK cells can survive a lethal challenge of mouse adapted *Ebolavirus* when pre-treated 1-3 days prior with Ebola VLPs (Warfield *et al.*, 2004). However, mice that are deficient in NK cells due to NK cell removal by anti-Asialo GM1 injections, succumb and die even when pre-treated with VLPs (Warfield *et al.*, 2004). Stimulated NK cells traffic to the mediastinal lymph nodes and spleen where they produce cytokines such as IFN-γ, TNF-α, IL-4, IL-5, IL-6, IL-13 and MIP-1α (Warfield *et al.*, 2004) and kill virus infected cells. The cytokines the NK cells produce in the lymph nodes and spleen are taken up by other immune cells such as $CD4^+$ T cells, which are then able to become activated.

The protection with VLPs seems to correspond to the idea that NK cells fight an *Ebolavirus* infection using perforin. Mice that are deficient in perforin, when given VLPs a few days prior to challenge with mouse-adapted *Ebolavirus* do not survive infection (Warfield *et al.*, 2004). However, mice that are deficient in IFN-γ can survive a lethal challenge of mouse adapted *Ebolavirus* (Warfield *et al.*, 2004) when vaccinated with VLPs. This suggests that cells that perform perforin-mediated apoptosis are needed to fight this infection even though IFN-γ did not play a significant role in this set of experiments. This data suggests that because of the rapid time to protection from viral challenge, these perforin containing cells must be acting from the innate immune system; hence NK cells might be the source.

Vaccine development to fight infectious diseases is based on targeting the adaptive immune system and inducing the generation of B and/or T memory lymphocytes

specific for the desired infectious agent. This may not be the case in recent studies of Vesicular Stomatitis Virus (VSV) based vaccine against the *Ebolavirus*. Both mice and guinea pigs have survived a lethal dose of mouse-adaptive and guinea pig-adapted *Ebolavirus* respectively, when vaccinated with VSVΔG/ZEBOV GP 30 minutes to one day after challenge (Feldmann *et al.*, 2007; Geisbert and Jahrling, 2003; Jones *et al.*, 2005). Non-human primate survivors, when given the *Ebolavirus* followed by VSVΔG/ZEBOV GP, had an increase in the percentage of NK cells in the PBMCs six days after infection (Feldmann *et al.*, 2007). The increase in NK cells seen in these experiments, suggest that the recombinant virus is either directly or indirectly stimulating NK cells in order to protect the animal from the viral challenge.

In addition to activating NK cells, non-human primates vaccinated with VSVΔG/ZEBOV GP develop low to moderate levels of IgG non-neutralizing antibodies against *Zaire ebolavirus* glycoproteins (Jones *et al.*, 2005). These circulating antibodies could be used by NK cells in ADCC during the first few days of the innate immune response. Although there is no direct evidence of this fact, these antibodies may be able to bind to infected cells that express the Ebola GP on their surface, thus causing them to be recognized by CD16 (the Fc_γ receptor) found on NK cells. By binding antibodies in enough quantity to the activating receptor CD16, the NK would become activated and kill the virally infected cells. Thus another possible way NK cells helps to protect the infected individual during the first few days of infection. Taken all together, the data suggests that NK cells could have become highly activated within a short period of time to kill viral infected cells, thereby allowing the non-human primates time to mount an adaptive immune response.

1.8 Ebola Therapeutics and Prophylaxis

Currently, there are no approved therapies for an Ebola infection. Early during the first Ebola outbreaks, the virus spreads easily in the population because of the lack of protective gear, basic equipment and other resources imperative to provide care for infected patients (Hewlett and Hewlett, 2005). For patients, only supportive therapy is offered to maintain their blood volume, electrolyte balance and manage shock, cerebral edema, renal failure, coagulation disorders, cardiovascular collapse and secondary infections (Feldmann *et al.*, 2005; Mupapa *et al.*, 1999). Studies for the treatment of Ebola haemorrhagic fever are currently ongoing. Many options are being investigated including neutralizing antibodies, different types of viral infection inhibitors and host immune modulators.

Human convalescent blood has been utilized in both human and non-human primate Ebola infections. However, the effect of convalescent blood is not certain. Eight patients infected with Ebola in the 1995 Kikwit outbreak in the Democratic Republic of the Congo were transfused with total blood taken from previous survivors of an Ebola outbreak (Keller and Stiehm, 2000; Mupapa *et al.*, 1999; Wilson *et al.*, 2001a). The whole blood given to the eight patients contained IgG antibodies specific to Ebola along with coagulation factors and shock mediators found in the blood of the survivors (Mupapa *et al.*, 1999; Wilson *et al.*, 2001a). Seven out of the eight patients given the convalescent blood survived the infection. However, these patients were also given better care than previous patients. They received infusions of glucose, electrolytes, antibiotics, anti-malaria drugs and food (Mupapa *et al.*, 1999). Therefore, it is not clear if the convalescent blood alone was responsible for the survival of these seven patients. In

another study, non-human primates transfused with convalescent blood from Ebola immune rhesus macaques following a challenge of the Ebola virus, developed viremia and clinical signs of haemorrhagic fever (Jahrling *et al.*, 2007). All of these primates died from the infection without a delay in death (Jahrling *et al.*, 2007). These data suggest that Ebola convalescent serum is not a sufficient treatment for survival of Ebola haemorrhagic fever.

Many studies using Ebola GP specific neutralizing monoclonal antibodies from different species have been performed on different animal models to determine whether antibodies could be used as a treatment against Ebola infection (Feldmann *et al.*, 2005). Whilst the results from these studies have shown a reduction in viral burden, survival rates after challenge have been diverse in the different animal models (Feldmann *et al.*, 2005). Horses, goats and sheep have been observed to be totally resistant to an *Ebolavirus* infection and, therefore, can be used to make antibodies against this virus (Kudoyarova-Zubavichene *et al.*, 1999). A British scientist received goat anti-Ebola IgG and recombinant human IFN-α2 after sticking himself with a needle contaminated with *Sudan ebolavirus*. He came down with signs of infection, but survived (Jahrling *et al.*, 1999; Kudoyarova-Zubavichene *et al.*, 1999; Mupapa *et al.*, 1999). There were no virus or Ebola antibodies found in his blood after his recovery. However there was no search for Ebola antigen in his blood right after the needle stick (Kudoyarova-Zubavichene *et al.*, 1999; Wilson *et al.*, 2001a).

Goat anti-Ebola IgG has also been given to guinea pigs with some positive effect However, protection was seen only when the antibodies were given to these animals within 72 hours post challenge (Keller and Stiehm, 2000; Wilson *et al.*, 2001a). Other

studies which used neutralizing mAbs against the Ebola GP showed complete protection in mice if given 2 days after mouse adapted *Ebolavirus* challenge (Takada *et al.*, 2007). However, as was found in the previous studies, protection was only partial when the mAbs were given 3 to 4 days post challenge (Takada *et al.*, 2007). Partial protection and delayed death was seen when these same mAbs were used in guinea pigs (Takada *et al.*, 2007). At the same time, a dose dependent protection was seen in guinea pigs when using human mAbs (Parren *et al.*, 2002). Better protection was observed in baboons which received equine anti-Ebola IgG after infection with Ebola. 80% of these animals survived when IgG was given 1 hour after infection (Jahrling *et al.*, 1999; Keller and Stiehm, 2000; Kudoyarova-Zubavichene *et al.*, 1999; Margaret A. Keller, 2000; Natalya M. Kudoyarova-Zubavicheme, 1999; P.B. Jahrling, 1999). Together, these studies suggest a role in protection for anti-Ebola antibodies. However, the failure to completely protect animals from death deems antibody therapy as insufficient.

Passive immunization with equine hyperimmune serum has also shown potential in some animal models. Only partial protection and a delay in death were seen in the mouse model, no matter how long after infection the serum was given (Jahrling *et al.*, 1999; Wilson *et al.*, 2001a). Guinea pigs were completely protected using equine IgG if given before viremia has developed (Jahrling *et al.*, 1999; Wilson *et al.*, 2001a). In the non-human primate model, equine hyperimmune serum was not completely protective. Cynomolgus macaques show a delay in the onset of illness and viremia, but all macaques died from the infection (Jahrling *et al.*, 1999; Jahrling *et al.*, 1996; Keller and Stiehm, 2000; Wilson *et al.*, 2001a). When given IFN-α2b in combination with equine IgG, death is also delayed, but these animals still succumb to disease (Jahrling *et al.*, 1996).

Therefore, treatment with antibodies against the *Ebolavirus* seem to aid in delaying death in these animals, possibly by ADCC, but these antibodies are not sufficient for complete survival of the animals.

Other studies have used different types of viral infection inhibitors and host immune modulators in an attempt to treat Ebola infections. Nematode-derived anticoagulation protein (rNAPc2) targets the extrinsic blood coagulation pathway. 33% of non-human primates survived an Ebola infection when treated with rNAPc2 (Feldmann *et al.*, 2005; Stroher and Feldmann, 2006). IFN-α treatments in mice, guinea pigs and non-human primates delayed the onset of viremia and death, but did not protect the animals from the infection (Feldmann *et al.*, 2005). While S-adenosylhomocysteine hydrolase inhibitors were able to increase IFN-α and protect mice, they had no effect in non-human primates (Feldmann *et al.*, 2005; Wilson *et al.*, 2001a). Recombinant human activated protein C (rhAPC), major physiological anticoagulant, was able to lower viral loads, decrease activation of coagulation and increase the mean time to death in rhesus macaques, but it was only partially protective in these animals (Hensley *et al.*, 2007).

A recent study performed by Geisbert *et al*, inoculated rhesus macaques with 1000 PFU of SEBOV intramuscular followed by an intramuscular injection of ~$2x10^7$ PFU of VSVΔG/SEBOV GP 20 to 30 minutes later (Geisbert *et al.*, 2008). All the NHPs given the post-exposure treatment developed fever by day 6 PI, but all survived the SEBOV challenge. Similar to post-exposure treatment with the VSVΔG/ZEBOV GP virus (Feldmann *et al.*, 2007), these NHP receiving VSVΔG/SEBOV GP post challenge developed IgM and IgG titres by day 6 and 10 respectively. This data indicates that the recombinant VSV viruses are able to help protect NHPs from a lethal challenge of

Ebolavirus. However, more data must be generated to determine the method of protection post-exposure to the virus challenge.

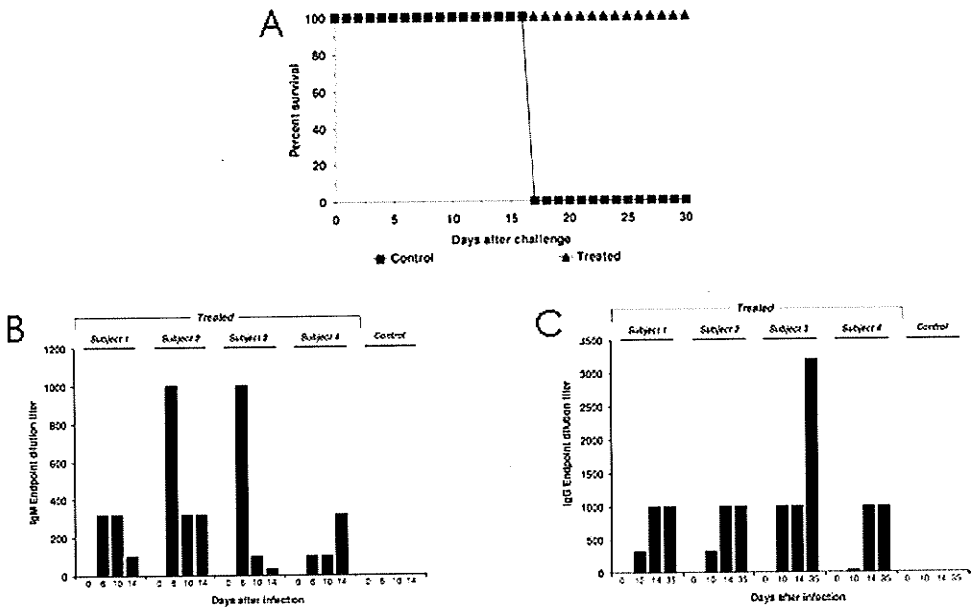

Figure 1.5: Experimental results from a Post-exposure study of VSVΔG/SEBOV GP against *Sudan Ebolavirus* in NHPs.
NHPs were injected with VSVΔG/SEBOV GP i.m 20 – 30 minutes subsequent to an i.m lethal injection of SEBOV. All NHPs receiving VSVΔG/SEBOV GP post-challenge (A) survived and generated (B) IgM and (C) IgG titres. (Geisbert *et al.*, 2008).

Most of the above treatments have not shown efficacy in all animal models. More importantly, most of these treatments were not able to fully protect non-human primates, the model which most closely resembles an *Ebolavirus* infection in humans. This being the case, a combination of treatment methods may be the most effective way to treat an Ebola infection during an outbreak (Feldmann *et al.*, 2005; Stroher and Feldmann, 2006) until a more effective treatment is made available for human use. However, treatment of the disease should include an approach that can induce the host innate immune system, such as the rVSV virus may accomplish, in order to slow down infection and build a proper response that can clear the virus from the infected individual.

1.9 Objectives and Hypothesis

Since the time to death from an Ebola infection is very short, the VSVΔG/ZEBOV GP virus used for post-exposure treatment must induce a response from the innate immune system rather than the adaptive immune system (Daddario-DiCaprio *et al.*, 2006b; Feldmann *et al.*, 2007; Geisbert *et al.*, 2008). This therapeutic appears to activate NK cells and build time for a viral clearance response by the adaptive immune system. The activities of NK cells seem to be very important within the discussed platforms for the model to survive infection. Without NK cells, some models did not survive challenge. Determination of the specific activities of NK cells during these vaccination periods must be studied further, however the importance of NK cells can not be denied.

The present study was designed to discover the role of natural killer cells in the recombinant VSVΔG/ZEBOV GP therapeutic platform. To determine the NK cell role, both primary splenic murine NK cells and a human NK cell line were used to establish a cell mediated cytotoxicity assay against target cells infected with VSVΔG/ZEBOV GP. Since the method of purification of primary splenic murine NK cells had to be established, the human NK cell line was utilized to set up the NK cell cytotoxicity assay. Once the assays were working, the murine NK cells were to be used in the same assays to eventually move the experiments *in vivo*. The read out for the cytotoxicity assay was by flow cytometry since it could be utilized easily both in CL2 and CL4 conditions.

The hypothesis for this project was that NK cells would lyse VSVΔG/ZEBOV GP infected cells more frequently than uninfected cells. This would possibly occur because the target cell would decrease the amount of MHC class I on the surface causing a loss in

an inhibition signal to the NK cells. The expression Ebola GP on the surface of the target cell would also give an additional activation signal to the NK cell to enhance the cytotoxicity against the infected target cell.

1.10 Significance of the Study

The present study was used to determine one of the pathways of protection of the VSVΔG/ZEBOV GP therapeutic post-exposure to an infection with the *Ebolavirus*. Once the manner of protection is resolved, moving to clinical trials of the vaccine would be less complicated. Since protection with this recombinant virus was seen utilized as both a vaccine and as a therapeutic against the *Ebolavirus*, it could be used to vaccinate humans and non-human primates in Africa against Ebola. The recombinant virus could also be used in an outbreak situation as a treatment for care givers to prevent transmission of the virus from an infected individual. Finally, this therapeutic could be used against a bioterrorist attack using the *Ebolavirus*.

2. Materials and Methods

2.1 Cell Lines

Vero E6 (green African monkey kidney) cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, U.S.A, Cat# CRL-1586). The Vero E6 cells were cultured in supplemented Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, Oakville, Ontario, Canada, Cat# D5796) now known as DMEM-10 (see Appendix 3 for recipe). The Vero E6 cells were maintained by splitting them 1:8 every four days when they were 100% confluent. Briefly, adherent cells were washed once with PBS. Two milliliters of 0.25% Trypsin-3.8 g/L EDTA (Invitrogen, Gibco, Cat# 25200) was added to the cells and the flask was incubated at 37°C, 5% CO_2, until the cells sloughed off after gentle tapping of the flask sides. Cells were resuspended in 3 mL in DMEM-10 and the 1:8 split was performed. Cells were cultured in 20 mL of DMEM-10 in a 75 cm^2 polystyrene Cell Culture Flasks (Corning Incorporated, Corning, New York, USA, Cat# 43064).

293T (human kidney epithelial cells) cells were provided by Hans Schnittler (TU-Dresden, Germany). These cells were cultured in DMEM-10. The 293T cells were maintained by splitting them 1:10 every four days when they were approximately 90% confluent. The 293T cells were split and cultured as described for the Vero E6 cells.

NK-92MI (human lymphoblast NK) cells were purchased from ATCC (Cat# CRL-2408). They were cultured in supplemented Alpha Modified Minimum Essential Medium Eagle containing sodium bicarbonate (αMEM, Sigma-Aldrich, Cat# M4526), now known as αMEM-12 (Appendix 3). The NK-92MI cells were maintained by splitting them 1:2 every four days when cells were approximately at a 10^6 cells/mL

concentration. Briefly, suspension cells were centrifuged at 300 x g for 5 minutes (min) at 4°C. The media was removed and the pellet was resuspended in 5 mL of αMEM-12 and the 1:2 split was performed. Cells were cultured in 20 mL of αMEM-12 in 75 cm^2 polystyrene Tissue Culture Flasks (Sarstedt Inc., Newton, North Carolina, Cat# 83.1813.500). These cells were used at passage 15-30.

K562 (human erythroleukemia) cells (Cat# CCL-243), Daudi (human B lymphoblast) cells (Cat# CCL-213) and YAC-1 (mouse T lymphoblast) cells were procured from ATCC (Cat# TIB-160). These 3 cell lines were cultured in supplemented RPMI medium 1640, now known as RPMI-10 (Appendix 3). The cell lines were maintained by splitting them 1:8 every four days when the cells were approximately at a 10^6 cells/mL concentration. These cell lines were split and cultured as described for the NK-92MI cells. These cell lines were used at passage 15 to 25.

RAW 264.7 (mouse macrophage) cells were obtained from ATCC (Cat# TIB-71). They were cultured in DMEM-10 and maintained by splitting them 1:10 every four days when the cells were approximately 100% confluent. Briefly, media was discarded and 5 mL of DMEM-10 was added to the cells. The cells were removed by scraping the bottom of the flask with a cell scraper. The cell suspension was used to wash the bottom of the flask to remove any remaining adherent cells and the 1:10 split was performed. RAW 264.7 cells were cultured as described for the Vero E6 cells.

All the mammalian cell lines were maintained in a 37°C, 5% CO_2 humidified incubator.

MAX Efficiency Stbl2 Competent Cells were purchased from Invitrogen (Cat# 10268-019). *Escherichia coli* (*E. coli*) XL1-Blue competent cells were cultured at the

National Microbiology Laboratories (NML, Winnipeg, Manitoba, Canada) by growing a 0.5 mL overnight culture of *E. coli* XL1-Blue in 50mL of Luria Broth (LB) at 37°C with shaking until the OD_{660} was within 0.5 – 0.8. Cells were then incubated for 20 min on ice and centrifuged at 18,000 x g for 10 min at 4°C. The bacterial pellet was resuspended in 5 mL of TSS buffer (Appendix 3) and 1 mL aliquots were stored at -80°C.

2.2 Viruses

2.2.1 Wild Type Viruses

Zaire ebolavirus (ZEBOV) strain Kikwit, was provided by the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID, Frederick, Maryland, USA). Plamids for the Vesicular Stomatitis Virus (VSV) was donated from John Rose of Yale University (New Haven, Connecticut, USA). Stock preparations of both viruses were grown in T-150 cm^2 cell culture flasks (Corning Incorporated, Cat# 420825) seeded with Vero E6 cells. Absorption of the virus on Vero E6 cells was performed with either $4x10^4$ $TCID_{50}$/mL or $4x10^3$ $TCID_{50}$/mL in 10 mL of DMEM and incubated for 1 hour at 37°C, 5% CO_2, while rocking the flask every 15 min to ensure full coverage of all cells in the flask. The virus inoculum was removed and 35 mL of DMEM-2 (Appendix 3) was added to the cells. The flask was left at 37°C, 5% CO_2, until 80% cytopathic effect (CPE). The remaining cells were removed from the flask with a cell scraper. To remove the cellular debris, the cells and supernatant were centrifuged at 500 x g for 10 min at 4°C. The supernatant was collected and heat inactivated FBS was added to the supernatant at a final concentration of 10%. Aliquots of virus were stored at -80°C. Handling of the *Ebolavirus* was performed under CL4 conditions. Handling of VSV was performed under enhanced CL2 conditions. Experiments with both viruses were conducted in a biosafety

cabinet (BSC) as outlined in the Health Canada Laboratory Biosafety Guildlines (http:www.phac-aspc.gc.ca/publicat/lbg-ldmbl-04/index.html).

2.2.2 Recombinant Viruses

VSVΔG/ZEBOV GP strain Kikwit, VSVΔG/Sudan (SEBOV) GP strain Boniface and VSVΔG/Marburgvirus (MARV) GP strain Musoke were constructed and cultured at the NML (Roberts *et al.*, 1999). Please see section 2.5 for construction of the recombinant viruses. All recombinant viruses were cultured and handled as per wild type viruses under enhanced CL2 conditions.

2.3 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was used for obtaining ZEBOV and SEBOV Gulu GP cDNA for cloning (section 2.6). RT-PCR was performed using a Titan One Tube RT-PCR kit (Roche, Laval, Quebec, Canada), a Robust I RT-PCR kit (Finnzymes, Espoo, Finland) or an OneStep RT-PCR kit (Qiagen, Mississauga, Ontario, Canada). RT-PCR reactions were carried out in a Perkin Elmer GeneAmp PCR System 2400 thermocycler or a Biometra T1 thermocycler. In using the Titan kit, two 25 μL reaction mixtures were prepared and combined. Reaction mixture 1 contained: 12.5 μL of sterile water, 4 μL of 10 mM dNTP mix, 2.5 μL of 5 mM DTT, 2 μL of each 10 mM forward and reverse primers and 2 μL of RNA (1 μg/μL). Reaction mixture 2 contained: 14 μL of sterile water, 10 μL of 5x buffer and 1 μL of enzyme mix. The Robust kit was made to make 1 reaction mixture containing: 31.5 μL of sterile water, 5 μL of 10x buffer, 1.5 μL of 50 mM $MgCl_2$, 1 μL of 10 mM dNTP mix, 1 μL of 5 unit/μL reverse transcriptase, 2 μL of 1 unit/μL DNA polymerase, 3 μL of each 10 μM forward and reverse primers and 2 μL RNA (1 μg/μL). The Qiagen kit was used to make 1 reaction mixture containing: 28 μL of sterile water,

10 μL of 5x buffer, 2 μL of 10 mM dNTP mix, 3 μL of each 10 μM forward and reverse primers, 2 μL enzyme mix and 2 μL of RNA (1 μg/μL). All reactions were set up on ice.

An example thermocycle protocol for a RT-PCR reaction for ZEBOV GP (2031 bps utilizing primer set EBOVGP95140rXhoI and EBOVGP952171fNheI) included:

Step	Temperature	Time
RT-PCR	50°C	30 min
Initial Denaturation	94°C	2 min
Denature	94°C	30 seconds
Anneal	55°C	30 seconds
Extend	72°C	2.5 min
Final Extension	72°C	7 min
Pause	4°C	infinitely

Each protocol was run for 40 cycles between the denaturation and extension steps.

2.3.1 Real Time RT-PCR

Real Time RT-PCR was used to determine virus titre after mammalian cell line infections. Real time RT-PCR was performed using a QuantiTect Probe RT-PCR kit (Qiagen, Cat# 204456) in order to quantify amounts of RNA in a sample over time. This assay was carried out as per the manufacturer's protocol. The kit contained a 12.5 μL of reaction mixture to which 8.5 μL of sterile water, 0.5 μL of forward and reverse primer probes, 2 μL of RNA and 0.5 μL of a reverse transcriptase enzyme were added. All reactions were set up on ice.

Real Time RT-PCR was run on a Cepheid Smart cycler and analyzed using Smart Cycler software. An example smart cycle protocol for a real time RT-PCR reaction for ZEBOV GP included:

Step	Temperature	Time
RT-PCR	50°C	30 min
Activation	95°C	1.5 min
Denature	95°C	15 seconds
Anneal	60°C	60 seconds
Pause	4°C	infinitely

Each protocol was run for 45 cycles between the denaturation and extension steps.

2.3.2 RT-PCR Product Anaylsis

RT-PCR amplicons were run on a 0.8% agarose gel to determine the size and quality of the samples. The gels, stained with ethidium bromide, were run in Tris/acetate/EDTA electrophoresis buffer and ran for 30 – 45 min at 120 – 140 volts. At the end of the run, the DNA was visualized with a MacroVue UV-25 Hoefer transilluminator or an Alpha Innotech MultiImage Light Cabinet using AlphaEase FC software (version 4.1.0, Genetic Technologies Inc., Miami, Florida, U.S.A).

2.4 Polymerase Chain Reaction (PCR)

PCR was used to obtain SEBOV Boniface GP cDNA for cloning. PCR was carried out using a Pfu Ultra II Fusion HS kit (Stratagene, Cedar Creek, Texas, USA) in either the Perkin Elmer or the Biometra thermocyclers. The reaction mixture contained 70 µL of sterile water, 10 µL of 10x buffer, 4 µL of 10mM dNTP mix, 6 µL of each 10 µM forward and reverse primers, 2 µL of DNA (1 µg/µL) and 2 µL of enzyme mix. All reactions were set up on ice.

An example thermocycle protocol for a PCR reaction for SEBOV GP Boniface (2362 bps utilizing primer set SudanSt.B120FB and SudanSt.B2149R) included:

Step	Temperature	Time
Initial Denaturation	95°C	2 min
Denature	94°C	30 seconds
Anneal	55°C	30 seconds
Extend	72°C	2.5 min
Final Extension	72°C	7 min
Pause	4°C	infinitely

Each protocol was run for 40 - 45 cycles between the denaturation and extension steps.

2.4.1 Product Analysis

PCR amplicons were run on a 0.4 – 0.8% agarose gel to determine the size and quality of the samples. The gels, stained with ethidium bromide, were run in Tris/acetate/EDTA electrophoresis buffer and ran for 30 – 45 min at 120 – 140 volts. At the end of the run, the DNA was visualized with a MacroVue UV-25 Hoefer transilluminator or an Alpha Innotech MultiImage Light Cabinet using AlphaEase FC software (version 4.1.0, Genetic Technologies Inc., Miami, Florida, U.S.A).

2.5 Construction of Recombinant Viruses

Briefly, the glycoprotein of ZEBOV Kikwit was amplified by reverse transcription polymerase chain reaction (RT-PCR) from virus stocks with primers including the restriction sites XhoI and NheI (see Appendix 1 for primer sequences and section 2.3 for detailed RT-PCR methodology). Primers were generated by Operon Biotechnologies, Inc. (Huntsville, Alabama, USA). The glycoprotein of SEBOV Boniface was amplified by PCR using a pCAGGS/SEBOV GP vector as a template (see section 2.4 for detailed PCR methodology). This template plasmid was kindly donated by Ayato Takada (University of Tokyo, Tokyo, Japan). Primers for SEBOV Boniface included the restriction sites Kpn1/BfuAI/XhoI and SphI/BfuAI/NheI (Appendix 1). The ZEBOV and SEBOV glycoprotein gene amplicons were purified and digested with XhoI

and NheI and digested with BfuAI, respectively. A pCAGGS.MCS vector (Figure 2.1) was digested with XhoI and NheI. Each glycoprotein cDNA was ligated into the cut pCAGGS.MCS vector. The inserted glycoproteins were sequenced to confirm the correct insertion using primers (Appendix 1) generated by the DNA Core at the NML.

The *Ebolavirus* GP genes from the different Ebola species were to be subcloned into the VSV XN2 plasmid (Figure 2.1) for future virus rescue. The *Marburgvirus* glycoprotein insert was removed from an existing VSVΔG/MARV GP plasmid using the restriction enzymes XhoI and NheI to obtain an empty VSV XN2 plasmid (Figure 2.2). The pCAGGS/ZEBOV GP Kikwit and pCAGGS/SEBOV GP Boniface vectors were also digested with XhoI and NheI to remove each Ebola glycoprotein cDNA. The glycoprotein cDNA of ZEBOV Kikwit and SEBOV Boniface were ligated into the cut VSV XN2 vector and sequenced for confirmation of the sequence. The resulting recombinant VSV (rVSV) plasmids were rescued (section 2.13).

2.6 Cloning

See Appendix 1 for a list of primers used for cloning and sequencing. Primers for ZEBOV Kikwit, SEBOV Boniface and SEBOV Gulu were based upon Genbank sequences AY354458, U28134 and AY344234 respectively. See Appendix 2 for nucleotide sequences of the different strains of the Ebola glycoprotein segment.

The presence of RT-PCR or PCR products was confirmed by agarose gel electrophoresis. The inserts were PCR purified using a QIAquick PCR Purification kit from Qiagen (protocol as directed by the manufacturer) and eluted in 30 μL of sterile water. The inserts and vectors were digested with XhoI and NheI enzymes at 37°C for 3 hours (Figure 2.2). The digests were run on an agarose gel and gel extracted using a

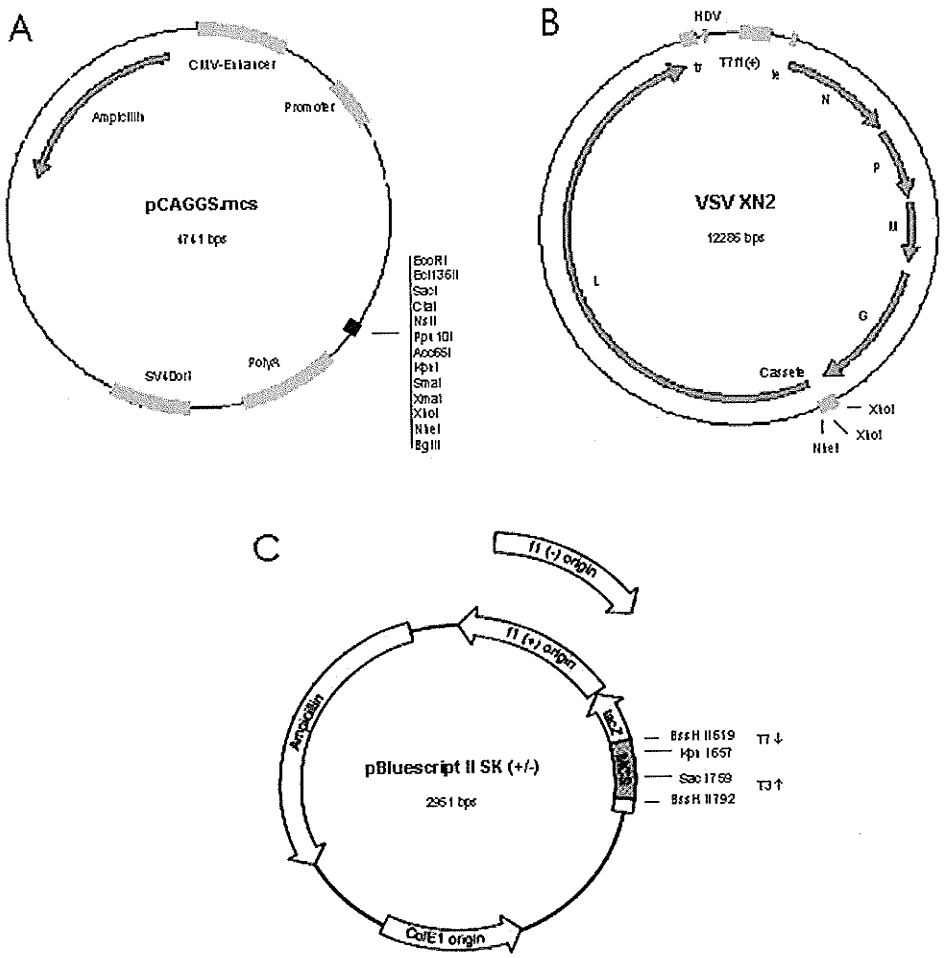

Figure 2.1: Vectors used for cloning and rescuing of the recombinant viruses.
Vector maps of backbones used for cloning and virus rescue (A) pCAGGS.MCS, (B) VSV XN2 and (C) pBluescript II SK (+) plasmids. The pBluescript II SK constructs were created with either the VSV N, P or L genes incorporated into the multiple cloning site. Note: Sequence size for VSV XN2 is without VSV G included. All mentioned restriction enzymes were purchased from New England Biolabs (Ipswich, Massachusetts, USA).

QIAquick Gel Extraction kit from Qiagen (protocol as per the manufacturer directions). The cut pCAGGS vector (Figure 2.1) was used directly from this step for ligation. The cut VSV XN2 (Figure 2.1) vector was dephosphorylated using Shrimp Alkaline Phosphatase (SAP, Roche, Cat# 1758250) for 1 hour at 37°C, 30 min at 50°C then 30 min at 72°C. Ligation of insert and vector was carried out overnight at 16°C (pCAGGS) or

14°C (VSV XN2) using T4 DNA Ligase HC (Roche, Cat# 10677520) at 1:3, 1:6 and 1:10 vector to insert ratios.

2.7 Transformations

2.7.1 pCAGGS Vector Transformations

The DNA ligation mix and 50 µL of *E. coli* XL1-Blue competent cells were placed on ice for 10 min. The ligation mix and the competent cells were then mixed together and left on ice for an additional 10 min. The competent cell/DNA mixture was then heat shocked at 42°C for 45 seconds to allow *E. coli* to take up the DNA. The cells were then placed on ice while adding 200 µL of LB broth. This mixture was incubated for 1 hour at 37°C while being shaken to allow *E. coli* cells to make ampicillin resistant proteins, then centrifuged at 4°C at 6000 x g for 1 min and the pellet was resuspended in150 µL of LB broth. The culture was then spread on a LB agar plate containing 100 µg/mL ampicillin (LB-Amp) and incubated overnight, upside down at 37°C.

2.7.2 VSV XN2 Vector Transformations

The DNA ligation mix, 50 µL of MAX Efficiency Stbl2 Competent Cells and a 15 mL polypropylene centrifuge tube (Corning Incorporated, Cat# 430052) were placed on ice for 10 min. The competent cells and the DNA were mixed together in the 15 mL tubes and left on ice for an additional 30 min. The mixture was then heat shocked at 42°C for 45 seconds, then placed on ice for another 3 min. One milliliter of Super Optimal Broth (S.O.C) medium (Invitrogen, Cat# 15544-04) was added to the mixture and the tube was incubated at room temperature while being shaken for 90 min. Then the culture was centrifuged at 4°C, 6000 x g for 1 min and the pellet was resuspended in 150 µL of

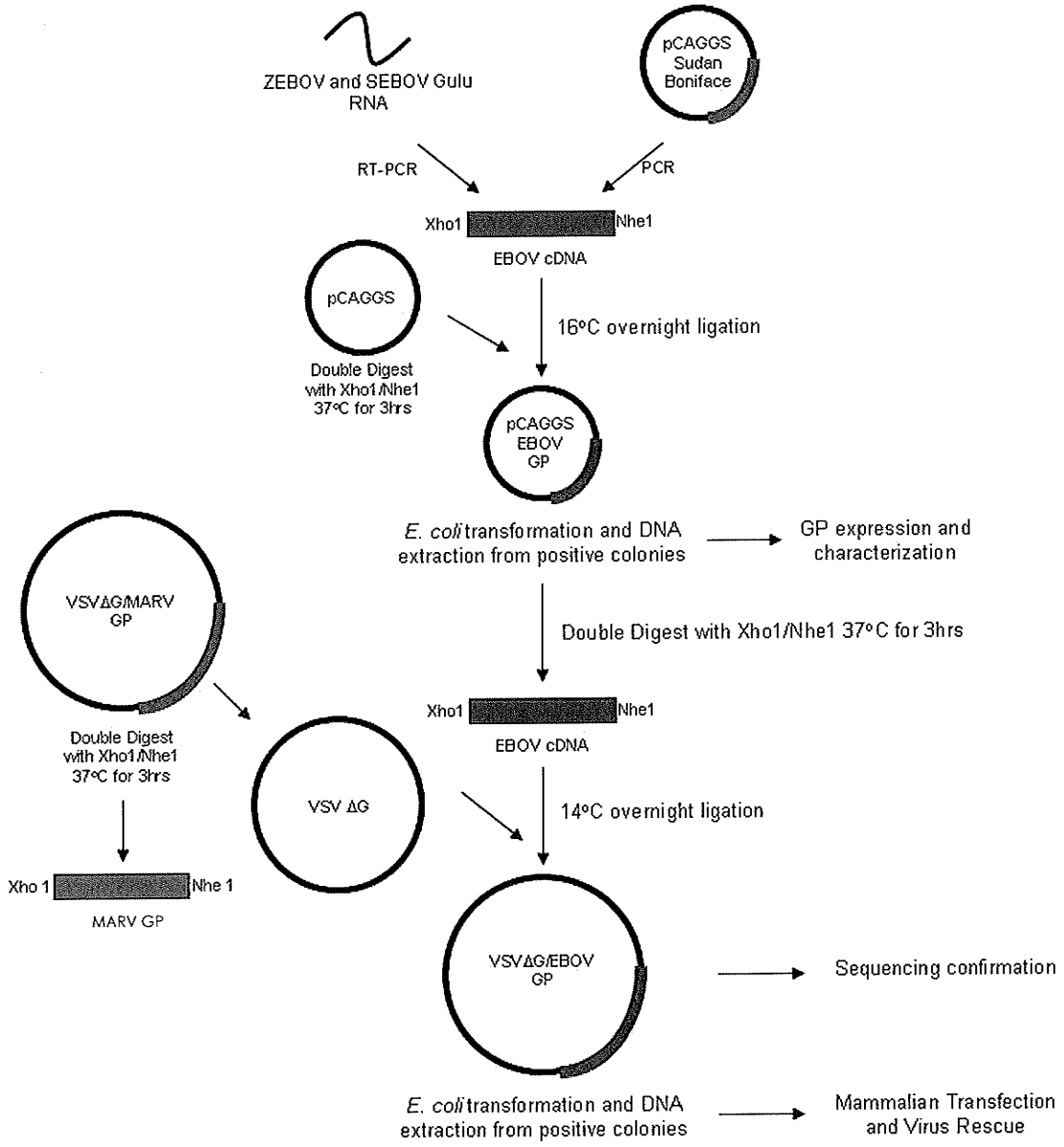

Figure 2.2: Cloning of the recombinant viruses.
A flow chart for the cloning of VSVΔG/EBOV GP (Zaire and Sudan) from viral RNA or DNA plasmid.

S.O.C medium. The culture was then spread on a LB-Amp plate and incubated upside down at room temperature for 2 – 3 days.

2.7.3 Obtaining DNA Plasmids

Once colonies were visible on the LB-Amp plate, each colony was picked and placed in 3 mL of LB-Amp broth. The colonies were incubated while being shaken

overnight at 37°C for pCAGGS plasmids or for 2 – 3 days at room temperature for VSV XN2 plasmids. When the cultures were cloudy, a mini preparation of DNA was carried out using a QIAprep Spin Miniprep Kit (Qiagen, Cat# 27106) as per the manufacturer's directions. A small sample of each preparation was digested with XhoI and NheI to ensure insertion of the glycoprotein open reading frame into the corresponding vector. Each digestion was run on an agarose gel plus ethidium bromide staining and visualized by UV exposure to confirm the correct size of the gene fragment. Five hundred microliters of any culture containing the correct gene fragment size were added to 500 mL of LB-Amp broth and incubated while being shaken overnight at 37°C for pCAGGS plasmids or for 2 – 3 days at room temperature for VSV XN2 plasmids.

The large scale preparations were centrifuged at 6000 x g for 15 min at 4°C. DNA was obtained using a QIAfilter Plasmid Maxi Kit (Qiagen, Cat# 12263) as per the manufacturer's directions. The DNA obtained was sent for sequencing at the DNA Core Facilities at the NML, along with forward and reverse primers that span the length of the corresponding glycoprotein open reading frame.

The sequences received from the DNA Core were analyzed against the Ebola glycoprotein nucleotide sequences found on GenBank (Appendix 2) using computer programs Chromas (version 1.45, Technelysium Pty Ltd, Eden Prairie, Minnesota, U.S.A) and Sci Ed Central Clone Manager 7 (version 7.04, Sci-Ed Software, Cary, North Carolina, U.S.A). The plasmids containing no mutations were used for virus rescue.

2.8 Site Directed Mutagenesis

Site directed mutagenesis was performed on a point mutation found in the ZEBOV GP Kikwit (Appendix 2) by using a QuikChange Site-Directed Mutagenesis Kit

(Stratagene, Cat# 200518) as per the manufacturer's protocol. This method was performed in order to make the sequence of the cloned cDNA glycoprotein identical to that of the virus stock used at the NML. Briefly, a sample reaction was concocted of sterile water, a 10x buffer, 5 – 50 ng of the plasmid to be mutated, dNTPs, Pfu Turbo polymerase and forward and reverse primers that included the point mutation (Appendix 1). A PCR reaction was performed using a 12 cycle reaction. The resulting plasmids were transformed and cultured in *E. coli* XL1-Blue competent cells. DNA obtained from mini preparations of these cultures were sent to DNA Core for sequencing. Clones positive for the mutation were grown in larger cultures for maxi preparations to obtain large amounts of plasmid DNA.

2.9 Transfections for Protein Expression

A Costar 6-well cell culture polystyrene plate (Corning Incorporated, Cat# 3516) was coated with poly-D-lysine hydrobromine (Sigma, Cat# P7886) and incubated at 37°C for 30 min. The poly-D-lysine was then removed and the wells were washed twice with sterile water. A confluent layer of 293T cells in a T75 cm^2 flask was then split (1:3 dilution from 5 mL), and seeded into a poly-D-lysine coated 6-well plate. The plate was placed in a 37°C incubator, 5% CO_2, overnight. The cells were to be used the next day when they are approximately 90% confluent.

A tube containing 3.6 μg of plasmid DNA in 90 μL of 1x Opti-MEM (Invitrogen Gibco, Cat#3198) was added to a tube of 3.6 μL of a 1 mg/mL Lipofectamine 2000 Reagent (LP-2000, Invitrogen, Cat# 11668-019) in 90 μL of Opti-MEM. The tube was incubated at room temperature for 15 min, and then 720 μL of Opti-MEM was added. The media was removed from the seeded 6-well plate and the cells were washed once

with Opti-MEM. The 900 µL of the DNA/LP-2000 mixture was then added per well and the plate was incubated in a 37°C incubator, 5% CO_2, until approximately 90% cell cytotoxicity was seen, usually in 48 hours. The cells and supernatant were collected and the supernatant was stored at -20°C. The cells and supernatant were examined for the presence of EBOV GP.

For a 6-well plate, 200 µL of 1x SDS-gel loading buffer (GLB, diluted from 4x stock solution, 25 mL of 0.5 M Tris HCl pH 6.8, 4 g SDS, 20 mL glycerol, 0.2 g bromophenol blue and 5 mL sterile water) was added to each well. If reducing conditions were required, 800 µL of βME (into the 4x stock solution) was added to the SDS-GLB. The plate was put on ice for 5 min to inhibit any protease activity. After incubation, the lysates were transferred to a 2 mL screw-cap microtube. For supernatant samples, 15 µL of the supernatant was added to 5 µL of 4x SDS-GLB with βME in a 2 mL screw-cap tube. All samples were then boiled for 5 min at 99°C and then centrifuged at 6000 x g for 1 min.

2.10 Protein Gel Electrophoresis and Western Blot

To perform sodium dodecyl sulfate-protein acrylamide gel electrophoresis (SDS-PAGE), the 10% resolving gel and the 4% stacking gel were made fresh before the addition of the samples. The samples in SDS-GLB were added to each well of the SDS-PAGE gel. A lane of the SDS-PAGE gel contained Magic Mark XP Western Blot Standard (Invitrogen, Cat# LC5602) and another contained SeeBlue Plus 2 Prestained Standard (Invitrogen, Cat# LC5925). The loaded gel was run by electrophoresis at 168V for 1.5 - 2 hours until the buffer front ran off the gel.

To transfer the proteins from the SDS-PAGE gel to a polyvinylidene fluoride (PVDF) transfer membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom, Cat# RPN303F), the gel was sandwiched between two filter pads and the membrane. The first filter pad and gel were soaked in anode buffer (Appendix 3). The PVDF membrane was incubated in methanol for 5 min and then in anode buffer. The final filter pad was wet in cathode buffer (Appendix 3) and laid on top of the gel. The sandwich was placed in a Trans-Blot SD semi-dry transfer cell and run at 60 mAmps/gel for 2 hours.

Following the protein transfer, the membrane was incubated overnight in a phosphate buffered saline (PBS) solution containing 5% skim milk (Fisher, Cat# NC9711290) and 0.1% Tween-20 (v/v) (Fisher, Cat# NC9713642) at 4°C to reduce any non-specific binding of antibodies to the membrane. The next morning, the membrane was washed twice with PBS/0.1% Tween-20 (v/v) then incubated with the primary antibody (1°) for 1 hour at room temperature, washed twice with PBS/0.1% Tween-20 (v/v) and incubated with the secondary antibody (2°), coupled to horse radish peroxidase (HRP), for 1 hour at room temperature. To detect proteins on the membrane, chemiluminescene was performed using an ECL Plus Western Blotting Detection System Kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom, Cat# RPN2132) as per the manufacturer's directions. Briefly, an ECL substrate was incubated on the membrane for 5 min and visualized on a Hyperfilm ECL chemiluminescence film (GE Healthcare, Cat# 28906839). See Table 2.1 for a list of 1° and 2° antibodies used in all assays.

2.11 Immunofluorescence Assay (IFA)

IFA was performed on both adherent and suspension cells. Adherent cells were either grown directly on a cover slip in a plate or on a plate. Suspension cells were centrifuged onto a microscope slide using a cytospin cassette. Briefly, a filter pad was placed on top of a microscope slide and put into the cytospin loading cassette. One hundred microliters of PBS was placed into a cut out circle on the filter pad and the cassette was centrifuged at 70 x g for 1 min at 4°C. One hundred microliters of a cell suspension (~ 0.5 x 10^6 cells/mL) was placed in the circle and centrifuged at 70 x g for 3 min at 4°C. The filter paper was discarded and the microscope slide was air dried.

To perform the IFA, the cells were first fixed with a 4% paraformaldehyde (PFA) solution for 15 min at room temperature. In the case of the suspension cells, the cells were fixed before adhering them to the microscope slide. Cells were washed 3x with PBS and incubated with the 1° antibody for 1 hour at 37°C. Afterwards, the cells were washed 3x with PBS and then incubated with the 2° antibody (table 2.1) for 1 hour at 37°C. The cells were washed 3x with PBS. In some cases, one drop of ProLong Gold antifade reagent with DAPI (Invitrogen Molecular Probes, Cat# P36031) was added to the cells in order to stain the cell nuclei. If the antifade reagent was used the cover slip was sealed on a microscope slide with nail polish. If not, the cells were fixed with 4% PFA for 5 min, washed and visualized under a fluorescence microscope. The Axiovision 4 (version 4, Zeiss, München, Germany) software was utilized to detect and capture fluorescent images.

Table 2.1: Primary and secondary antibodies used for all assays.

Antibody	Isotype	Flourochrom	Amount Used	Assay Used	Purchase Company	Catalogue Number
Anti-VSV GP	IgG1	---	1:2000	WB, IFA, FACS	Sigma-Aldrich	V-5507
ZGP12/1.1	IgG2a	---	1:5000, 1:2000	WB/ IFA, FACS	Ayato Takada (U of Tokyo)	---
EGP42/3.7	IgG1	---	1:5000	WB	Ayato Takada	---
Anti-ZEBOV GP 5D2	IgG_{2a}, κ	---	1:200	IFA	Xiangguo Qiu (NML)	---
Rat anti-mouse F4/80	IgG2b	FITC	5μL	FACS	Cedarlane	MCA497F
Rat anti-mouse CD49b	IgM, κ	FITC	1μL	FACS	BD	553857
Rat anti-mouse CD19	IgG_{2a}, κ	PE	1.25μL	FACS	Groovy Blue Genes	115507
Rat anti-mouse CD49b	IgM, κ	PE	1.25μL	FACS	Groovy Blue Genes	108907
Mouse anti-mouse H-$2D^d$	IgG_{2a}, κ	PE		FACS	BD	553580
Rat anti-mouse CD4	IgG_{2a}, κ	PerCP-CY5.5	5μL	FACS	BD	550954
Rat anti- mouse CD8	IgG_{2a}, κ	PerCP-CY5.5	5μL	FACS	BD	551162
Hamster anti-mouse CD3	IgG, κ	APC-Cy7	5μL	FACS	BD	557596
Mouse anti-mouse NK-1.1	IgG_{2a}, κ	APC	2.5μL	FACS	Groovy Blue Genes	108709
Mouse anti-human IFN-γ	IgG1, κ	FITC	20μL	FACS	Groovy Blue Genes	502506
Mouse anti-human TNF-α	IgG1, κ	FITC	20μL	FACS	Groovy Blue Genes	502906
Mouse anti-human CD56	IgG1, κ	PE	20μL	FACS	BD	556647
Mouse anti-human perforin	IgG2b, κ	PE	20μL	FACS	Groovy Blue Genes	308106
Mouse anti-human CD95	IgG1, κ	PE-Cy5	20μL	FACS	BD	559773
Mouse anti-human CD107a	IgG1, κ	PE-Cy5	20μL	FACS	BD	555802
Mouse anti-human HLA-A,B,C	IgG1, κ	APC	20μL	FACS	BD	555555
Rat Isotype	IgM, κ	FITC	1μL	FACS	BD	553942
Rat Isotype	IgG_{2a}, κ	PE	1.25μL	FACS	BD	553930
Rat Isotype	IgG_{2a}, κ	PerCP-CY5.5	5μL	FACS	BD	550765
Mouse Isotype	IgG_{2a}, κ	APC	2,5μL	FACS	Groovy Blue Genes	400221
Goat anti-mouse	IgG (H+L)	Alexa Fluor 488	1:500	IFA, FACS	Invitrogen Molecular Probes	A11001

2.12 Protein Deglycosylation

Transfected cells (section 2.9) were harvested with 1x SDS-GLB containing βME, boiled for 5 min at 99°C and centrifuged at 6000 x g for 1 min. The proteins were deglycosylated using an Enzymatic Deglycoslyation kit from ProZyme Glyko (San Leandro, California, USA, Cat# GK80110) as per the manufacturer's protocol. Briefly, 2 μL of sample was incubated in a denaturation solution for 5 min at 100°C. A detergent was added to the tube before the addition of the enzymes. The following enzymes were then added in order to remove sugar complexes from the protein: N-Glycanase, Sialidase A, O-Glycanase, β(1-4)-Galactosidase and β-N-Acetylglucosaminidase. The mixture was then incubated at 37°C for 3 hours before running the samples on a SDS-PAGE protein gel for visualization.

2.13 Virus Rescue

A 6-well plate was seeded with a 1:1 mixture of 293T cells and Vero E6 cells to obtain a 90% confluent monolayer. The cells were transfected with the full length VSV genome plasmid and the VSV helper plasmids (Figure 2.3): pCAGGS/T7, pBluescript SK (+) (pBS)/VSV P, pBS/VSV L and pBS/VSV N (Figure 2.1). Please refer to Table 2.2 for DNA amounts used for specific virus rescues. The following reaction mix was prepared for each well of a 6-well plate. One tube of DNA mixtures in 90 μL of Opti-MEM and a second tube of 14 μL of LP-2000 in 90 μL of Opti-MEM were incubated for 5 min before mixing them together and incubated for 15 min. Seven hundred and twenty microliters of Opti-MEM was then added to the tube. Media from the 6-well plate was removed and the DNA mixture was added before incubating at 37°C, 5% CO_2, for 24

hours. One milliliter of DMEM-2 without antibiotics was added to each well, and the plate was incubated for an additional 48 hours.

Supernatant was removed and centrifuged at 300 x g for 5 min. For the blind passage, 500 μL of transfection supernatant was added per well to a 90% confluent Vero E6 6-well plate with media removed. The remaining supernatant was stored at -80°C. The plate was incubated for 1 hour at 37°C, 5% CO_2, and was shaken every 15 min. Two milliliters of DMEM-2 (without antibiotics) was then added to each well and the plate was incubated at 37°C, 5% CO_2, until CPE was seen. Supernatant and cell lysates were then checked for infectious virus by another blind passage on Vero E6 cells, as well as by Western blot and RT-PCR.

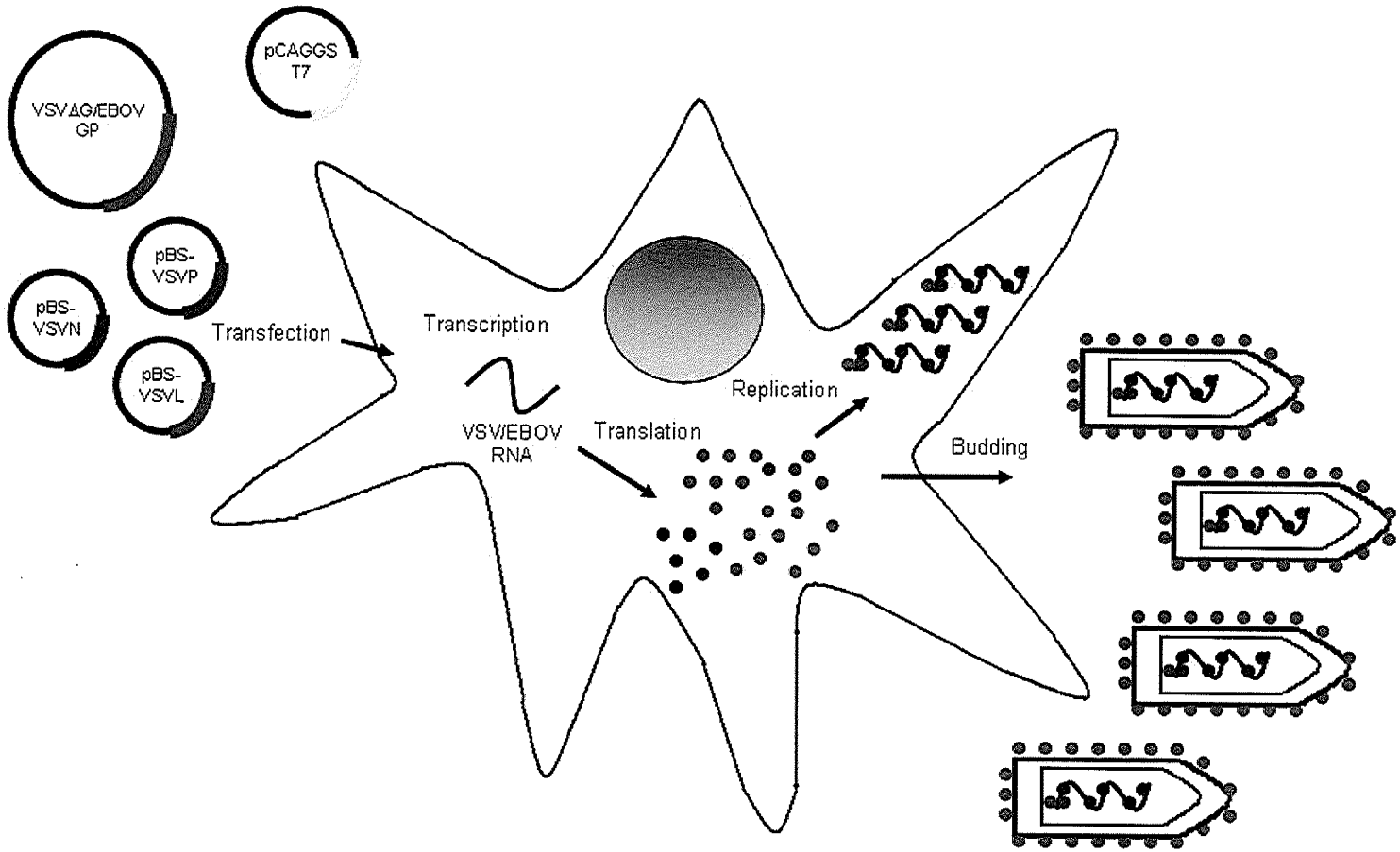

Figure 2.3: Virus Rescue.
The reverse genetics method constructs recombinant VSV viruses from the transfection of tissue culture cells with VSVΔG/EBOV GP plasmids and VSV helper plasmids. The VSV helper plasmids are added to the transfection to make VSV proteins for viral replication. The final assembled virus resembles a Rhabdovirus expressing Ebola glycoproteins on its surface.

Table 2.2: Quantities of DNA plasmid per well for virus rescue.

Construct	DNA Amount
Wild Type VSV	
pBS-VSV N	0.625μg
pBS-VSV P	1.56μg
pBS-VSV L	0.75μg
pCAGGS-T7	2.5μg
VSV	2.5μg
VSVΔG/EBOV GP	
pBS-VSV N	0.5μg
pBS-VSV P	1.25μg
pBS-VSV L	0.6μg
pCAGGS-T7	2.0μg
VSVΔG/EBOV GP	2.0μg

2.14 Viral Infection

Viral infections in cell culture for cell death assays were performed as followed. Cells were split the day prior to infection. Virus diluted in unsupplemented RPMI was added to the cells, either directly to the plate of adherent cells or in a tube of suspension cell. Cells were incubated for 1 hour at 37°C and were shaken every 15 min. For suspension cells, the tube was finger flicked rather than vortexed to ensure gentle treatment of the cells. After incubation, RPMI-10 was added to cells. The cells were then incubated at 37°C, 5% CO_2, until needed or CPE was seen, depending on the experiment.

2.15 Virus Titration by Plaque Assay

Plaque assays were performed to determine virus titres. Vero E6 cells were split 1:2 the day prior and seeded in a 12-well plate so that they were 100% confluent for this assay. Virus dilutions, from 10^{-2} to 10^{-7}, were prepared in 15 mL tubes with unsupplemented DMEM. An initial 1/100 dilution of the virus stock was added to the first tube containing 5 mL of unsupplemented DMEM, followed by a 10 fold serial

dilution. The media from the 12-well plate of Vero E6 cells was removed and 250 µL of each dilution was added in duplicate to each well. The cells were incubated for 1 hour at 37°C, 5% CO_2, and were shaken every 15 min.

The virus inoculum was removed from the wells and 2 mL of an agarose overlay, 1:1 ratio of a sterile 2% UltraPure low melting point (LMP) agarose (Invitrogen, Cat# 15517-022) and 2x Modified Eagle Medium (MEM, Invitrogen Gibco, Cat# 11935) supplemented with 4% heat inactivated FBS, was added to each well. The plate was incubated at room temperature until the overlay solidified and then incubated inverted at 37°C, 5% CO_2, until large plaques were seen in the cell monolayer. At that point, 2 mL of a working solution of crystal violet (2% crystal violet w/v, 2 parts ethanol, 1 part 37% formaldehyde and 7 parts water) was added to each well to fix the cells and inactivate the virus. The plate was then incubated at room temperature overnight. Afterwards, the overlay was removed and plaques counted.

The virus titre was determined at the dilution where there were $\geq$ 20 - 200 countable plaques in the well. The following equation was used to calculate the virus titre:

$$\text{Plaque Average} \times \frac{1}{\text{dilution}} \times \frac{1}{\text{volume}} = \text{PFU/mL}$$

Where volume used is equal to the amount of virus used to infect cells in milliliters.

2.16 RNA Extraction

One hundred and forty microliters of supernatant or cell lysate from a viral infection was incubated in 560 µL of AVL buffer from a QIAamp Viral RNA Mini Spin kit (Qiagen, Cat# 52906) for 10 min at room temperature. RNA extraction was performed

as per the manufacturer's directions. The final elution of RNA was completed in 60 μL of sterile water and stored at -80°C until needed.

2.17 Antibody Staining for FACS Analysis

2.17.1 Surface Staining

Cells were put into suspension in PBS and washed by centrifugation twice at 300 x g for 5 min at 4°C. PBS was removed and the tube was blotted on a clean kimwipe. In the remaining PBS, the cells were blocked with human γ-globulin (Sigma-Aldrich, Cat# G4386) for human cells or rat anti-mouse CD16/CD32 (Table 2.1) for mouse cells, to prevent unspecific binding of antibodies for 10 min at room temperature. A 1° or fluorescently labeled antibody was added in PBS to a total volume of 100 μL and added to the cell and incubated at 4°C for 30 min. The cells were then washed twice in PBS. If a 2° antibody was needed, it was added to 100 μL of PBS and the cell/antibody mix was incubated at 4°C for another 30 min. The cells were washed again and fixed with 4% PFA overnight at 4°C.

Ten thousand cell events were acquired on either the BD LSR II FACS machine or the BD FACSCalibur, using FACS Diva (version 5.0.2, BD Biosciences, Mississauga, Ontario, Canada,) for the LSR II or Cell QuestPro (version 4.0, BD Biosciences) for the FASCalibur. To analyze the data, FloJo (version 7.2.4, Tree Star Inc., Ashland, Oregon, U.S.A) was utilized.

2.17.2 Intracellular Staining

Cells were stimulated with 50 ng/mL phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, Cat# P8139) and 1 μM Ionomycin Calcium Salt (Sigma-Aldrich, Cat# I-0634) for 12 hours, or 24 hours with recombinant virus at 37°C, 5% CO_2. Six hours into

the incubation, 1 μL of a 100 μg solution of GolgiPlug was added to inhibit proteins from being transported to the cell surface. Following the full incubation period, cells were surface stained then permeablized and fixed using a BD Cytofix/Cytoperm kit (BD Biosciences, Cat# 555028) as per the manufacturer's the directions. The cells were then incubated with an intracellular antibody in 100 μL PBS at 4°C for 30 min, washed once, and fixed with 4% PFA overnight at 4°C. Cells were acquired on a flow cytometer the next day.

2.18 Cytokine Enzymes-Linked ImmunoSorbent Assay (ELISA)

Cells were infected with recombinant virus for 0 to 48 hours. Following infection, supernatant was removed from the cells by centrifugation at 300 x g for 5 min at 4°C. Supernatants were frozen at -80°C until all samples could be run at the same time. ELISA kits for Interferon IFN-α (Cat # 41100-1), IFN-β (Cat# 41410-1), and IFN-γ (Cat# 41500-1) were purchased from Cedarlane (Burlington, Ontario, Canada), and ELISA kits for TNF-α was purchased from R&D Systems (Minneapolis, Minnesota, USA, Cat# DTA00C) and followed as per the manufacturer's protocol. Briefly, infection supernatant was put into a 96-well plate coated with antibodies against the cytokine of interest and incubated at room temperature for 1 - 2 hours. Supernatant was then removed and washed. A HRP linked to an anti-cytokine antibody was added to each well and incubated at room temperature for 1 hour. The HRP solution was then removed and the plate was washed. A 3.3', 5.5'-tetramethylbenzidine (TMB) substrate was added to the wells and incubated for 15 min at room temperature or until color developed. The reaction was stopped and the plates were run on an ASYS UVM 340 ELISA plate reader

at an optical density (OD) of 450nm using DigiRead software (version 1.3.0.0, ASYS Hitech GmBH, Cambridge, United Kingdom).

OD readings were converted to protein concentrations based upon the best fit linear line of the standard curve obtained from running a standard cytokine sample simultaneously with infection supernatants.

2.19 Natural Killer Cell Mediated Cytotoxicity (CMC) Assay

The NK CMC assay was adapted from the fluorometric antibody-dependent cellular cytotoxicity assay (Gomez-Roman *et al.*, 2006; Gomez-Roman *et al.*, 2005; Sheehy *et al.*, 2001). Target cells, either uninfected or infected with virus at a multiplicity of infection (MOI) of 1 or 10, were incubated for 24 hours at 37°C, 5% CO_2 (Figure 2.4). Supernatant was removed from the cells by centrifugation at 300 x g for 5 min at 4°C. The cells were subsequently washed twice with plain DMEM at 300 x g for 5 min at 4°C. Staining of the cells was based upon a concentration of 10^7 cells per 500 µL of DMEM. A final concentration of 1.5 µM of Vybrant CFDA SE cell tracer (CFSE, Invitrogen Molecular Probes, Cat# V12883) and a final concentration of 5 µM of PKH-26 Red Fluorescent Cell Linker (Sigma Aldrich, Cat# P9891) was added to the cells in DMEM and incubated at 37°C, 5% CO_2, for 8 min, while shaking the tube every 2 min. Following incubation, an equal volume of cold heat inactivated FBS was add to the cell/dye mixture. An equal volume of DMEM-10 was added to the cells and centrifuged at 300 x g for 5 min at 4°C. The cells were washed once more with DMEM at 300 x g for 5 min at 4°C and then resuspended in DMEM-10.

Effector cells (NK-92MI at passage 15 - 30, or primary murine NK cells stimulated with recombinant IL-2 for 14 days) were taken from culture and washed once

with DMEM at 300 x g for 5 min at 4°C. One hundred microliters of stained target cells and 100 µL of effector cells were added together in each well of a 0.5 mL polypropylene

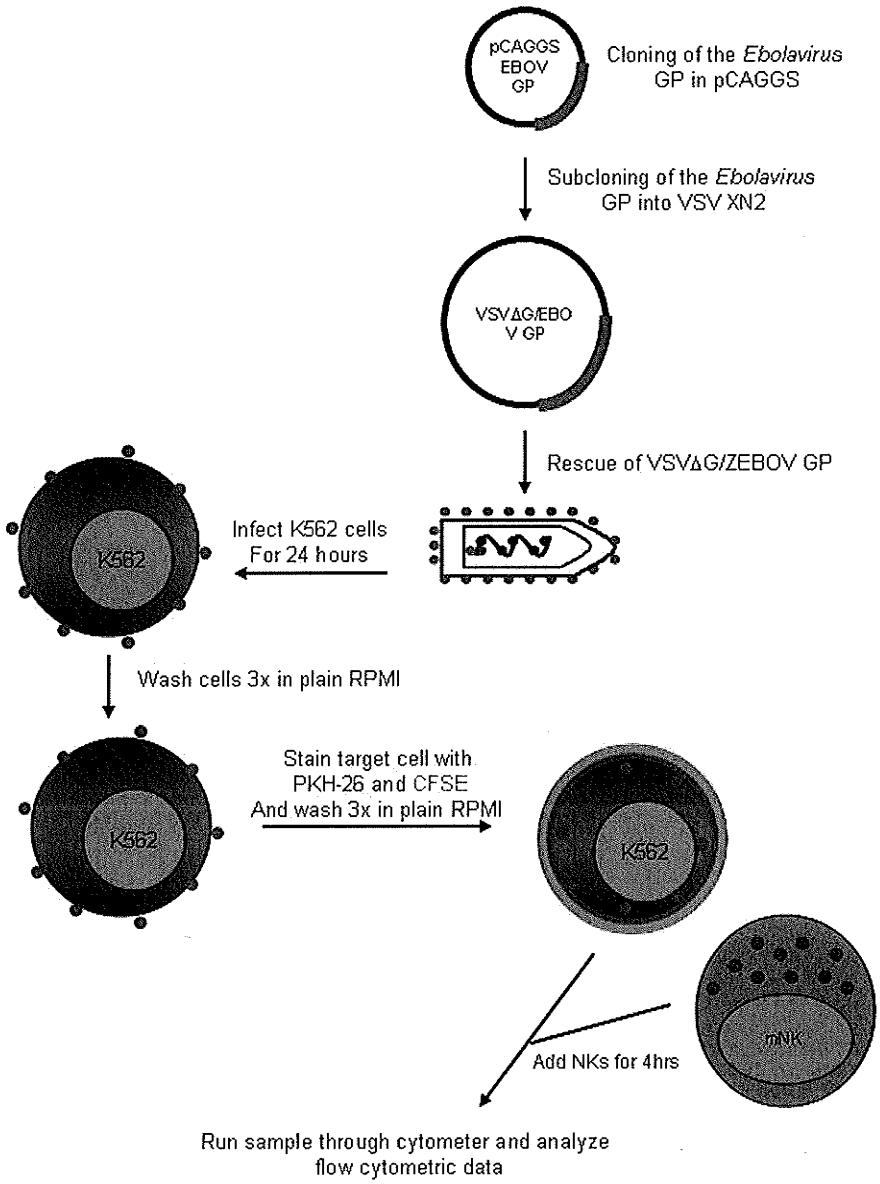

Figure 2.4: General outline for NK cell mediated cytotoxicity assay. The general steps outlined here were used for the cloning and rescue of VSVΔG/EBOV GP virus. The recombinant virus rescued was employed in the NK cell mediated cytotoxicity assay for the determination of the role of NK cells in the *Ebolavirus* post exposure therapeutic.

assay plate (Corning Incorporated, Cat# 3956). Five hundred million target cells were used per well at an effector to target (E:T) ratio of 1:1, 3:1 while $1.5x10^6$ target cells were used per well for an E:T ratio of 0.33:1. Three lanes of uninfected and infected (MOI 1 and 10) cells were placed with NK cells and three lanes of uninfected and infected (MOI 1 and 10) cells were placed without NK cells but with 100 μL of DMEM-10 (Figure 2.5). Time 0 samples were removed and placed in a second plate. Both plates were covered and centrifuged at 300 x g for 2 min at 4°C. The first plate was placed at 37°C, 5% CO_2, for 4 hours. Media was removed from the time 0 samples and 4% PFA was added to the well. This plate was placed at 4°C.

Following incubation, both plates were centrifuged at 300 x g for 5 min at 4°C. Media was removed from the 4 hour samples, resuspended in 4% PFA and samples were moved to the 4°C plate and stored at 4°C overnight. The next day, each sample was placed in a 5 mL polystyrene round-bottom tube and read on the BD FACSCalibur.

Using the Cell QuestPro program, a gate was placed on the unstained target cell population using forward and side scatter axis, excluding any dead cells. The CFSE, in FL1, and PKH-26, in FL2, were compensated for based upon single stained target cells. Samples were run acquiring 10,000 events in the parent gate and all events were saved.

The FlowJo program allowed analysis of each cell treatment. The first forward/side scatter gate was based upon live cells in the time 0 samples to determine the cut off point of live and dead target cells. The quadrant gate was then based upon these samples. Subsequent 4 hour samples were gated based upon this scheme (Figure 2.6).

The percent of cytotoxicity was determined by the shift of target cells from the live cell quadrant to the dead cell quadrant (Figure 2.5). To calculate percent cytotoxicity, the following equation was used:

$$\% \text{ Cytotoxicity} = \frac{\text{Dead Target Cells}}{\text{Total Target Cells}} \times 100\%$$

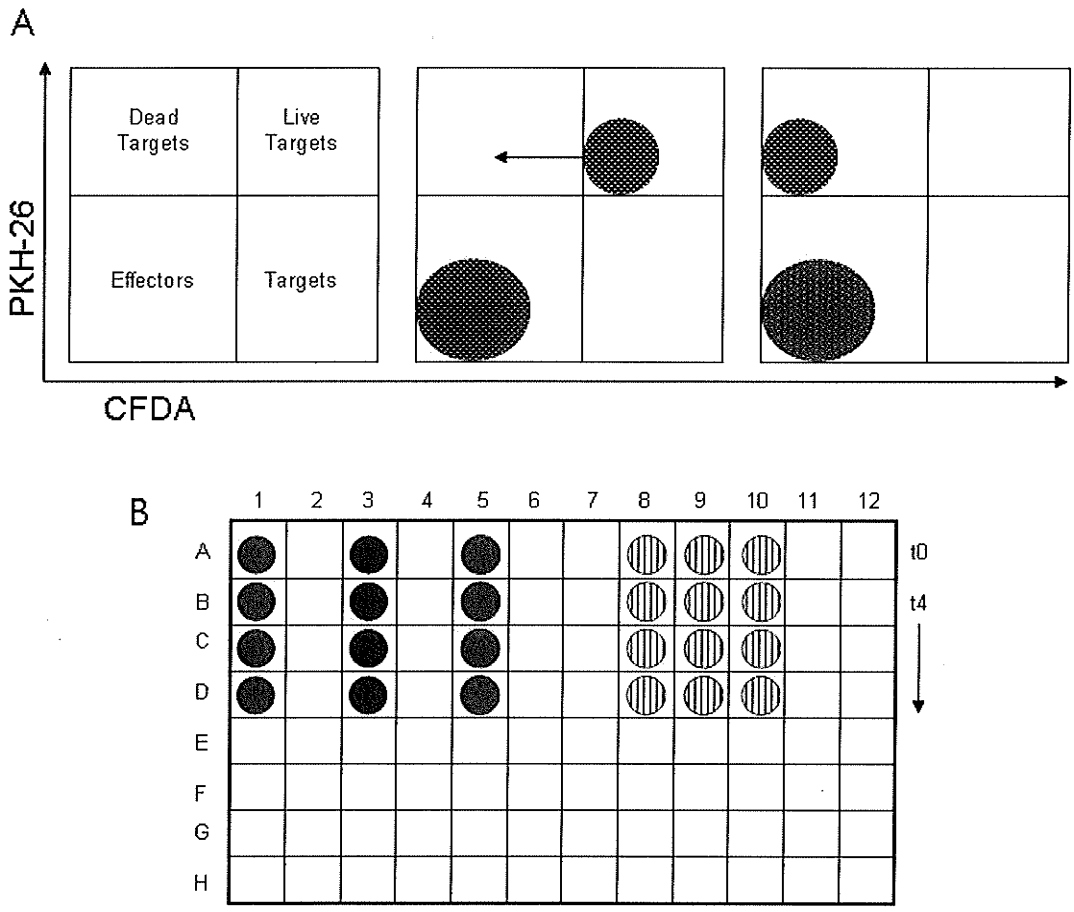

Figure 2.5: NK cell mediated cytotoxicity assay layout.
(A) Quadrant scheme shows shift of live target cells to dead target cells from 0 - 4 hour incubation of assay at 37°C, 5% CO_2. (B) In experimental plate layout, green circles (●) are uninfected target cells, purple circles (●) and red circles (●) are VSVΔG/ZEBOV GP Kikwit MOI of 1 or MOI of 10 infected target cells respectively. Solid circles are experiments with NK cells addition. Vertical lined circles are experiments without NK cell addition.

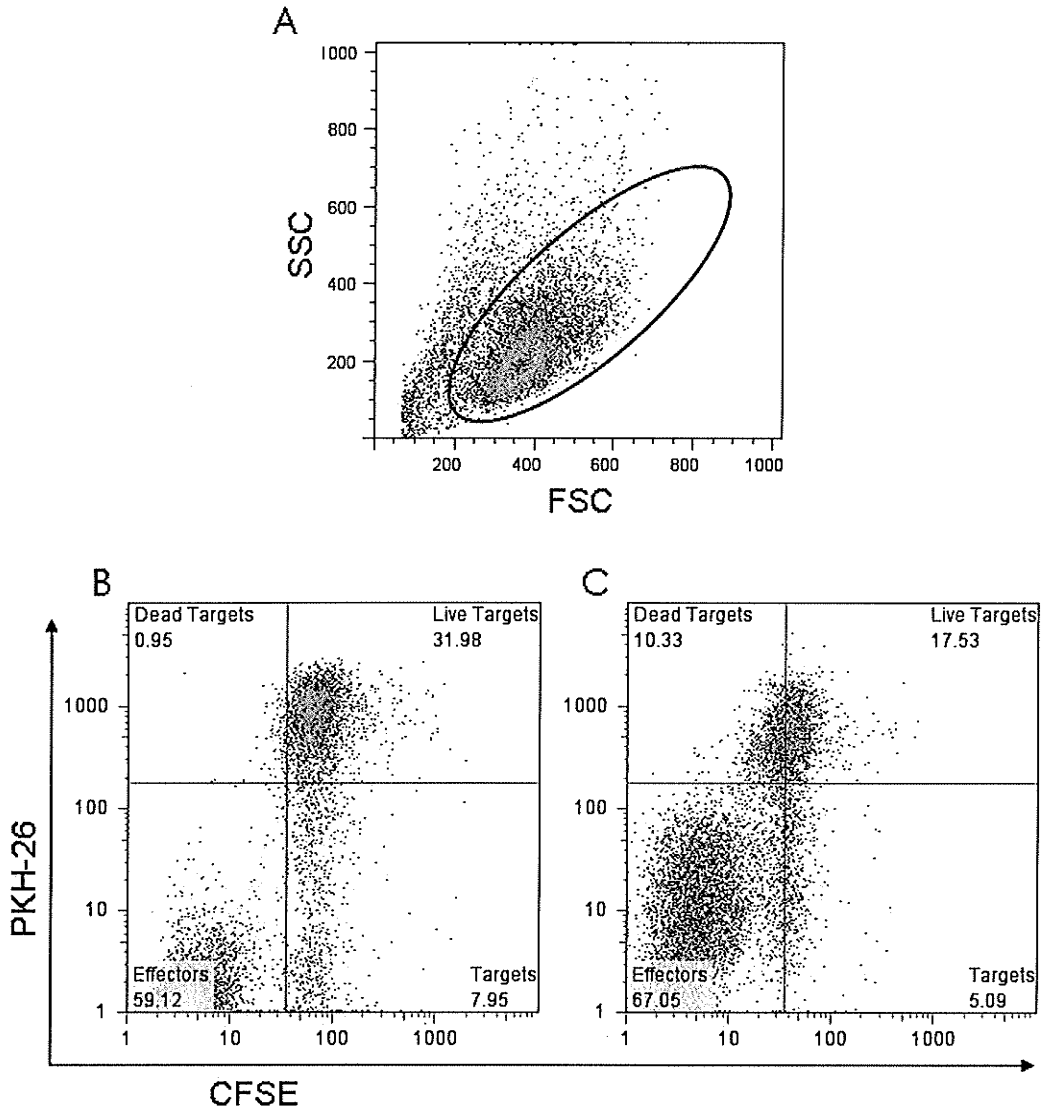

Figure 2.6: Gating strategy for the NK cell mediated cytotoxicity assay.

Cell population for quadrant graphs (B and C) were based upon (A) side-forward scatter plots from 0 hours after NK cell addition. (B) represents 0 hours after NK cell addition, and (C) represents 4 hours after NK cell addition (3:1 E:T ratio). Quadrant position is based upon 0 hours. The live target cells, stained with PKH-26 and CFSE are located in the upper right quadrant. As the effector cells (lower left quadrant) lyse the target cells, the target cells lose the CFSE dye and move to the dead target quadrant (upper left). Live target cells that do not take up the PKH-26 stain, are found in the lower right quadrant and will lose CFSE as the effector cells lyse them.

2.20 Animal Care

Six to eight week old C57Bl/6 and Balb/c mice (AUD# H-06-011), utilized for spleen extraction were purchased from Charles Rivers (Saint-Constant, Quebec, Canada). All mice work was performed in CL2 at the National Microbiology Laboratory. Animal work was approved by the Canadian Science Centre for Human and Health Animal Care Committee following the guidelines of the Canadian Council on Animal Care.

2.21 Natural Killer Cell Purification from Mouse Spleens

Either Balb/c or C57Bl/6 male mice were used at age 6 – 8 weeks old. The mice were euthanized and the spleen was removed from each mouse. The excised spleen was placed in PBS supplemented with a bovine serum albumin stock solution (BSA, Miltenyi Biotec, Auburn, California, Cat# 130-091-376), now known as PBS-10, on ice. The spleen was then homogenized between the frosted ends of two sterile microscope slides and run through a 40 μm nylon cell strainer. The homogenate was washed in a 50 mL conical tube in 15 mL of PBS-10 at 200 x g for 5 min at 4°C. The pellet was resuspended in 2 mL PBS-10 and 16 mL of a red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, ph 7.6, NML) was added and vortexed. This mixture was incubated for 5 min at room temperature. Approximately 2 mL of heat inactivated FBS was added to the buffer and the cells were centrifuged at 300 x g for 10 min at 4°C. Then the cells were washed twice more with 18 mL of PBS-10 and centrifuged at 300 x g for 10 min at 4°C. Finally, the pellet was resuspended in 4 mL PBS-10. One hundred microliters of suspension was removed for FACS analysis.

Prior to spleen extraction, two nylon wool columns (Polysciences Inc., Warrington, Pennsylvania, USA, Cat# 21759) were washed with warm 5 mL of αMEM

supplemented with the BSA stock solution, now known as αMEM-10B, to ensure that all of the nylon wool has been wet with media. Ten milliliters of warm αMEM-10B was added to the column and incubated for 1 hour at 37°C. The media was then drained from the column until the media reached the top of the nylon wool. Two milliliters of the spleen homogenate was added to each column and drained until the homogenate reached the top of the nylon wool. One milliliter of warm αMEM-10B was added to the column and drained as above. Five milliliter of warm αMEM-10B was then added on top of the nylon wool and the column was incubated at 37°C, 5% CO_2, for 1 hour.

Following incubation, adherent cells bound to the nylon wool (Julius *et al.*, 1973) and non-adherent cells passed through. The column was washed twice with 5 mL of warm supplemented αMEM without knocking or plunging the column. The cell suspension and washes were collected and centrifuged at 300 x g for 10 min at 4°C. A sample of the suspension was removed to count cells utilizing a hemocytometer (VWR Scientific, West Chester, Pennsylvania, U.S.A, Cat# 15170-208). Briefly, 10 µL of the cell suspension was added to 80 µL of PBS in a press cap tube. Ten microliters of a 0.4% trypan blue stain (Invitrogen, Cat# 15250-061) was added to the tube. 10 microliters of the stained cell mixture was added to a hemocytometer and visualized under a microscope. The cells were counted in the 4 corner quadrants of the hemocytometer and an average was taken. The following equation was utilized to determine the number of cells in the original tube:

$$\text{Total cell Number} = \text{Cell Average} \times 10 \times 10^4 \times \text{Total Cell Volume Used}$$

The remaining cells were magnetically labeled using a negative mouse NK Cell Isolation Kit (Miltenyi Biotec, Cat# 130-090-864) according to manufacturer's direction. Briefly, all cells types, except NK cells, were labeled with a biotin labeled antibody specific to the cell type. Anti-biotin labeled microbeads were added to the cells. The microbead labeled mixture was run through a column with a magnetic field. The cells bound to the microbeads, stuck to the column owing to the magnetic field. The unlabeled NK cells flowed through the column and were collected in a tube.

The cells were washed twice with PBS-10 at 300 x g for 5 min at 4°C. A sample was removed for FACS analysis and to count the cells. The remaining cells were resuspended at a concentration of $0.5x10^6$ cell/mL of HyQ RPMI-1640 Medium (HyClone, Logan, Utah, USA, Cat# SH30255.01) containing 25 mM HEPES, 2mM L-glutamine and supplemented with 10% heat inactivated FBS and 1 μL of 1000 units/mL of human IL-2 (AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Allergy and Infectious Disease, National Institutes of Health, Contributed by M. Gatley, Hoffman-LaRoche, Nutley, New Jersey, USA).

The NK cells were cultured for 3 days at 37°C, 5% CO_2, to which fresh media with IL-2 was added. On day 5 - 7, cells were split 1:2 adding fresh media supplemented with IL-2. Primary mouse NK cells were cultured up to 2 weeks before signs of cell death was seen.

2.22 Statistics

Statistical tests for the NK cell mediated cytotoxicity assays were performed utilizing an unpaired 2 tailed t-test with the GraphPad Prism 4 (version 4.03, GraphPad Software Inc., La Jolla, California, U.S.A) software.

3. Results

3.1 Cloning of the Full Length Recombinant VSV Plasmids

3.1.1 Cloning Ebola GP into the pCAGGS Expression Vectors

The *Ebolavirus* glycoproteins (GP) were to be cloned into the mammalian expression vector, pCAGGS, to determine proper expression and sequence of the Ebola GP gene prior to subcloning and subsequent virus rescue. The *Zaire ebolavirus* strain Kikwit (Zaire Kikwit) and *Sudan ebolavirus* strain Boniface (Sudan Boniface) and Gulu (Sudan Gulu) were to be cloned for rescue. Each recombinant virus was generated as described recently by Garbutt *et al* (Garbutt *et al.*, 2004). However, only Zaire Kikwit was used in the NK cell mediated cytotoxicity assays to optimize the protocol of the assay. Briefly, the GP genes were cloned by RT-PCR utilizing primers (see Appendix 1 for sequences) designed to flank the GP open reading frame. Each primer set included restriction enzymes recognition sites to accommodate subsequent insertion into the pCAGGS expression vector (Figure 2.1). RT-PCR performed obtained a 2030 bp gene for Zaire Kikwit and a 2029 bp gene for Sudan Gulu (Appendix 2).

Each GP gene was sequenced using the primers found in Appendix 1 to ensure that an 8 adenine (A) nucleotide sequence was located in the gene. This 8A sequence was originally a post-transcriptional edit of the full length GP gene that primarily makes a 7A sequence encoding for sGP. When the extra A is added to the sequence, a frame shift occurs causing a change in the GP reading frame and ensures translation of the structural GP_1 and GP_2 genes (Figure 1.3).

The nucleotide sequences for Zaire Kikwit (reference # AY354458) and Sudan Gulu (reference # AY316199) were obtained from PubMed. These sequences were used

as reference when analyzing the sequence results of the pCAGGS Ebola GP clones of the Winnipeg strain of the viruses used at the NML. Two point mutations were observed in the GP gene of the Zaire Kikwit genomic RNA, which changed the coding sequence from a cytosine to a thymine nucleotide at each site (Appendix 2). The mutation at position 1289 was found in the pCAGGS ZEBOV Kikwit GP clone. The mutation at position 1631 was changed in the pCAGGS ZEBOV Kikwit GP clone by site-directed mutagenesis so that the vaccine GP sequence would match the GP sequence of the challenge wild type *Ebolavirus*. This process was carried out even though the PubMed reference sequence had a different nucleotide sequence at this site. In the pCAGGS SEBOV Gulu GP clone, a three nucleotide mutation was located starting at the 2012 nucleotide site. The original triple thymine sequence was changed to three adenine nucleotides (Appendix 2).

Following sequencing, the cDNA GP genes generated were cloned into the pCAGGS multiple cloning site. Identification of pCAGGS vector (5000 bps) containing the Ebola GP (2000 bps) was determined by agarose gel electrophoresis and confirmed by DNA sequencing (Figure 3.1-A). Positive clones were grown in a larger preparation for resequencing, characterization and subcloning.

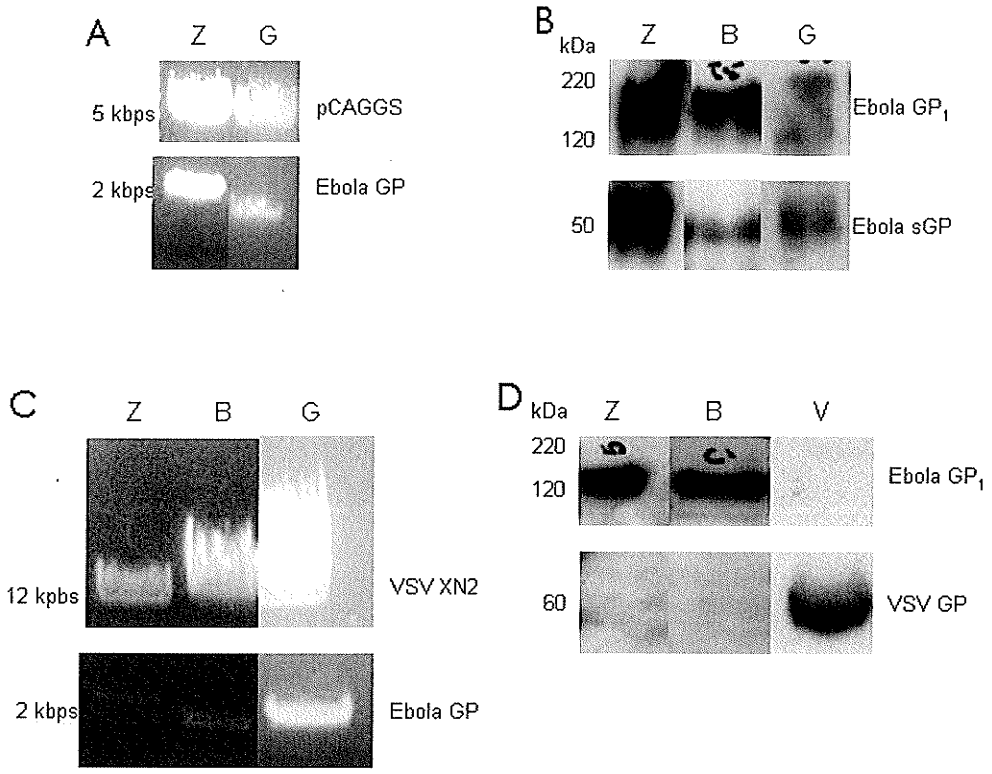

Figure 3.1: Restriction digests and protein expression for EBOV GP clones.
Double digestions of (A) pCAGGS and (C) VSV EBOV GP constructs to determine positive clones of EBOV GP. Western blots for transfections of (B) pCAGGS constructs and successful (D) recombinant virus rescues are also shown. Primary antibodies ZGP12/1.1 (1:5000) for Zaire Kikwit or EGP42/3.7 (1:5000) for Sudan or anti-VSV GP (1:2000) for VSV wild type, followed by a HRP conjugated goat anti-mouse antibody (1:10,000) was utilized for western blots. M = marker, Z = Zaire Kikwit, B = Sudan Boniface, G = Sudan Gulu and V = VSV wild type.

3.1.2 Characterization of the pCAGGS-*Ebolavirus* GP Vector

To determine whether the EBOV GP gene was expressed properly in the pCAGGS expression vector, the positive pCAGGS EBOV GP clones were transfected into 90% confluent 293T cells. Immunoblot analysis of the transfection supernatant revealed a band at approximately 140 kDa indicative of Ebola GP_1 and a band at approximately 50 kDa, indicative of Ebola sGP (Figure 3.1-B). By visualizing the cells

with a fluorescent microscope, Ebola GP was seen on the surface of the cells (Figure 3.2-B and C). The phase contrast picture was included to demonstrate that the staining pattern of the Ebola GP matched the outer surface of the cells (Figure 3.2-A).

Earlier work with Zaire Mayinga had shown the GP to be heavily glycosylated with both N-linked and O-linked sugars (Dowling *et al.*, 2007; Feldmann *et al.*, 1994; Jeffers *et al.*, 2002). To confirm proper glycosylation of the Ebola GP constructs, 293T cells were transfected with either pCAGGS Zaire Kikwit GP or pCAGGS Sudan Gulu GP for 48 hours. Following harvesting, the cell lysates were treated with several deglycosylation enzymes in order to remove complex sugar residues from proteins. The enzymes N-glycosylase, Sialidase A and O-glyconase remove N-linked, A-linked and O-linked sugars from proteins, respectively. When treated with the enzymes, Zaire Kikwit, Sudan Gulu and the positive control *Zaire Ebolavirus* strain Mayinga (Zaire Mayinga), showed similar shifts in weight (Figure 3.3). By visualizing and confirming the glycosylation pattern of the EBOV GP it was determined that the EBOV GP was processed correctly processed through the Endoplasmic Reticulum-Golgi apparatus secretory system of the 293T cells and was therefore suitable for subcloning into the VSV XN2 for subsequent recombinant virus rescue.

3.1.3 Subcloning Ebola GP into the VSV XN2 Vector

The VSV XN2 vector is the backbone of the recombinant virus used in this project. It contains the full gene sequence of VSV, except for the VSV G open reading frame which is to be replaced with the GP open reading frame of the *Ebolavirus*. This vector allows rescue of a virus that is morphologically similar to a rhabdovirus, but

expresses the EBOV GP on its surface instead of the VSV G. The positive pCAGGS Zaire Kikwit GP clone and pCAGGS

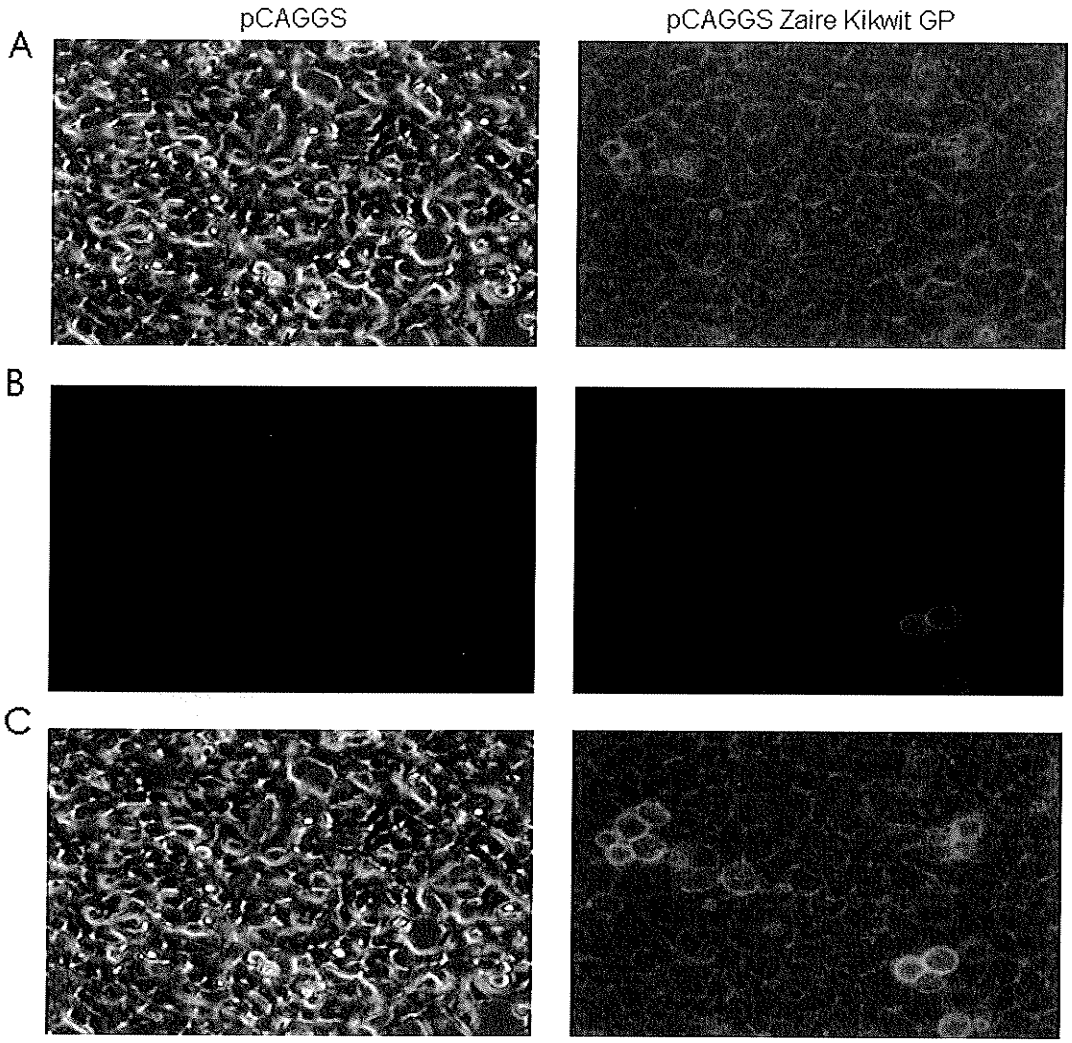

Figure 3.2: Transfection of pCAGGS Zaire Kikwit GP into 293T cells.
Fluorescent microscopy of 293T cells transfected with pCAGGS or pCAGGS Zaire Kikwit GP shows GP positive cells in (A) phase contrast, (B) FITC channel and (C) overlay of both channels of pCAGGSZaire Kikwit GP transfected cells only. The cells were probed without permeablization with ZGP12/1.1 antibody (1:5000) followed by a FITC conjugated goat anti-mouse antibody (1:10,000). The IFA shows cells at 200x magnification.

Sudan Gulu GP clone were digested to remove the EBOV GP for subsequent subcloning into the VSV XN2 expression vector. The GP of Sudan Boniface, 2362 bp, was obtained

by PCR of a pCAGGS SEBOV Boniface GP vector. The Sudan Boniface GP nucleotide sequence included the 8A sequence for the GP_1/GP_2 frame shift and seven point mutations (Appendix 2) not seen in the Sudan Boniface GP sequence found on PubMed

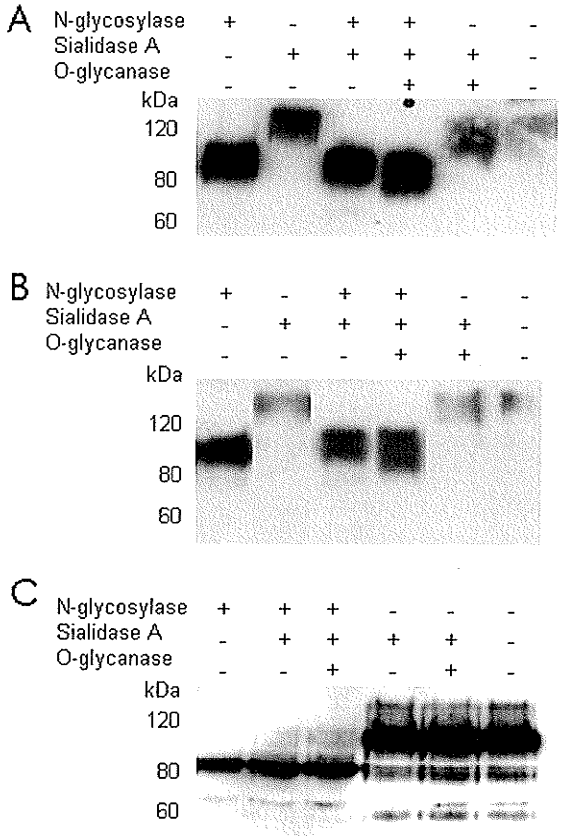

Figure 3.3: Characterization of EBOV GP.
Western blot results for the deglycosylation of (A) pCAGGS Zaire Mayinga GP, (B) pCAGGS Zaire Kikwit GP and (C) pCAGGS Sudan Gulu GP in 293T transfected cells. Primary antibodies ZGP12/1.1 (1:5000) for Zaire Kikwit or EGP42/3.7 (1:5000) for Sudan Gulu, followed by a HRP conjugated goat anti-mouse antibody (1:10,000) was utilized for western blots.

(reference # U28134). These mutations include nucleotide 177 (adenine to cytosine), nucleotide 283 (cytosine to adenine), nucleotide 285 (adenine to guanine), nucleotide 345 (adenine to guanine), nucleotide 543 (cytosine to thymine), nucleotide 608 (cytosine to thymine) and nucleotide 1414 (guanine to thymine). However, these mutations were

found in the original pCAGGS SEBOV Boniface GP plasmid obtained from Ayato Takada.

Each Ebola GP gene was subcloned into the digested VSV XN2 vector. Identification of VSV XN2 (12,000 bps) containing the EBOV GP (2000 bps) was determined by agarose gel electrophoresis and confirmed by DNA sequencing (Figure 3.1-C). Positive clones were grown in a larger preparation for sequencing and virus rescue. The DNA of positive VSVΔG/EBOV GP clones was sequenced using primers found in Appendix 1, to ensure that there were no differences between the pCAGGS clones and the VSV XN2 clones.

3.2 Rescue of the Recombinant VSVΔG/EBOV GP Virus

The reverse genetics system is a method to generate infectious viral particles entirely from cloned cDNA (Muhlberger *et al.*, 1999; Neumann *et al.*, 2002). Mammalian cells are transfected with plasmids encoding viral proteins (Figure 2.3). Once inside the cells, the plasmids are transcribed and translated by host proteins. The viral proteins assemble, replicate and bud from the host cell to make viral particles (see section 2.13 for rescue protocol). The VSVΔG/EBOV GP plasmids were used to rescue the VSVΔG/EBOV GP viruses used in the NK cell mediated cytotoxicity assays. The VSVΔG/ZEBOV Kikwit GP and VSVΔG/SEBOV Boniface GP viruses were rescued, however, only the VSVΔG/ZEBOV Kikwit GP virus was used in the cytotoxicity assays. The VSVΔG/SEBOV Boniface GP virus was used in a post-exposure protection study against the *Sudan ebolavirus* in non-human primates conducted by Geisbert *et al* (Geisbert *et al.*, 2008). The VSVΔG/SEBOV Gulu GP virus is in the process of being rescued. A 1:1 mixture of 293T cells and Vero E6 cells were transfected with the

VSVΔG/EBOV GP clones and VSV helper plasmids in the concentrations found in Table 2.2. A mixture of these two cell types were employed in the transfection because 293T cells are easily transfected by the plasmids, however they do not survive long enough to make large volumes of virus. The Vero E6 cells were utilized because they are highly susceptible to infection by the recombinant viruses and would propagate the recombinant virus more readily than 293T cells. VSV wild type was used as a positive control for the rescue attempt. The infectious supernatant was run on a 10% SDS-PAGE gel to confirm rescue and GP expression of the VSVΔG/EBOV GP or the VSV wild type viruses. Immunoblot analysis revealed a band at approximately 140 kDa indicative of Ebola GP_1 and a band at approximately 60 kDa indicative of VSV GP, respectively (Figure 3.1-D).

A RNA extraction was performed on the infectious supernatant in order to confirm the findings from the immunoblots and blind passages. Using primer sets found in Appendix 1 directed to each specific Ebola GP, RT-PCR was performed on the extracted RNA to confirm the presence of VSVΔG/EBOV GP virus.

3.3 Determination of Target Cell Susceptibility for the Human NK Cell Mediated Cytotoxicity Assays

To perform the NK cell mediated cytotoxicity assays, a target cell had to be chosen. Firstly, the cell line was an established line that had been successfully utilized in cytotoxicity assays. Secondly, the cell line used had to be able to be infected by the VSVΔG/EBOV GP virus since many of the cell lines used in killing assays are lymphoblast and lymphocytes are not infectable by Ebola.

The Daudi human B cell and K562 erythroleukemic human lymphoblast cell lines are known to be partially susceptible or susceptible to NK cell killing respectively. Therefore, they were assessed for susceptibility to infection with the VSVΔG/ZEBOV

GP strain Kikwit virus (VSVΔG/ZEBOV GP). Both suspension cells lines were infected with a MOI of 1 of VSVΔG/ZEBOV GP virus and supernatant was harvested at 1, 4, 8, 12 and 24 hours post infection. Zaire Kikwit GP was detected by RT-PCR and by western blot analysis. At no time point was RNA or viral protein produced by the Daudi cell line (Figure 3.4-A). Both RT-PCR and western blot analysis verified that Zaire Kikwit RNA and Ebola GP were produced by the K562 cell line (Figure 3.4-B) beginning at 4 and 8 hours of infection respectively. Zaire Kikwit RNA is detectable in the supernatants of both Daudi and K562 infected cells due to the cells not being washed following the 1 hour viral absorption period (Figure 3.4).

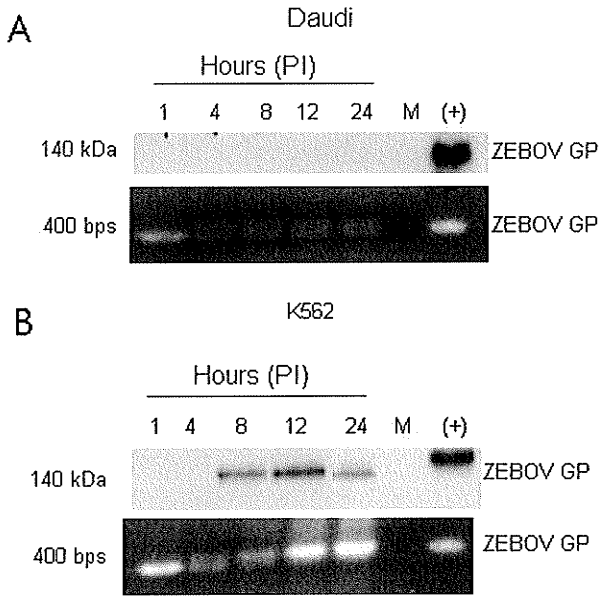

Figure 3.4: Confirmation of virus growth in human cell lines.
Western blots (upper panels) of the cell lysates and RT-PCR (lower panels) of supernatants of VSVΔG/ZEBOV Kikwit GP infected (A) human Daudi lymphoblast cell line and (B) human K562 lymphoblast cell line show production of ZEBOV GP viral proteins and viral RNA only in the K562 cells beginning at 8h after infection. Primary antibody ZGP12/1.1 (1:5000) followed by a HRP conjugated goat anti-mouse antibody (1:10,000) was utilized for western blots. M = Mock cells with no virus and (+) = VSVΔG/ZEBOV Kikwit GP RNA or cell lysate from Vero E6 infected cells.

To optimize the conditions for the VSVΔG/ZEBOV GP virus infection of the K562 cell line, an infection was carried out at MOIs of 0.1, 1 and 10 for 24 hours. After IFA and visualization with a fluorescent microscope, Ebola GP was seen on the surface of the VSVΔG/ZEBOV GP infected K562 cells while uninfected cells did not express Ebola GP (Figure 3.5). Therefore, the K562 cell line was selected for the NK cell mediated cytotoxicity assay.

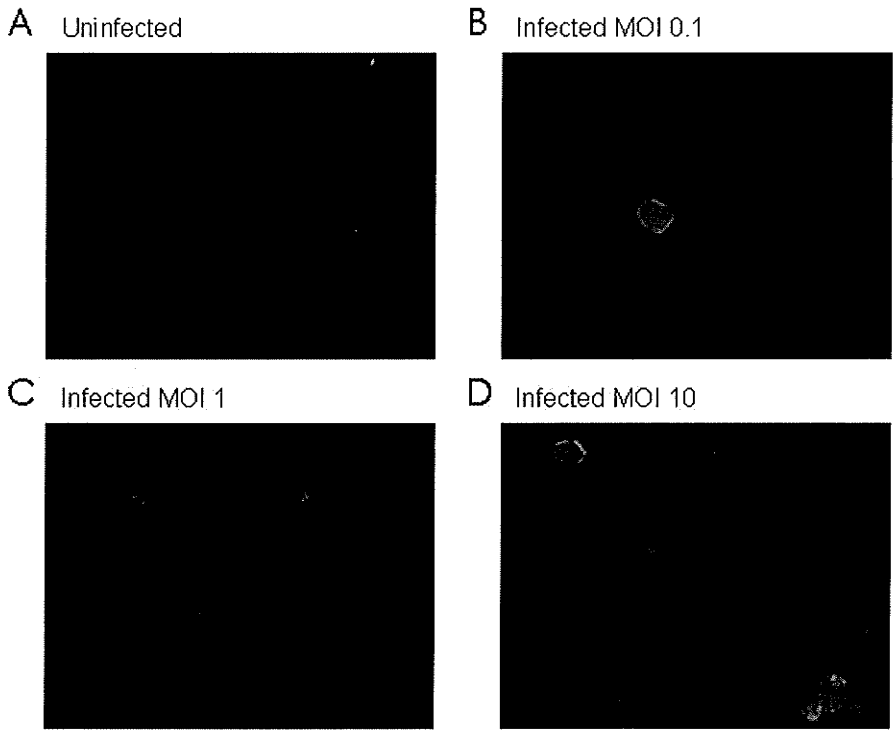

Figure 3.5: Immunofluorescence confirming ZEBOV GP expression in K562 cells.
The K562 cells were either (A) uninfected or infected for 24h with VSVΔG/ZEBOV Kikwit GP at MOIs of (B) 0.1, (C) 1, and (D) 10. The cells were probed without permeablization with ZGP12/1.1 antibody (1:2000) followed by a FITC conjugated goat anti-mouse antibody (1:10,000) and the nucleus was stained with DAPI. Magnification of the IFA was at 400x.

Since the recombinant virus is a combination of the VSV and the *Ebolavirus*, the K562 cells were also infected with VSV wild type strain Indiana (provided by J. Rose,

Yale University School of Medicine, New Haven, Connecticut, U.S.A) at a MOI of 1 for 24 hours. In IFA, VSV GP was seen in and/or on the surface of the K562 cells whether they were permeablized (Figure 3.6-D) or not (Figure 3.6-B). The infection of the K562 cells with VSVΔG/ZEBOV GP virus or VSV wild type virus was further verified by FACS analysis. Following infection with both viruses, cells were stained positively for the respective viral GP on the surface of the K562 cells (Figure 3.7). Therefore, these data confirm the infectability of the K562 cells and their usefulness for the NK cell cytotoxic killing assays.

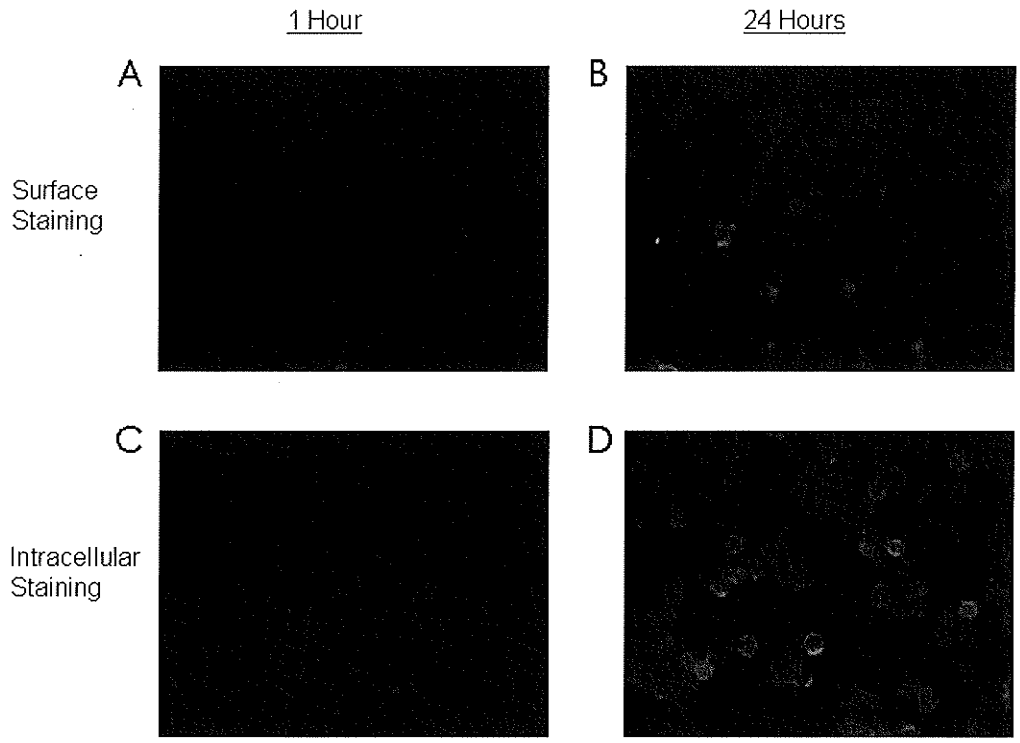

Figure 3.6: Fluorescent microscopy confirming VSV wild type infection of K562 cells.
K562 cells were infected with VSV wild type at a MOI of 1 for (A, C) 1h or (B, D) 24 hours. Cells were (A, B) surfaced stained or (C, D) permeablized and stained for VSV GP. The K562 cells were probed with an anti-VSV G antibody (1:2000) followed by a FITC conjugated goat anti-mouse antibody (1:10,000) and

the nucleus was stained with DAPI. Cells were visualized at a 400x magnification.

3.4 Stimulation of the NK-92MI Cell Line

3.4.1 Viral Infection of the NK-92MI Cell Line

Lymphocytes, including NK cells, are not normally infected by *Ebolavirus* (Baize *et al.*, 1999; Bray and Geisbert, 2005; Geisbert *et al.*, 2003; Ignatiev *et al.*, 2000; Reed *et al.*, 2004; Sanchez *et al.*, 2004). Therefore, it had to be established that the human NK cell line to be used in these killing assays would also not be infected by *Ebolavirus* or the VSVΔG/ZEBOV GP virus. The NK-92MI cell line was chosen because unlike the parent cell line NK-92, it was able to synthesize its own IL-2 for cell growth and proliferation (Tam *et al.*, 1999). It was confirmed that NK-92MI was not susceptible to *Ebolavirus* strain Kikwit, VSVΔG/ZEBOV GP virus and was susceptible to VSV wild type. The NK-92MI cells were infected with *Zaire ebolavirus* strain Kikwit at a MOI of 0.1 and 10 in CL4. Samples of the supernatants were taken at 1, 24, 48 and 72 hours post infection. Supernatants of uninfected NK-92MI cells were also harvested at the same time points. RT-PCR was performed on all the supernatant samples using the primer sets found in Appendix 1. Input viral RNA was detected in all supernatant samples infected with *Zaire ebolavirus* at MOIs of 0.1 and 10 at all time points taken (Figure 3.8-A). In addition, real time RT-PCR performed on the same supernatants demonstrated no increase in the amount of virus found in the supernatant at any time point taken after infection (Figure3.8-B). To verify that no virus replication occurred in the NK-92MI cells, western blot analysis on the supernatants and cell lysates was performed. No Ebola GP protein was seen by immunoblot at any MOI or time point taken (Figure 3.8-C). This suggests that the RNA amplified from the infection was input virus that bound to the cells, which

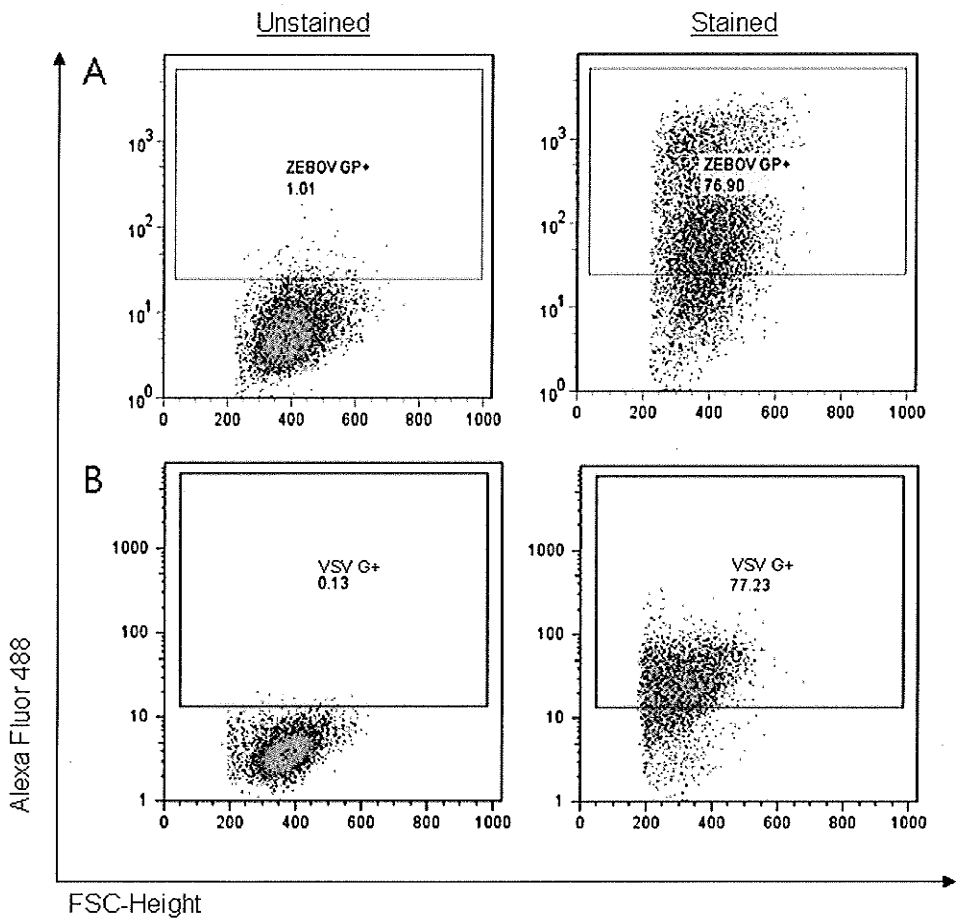

Figure 3.7: FACS analysis of an infection of K562 cells by VSVΔG/ZEBOV GP and VSV wild type.
Intracellular FACS data showing K562 cells infected with (A) VSVΔG/ZEBOV Kikwit GP or (B) VSV wild type for 24 hours and stained for ZEBOV GP or VSV GP, respectively. The cells were probed with ZGP12/1.1 antibody (1:2000) for ZEBOV GP and an anti-VSV G antibody (1:2000) for VSV wild types followed by a FITC conjugated goat anti-mouse antibody (1:10,000).

was not able to infect the cells to produce progeny virus. Thus, the NK-92MI cell line is similar to primary NK cells, since regarding their resistance to the *Ebolavirus*.

The NK-92MI cell line was then infected with the VSVΔG/ZEBOV GP virus at MOIs of 0.1, 1, 5 or 10. Supernatant was taken from the cells at 1, 24, 48 and 72 hours

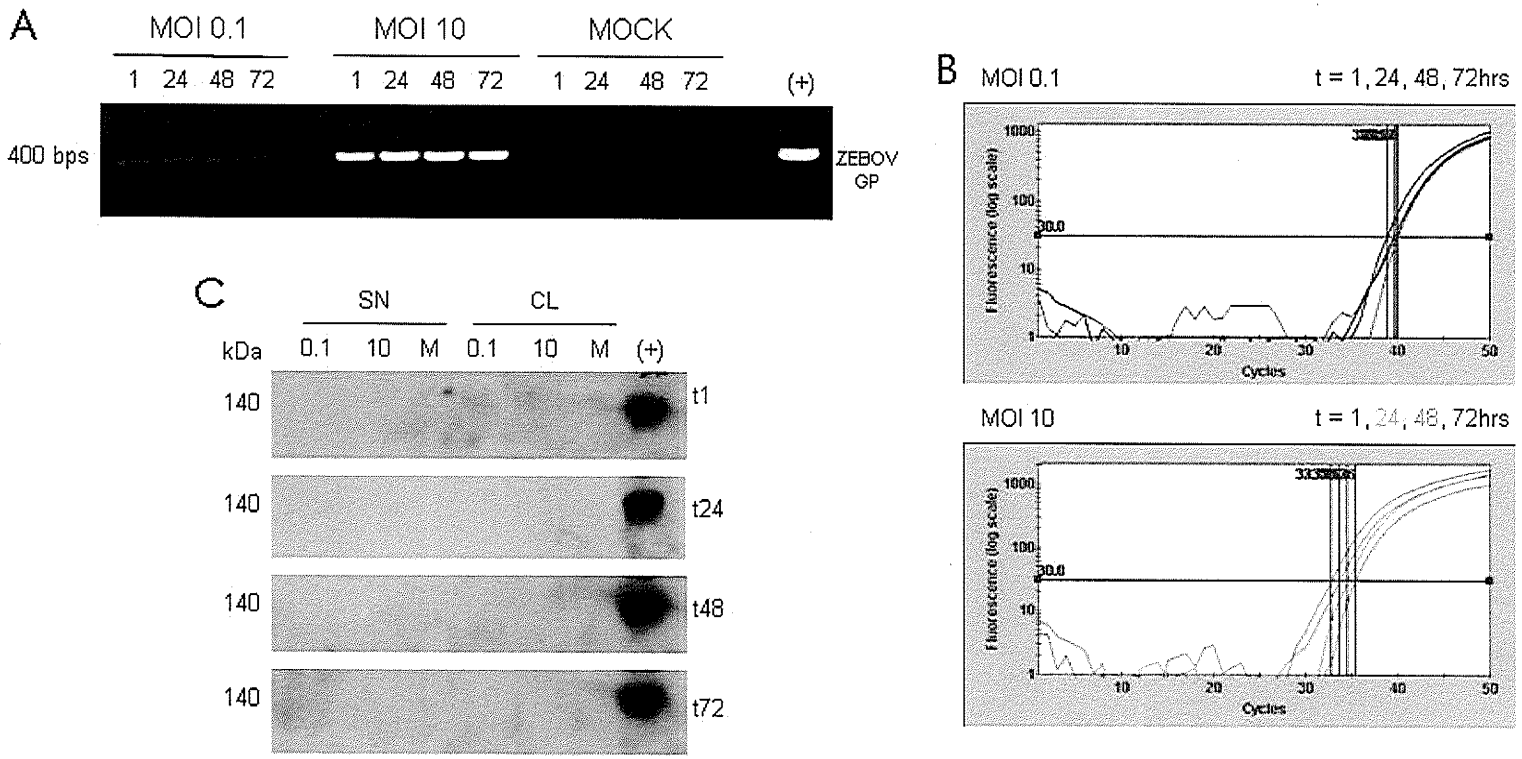

Figure 3.8: Infection of NK-92MI cells with ZEBOV performed at MOIs of 0.1 and 10.
(A) RT-PCR performed on supernatants (SN) of NK-92MI infected cells shows a constant quantity of ZEBOV genome at both MOIs. In addition, (B) real time RT-PCR performed on the same SN do not show an increase in viral genome at either MOI over 72 hours of infection, indicating the detection of input virus. (C) Western blots of the SN and cell lysate (CL) from ZEBOV infected NK-92MI cells do not show production of viral proteins. Primary antibody ZGP12/1.1 (1:5000) followed by a HRP conjugated goat anti-mouse antibody (1:10,000) was utilized for western blots. M = Mock cells with no virus, (-) = H_20 and (+) = ZEBOV Kikwit RNA or VSVΔG/ZEBOV Kikwit GP CL from Vero E6 cells.

post infection. RT-PCR performed on the supernatants did not show any viral RNA at any time point of infection, at any MOI (Figure 3.9-A). This difference from the ZEBOV infection, maybe due to the shape of the natural long filament ZEBOV particle wrapping around the NK cell, while the Rhabdovirus shaped VSVΔG/ZEBOV GP particle does not have the capacity to achieve this type of binding. The supernatants were also passaged onto 100% confluent Vero E6 cells to determine if any infectious particles were found in the supernatant. The plaque assay determined that none of the supernatant samples at any time point or MOI contained infectious virus able to infect Vero E6 cells at a detectable level. Immunoblots performed on the supernatants and cell lysates of the infected NK-92MI cells did not show any Ebola GP production (Figure 3.9-B). To confirm that VSVΔG/ZEBOV GP does not infect NK-92MI cells, an IFA was performed on the cells. The cell line was infected with VSVΔG/ZEBOV GP at a MOI of 1 and cultured for 24 hours. When visualized under a fluorescent microscope, no Ebola GP was seen on or in the NK-92MI cells within 24 hours of infection (Figure 3.10-B and C). Staining observed on uninfected cells (Figure 3.10-A) showed unspecific binding since the binding pattern did not complement the cell surface. Thus the infections performed on the NK-92MI cells strongly suggest *Ebolavirus* or VSVΔG/ZEBOV GP can not infect or replicate in this cell line.

It has been found that NK cell lines can be infected by VSV wild type (Zaczynska *et al.*, 2008). For this reason, it was expected that NK-92MI could have been infected by VSV wild type. The NK-92MI cell line was infected with VSV wild type at MOIs of 0.1, 1, 5 and 10. Samples of the supernatants were taken at 1, 4, 48 and 72 hours post infection. RT-PCR performed on the supernatants illustrated VSV RNA 24 hours post

infection (Figure 3.11-A). VSV G was detected in the cell lysate, but not the supernatant, starting at 24 hours post infection as assayed by immunoblot (Figure 3.11-C). Supernatant from the VSV wild type infection was passaged onto 100% confluent Vero E6 cells and a plaque assay was performed. VSV wild type viral titres were calculated

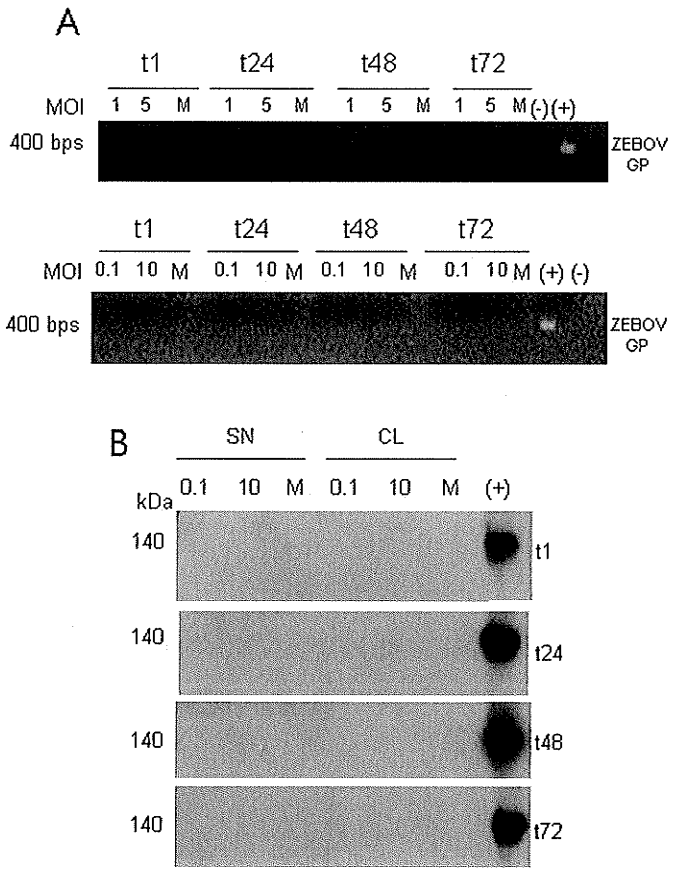

Figure 3.9: VSVΔG/ZEBOV Kikwit GP infection of NK-92MI cells performed at MOIs of 0.1, 1, 5 and 10.

RT-PCR performed on supernatants (SN) of NK-92MI infected cells do not show replication of VSVΔG/ZEBOV Kikwit GP. (B) Western blots of SN and cell lysate (CL) of VSVΔG/ZEBOV Kikwit GP infected NK-92MI cells do not show production of viral proteins. Primary antibody ZGP12/1.1 (1:5000) followed by a HRP conjugated goat anti-mouse antibody (1:10,000) was utilized for western blots. M = Mock cells with no virus, (-) = H_20 and (+) = VSVΔG/ZEBOV Kikwit GP RNA or CL from Vero E6 cells.

for each time point, at each MOI. Virus titres for the infection of NK-92MI cells increased over time for each MOI (Figure 3.11-B).

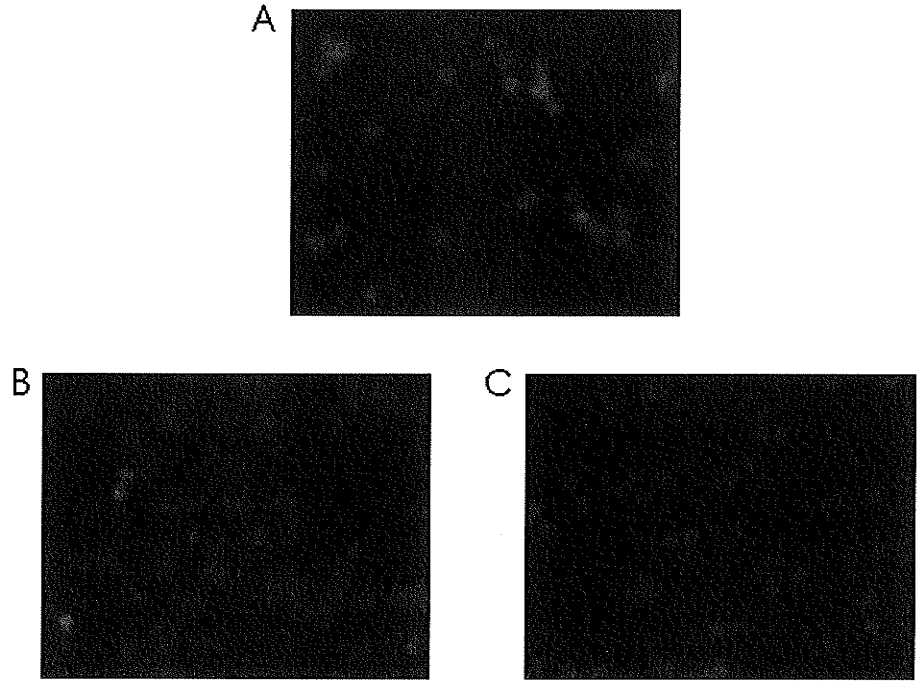

Figure 3.10: Fluorescent microscopy of NK-92MI cells infected with VSVΔG/ZEBOV Kikwit GP.
NK-92MI cells were either (A) uninfected or (B, C) infected with VSVΔG/ZEBOV Kikwit GP at a MOI of 1 for 24h. Cells were (A, B) surfaced stained or (C) permeablized and stained for ZEBOV GP. The cells were probed with ZGP12/1.1 antibody (1:2000) followed by a FITC conjugated goat anti-mouse antibody (1:10,000) and the nucleus was stained with DAPI at magnification of 400x.

Confirmation of the VSV wild type infection of NK-92MI cells was performed by IFA (Figure 3.12). The ability of VSV wild type to infect the NK-92MI cells indicated that the change of the glycoprotein from VSV to Ebola altered the tropism of the VSV virus. The ligand of viral entry for the *Ebolavirus* into targeted cells is the Ebola glycoprotein (Marzi *et al.*, 2007). Thus, seeing that VSVΔG/ZEBOV GP was not able to infect the

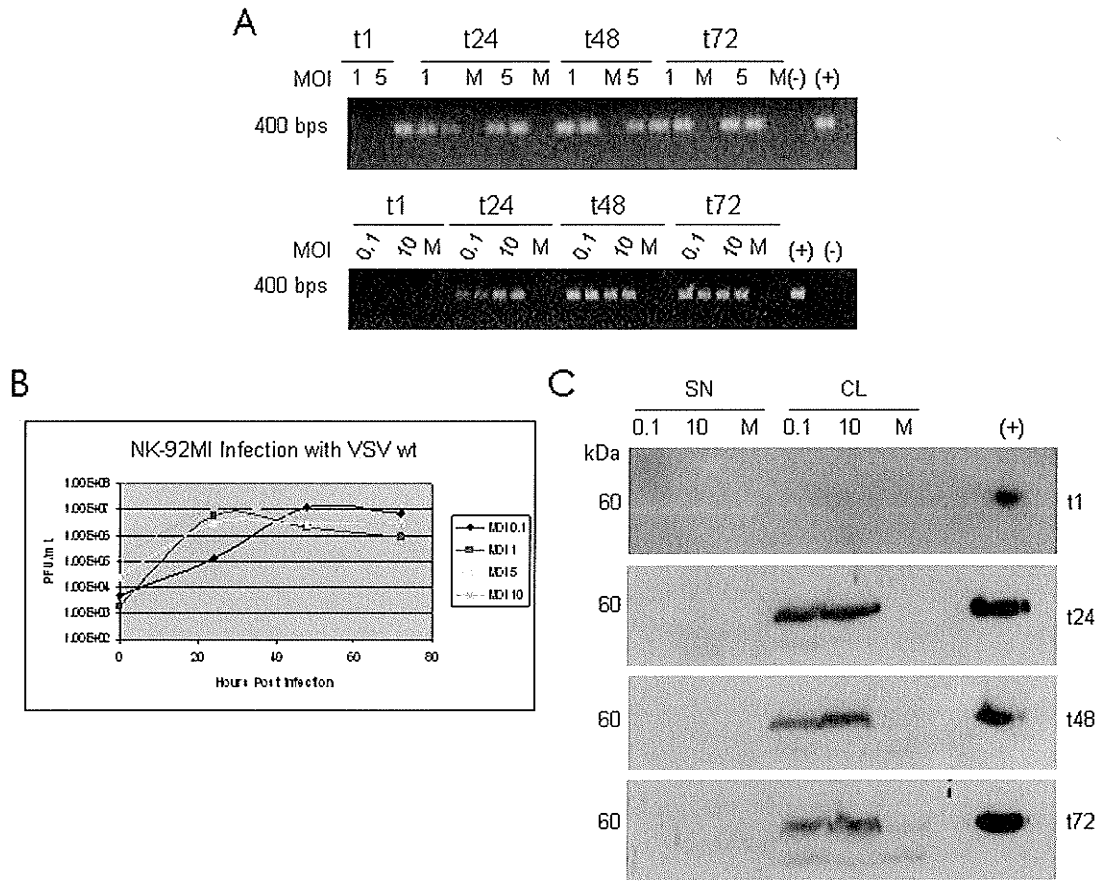

Figure 3.11: VSV wild type (wt) infection in NK-92MI cells performed at MOIs of 0.1, 1, 5 and 10.

(A) RT-PCR performed on supernatants (SN) of NK-92MI infected cells show replication of VSV wt genome after 24h of infection at different MOIs of 1 and 5 (top panel) and 0.1 and10 (lower panel). (B) Plaque assay results for dilutions of supernatant of infected NK-92MI cells show increases in viral titre with peak VSV wt titre at 24h. (C) Western blots of SN and cell lysate (CL) of VSV wt infected NK-92MI cells show production of viral proteins after 24h of infection. Primary antibody anti-VSV G (1:2000) followed by a HRP conjugated goat anti-mouse antibody (1:10,000) was utilized for western blots. M = Mock cells with no virus, (-) = H_20 and (+) = VSV wild type RNA or CL from Vero E6 cells.

NK-92MI cells, it suggests that NK cells, as all known lymphocytes, lack the Ebola receptor needed for infection.

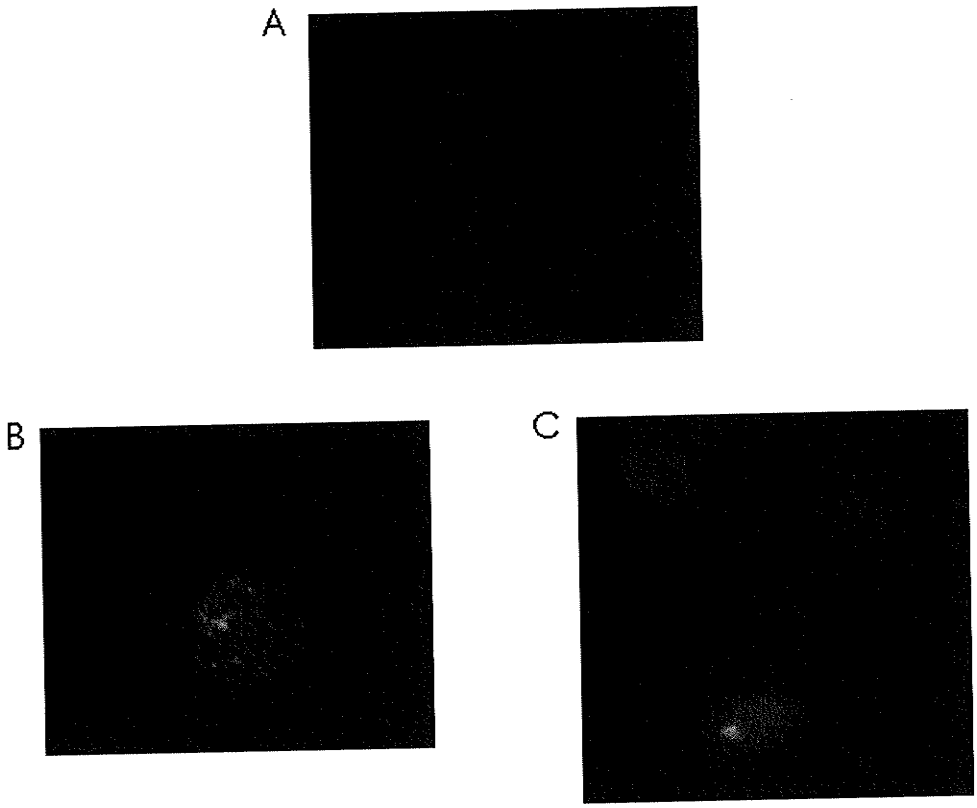

Figure 3.12: VSV wild type infection of NK-92MI cells.
NK-92MI cells infected with VSV wild type at a MOI of 1 for 24h expressed VSV G. (A) Uninfected or infected cells were (B) surfaced stained or (C) permeablized and stained for VSV G. The cells were probed with an anti-VSV G antibody (1:2000) for VSV wild types followed by a FITC conjugated goat anti-mouse antibody (1:10,000) at a magnification of 1000x for infected cells or 400x for uninfected cells.

3.4.2 Chemical Stimulations of the NK-92MI Cell Line

The NK-92MI cell line has an activated profile (Gong *et al.*, 1994), therefore, it was determined if these cells could be further activated and behave as normal activated NK cells. The cell line was stimulated with the mitogens phorbol-12-myristate-13-acetate (PMA) and ionomycin in combination for 5 hours at 37°C. The NK-92MI cells were able to secrete IFN-γ and TNF-α upon stimulation (Figure 3.13-A and B). A decrease in the amount of perforin found in the cells was also observed in the cells when stimulated with

PMA and ionomycin for five hours, suggesting degranulation into the medium (Figure 3.13-C). This indicates that the NK-92MI cell line was able to be activated and secreted cytokines and degranulate following chemical stimulation.

3.5 Development of the Human NK Cell Mediated Cytotoxicity Assay

3.5.1 Optimization of the Human Target Cell Infection

We determined the optimal conditions for the infection of the target cells with different MOIs of VSVΔG/ZEBOV GP and by determining the infection by western blot, IFA and flow cytometry. The K562 cells were infected with MOIs of 0.1 and 1 of VSVΔG/ZEBOV GP. Cell lysates from 1, 4, 8, 12 and 24 hours after infection were run on a SDS-PAGE gel. Immunoblots demonstrated Ebola GP only in the MOI of 1 infection 8 hours post infection (Figure 3.14-A and B). In addition, to ensure that the infection with VSVΔG/ZEBOV GP did not kill all of the target cells during the incubation time, the K562 cells were infected with a MOI of 1 and samples taken over a 72 hour period were stained with a live/dead fluorescent dye. FACS analysis of the infected cells showed an increase in cell death over the 72 hour period of infection (Figure 3.14-C). Cell death was observed in uninfected cells; however, more death was seen in the infected cells, especially after 24 hours. Therefore, it was determined that the infection period of the K562 cells with VSVΔG/ZEBOV GP could not exceed 24 hours, since the cells would die due to the infection rather than the action of the NK cells. A MOI of 1 for 8 hours was chosen for the infection of K562 cells for the NK cell mediated cytotoxicity assay since at 8 hours viral products were detected and cell death due to the virus was similar to that of non-infected cells at this time point and virus dose.

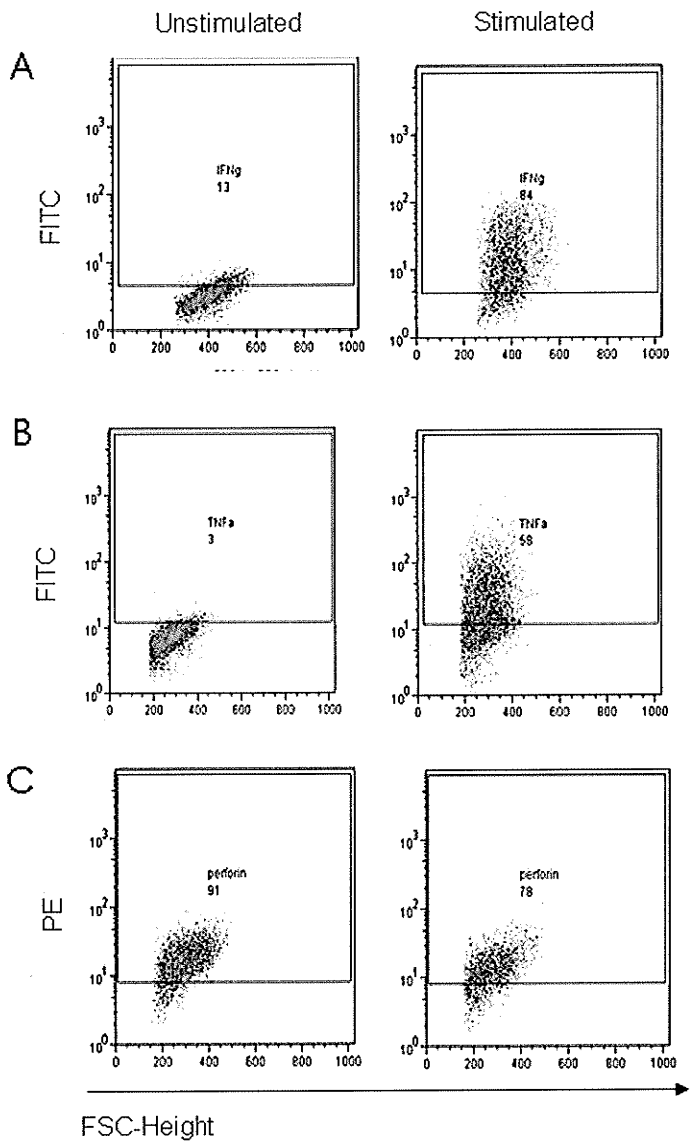

Figure 3.13: FACS analysis of a PMA/ionomycin stimulation of NK-92MI cells for 5h at 37°C.

NK-92MI cells were able to produce (A) IFN-γ and (B) TNF-α from NK-92MI cells after stimulation with 0.0005 μg/mL PMA and 0.375 μg/mL ionomycin. (C) Intracellular perforin levels in cells following stimulation with PMA/ionomycin decreased, probably due to the release of perforin into the media.

3.5.2 Optimization of the Human Target Cell Staining

A NK cell mediated cytotoxicity assay utilizing flow cytometry instead of chromium release was utilized in order to perform the assay safely in both CL2 and CL4 without radioactive waste. In the fluorescent cytotoxicity assay, the target cells were stained with PKH-26 to distinguish them from the NK effector cells. The K562 cells were stained with increasing concentrations of the PKH-26 dye for 2, 5 or 10 min at 37°C. Not all of the target cell population was stained at a concentration of 2.5 μM of PKH-26 during any incubation period (Figure 3.15). Virtually all of the cells were stained brightly at 5 μM and 10 μM for all incubation periods tested. However a slightly higher percentage of cells were stained at a concentration of 5 μM in a 5 min incubation period (Figure 3.15). Thus, for the cytotoxicity assay, the target cells were to be stained with 5 μM of PKH-26 for 5 min.

The K562 cells were also stained with a decreasing concentration of the CFDA SE cell tracer dye (CFSE), starting at 0.5 μM to 0.1 μM for 5 min at room temperature. CFSE was utilized to distinguish the difference between live target cells and dead target cells. The CFSE at 0.5 μM stained all the cells very brightly. The intensity of the dye decreased slightly as the concentration decreased (Figure 3.16). However, a higher concentration of CFSE was used for staining of the K562 cell line because the combination of both PKH-26 and CFSE together decreased the intensity of the CFSE dye further. Thus, after several trials of increasingly higher concentrations of CFSE plus PKH-26 at a concentration of 5 μM, a final CFSE concentration of 1.5 μM was selected for all future studies (Figure 3.16).

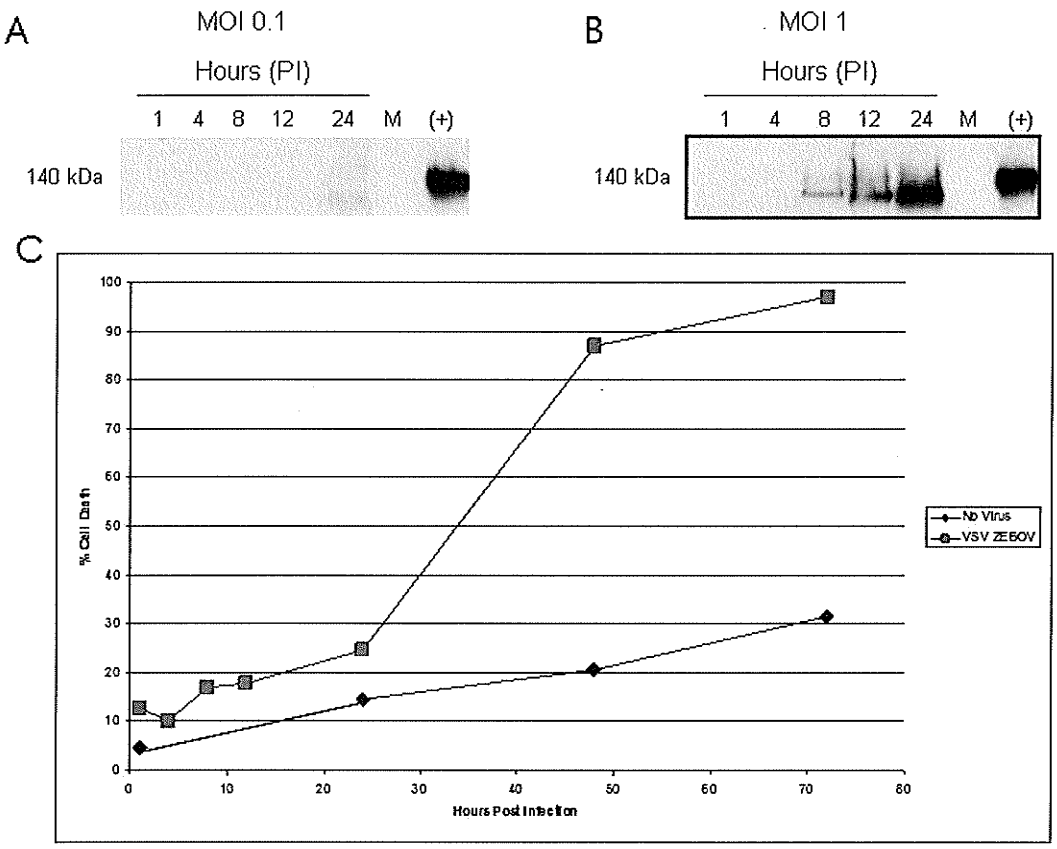

Figure 3.14: VSVΔG/ZEBOV Kikwit GP infection of the K562 cell line.
Infection of K562 cells with VSVΔG/ZEBOV Kikwit GP show no viral protein using a (A) MOI of 0.1, but shows viral protein at a (B) MOI of 1 after 8h after infection. Primary antibody ZGP12/1.1 (1:5000) followed by a HRP conjugated goat anti-mouse antibody (1:10,000) was utilized for western blots. (C) A flow cytometric cell death assay shows acceptable cell death after 24h due to the virus infection. M = Mock cells with no virus and (+) = VSVΔG/ZEBOV Kikwit GP cell lysate from infected Vero E6 cells.

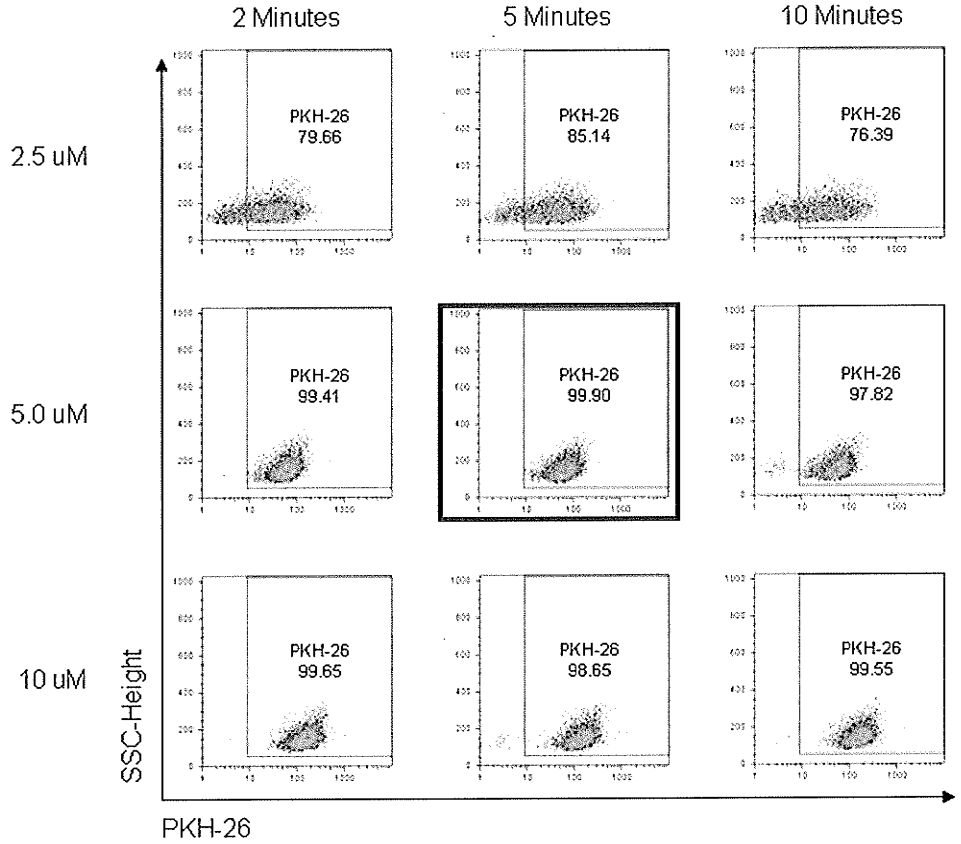

Figure 3.15: Staining K562 cells with PKH-26.
To optimize the staining of K562 cells with PKH-26 Red Fluorescent Cell Linker dye, several concentrations and 37°C incubation periods were utilized. A final concentration of 5 μM of PKH-26 in a 5 min incubation period was utilized.

3.5.3 Determination of the Optimal Conditions for the Human NK Cell Mediated Cytotoxicity Assay

VSV wild type has been used in NK cytotoxicity assays that have demonstrated an increase in target cell killing after the target cells have been infected (Schattner and Rager-Zisman, 1986). K562 target cells were infected with VSV wild type at MOIs of 1 and 10 for an 8 hours infection period or incubated in RPMI media supplemented with 3% FBS (RPMI-3) as a control. Following staining, the treated K562 cells were either

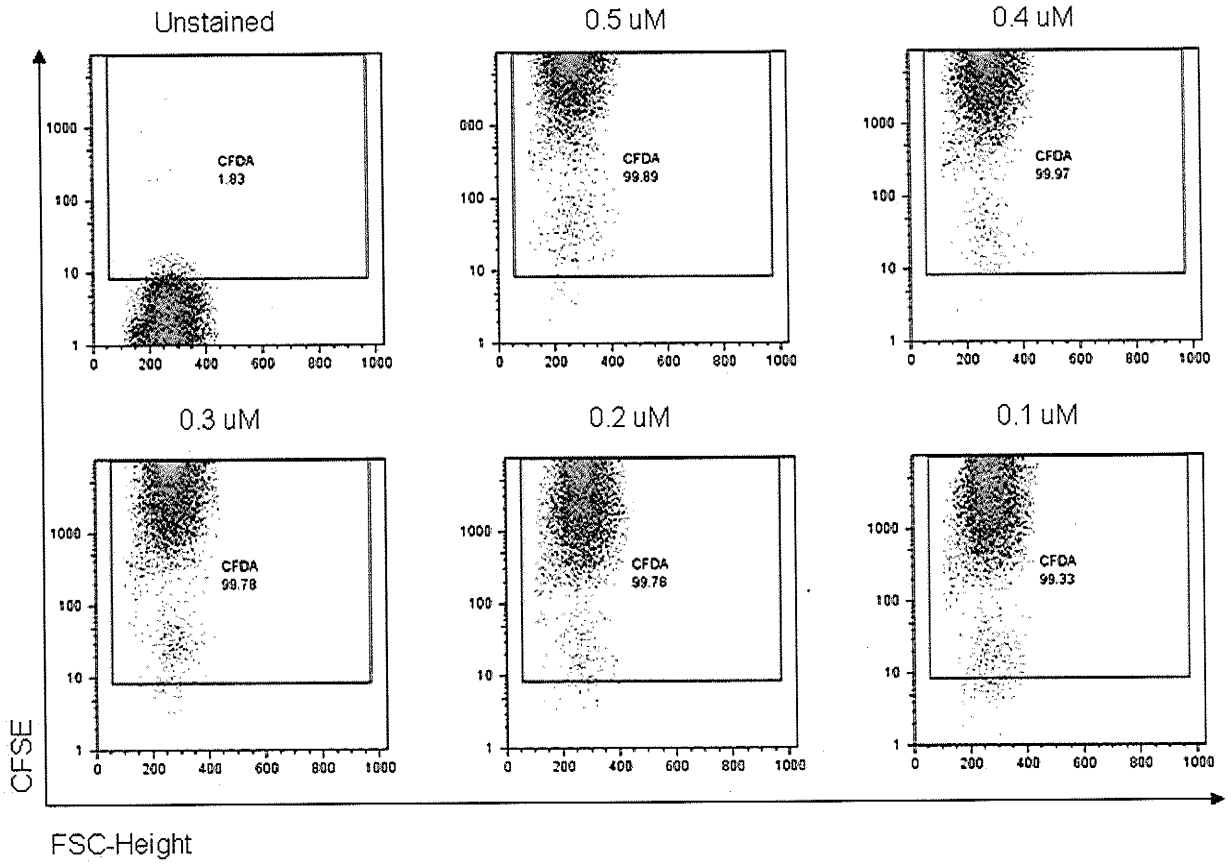

Figure 3.16: Staining K562 cells with CFSE.
To optimize the staining of K562 cells with the Vybrant CFDA SE cell tracer dye (CFSE), several concentrations of CFSE were utilized for an incubation period of 8 min at 37°C. A final concentration of 1.5 μM was utilized for the NK cell mediated cytotoxicity assay due to a change in the intensity of the CFSE dye when stain in combination with PKH-26.

incubated with NK-92MI cell, or left for the same time without NK cells, at a 3:1 E:T ratio for 4 hours as shown in Figure 3.17. There was significantly more target cell lysis found in cells infected with VSV wild type at a high MOI than in uninfected cells (Figure 3.17). Uninfected cells were killed more than infected cells at low MOI; however, it was not statistically significant. There was no difference in cell death observed in the uninfected cells incubated with NK cells compared to incubation in media alone. This suggested that conditions for the assay were not optimal. However, this data was still able to demonstrated that the cytotoxicity assay would work utilizing the NK-92MI cell line and flow cytometry.

The K562 target cells were then either incubated with or without VSVΔG/ZEBOV GP for 8 hours. The target cells were then added to the NK-92MI effector cells at 12:1, 6:1, or 3:1 effector to target (E:T) ratios. Samples of each treatment was taken at 2 and 4 hours after addition of the NK-92MI cells. As the E:T ratio increased, more target cells were lysed by the NK-92MI cells (Figure 3.18). However, there was no difference seen in the amount of target cell lysis between uninfected cells to infected cells (Figure 3.18). Since no difference was seen, it was determined that the K562 cells must require a longer incubation time with the VSVΔG/ZEBOV GP virus than was required for VSV wild type prior to NK cell addition. Several incubation periods were utilized to increase the amount of cell lysis performed on the VSVΔG/ZEBOV GP infected cell compared to non-infected cells. No difference in target cell lysis was seen between uninfected and infected cells at 12 hours after virus infection. At 24 and 48 hours of VSVΔG/ZEBOV GP infection of the K562 cells, there was an increase in the amount of killing of the infected target cells performed by the NK-92MI

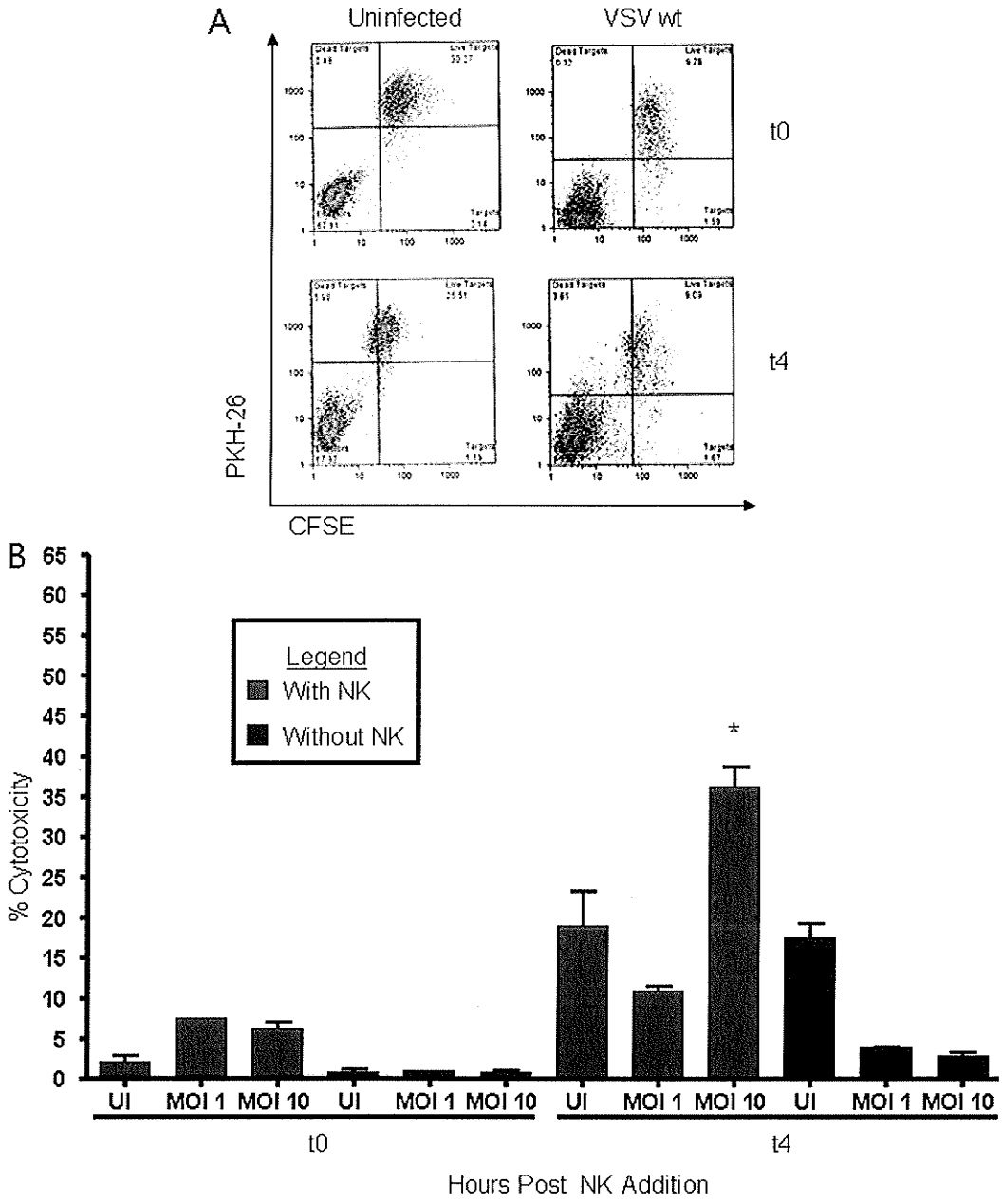

Figure 3.17: NK-92MI cell mediated cytotoxicity of VSV wild type infected K562 cells

K562 cells were infected with VSV wild type for 8 hours and added to NK-92MI cells a 3:1 effector to target ratio. (A) Demonstrates a sample of raw data acquired by flow cytometry and analyzed with FlowJo. (B) Illustrates the mean data of 8 CMC assays. VSV wild type infected cells at a high MOI were lysed more than uninfected K562 cells or cells infected at a low MOI. UI = uninfected, MOI of 1 and MOI of 10 = target cell infected with VSV wild type at each respective MOI. * P values between UI and MOI of 10 = 0.003. n = 8. Error bars represent SEM.

cells (data not shown. The 24 hour time point is discussed in more detain in section 3.6). However, since there was no difference in the amount of killing observed between 24 and

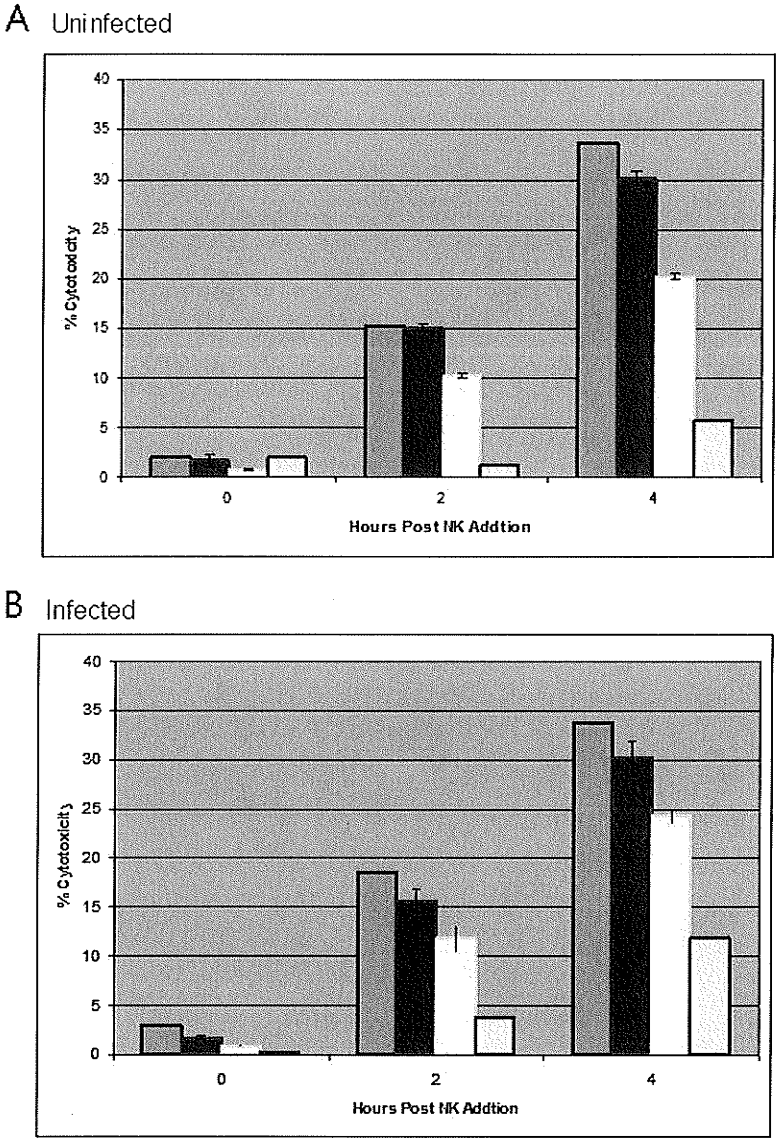

Figure 3.18: Lysis of K562 target cells by NK-92MI effector cells.
K562 cells were incubated with either media or a MOI of 1 of VSVΔG/ZEBOV Kikwit GP for 8 hours at different effector to target (E:T) ratios (12:1, 6:1, 3:1, No virus + no NKs). The percent cytotoxicity of NK-92MI cells on (A) uninfected K562 cells or (B) VSVΔG/ZEBOV Kikwit GP infected K562 cells escalates as the E:T ratio increases, compared to K562 cells that were not incubated with NK-92MI effector cells. Error bars represent SD.

48 hours of target cell infection, and the fact that cell viability became an issue following virus infection for 48 hours (Figure 3.14), 24 hours was chosen as an optimal infection period for NK cell mediated cytotoxicity assays with VSVΔG/ZEBOV GP.

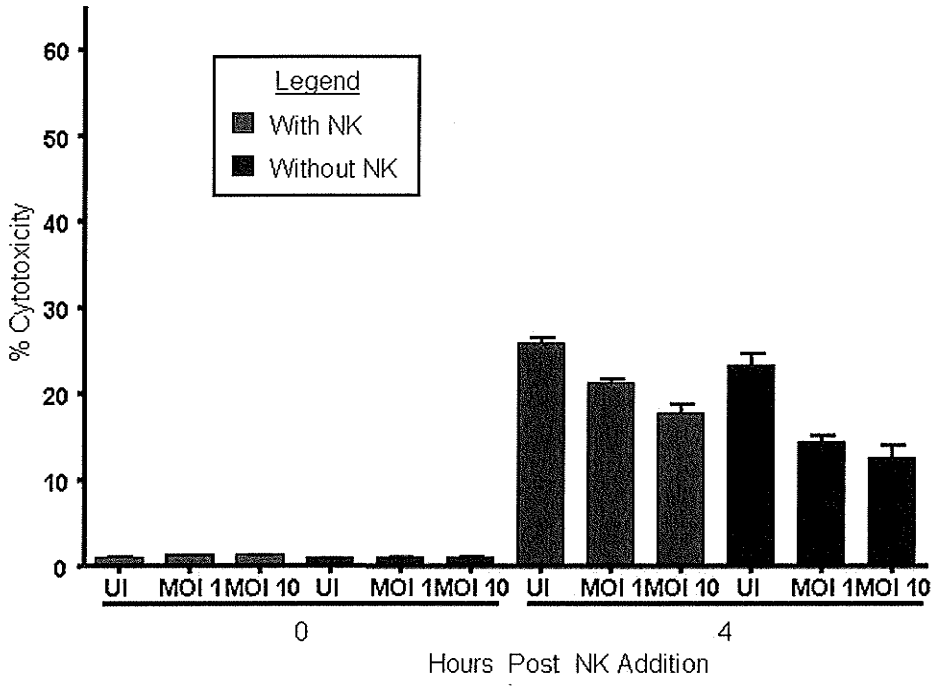

Figure 3.19: NK-92MI cell mediated cytotoxicity assay with an E:T ratio of 0.33:1.
K562 cells were infected with VSVΔG/ZEBOV Kikwit GP for 24 hours followed by an addition of NK-92MI cells at a 0.33:1 effector to target ratio. At this low E:T ratio, there is no additional target cell lysis observed of infected target cells. UI = uninfected, MOI of 1 and MOI of 10 = target cell infected with VSVΔG/ZEBOV Kikwit GP at each respective MOI. No statistically significant differences were observed. n = 9. Error bars represent SEM.

3.6 Human NK Cell Mediated Cytotoxicity Assay

K562 target cells were infected at MOIs of 1 or 10 with VSVΔG/ZEBOV GP or not. The concentration of FBS in the media was increased due to a lack of target cell stability at a lower concentration of FBS. Following infection, the target cells were stained with PKH-26/CFSE and either incubated in unsupplemented media or the NK-

92MI cells were added at 0.33:1, 1:1 and 3:1 E:T ratios for 4 hours. At the 0.33:1 E:T ratio, only background killing of the target K562 cells was observed whether the target cells were infected or not (Figure 3.19). The fact that there were no significant differences in the percent killing of infected and non-infected target cells was probably due to not having enough effector cells to lyse the infected cells more than the natural killing that occurs of the K562 cells.

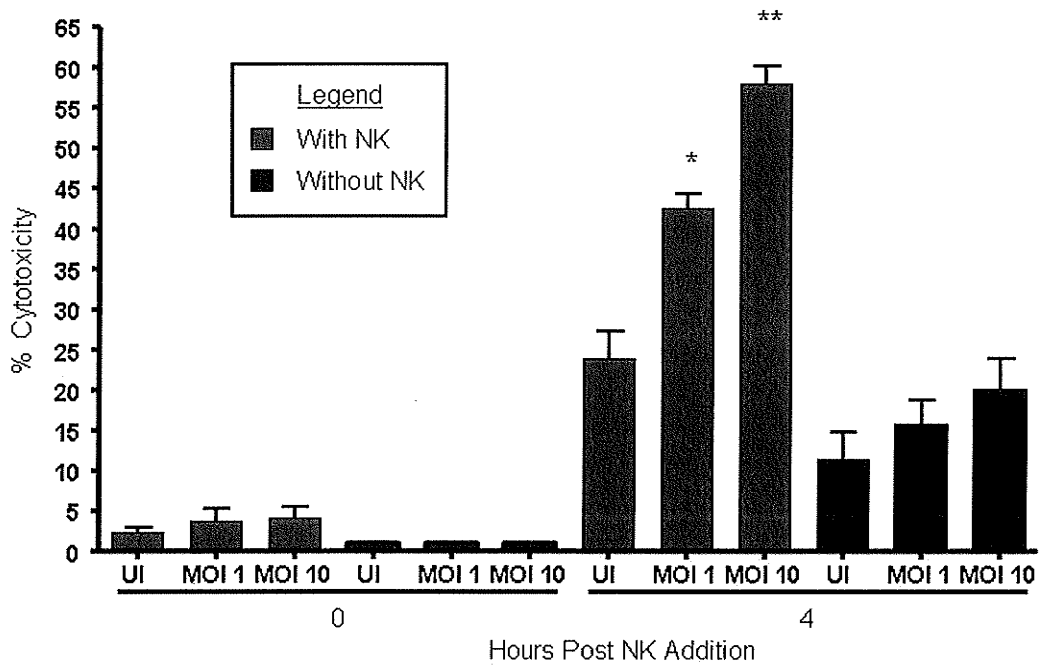

Figure 3.20: NK-92MI cell mediated cytotoxicity assay with an E:T ratio of 1:1.
K562 cells were infected with VSVΔG/ZEBOV Kikwit GP for 24 hours followed by an addition of NK-92MI cells at a 1:1 effector to target ratio. Progressively more infected cells were lysed by the NK-92MI effector cells at this E:T ratio. UI = uninfected, MOI of 1 and MOI of 10 = target cell infected with VSVΔG/ZEBOV Kikwit GP at each respective MOI. * = P values between UI and MOI of 1 = 0.0002. ** = P values between UI and MOI of 10 < 0.0001. n = 9 Error bars represent SEM.

At the 1:1 E:T ratio, there was significantly more target cell lysis of cells that were infected with VSVΔG/ZEBOV GP than in uninfected cells (Figure 3.20). Cells

infected at a higher MOI experienced more lysis than cells infected at a lower MOI at the 1:1 E:T ratio (Figure 3.20). Since more infected cells were killed than uninfected, this would suggest that the infection with VSVΔG/ZEBOV GP caused enough change in the phenotype of the target cells to cause the NK cells to lyse them more than the uninfected cells. Given that more cells were lysed at a higher MOI of VSVΔG/ZEBOV GP, this could indicate that a 1:1 effector to target ratio would provide better protection against an infection than having less effectors for every infected cell.

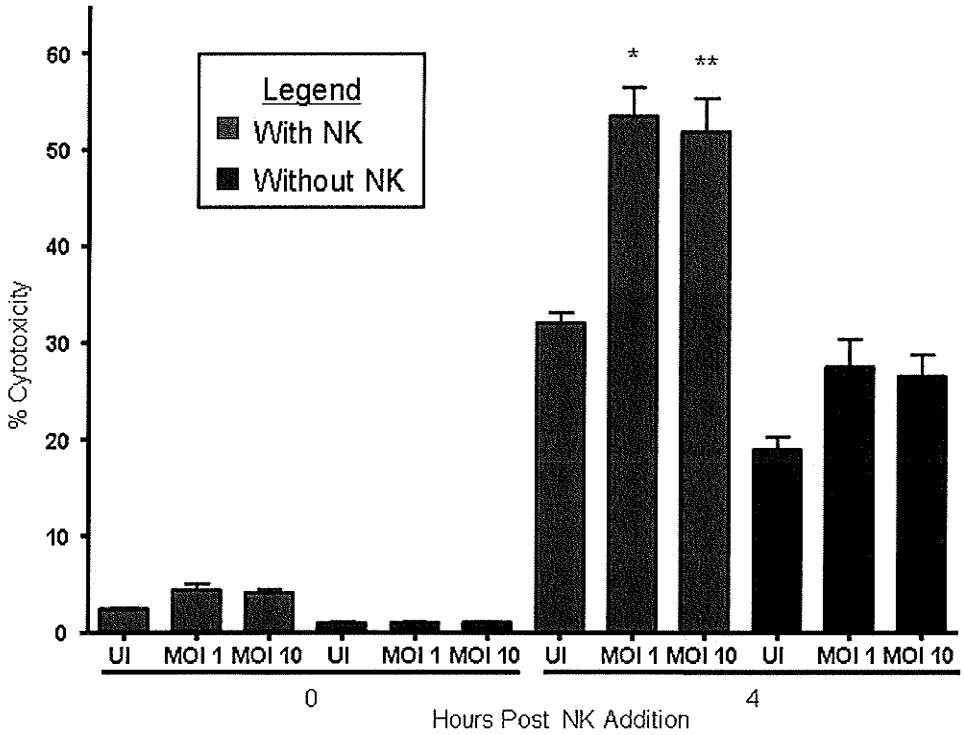

Figure 3.21: NK-92MI cell mediated cytotoxicity assay with an E:T ratio of 3:1.

K562 cells were infected with VSVΔG/ZEBOV Kikwit GP for 24 hours followed by an addition of NK-92MI cells at a 3:1 effector to target ratio. Significantly more VSVΔG/ZEBOV Kikwit GP infected cells were lysed than uninfected cells. However, no difference is observed from a high to a low MOI. UI = uninfected, MOI of 1 and MOI of 10 = target cell infected with VSVΔG/ZEBOV Kikwit GP at each respective MOI. * = P values between UI and MOI of 1 < 0.0001. ** = P values between UI and MOI of 10 < 0.0001. n = 9. Error bars represent SEM.

At a 3:1 E:T ratio, there was also significantly more target cell lysis in cells that were infected with VSVΔG/ZEBOV GP than in uninfected cells (Figure 3.21). Unlike the 1:1 E:T ratio, there was no difference in the amount of target cell lysis in cells infected at a higher or lower MOI (Figure 3.21). Seeing as there was no increase in the amount of target cell lysis as the MOI increased, this could suggest that there is a saturation of the effector cells with target cells for this system. This implies that having more effector cells does not necessarily mean that more target cell lysis would occur, especially seeing that approximately the same percentage of cytolysis is observed at the 1:1 high MOI and at the 3:1 infection at both MOIs.

3.7 Effects of VSVΔG/ZEBOV GP Virus on Target and Effector Cells

3.7.1 Effects of VSVΔG/ZEBOV GP on K562 Cells

Generally, in order for NK cells to kill a target cell, changes in the phenotype of the target cell, such as expression of certain surface receptors or an increase in the secretion of soluble products, must occur to enable the NK cell recognize and lyse the infected target cell. The infection of K562 cells with VSVΔG/ZEBOV GP caused an increase in the expression of Ebola GP on the surface of the cell (Figure 3.22-A). This infection also caused an overall slight increase in the amount of Human Leukocyte Antigens (HLA) on the cell surface (Figure 3.22-B). However, since not all the cells become infected with VSVΔG/ZEBOV GP within the 24 hour period, a closer look was taken at the HLA molecules on the K562 cells that expressed Ebola GP on the cell surface after the 24 hour infection period. By FACS analysis of the VSVΔG/ZEBOV GP infected cells, gating was performed on target cells expressing ZEBOV GP or not,

followed by determination of the level of HLA on these two different populations. It was

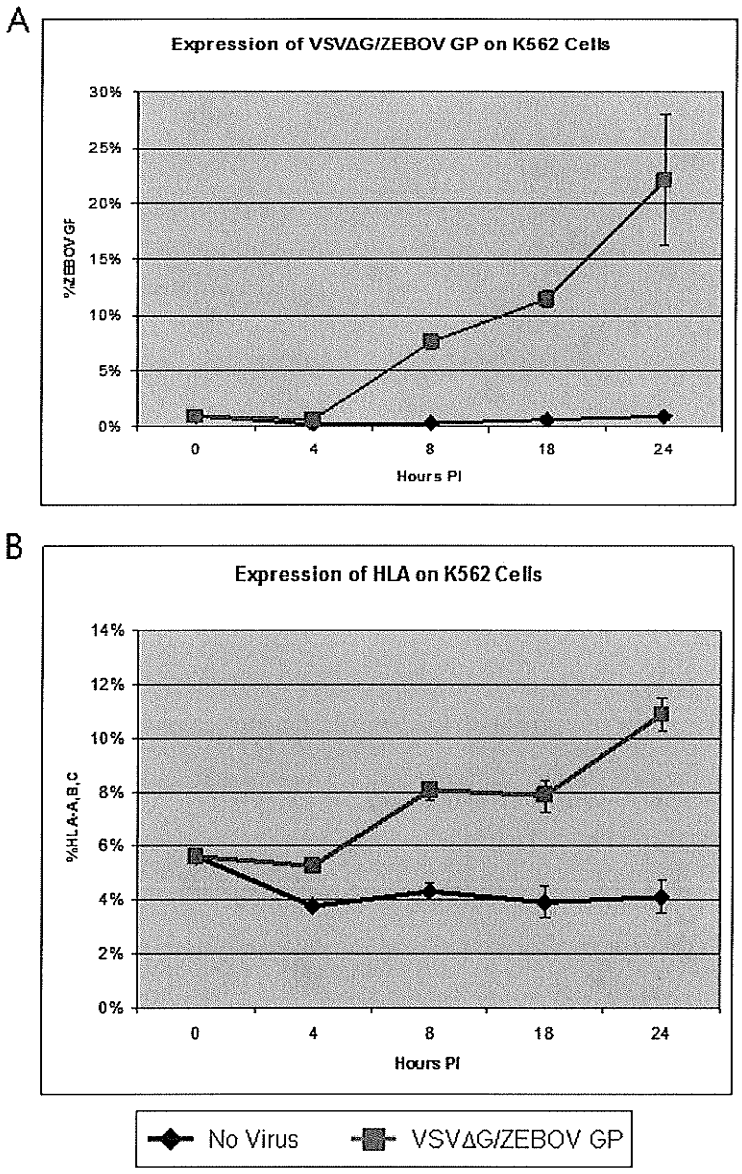

Figure 3.22: Cell surface expression of ZEBOV GP and HLA on K562 cells after infection with VSVΔG/ZEBOV Kikwit GP.
K562 cells were incubated with VSVΔG/ZEBOV Kikwit GP at a MOI of 1 for 24 hours. Cells were taken before infection, 4, 8, 18 and 24 hour PI for flow cytometry. Cells were probed without permeablization with Mouse anti-human HLA-A,B,C and ZGP12/1.1 (1:2000), followed by a Goat anti-mouse Alexa Fluor 488 antibody (1:500) and run on the FACS Calibur. There is an increase in expression of (A) ZEBOV GP and an overall increase in (B) the percentage of HLA on K562 cells following a 24 hour infection of VSVΔG/ZEBOV Kikwit GP. n = 6. Error bars represent SD.

found that there was a significant decrease in the amount of HLA expressed on the surface of the cells over the 24 hour infection period (Figure 3.23). It was observed that the target cells not expressing ZEBOV GP maintained the amount of HLA on their cell surface, similar to cells not exposed to VSVΔG/ZEBOV GP at all. This data suggests the VSVΔG/ZEBOV GP virus may only infect cells that expressed a higher level of HLA on their surface. The infection caused these K562 cells to reduce the amount of HLA on the cell surface. This decrease could be due to the virus attempting to conceal the infected cell from the host immune system. However, since NK cells search for cells that have altered their phenotype, this decrease in HLA sends signals to the NK cell to lyse the cell. It should be noted here, that only a fraction of K562 cells normally express HLA. The decrease in HLA observed in Figure 3.23 represents a very small population of K562 cells. However, data seen in later experiments with a mouse macrophage cell line (section 3.11) supports data seen here and will be discussed shortly.

Cytokines secreted from the infected target cells can also have an effect on the function of NK cells. Therefore, the supernatants from VSVΔG/ZEBOV GP infected K562 cells were examined for secretion of IFN-α and IFN-β by ELISA just before infection and 4, 8, 18 and 24 hours after infection. No IFN-α secretion was detected from either the VSVΔG/ZEBOV GP infected K562 cells or the uninfected K562 cells (data not shown). However, both the VSVΔG/ZEBOV GP infected cells and the uninfected cells secreted IFN-β over a 24 hour infection period (Figure 3.24). The level of IFN-β continued to increase in both treatments of the K562 cells, but there was a drastic decrease in the concentration of IFN-β secreted by the VSVΔG/ZEBOV GP infected K562 cells at the 24 hour post infection time point (Figure 3.24).

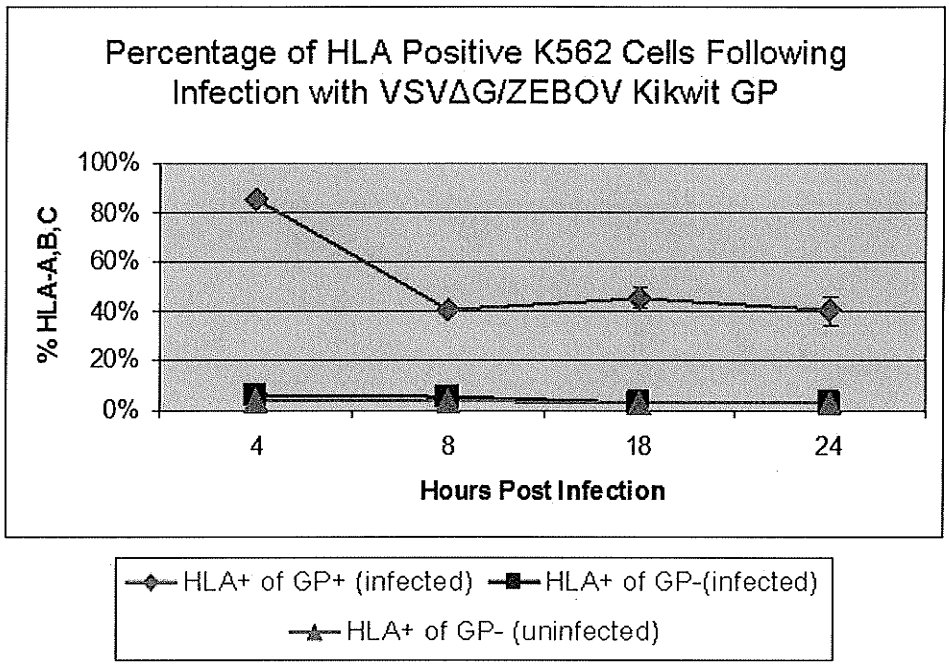

Figure 3.23: Percentage of HLA on K562 cells expressing ZEBOV GP after an infection with VSVΔG/ZEBOV Kikwit GP.
K562 cells were infected with VSVΔG/ZEBOV GP or not for 24 hours. Following infection, cells were stained with antibodies for ZEBOV GP and HLA for FACS analysis. Only a small percentage of K562 cells express the ZEBOV GP following infection with VSVΔG/ZEBOV GP. Of the K562 cells that do express ZEBOV GP, there is a decrease the amount of HLA on their cell surface during a 24 hour infection period while cells not expressing GP maintain their amount of surface HLA. Error bars represent SD. n = 6.

This drop in IFN-β secretion could be due to the VSVΔG/ZEBOV GP virus attempting to inhibit the effects of IFN-β on the immune system, such as increasing expression of HLA and activating NK cells (Salazar-Mather and Hokeness, 2006). This decrease in IFN-β could also be due to an increase in the IFN-β receptor on the target cells. An increase in the IFN-β receptor would cause an uptake of the cytokine in an autocrine mechanism, thus less IFN-β would be detectable in the supernatant. However,

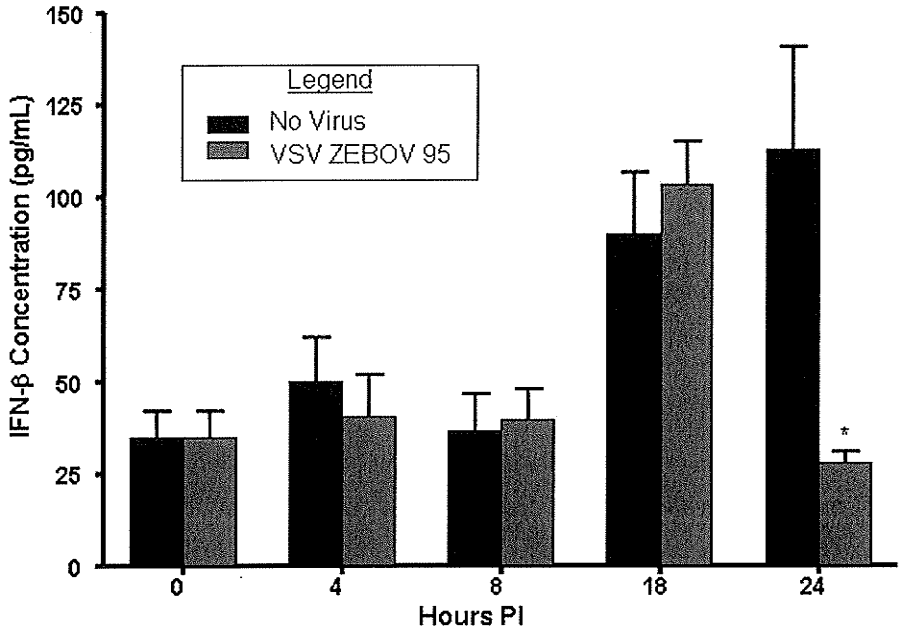

Figure 3.24: Secretion of IFN-β following infection of K562 cells with VSVΔG/ZEBOV Kikwit GP.
Supernatant from K562 cells show secretion of IFN-β over a 24 hour infection period, with a decrease in IFN-β secretion following 24 hours of infection. * = P values between uninfected and infected at 24 hours post infection = 0.0016. n = 6. Error bars represent SEM.

since the infectious supernatant is removed from the K562 cells before the NK cell mediated cytotoxicity assay is performed; this decrease in the secretion of IFN-β may not have a direct effect on the cytotoxicity assay. These possiblilities are discussed further in sections 4.3.1 and 4.6.

3.7.2 Effect of VSVΔG/ZEBOV GP on NK-92MI Cells

Although VSVΔG/ZEBOV GP does not productively infect NK-92MI cells, an attempt was made to determine whether this virus could stimulate these cells into secreting different cytokines. Therefore, NK-92MI cells were either exposed to VSVΔG/ZEBOV GP at a MOI 1 or incubated in plain RPMI-10 for 24 hours. Supernatants from both treatments of the NK-92MI cells were examined for TNF-α and

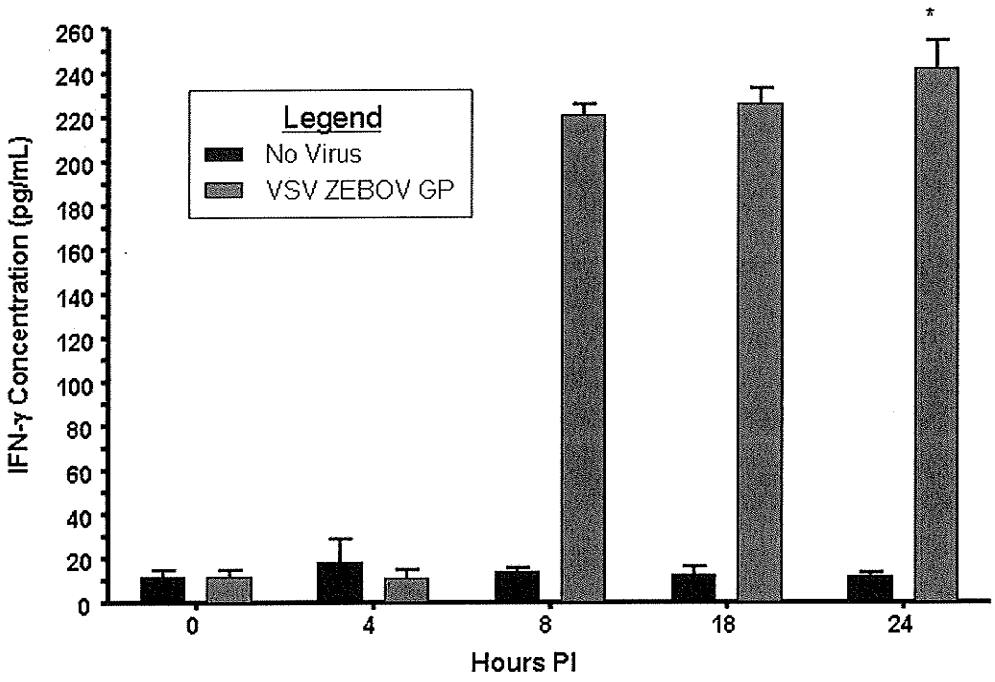

Figure 3.25: Secretion of IFN-γ following exposure of NK-92MI cells to VSVΔG/ZEBOV Kikwit GP.
Supernatant from NK-92MI cells stimulated with the recombinant virus show secretion of IFN-γ over a 24 hour infection period. * = P value for IFN-γ = 0.0429. n = 6. Error bars represent SEM.

IFN-γ by ELISA (two cytokines known to be secreted by NK cells upon their activation) at times 0, 4, 8, 18 and 24 hours after incubation. However, no significant amount of TNF-α was secreted from either the VSVΔG/ZEBOV GP exposed NK-92MI cells or the unexposed NK-92MI cells at any time point (data not shown), but small quantities of IFN-γ was observed to be secreted from both treated NK-92MI cells after four hours of infection. However, after 8 hours of incubation with the virus, the VSVΔG/ZEBOV GP exposed cells began to secrete massive amounts of the cytokine (Figure 3.25). This confirmed the theory that the VSVΔG/ZEBOV GP virus is able to stimulate the NK-92MI cells to secrete cytokines. Although early secretion of IFN-γ was not detectable in

this ELISA assay, IFN-γ secretion would be very important in a mouse model of a VSVΔG/ZEBOV GP infection in order to illicit an early immune response to the virus.

3.8 Purification of Murine Splenic Natural Killer Cells

One goal of this thesis project was to use primary NK cells to determine the role of NK cells in the VSVΔG/ZEBOV GP virus post-exposure therapeutic. This was a project goal in order for the group to perform future *in vivo* murine experiments to further define the role of NK cells in the post-exposure platform of the VSVΔG/ZEBOV GP virus. To achieve this goal, a pure population of primary NK cells had to be obtained from a mouse and cultured to acquire large amounts of NK cells for the cytotoxicity assays. Contaminating cells, such as macrophages, could account for an increase in target cell lysis during the cytotoxicity assay. Thus, the population of NK cells had to be pure. Both C57Bl/6 and Balb/c mice were euthanized and NK cells were purified from their spleens utilizing a negative selection NK purification kit. Since C57Bl/6 mice have two NK cell markers, NK1.1 and CD49b, these mice were first used to optimize the purification of the murine NK cells. Once the purification assay was established, spleens from Balb/c mice were obtained for the purification seeing that Balb/c mice were utilized in *in vivo* experiments with VSVΔG/ZEBOV GP (Feldmann *et al.*, 2007; Jones *et al.*, 2005).

To test the purity of the purified NK cell population, the splenic cells were stained with monoclonal antibodies (Table 1.1). This procedure was done to determine the gating scheme of the different splenic cells. Figure 3.26 demonstrates the gating scheme of each cell type. Approximately 65% of the spleen was made up of CD 19^+ B cells, 27% CD 3^+ T cells, 2% F4/80^+ bearing macrophages, and 5% CD49b$^+$ NK cells. Two different

flurochromes were utilized for CD49b for multiple staining purposes. By creating this gating scheme it could be determined the percentage of each cell type that may still exist in the purified NK cell population.

The purification method established obtained a roughly 80% to 95% pure population of NK cells (Figure 3.27). NK cells require IL-2 or IL-15 for survival and proliferation (Azeredo *et al.*, 2006; Blom and Spits, 2006; Colucci *et al.*, 2003; Di Santo, 2006; Vosshenrich *et al.*, 2005; Williams *et al.*, 1997). However, a high concentration of IL-2 seems to be sufficient to grow NK cells ex vivo (Klingemann and Martinson, 2004; Meehan *et al.*, 2008). Therefore, the purified NK cells were cultured with recombinant human IL-2 for approximately 14 days before they could be used in a cytotoxicity assay. When first cultured, the NK cells were floating, round shaped cells. However as time progressed, the cells started to elongate, adhere to the plate and proliferate rapidly (Figure 3.28-A, B and C). After about 3 days in culture, the NK cells began to proliferate at an exponential rate (Figure 3.28-D). The cells were split 1:2 when they were confluent.

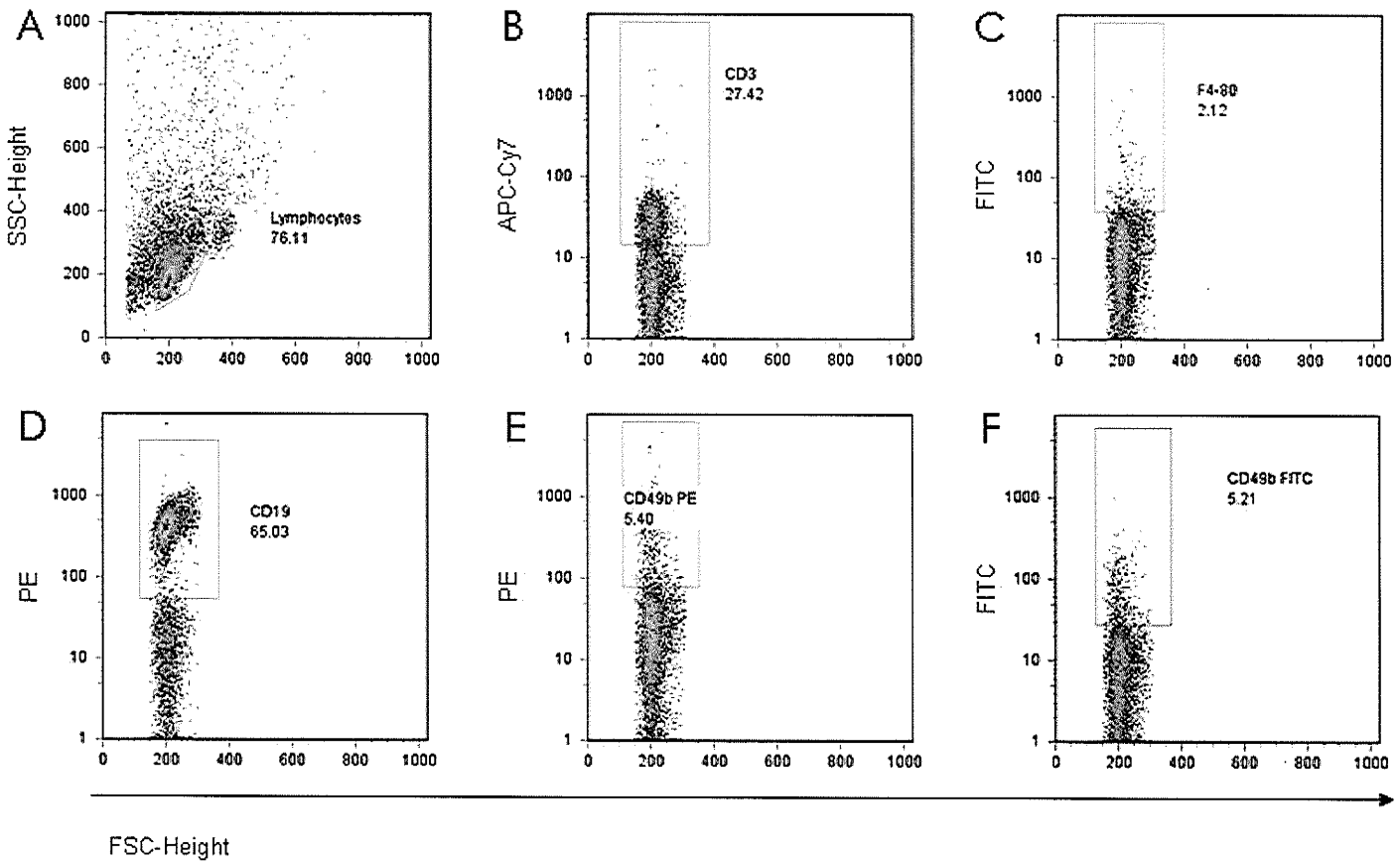

Figure 3.26: Staining scheme for the purification of murine splenic NK cells from Balb/c mice.
Panels B-F were based upon the lymphocyte gate in panel A. Each antibody was gated based upon an unstained population, single stained whole spleen and isotype stains. Each panel shows the single stain for each splenic cell type including (B) T cells, (C) macrophages, (D) B cells and (E and F) NK cells.

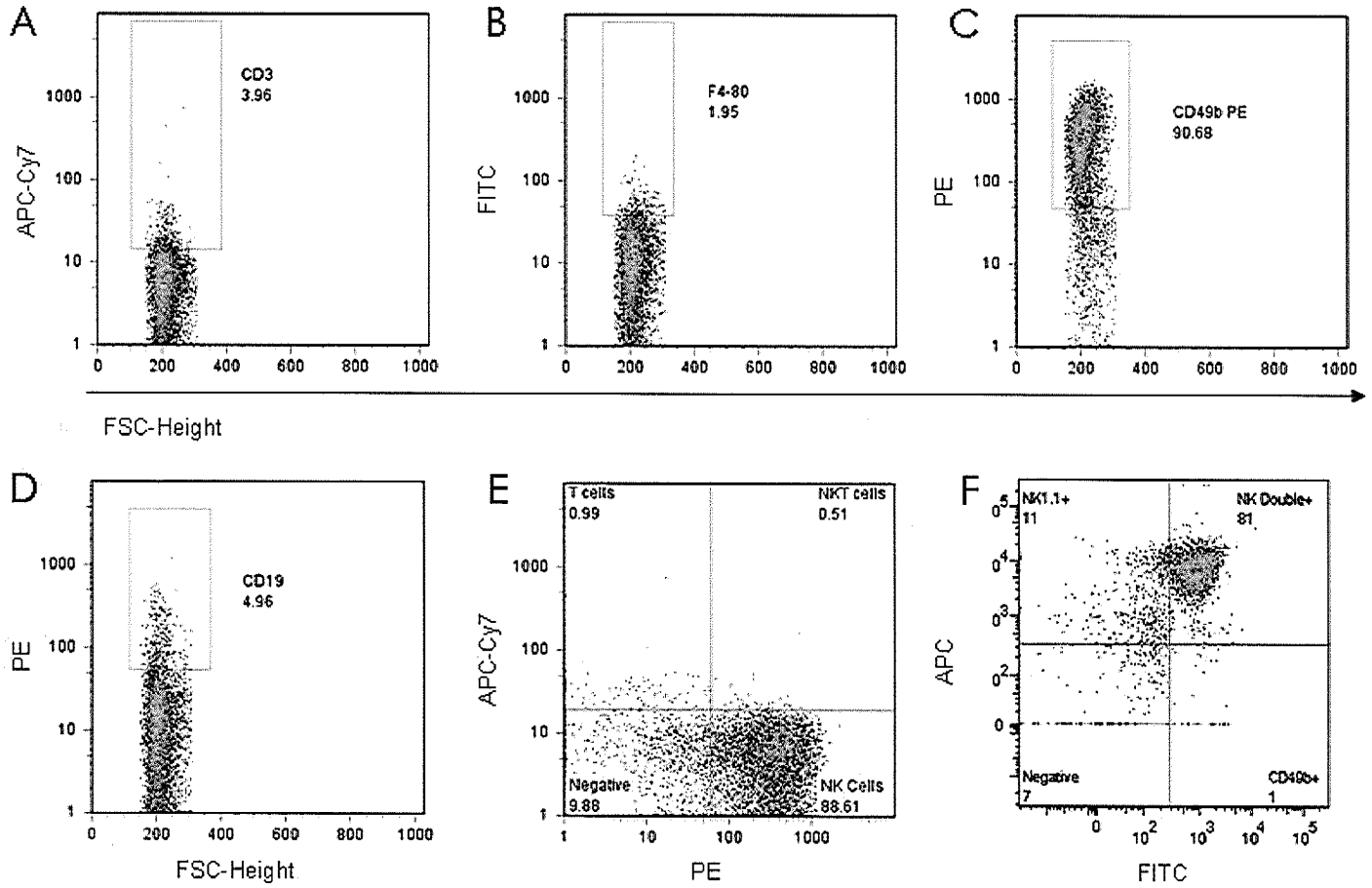

Figure 3.27: Staining scheme after the purification of NK cells from a Balb/c mouse spleen.
Single stains for (A) T cells, (B) macrophages, (C) NK cells and (D) B cells were performed on a sample of the purified spleen. Panel E shows typical results of the purity of NK cells based upon CD3 and CD49b cell surface markers and panel F shows purity results based upon NK1.1 and CD49b.

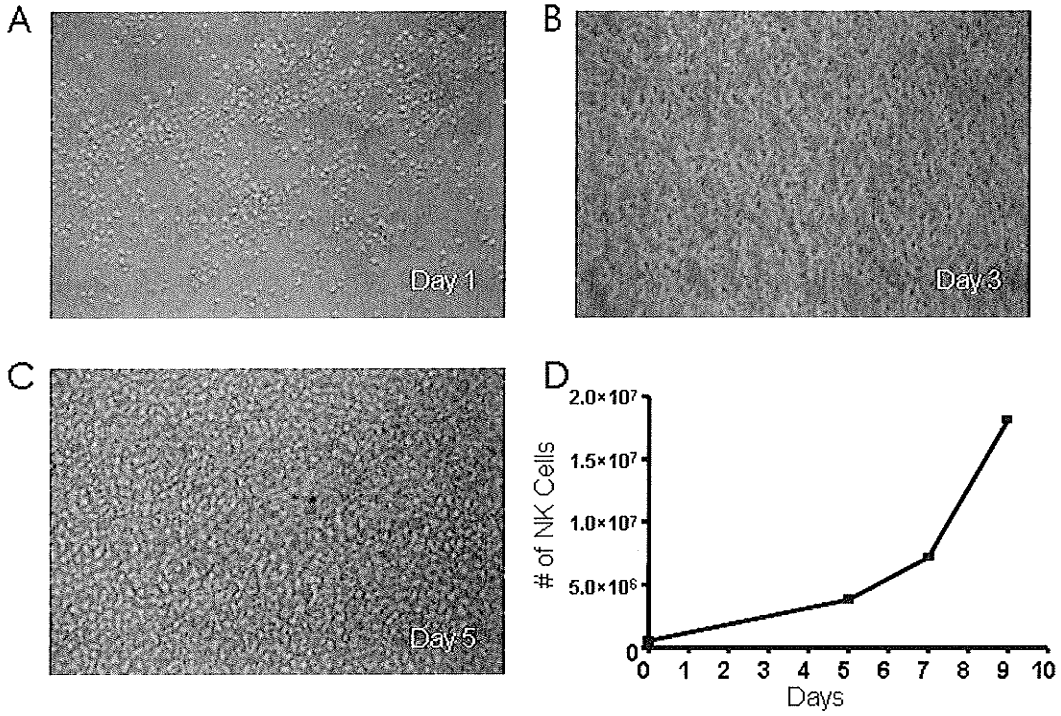

Figure 3.28: Proliferation of Balb/c splenic NK cells.
Microscopy of murine NK cells (A) 1 day, (B) 3 days and (C) 5 days after the first addition of 1000 units/mL of recombinant human IL-2 demonstrate rapid proliferation and a change in cell morphology over time. (D) The proliferation of murine NK cells over a 9 day period illustrates an exponential growth curve of the cells. Pictures are taken at 4x magnification.

3.9 Development of the Murine NK Cell Mediated Cytotoxicity Assay

3.9.1 Determination of the Murine Target Cell

Similar to the human cytotoxicity assay, a murine target cell needed to be chosen that could be infected with VSVΔG/ZEBOV GP and was susceptible to NK cell lysis. Two murine target cells were tested, a YAC-1 T lymphoblast cell line and a RAW 264.7 macrophage cell line. The YAC-1 T cell line was selected because they have been used in NK cytotoxicity assays by other groups The RAW 264.7 macrophage cell line was also tested in the murine cytotoxicity assays for three reasons. First, macrophages and dentritic cells are the first targets of the *Ebolavirus* (Bray and Geisbert, 2005; Geisbert *et*

al., 2003; Mahanty and Bray, 2004; Reed *et al.*, 2004). Second, these cells can be infected by VSVΔG/ZEBOV GP. Third, the RAW 264.7 cells come from Balb/c mice, the same strain of mice used for the NK cell purification. This final motive for using these cells was that they allowed for lower background lysis of uninfected cells because of mismatched cell haplotypes.

YAC-1 cells did not show signs of infection either by RT-PCR of the supernatants or immunoblots of the cell lysates during a 24 hour infection period (Figure 3.29-A). The weak bands observed in the RT-PCR of the supernatants were determined to be primer dimmers. The RAW 264.7 cell line was highly infected with mRNA production at 8 hours after infection by RT-PCR and with Ebola GP expression as early as 8 hours after infection by western blot (Figure 3.29-B). Production of Ebola GP on the cell surface was verified by IFA, when the RAW 264.7 cells were infected for 24 hours with VSVΔG/ZEBOV GP. Visualization under a fluorescent microscope demonstrated that Ebola GP on the surface of the RAW 264.7 cells, but did not show any Ebola GP on the uninfected cells (Figure 3.30). Since the RAW 264.7 cells were able to be infected by VSVΔG/ZEBOV GP, this cell line was chosen for the murine NK cytotoxicity assays.

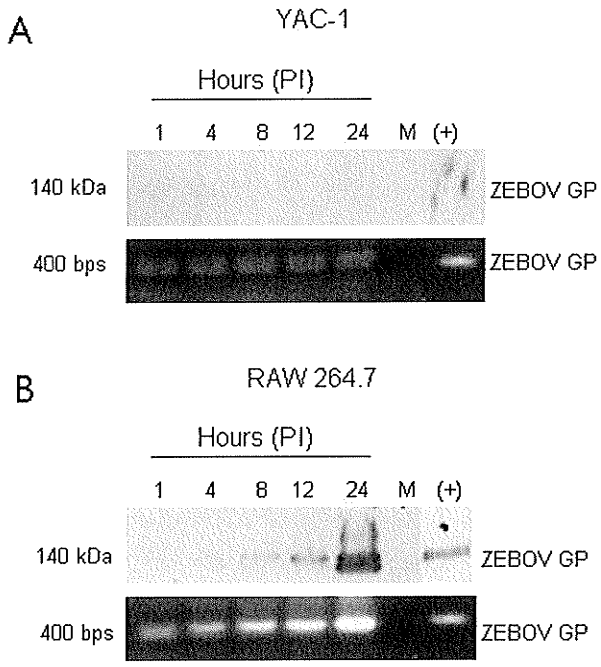

Figure 3.29: Confirmation of virus growth in murine cell lines.
Western blots (upper panels) of the cell lysates and RT-PCR (lower panels) of supernatants of VSVΔG/ZEBOV Kikwit GP infected (A) murine YAC-1 lymphoblast cell line and (B) murine RAW 264.7 macrophage cell line show production of ZEBOV GP viral proteins and viral RNA only in the RAW 264.7 cells beginning at 8h after infection. Primary antibody ZGP12/1.1 (1:5000) followed by a HRP conjugated goat anti-mouse antibody (1:10,000) was utilized for western blots. M = Mock cells with no virus and (+) = VSVΔG/ZEBOV Kikwit GP RNA or cell lysate from Vero E6 infected cells.

3.9.2 Optimization of the Protocols Utilized for the Infection of Murine Target Cells

As mentioned with the human K562 cell line, the amount of virus needed to infect the RAW 264.7 cells had to be determined. The RAW 264.7 cell line was incubated in unsupplemented DMEM-3 or infected with VSVΔG/ZEBOV GP or VSV wild type (positive control for infection) at either MOIs of 0.1 or 1 for 72 hours. Samples of the cells were taken from each treatment at 1, 8, 12, 24, 48 and 72 hours after infection (Figure 3.31). RAW 264.7 cells infected with VSVΔG/ZEBOV GP at both MOIs showed

acceptable amount of cell death within 24 hours. Since in the human NK cell killing assay an MOI of 1 was utilized, the same was chosen for the murine NK cell killing

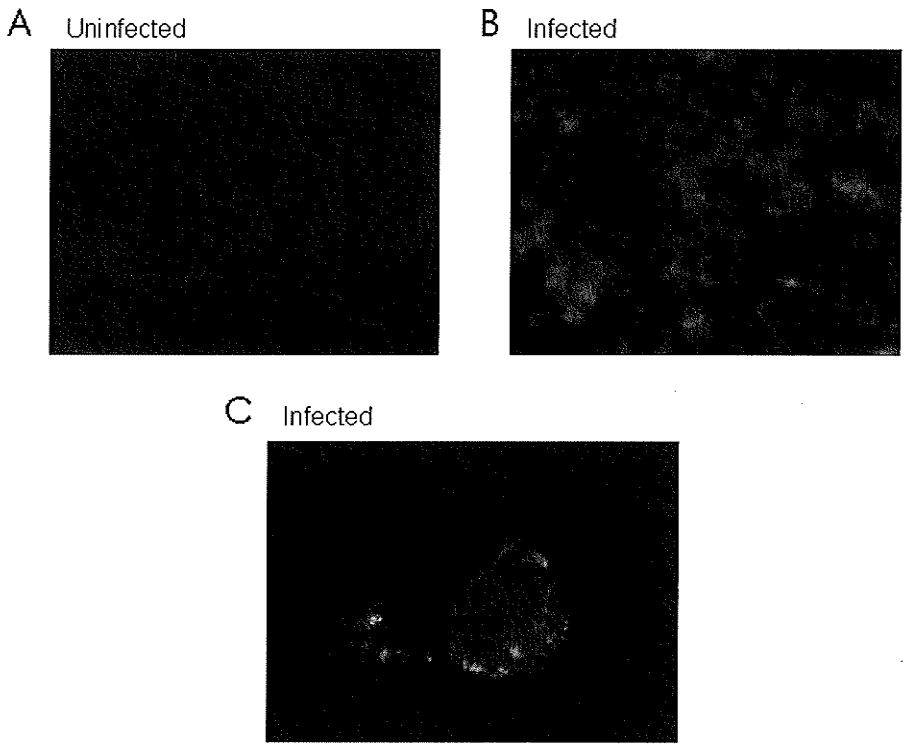

Figure 3.30: An infection of RAW 264.7 cells with VSVΔG/ZEBOV Kikwit GP.
Fluorescent microscopy of RAW 264.7 cells either (A) uninfected or (B, C) infected with VSVΔG/ZEBOV Kikwit GP at a MOI of 1 for 24h showed ZEBOV GP on the surface of the cells. The cells were probed with ZGP12/1.1 antibody (1:2000) followed by a FITC conjugated goat anti-mouse antibody (1:10,000). Pictures were taken at (A, B) 400x magnification or (C) 1000x magnification.

assays to make the two assays more comparable. However, all the cells infected with VSV wild type were virtually all destroyed within 24 hours of infection at both MOIs. Therefore VSV wild type was not used as a positive control in the murine cytotoxicity assays.

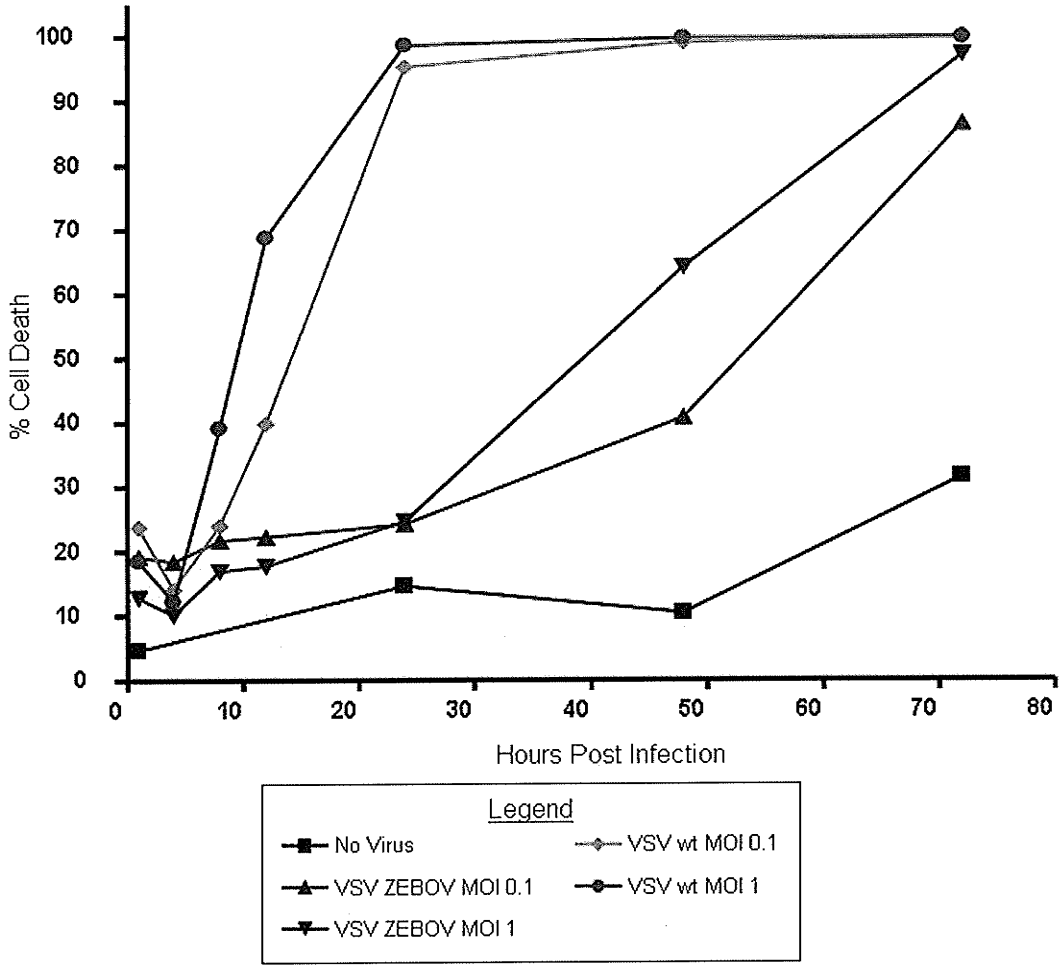

Figure 3.31: Infection of RAW 264.7 cells.
The RAW 264.7 cells were either infected with media, VSVΔG/ZEBOV Kikwit GP or VSV wild type (wt) at MOIs of 0.1 or 1 for 72 hours. A flow cytometric cell death assay shows increased cell death due to viral infection after 72h of infection.

The RAW 264.7 cells were stained with increasing concentrations of PKH-26 for 5 or 10 min at 37°C. Virtually all the cells were stained brightly at all concentrations of the dye for any incubation period tested (Figure 3.32). However, because a slightly higher percentage of cells were stained at a final concentration of 5 μM in a 5 min incubation period this was chosen to use for staining the RAW 264.7 cells.

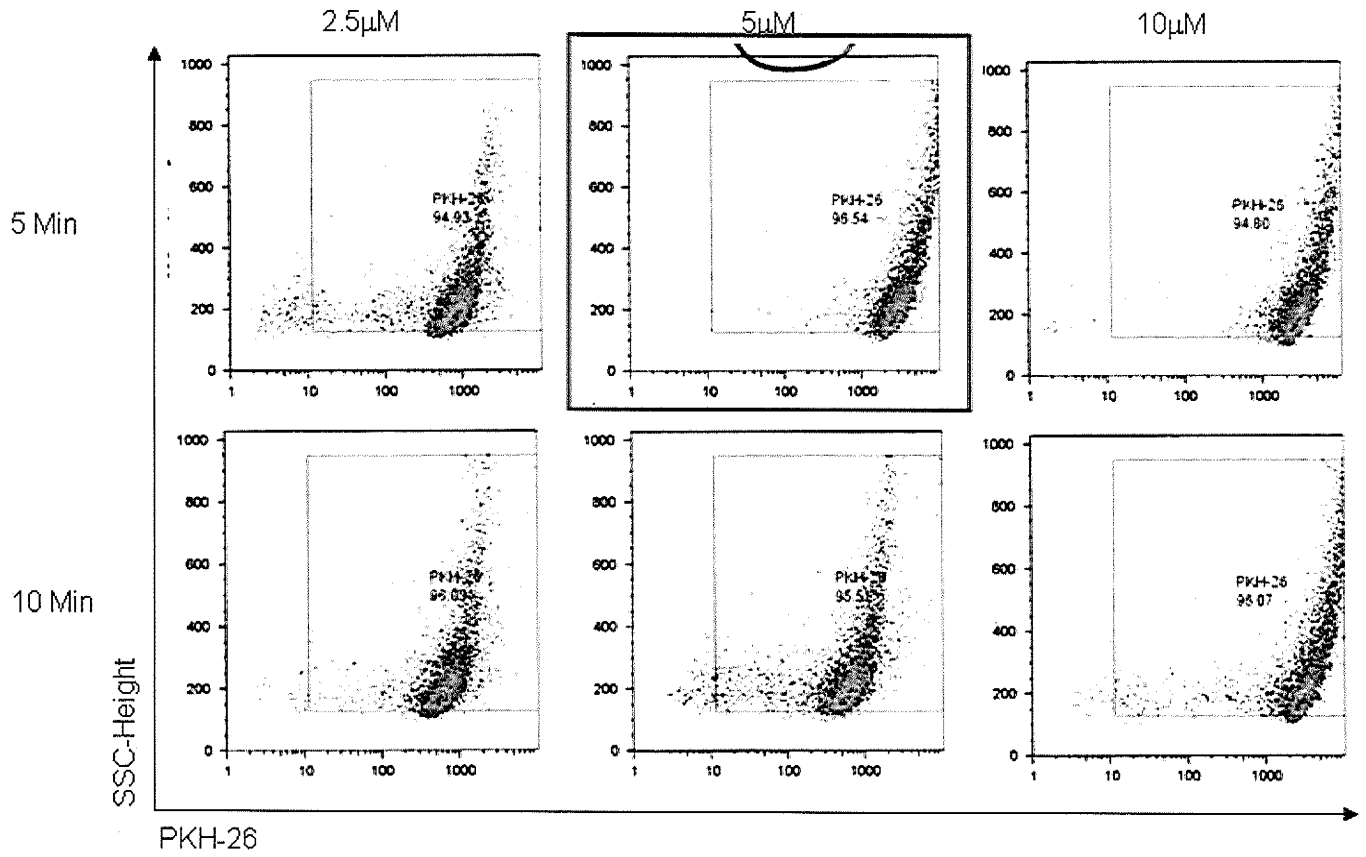

Figure 3.32: Staining of RAW 264.7 cells with PKH-26.
To optimize the staining of RAW 264.7 cells with PKH-26 Red Fluorescent Cell Linker dye, several concentrations and 37°C incubation periods were utilized. A final concentration of 5 µM of PKH-26 in a 5 min incubation period was utilized.

Various concentrations of CFSE were also tested on the RAW 264.7 cells with an incubation period of 5 or 10 min at room temperature. The different concentrations and incubation periods of the CFSE dye caused all the cells to be stained at approximately the same intensity (Figure 3.33). However, none of these concentrations were used for staining of the RAW 264.7 cells because similar to the human K562 cells, combining the PKH-26 and CFSE dyes together changed the intensity of the CFSE dye. Therefore, to create a better staining intensity and to make the murine target cell staining comparable to the human target cell staining, a final concentration of 1.5 μM was chosen for staining with CFSE.

3.10 Murine NK Cell Mediated Cytotoxicity Assay

The murine NK cells used for the cytotoxicity assay were cultured for 14 days with human recombinant IL-2. The effector cells were added to RAW 264.7 cells that were either incubated in unsupplemented DMEM-10 or infected with VSVΔG/ZEBOV GP at a MOI 1 for 24 hours. The cytotoxicity assay was conducted for 4 hours following staining of the RAW 264.7 cells with PKH-26/CFSE. As with the human NK cell mediated cytotoxicity assay, treated RAW 264.7 cells were also incubated without NK cells as a control for the assay. The IL-2 stimulated murine NK cells were added to the treated RAW 264.7 cells at a 3:1 E:T ratio. Here it was found that significantly more RAW 264.7 cells were lysed by the NK cells when they were infected by VSVΔG/ZEBOV GP than when they were uninfected (Figure 3.34). This suggests that the infection with VSVΔG/ZEBOV GP causes the RAW 264.7 cells to change their phenotype, which then conveys signals to the NK cells to lyse the target cells. This change in phenotype will be discussed in the next section.

3.11 Effects of the VSVΔG/ZEBOV GP Virus on RAW 264.7 Cells

As in the case of the human K562 cell infection, cell surface expression of Ebola GP and MHC class I on the RAW 264.7 cells following infection with the VSVΔG/ZEBOV GP virus was investigated. As expected, the infection of RAW 264.7 cells with VSVΔG/ZEBOV GP caused an increase in the expression of Ebola GP on the cell surface (Figure 3.35-A). This infection also caused a decrease in the amount of MHC class I on the cell surface (Figure 3.35-B). This decrease in MHC class I molecule could be due to the virus attempting to conceal the infected cell from cytotoxic T cells. However, by decreasing the MHC class I on the cell surface, NK cells are sent signals that the cell has been impaired and thus needs to be disposed of.

When specifically gating on target cells expressing ZEBOV GP or not, it was observed that there was a decrease in the percentage of MHC class I on both the target cells expressing ZEBOV GP and the target cells not expressing GP (Figure 3.35-C). The decrease in the percentage of MHC class I target cells not expressing ZEBOV GP is slightly larger than target cells expressing ZEBOV GP. However, the actual amount of cells that express ZEBOV GP at the 4 hour PI time point is very low. Therefore, the difference in the decrease of expression of MHC class I in these two populations may have been less if there were more events captured. This trend in the percentage of MHC class I on infected RAW 264.7 cells followed a similar decrease seen in RAW 264.7 cells not exposed to VSVΔG/ZEBOV GP. Therefore, this decrease could have been due to metabolic stress in the cells over time. However, because one of the population of infected RAW 264.7 cells did have a larger decrease in MHC class I than uninfected

target cells, this could be due to soluble proteins secreted from the cells and will be discussed next.

Soluble proteins secreted by the infected target cell can also cause an effect on the NK cell function. Here RAW 264.7 cells were infected with VSVΔG/ZEBOV GP at a MOI of 1 for 24 hours. Samples of the supernatants were taken at 0, 4, 8, 18 and 24 hours time points. The supernatants were analyzed by ELISA to determine whether IFN-α or IFN-β were being secreted by the RAW 264.7 cells, owing to the infection with VSVΔG/ZEBOV GP. No detectable IFN-α was produced at any time point during the infection by VSVΔG/ZEBOV GP or in uninfected RAW 264.7 cells (data not shown). However, VSVΔG/ZEBOV GP infected cells began to secrete detectable levels of IFN-β after 18 hours of infection, while uninfected cells did not produce any IFN-β (Figure 3.36).

The secretion of IFN-β from the infected cells could be a potent activator of anti-viral responses from the host immune system. However, since detectable levels of secretion occurs after 18 hours of infection and the infectious supernatant is removed before the addition of NK cells, this cytokine does not have an effect on the NK cell mediated cytotoxicity assay.

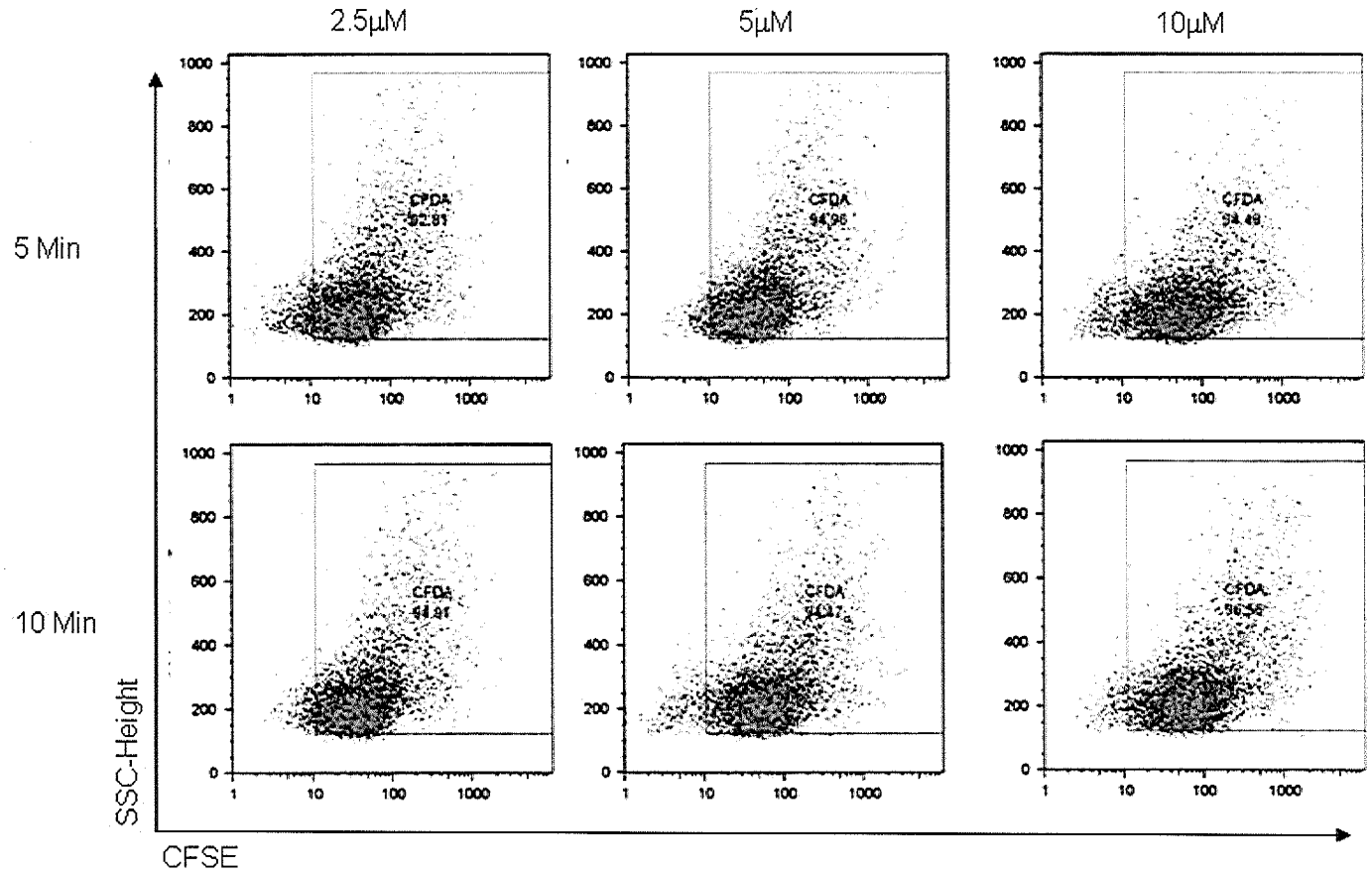

Figure 3.33: Staining of RAW 264.7 cells with CFSE.
To optimize the staining of 264.7 cells with the Vybrant CFDA SE cell tracer dye (CFSE), several concentrations of CFSE were utilized for an incubation period of 5 or 10 min at 37°C. A final concentration of 1.5 µM was utilized for the NK cell mediated cytotoxicity assay due to a change in the intensity of the CFSE dye when stain in combination with PKH-26.

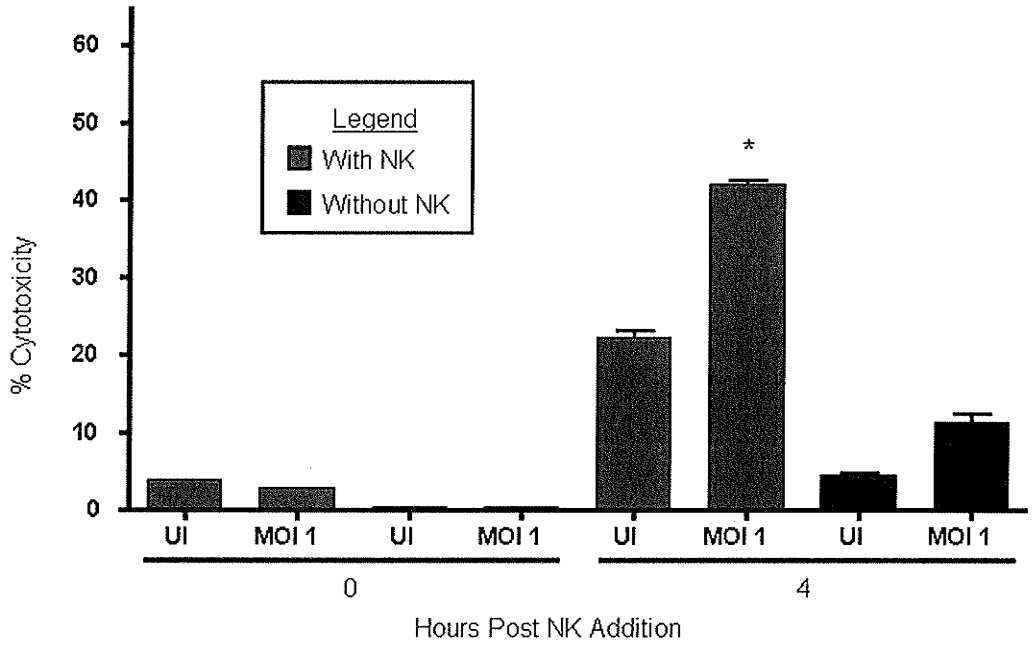

Figure 3.34: IL-2 stimulated murine NK cell mediated cytotoxicity of VSVΔG/ZEBOV Kikwit GP infected RAW 264.7 cells.

RAW 264.7 cells were infected with VSVΔG/ZEBOV Kikwit GP for 24 hours followed by addition of IL-2 stimulated murine NK cells at a 3:1 effector to target ratio. Significantly more VSVΔG/ZEBOV Kikwit GP infected RAW 264.7 cells were lysed than uninfected cells. UI = uninfected, MOI of 1 = target cell infected with VSVΔG/ZEBOV Kikwit GP at a MOI of 1. * = P values between UI and MOI 1 < 0.0001. n = 8. Error bars represent SEM.

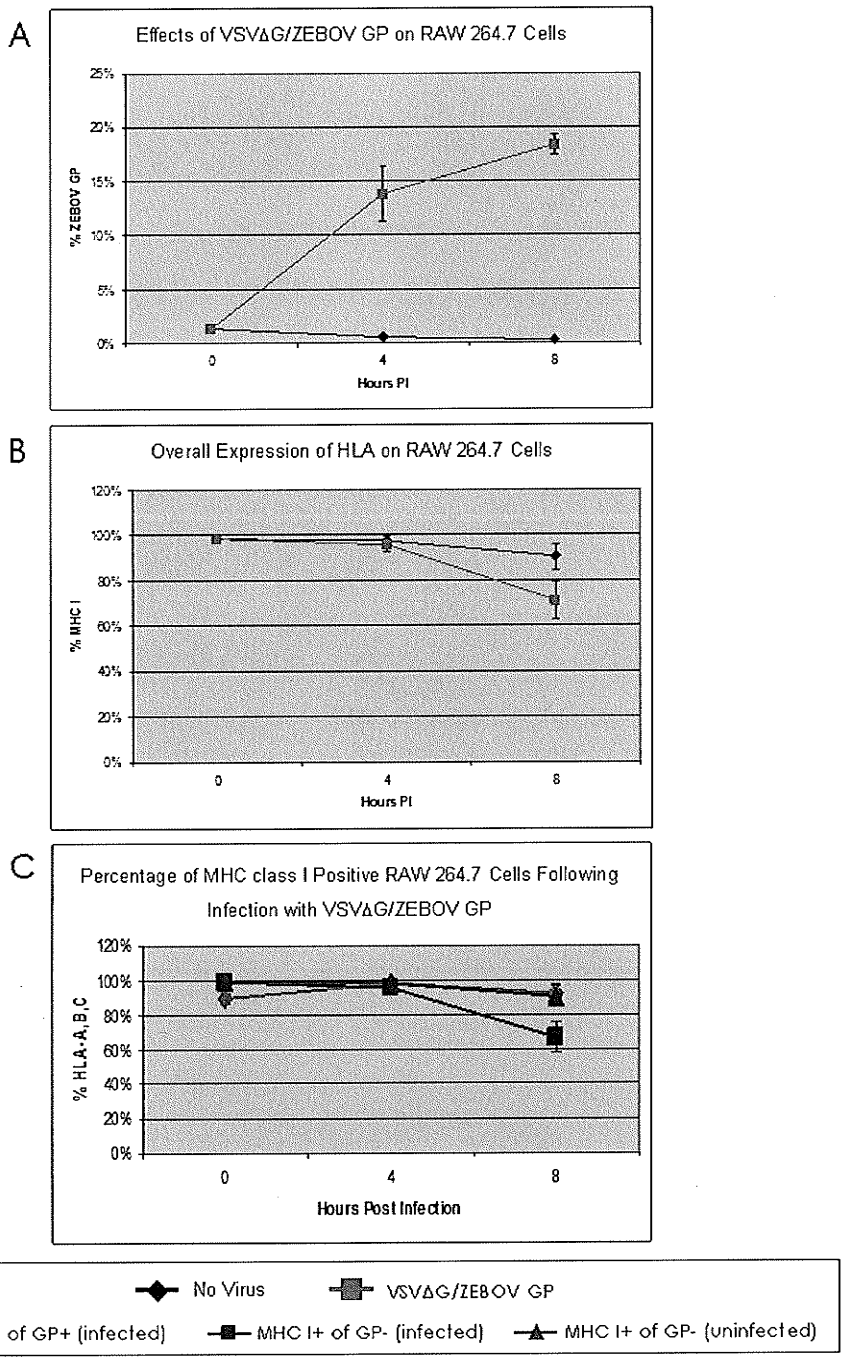

Figure 3.35: Cell surface expression of ZEBOV GP and MHC class I on RAW 264.7 cells after infection with VSVΔG/ZEBOV Kikwit GP.
There is an increase in expression of (A) ZEBOV GP and an decrease in (B) the percentage of MHC I on infected RAW 264.7 cells following a 24 hour infection of VSVΔG/ZEBOV Kikwit GP. When specifically observing the MHC I positive cells following infection with VSVΔG/ZEBOV Kikwit GP, there is a decrease in the percentage of MHC I in all treatments. n = 6. Error bars represent SD.

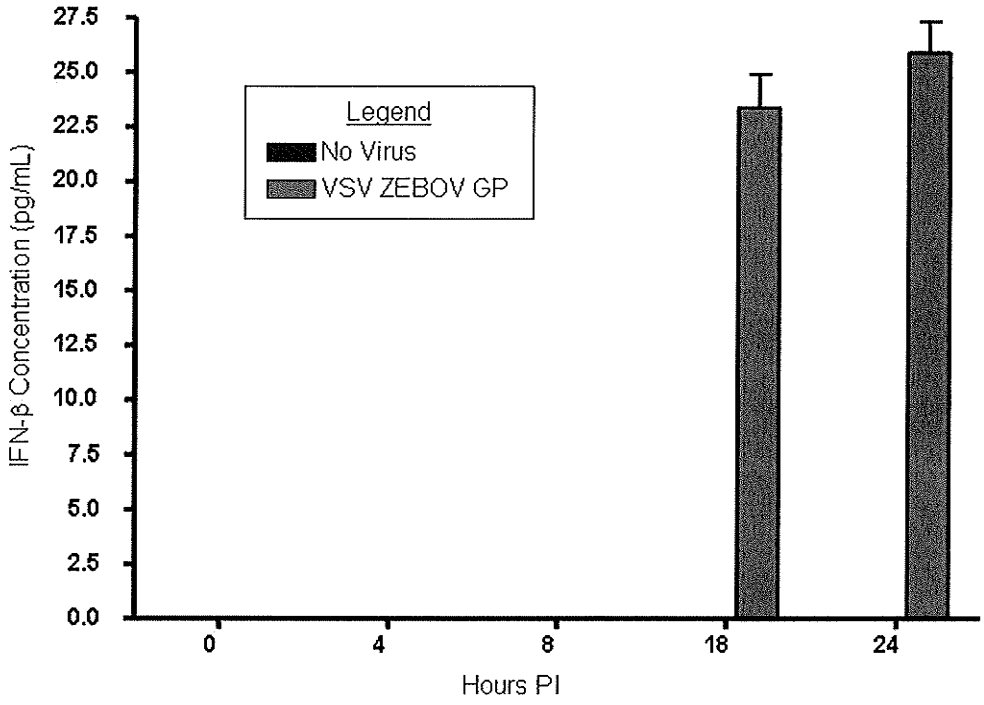

Figure 3.36: Secretion of IFN-β from VSVΔG/ZEBOV Kikwit GP infected RAW 264.7 cells

Supernatant from infected RAW 264.7 cells show secretion of IFN-β following 18 hours of infection. n = 6. Error bars represent SEM.

4 Discussion

4.1 Determinating of the Role of NK cells in the *Ebolavirus* Therapeutic

The goal of this study was to establish a NK cell mediated cytotoxicity assay for the recombinant VSVΔG/ZEBOV GP virus. The first aim of this study was to determine the effects of the VSVΔG/ZEBOV GP virus on the NK cell function and second, to determine the effects of the recombinant virus on target cells which leads to their destruction by NK cells. In order to understand the effects of the VSVΔG/ZEBOV GP virus on both NK cells and target cells, two methods had to be developed; 1.) the establishment of a NK cell mediated cytotoxicity assay and 2.) infection of NK or target cells with subsequent analysis of cell surface markers and cytokines.

The first method involved instituting a cytotoxicity assay for both human and murine NK cells. In this assay, target cell lines were infected with the recombinant viruses and then incubated with the NK cells. This allows for the study of the effects the recombinant virus has on the cytotoxic functions of NK cells. With this assay, one could then test the hypothesis that infection of target cells will cause NK cells to lyse them more frequently than uninfected cells.

The second method involved infecting NK cells or the target cell lines with the VSVΔG/ZEBOV GP virus. Following infection, the effects the recombinant virus had on the expression of cell surface molecules on both the target cells and the secretion of cytokines by either cell type could be measured over time. This allows one to investigate whether the infection of the target cells would alter the expression of the surface molecules of the target cells. Concomitantly, induction of cytokine secretions from both cell types could be assayed. The hypothesis is that altered surface expression and

cytokine secretions will cause the NK cells to become activated and lyse the target cells in the NK cell cytotoxicity assays.

4.2 Advantages of NK Cell Cytotoxicity Assay by Flow Cytometry Over Other Methods.

Originally, T cell and NK cell cytotoxic assays were performed utilizing a radioactive molecule release method, such as the most widely used chromium (^{51}Cr) release method. In these assays, the target cell was labeled with radioactive molecules such as ^{75}Selenium (^{75}Se), Tritium (^{3}H) or ^{51}Cr and incubated with effector cells (Fischer and Mackensen, 2003). Lysis of the target cells allowed release of the radioactive label into the supernatant, which was measured by a γ irradiation counter. However, this assay has drawbacks such as poor uptake and non specific release of the radioactive label, the length of the assay and the hazards of radioactivity (Gomez-Roman *et al.*, 2006; Pross *et al.*, 1981; Sheehy *et al.*, 2001; Slezak and Horan, 1989).

Due to the drawbacks of using radioactive molecule release assays, other methods of measuring cytotoxicity by flow cytometry have been established. These methods include using molecules such as lanthanide europium chelated to diethylenetriaminopentaacetate, CFDA, Cell Tracker Orange, and numerous dyes in the PKH family to stain the target cells (Burkett *et al.*, 2005; Fischer and Mackensen, 2003; Hatam *et al.*, 1994; Kim *et al.*, 2007; Kruger-Krasagakes *et al.*, 1992; Pacifici *et al.*, 1993; Provinciali *et al.*, 1992; Racz *et al.*, 1990; Volgmann *et al.*, 1989). Following incubation with effector cells, a live/dead stain such as propidium iodide (PI), 7 amino-actinomycin D, 4-methylumbelliferyl heptanoate or Annexin V was added to the culture to determine the amount of cell death. Other flow cytometric methods added the stain to the effector cell to determine the difference between the effector cells and the target cells,

followed by the addition of a live/dead stain after the completion of the assay (Flieger *et al.*, 1995; Hoppner *et al.*, 2002). These single staining assays were shown to be comparable or have higher sensitivity than the ^{51}Cr release assays (Kruger-Krasagakes *et al.*, 1992; Provinciali *et al.*, 1992; Racz *et al.*, 1990). Utilizing flow cytometry also allowed for longer incubation periods, direct cell counting of the remaining viable cells and avoided the use of radioactive labels (Flieger *et al.*, 1995; Volgmann *et al.*, 1989).

The flow cytometric method, fluorometric assessment of T lymphocyte antigen specific lysis (FATAL), was further developed by Slezak *et al* (1989). This assay involved a duel staining of the target cell with PKH-1 and PI before addition of the cytotoxic T cell. By utilizing this method, four subpopulations in the assay could be designated: live effectors, dead effectors, live targets and dead targets. The percent target lysis, the viability of the effector cells at the completion of the assay, and the viable E:T cell ratios could be calculated by using this duel staining technique (Slezak and Horan, 1989).

This method was later modified by Gomez-Roman *et al*, to observe ADCC in human PBMC from HIV patients. This rapid fluorometric ADCC assay (RFADCC) was adapted to stain the target cells with PKH-26 and CFSE. The PKH-26 was used to uniformly label the target cells, while the CFSE would be lost by the target cell upon membrane disruption (Gomez-Roman *et al.*, 2006; Gomez-Roman *et al.*, 2005). By utilizing these two dyes, only the target cells were stained and could be monitored for cell death following incubation with the PBMCs. Due to the lack of radioactivity labeling and the ability of flow cytometry to detect more than just target cell death in a single

experiment, this assay was therefore modified to observe NK cell activity in this thesis project.

Many different variables of the flow cytometric cytotoxicity assay for the *Ebolavirus* therapeutic had to be optimized to obtain consistent results; such as the amount of virus used to infect the cells. If too much virus was used, cell death would occur from viral infection and not NK cell killing. Since the VSVΔG/ZEBOV GP virus did not replicate rapidly in the target cells, based upon the flow cytometric live/dead assay (Figure 3.14-C and 31), a sufficient number of target cells were alive following infection at a MOI of 1 when the NK cells were added to the assay. This allowed an adequate amount of target cells to be infected such that a NK cytotoxic effect beyond background lysis was observed (Figure 3.20 and 3.21).

Another variable of the assay was the optimization of the target cell infection controls. Infected or uninfected target cells were incubated in media to determine the base line of target cell death during the four hour incubation period. This was necessary to determine the effects of the virus on the cells within the four hour NK cell killing period, determine whether the target cells were dying due to serum starvation following the four hour incubation period, as well as reveal whether the cells were dying due to the potential toxicity of the stains used. Initial experiments revealed that there was no difference in target cell death when they were incubated with or without NK cells. Therefore, it was determined that the incubation conditions of the target cells in the assay did not maintain the cell viability. To increase the viability of the cells, the percentage of FBS in the media was increased from 3% to 10%. This increase in FBS was sufficient during the killing assay. Other factors could play a role in the death of target cells such as the stage of life

of the cell and cell death due to previous injuries to the cells. However, the controls utilized in the assay accounted for these additional factors and allowed for observation of only the NK cell lysis of target cells.

The third question concentrated on the toxicity of the CFSE and PKH-26 fluorescent dyes used to stain the target cells. Both of these dyes have been used in numerous methods such as cell trafficking experiments (Wallace and Muirhead, 2007). In such experiments, stained cells were followed for a number of days with minimal cell death due to the staining of the cells. Experiments were performed here to determine the optimal concentration and duration of the staining protocol of the target cells for the NK cell cytotoxicity assay which did not adversely affect the outcome.

The final cytotoxicity assay issue that was addressed was the length of the infection period of the target cells. Cytotoxicity assays performed with VSV wild type determined that in order for NK cells to kill VSV wild type infected cells, both viral replication and glycoprotein expression had to be observed on the cell surface (Wallach, 1983). Hence experiments were performed to determine when VSVΔG/ZEBOV GP viral RNA and protein was expressed in the target cell lines. Viral RNA was detected 4 hours and viral glycoprotein was observed 8 hours post infection. The percent cytotoxicity caused by NK cells in assays performed after 8 hours of infection were inconsistent with each other in all target cell treatments. Seeing that the VSV wild type experiments were performed after 18 hours of infection (Wallach, 1983), it was anticipated that increasing the infection period with VSVΔG/ZEBOV GP would resolve any variability in the NK cell percent cytotoxicity. However, following 18 hours of infection, percent cytotoxicity of NK cells on any treatment of target cells continued to fluctuate. This variation may

have been due to differences in viral protein expression or the amount of cells infected. An increase of the infection period to 24 hours caused the percentage of NK cell cytotoxicity in all treatments to be consistent. Therefore, this final protocol was utilized in cytotoxic assays performed here.

4.3 VSVΔG/ZEBOV GP Effects on Different Cell Lines

4.3.1 The Effects of Viral Infection on the Target Cells

Previous studies have shown that target cells infected with VSV, herpes simplex virus or lymphocytic choriomengitis virus must have viral proteins on the cell surface, such as the VSV G, in order for cytotoxic T cells or NK cells to recognize the infected target (Chisholm *et al.*, 2007; Schattner and Rager-Zisman, 1986; Welsh and Oldstone, 1977; Zinkernagel *et al.*, 1978). Target cells infected with the VSVΔG/ZEBOV GP virus produced Ebola GP in increasing amounts on the surface of the target cells as the infection progressed (Figures 3.22 and 35). The expression of Ebola GP on the cell surface could be considered a stimulatory molecule on the target cell due to the increased cytotoxicity seen against the virally infected cells. Because of the shift in balance between inhibitory and activatory markers on the target cell surface, this would signal the NK cells to lyse the target cells and thereby control viral replication in the host (Doucey *et al.*, 2004; Smyth *et al.*, 2005). The increase in target cell cytotoxicity may not be dependent on additional changes in the target cell other than the expression of Ebola GP as long as the activation signal is greater than the inhibitory signal (Chisholm *et al.*, 2007; Golden-Mason and Rosen, 2006; Regunathan *et al.*, 2005).

Even though the increased cytotoxicity against infected target cells may not be dependent upon additional target cell changes, it was observed from human and murine

experiments performed, that target cells expressing the Ebola GP on the cell surface had a decrease in the surface expression of HLA (Figures 3.23 and 35). Studies have demonstrated that the Ebola GP downregulates the expression of HLA on infected cells (Simmons *et al.*, 2002; Sullivan *et al.*, 2005; Takada *et al.*, 2000). Thus the decrease in HLA on the cell surface may have been due to the expression of ZEBOV GP, seeing that VSV causes an increase in this molecule (Bi *et al.*, 1995; Christian *et al.*, 1996). The HLA antigen is a marker for self, and without it, the NK cells cannot recognize the cell as belonging to the host. Thus the NK cell will degranulate and kill the "non-self" cell. Therefore, as the VSVΔG/ZEBOV GP infection decreases the HLA antigen, it removes an inhibitory signal utilized by the NK cells. The expression of the stimulatory Ebola GP on the cell surface adds a stimulation signal to the NK cells. Therefore, the combination of the loss of inhibition and addition of stimulatory signals aids the NK cells to lyse the VSVΔG/ZEBOV GP infected cells more readily than the uninfected cells.

As mentioned earlier, within the culture of target cells infected with VSVΔG/ZEBOV GP, only a small percentage of target cells express ZEBOV GP on the cells surface. K562 GP expressing cells seem to be a population of cells expression a higher percentage of HLA on their cell surface (Figure 3.23). Therefore, it is possible that these cells become infected because they express more HLA, or that they express more HLA initially because they became infected. In order to determine the difference of these possibilities, further experiments on the levels of ZEBOV GP and HLA on the target cells would have to be performed at earlier time points than the 4 hours completed here. The percentage of cells infected at 4 hours PI was very low; therefore, the decrease observed may be an artifact of the assay. However, since the data shown here was executed

numerous times and consistently demonstrated this decrease in HLA over time, it is probable that the target cells infected with VSVΔG/ZEBOV GP do show this decrease in HLA following infection. The difference in expression ZEBOV GP on infected cells was also observed in the macrophage cells line RAW 264.7 and will be discussed next.

All the RAW 264.7 cells express high amounts of the MHC class I molecule (Figure 3.35). It was observed, as with the K562 cells, that there was a population of cells that expressed ZEBOV GP and a population that did not following infection with VSVΔG/ZEBOV GP. The RAW 264.7 cells not expressing ZEBOV GP had a larger decrease in the expression MHC class I than the target cells expressing ZEBOV GP (Figure 3.35-C). The difference seen in these two populations of infected cells could be due to the small amount of ZEBOV GP positive events acquired on the flow cytometer. Since the number of target cells not expressing ZEBOV GP had more events than the target cells not expressing, this difference may not have been significant. However, it is possible that these infected cells would continue to lose their MHC class I molecule over time, possibly due to the infection. The effects of the difference in ZEBOV GP expression of the two populations during the NK cell cytotoxicity assays will be discussed subsequently.

Within the cytotoxicity assay, the existence of two populations expressing ZEBOV GP or not could have caused the target cells not be lysed significantly more than cells not infected by VSVΔG/ZEBOV GP at all. However, this was not observed in the killing assays. The "infected" target cells not expressing ZEBOV GP on the surface may have been in the early stages of infection or may have had more expression of ZEBOV GP internally (comparing figures 3.7-A and 3.22) . The existence of ZEBOV GP within

the cells may have been enough of a signal in the target cell to decrease HLA on the target cell surface. This would cause these target cells to be susceptible to NK cell lysis. The existence of virus within these cells could also cause the target cells to generate and secrete cytokines such as IFN-β and IFN-α in order to fight the infection.

As IFN-β and IFN-α can cause NK cells to become activated and proliferate (Yokoyama *et al.*, 2004), supernatants from infected target cells were tested for these cytokines. If target cells were secreting these interferons during the VSVΔG/ZEBOV GP virus infection, it follows that NK cells would become activated in the NK cell cytotoxicity assay. Experiments demonstrate that K562 cells secreted IFN-β whether they were infected or not (Figure 3.24-A). Since IFN-β is a potent activator of antiviral pathways, the initial secretion of IFN-β would be helpful in an animal infection (Salazar-Mather and Hokeness, 2006). However, it was observed that 24 hours post infection, there was a sudden and significant decrease in the amount of IFN-β secreted from the infected K562 cells. The decrease could be due to the virus shutting down the production of IFN-β or an increase in the uptake of the cytokine by neighbouring uninfected K562 cells.

Some viruses have the ability to shut down the production of IFN-β. This is accomplished by different proteins in their genome, such as the *Ebolavirus* VP35 and VP24 proteins (Bray and Geisbert, 2005; Mohamadzadeh *et al.*, 2006; Peters, 2005). These Ebola proteins are not found in the therapeutic virus. Hence, this effect may come from the VSV component of the vaccine, as studies have demonstrated that the VSV matrix protein has the ability to inhibit type I interferon responses (Ahmad and Ahmad, 2003; Ferran and Lucas-Lenard, 1997; Waibler *et al.*, 2007). IFN-β has been

demonstrated to induce HLA antigen expression, activate NK cells and control the expression of cytokine and chemokines receptors on cells (Salazar-Mather and Hokeness, 2006). Consequently, by decreasing the amount of IFN-β that the infected cells can produce, the VSVΔG/ZEBOV GP virus altered the target cell profile thereby increasing the chances of cell survival. The reduction of HLA on the K562 target cells, possibly due to the decrease in IFN-β, caused the NK cells recognize these target cells as infected and therefore killed them. Since this decrease in IFN-β occurred after 24 hours, it could induce a decrease in the HLA expression of the target cells prior to incubation with the NK cells in the cytotoxicity assays performed. Hence the NK cells would lyse these infected cells at a higher frequency than the uninfected cells.

Infectious supernatant was removed from the target cells prior to the addition of the NK cells. Due to the properties of IFN-β on NK cells, the removal of this supernatant could have caused a decrease in possible activity of the NK cells. In order to determine this fact, studies could be performed that reused the infectious supernatant to monitor whether or not an increase in NK cell lysis is observed.

Infection of murine RAW 264.7 cells with VSVΔG/ZEBOV GP also resulted in production of IFN-β after 18 hours of infection. Unlike the human K562 cell infections, IFN-β was produced only from the infected RAW 264.7 cells (Figure 3.36). As was previously stated, IFN-β has been shown to induce MHC class I expression, activate NK cells and control the expression of cytokine and chemokines receptors on cells. In the NK cytotoxicity assay performed here, the secretion of IFN-β from the RAW 264.7 cells may not play the same role as it did with the human NK cytotoxicity assays. This could be due to the different cell lines used (macrophages rather than erythroleukemic cell) or that less

IFN-β was secreted from the infected RAW 264.7 cells than from the infected K562 cells. Due to this, any effect this cytokine may have had on surrounding targets cells may have been minimal.

Either model of IFN-β secretion from VSVΔG/ZEBOV GP infected target cells, human or murine, is believable due to the fact that the cells were of different lineages. For that reason, the target cells may have different effector functions within a viral infection. Macrophages are used as APCs and may have a greater role of presenting antigen during the infection with VSVΔG/ZEBOV GP. Thus the secretion of IFN-β from these cells may not be as important in this situation. Erythroleukemic cells within the infection may have a role in secreting cytokines that activate effector cells. Therefore, producing more IFN-β from the K562 cells than the RAW 264.7 cells may be due to the function of the cell type during an infection with VSVΔG/ZEBOV GP.

Regardless of the time or amount of IFN-β that is secreted from VSVΔG/ZEBOV GP infected target cells, this interferon may have relevance as a post-exposure therapeutic during an *Ebolavirus* infection. The secretion of IFN-β would induce MHC class I on the target cells to present viral proteins to T cells during an animal infection. The activation of NK cells due to the expression of GP and decreased expression of HLA in those infected cells then would increase the chances of a successful clearance of virally infected cells, both by the innate immune response and later from the adaptive immune response. Once NK cells are stimulated, they would lyse virus infected cells and secrete cytokines, such as IFN-γ, which would then activate other immune cells in the innate immune system as well as prime the adaptive immune response. Future experiments with

this assay should determine whether there is a correlation between the cytokines secreted by each cell type and the increased lysis of infected target cells.

4.3.2 The Effects of Viral Stimulation on NK-92MI Cells

Since the VSVΔG/ZEBOV GP virus does not infect NK-92MI cells, there was a possibility that it influenced the further activation of the NK-92MI cells. TNF-α and IFN-γ, two important cytokines which NK cells secrete, were investigated. When stimulated by VSVΔG/ZEBOV GP alone, NK-92MI cells did not secrete significant amounts of TNF-α within a 24 hour period. However, this viral stimulation caused the NK-92MI cells to secrete a substantial amount of IFN-γ after 8 hours of exposure (Figure 3.24-B). IFN-γ is an important modulator of both the innate and adaptive immune response. It can affect many different cells by stimulating the production of other cytokines, induce expression of HLA antigen, activate immune cells, as well as antiviral and antiproliferative effects (Grassegger and Hopfl, 2004; Ramirez *et al.*, 1992; Yu *et al.*, 2006). Since the production of IFN-γ occurred after 8 hours of exposure to the virus, the viral response could not have any effects on the NK cell cytotoxicity assays. However, during an animal infection, the secretion of IFN-γ in only 8 hours following infection with the VSVΔG/ZEBOV GP therapeutic could have a significant impact on the survival of the host. Not only does the infection with VSVΔG/ZEBOV GP cause a rapid secretion of IFN-γ, it would also aid in providing a faster immune response from other immune cells in both arms of immunity.

Direct stimulation of NK-92MI cells by the VSVΔG/ZEBOV GP virus was not expected. However, recent studies have shown that NK cells can recognize viral pathogens through toll-like receptors (TLRs) (Hart *et al.*, 2005). NK cells possess TLR-3

for double stranded RNA, and TLR-7 and -8 for single stranded RNA (ssRNA) (Girart *et al.*, 2007; Hart *et al.*, 2005; Lan *et al.*, 2007). Since VSVΔG/ZEBOV GP cannot replicate in NK cells, TLR-3 will not be discussed. TLR-7/8 however, are able to detect ssRNA, and as the genome of the *Ebolavirus* and VSVΔG/ZEBOV GP are ssRNA, these viruses might be detected by these TLRs. It is possible that minute amounts of the VSVΔG/ZEBOV GP virus are able to enter the cell via non-specific endocytosis and uncoat their viral genome, but they are not able to replicate. Therefore VSVΔG/ZEBOV GP viral RNA could bind to either TLR-7 or TLR-8 and stimulate the NK cells to secrete IFN-γ.

Since effects of VSVΔG/ZEBOV GP stimulation of the NK cells occurs after 8 hours of infection, it is improbable that the any effect the VSV virus has on the NK cells had a role in the cytotoxicity assays. It is more feasible that the effect the rVSV virus has on the target cells induces the NK cell cytolytic function. The decrease in HLA due to the virus infection and reduction of IFN-β secretion from the K562 cells, as well as the expression of ZEBOV GP on the target cell surface may all play a role in inducing higher NK cell cytotoxicity towards infected target cells. Future experiments should determine whether the effects of the IFN-β secretion or lack thereof from the target cells plays a role in the further stimulation of NK cells and target cell lysis.

4.4 The NK Cell Mediated Cytotoxicity Assay

A flow cytometric NK cell mediated cytotoxicity assay for target cells infected with the VSVΔG/ZEBOV GP virus was established. The assay was comparable to previous human and murine ^{51}Cr release assays for different virally infected cells, at higher MOIs than performed in the current project (Ahmad *et al.*, 2001; Arase *et al.*,

2002; Fletcher *et al.*, 1998; Prod'homme *et al.*, 2007; Tasca *et al.*, 2003). The human and murine cytotoxicity assays proved that cells infected with VSVΔG/ZEBOV GP are lysed more effectively than uninfected cells. As expected, increasing the E:T ratio induced an increase in the percent lysis of the target cells until a maximum level of cytotoxicity was attained (Figure 3.20 and 21). Cytotoxicity was dependent on the E:T ratio. A minimal amount of lysis was observed when the E:T ratio was at its lowest, demonstrating background killing of the assay (Figure 3.19).

The precise method in how NK cell interactions with the VSVΔG/ZEBOV GP virus might contribute to protection in the post-therapeutic is unclear. Studies observing VSVΔG/EBOV GP as a post-therapeutic have not concentrated on NK cells. However non-human primates that survived *Ebolavirus* infection all controlled the virus within the first 6 days following infection (Feldmann *et al.*, 2007; Geisbert *et al.*, 2008). In addition, VSVΔG/EBOV GP treated non-human primates had an increase in NK cell numbers and had early development of non-neutralizing antibodies in the serum (Feldmann *et al.*, 2007; Geisbert *et al.*, 2008). This suggests that effector functions of survival do not initially involve the adaptive immune system, but rather may be facilitated by NK cell functions such as ADCC and cytokine secretions. A swift activation of NK cells would facilitate a rapid removal of the infected target cells. The deletion of most of these infected cells would then allow the host time to mount an adaptive response against the infection, thus allowing recovery. Future assays utilizing the flow cytometric cytotoxicity assay should include neutralizing antibodies to determine a role for ADCC in the VSVΔG/EBOV GP therapeutic.

There are some shortcomings to the flow cytometric cytotoxic assay. Because of the quantity of cells required to be read by the flow cytometer, more target cells are necessary than if utilizing a ^{51}Cr release assay. So even though low E:T ratios were used for the VSVΔG/ZEBOV GP virus cytotoxicity assay, a considerable amount of NK cells are still essential to the assay. When obtaining murine NK cells, this requires either numerous mice to be euthanized or for the purified NK cells to be cultured in IL-2. Culturing the NK cells in IL-2 allows for proliferation of the small amount of NK cells obtained from the mouse spleen to attain enough NK cells to run the assay. However, the NK cells are now activated, and may lyse targets cell more spontaneously. This can be easily overcome with proper controls for the assay.

4.5 Model of the VSVΔG/EBOV GP Virus Post-Exposure Response to the *Ebolavirus*

Post-exposure experiments for both EBOV and MARV have been performed on different animal models with varying results. Experiments using *Ebolavirus* have been conducted in mice, guinea pigs and non-human primates. Mice were treated with VSVΔG/ZEBOV GP 30 minutes up to 24 hours post exposure to mouse-adapted ZEBOV and demonstrated full protection (Feldmann *et al.*, 2007). Guinea pigs were treated with VSVΔG/ZEBOV GP 1 hour and 24 hours following exposure to the guinea pig-adapted ZEBOV and were observed to be only partially protected (Feldmann *et al.*, 2007). Post-exposure experiments with recombinant VSV filovirus vectors have demonstrated that non-human primates have partial to full protection against *Ebolavirus* or *Marburgvirus* (Daddario-DiCaprio *et al.*, 2006b; Feldmann *et al.*, 2007; Geisbert *et al.*, 2008). Thus far, all animal models survive the infection in spite of developing signs of disease and loosing weight (Daddario-DiCaprio *et al.*, 2006b; Feldmann *et al.*, 2007; Geisbert *et al.*, 2008).

Because of the normally rapid death of the animals following an infection with Ebola, and the recovery of the animals subsequent to a post-exposure injection of VSVΔG/ZEBOV GP, a compartmentalized innate immune response is indicated as a consequence to the therapeutic. Therefore, as a major innate immune cell, the role of NK cells in this protection will be discussed here.

The VSVΔG/ZEBOV GP virus has the same cell tropism as Ebola (Garbutt *et al.*, 2004). Therefore, once the host is infected with either virus, similar pathways of infection would be observed (Figure 4.1). However, it is unlikely for a *Ebolavirus*-VSVΔG/ZEBOV GP superinfection to occur possibly due to processes a cell can undergo to protect itself during a viral infection, such as producing and secreting cytokines (Berry *et al.*, 2008; Turner and Moyer, 2008). The virus initially infects the monocytes/macrophages and DCs (Bray and Geisbert, 2005; Geisbert and Jahrling, 2003; Mahanty and Bray, 2004; Reed *et al.*, 2004). These cells travel to the local draining lymph nodes where additional cells are infected with the virus. Studies have demonstrated that Ebola replicates swiftly in the lymph nodes, spleen, liver, thymus, adrenal glands and lungs within 3 to 4 days of infection (Ebihara *et al.*, 2007; Gibb *et al.*, 2001). Studies have yet to illustrate replication of VSVΔG/ZEBOV GP within these organs; however, VSVΔG/ZEBOV GP viral replication is required for protection (Garbutt *et al.*, 2004). Because a systemic response is not noted prior to 6 days after infection (Daddario-DiCaprio *et al.*, 2006b; Feldmann *et al.*, 2007; Geisbert *et al.*, 2008), it is presumed that within these organs NK cell functions will be effective in facilitating protection.

Even though the VSVΔG/ZEBOV GP virus is given following an infection with the *Ebolavirus*, the recombinant virus would replicate more rapidly in the target cells (Garbutt *et al.*, 2004). Thus, VSVΔG/ZEBOV GP can induce an immune response before the *Ebolavirus* can induce a response in the infected cells. The VSVΔG/ZEBOV GP infected cells begin to express Ebola glycoprotein on the cell surface within hours of infection. They also initiate a decrease in HLA on the surface of the infected cells. Therefore, it was hypothesized that the increase in glycoprotein expression and the decrease in HLA induces a stimulatory signal and diminishes the inhibitory signal in the host NK cells, respectively. The NK cells begin lysing the VSVΔG/ZEBOV GP infected cells, and thus decrease the number of cell where the *Ebolavirus* can replicate. While NK cells lyse VSVΔG/ZEBOV GP infected cells, they also secrete cytokines such as IFN-γ that activate other immune cells such as macrophages, and prime the adaptive immune cells in order to acquire a faster and more direct attack against virally infected cells (Janeway *et al.*, 2001). The NK cell activation is mediated by contact with infected cells, cytokines secreted by the infected cells, and possibly by direct binding to the viral pathogen.

Seeing as Ebola inhibits T cell proliferation and activation, a T cell response may now be possible due to secretion of cytokines from the NK cells and the local presence of viral antigen following the VSVΔG/ZEBOV GP infection (Figure 4.1). Activated T cells secrete cytokines such as IFN-γ and IL-4, which would in turn activate NK cells, causing an increase in MHC for antigen presentation as well as activating B cells (Abbas and Lichtman, 2001; Janeway *et al.*, 2001). The secretions of these cytokines would recuit and activate other immune cells to combat virally infected cells.

Within the lymphoid organs, it is also possible that B cells are beginning to replicate due to the presence of viral antigen and the IFN-γ secreted by the NK cells (Figure 4.1) (Vetvicka *et al.*, 1988). These cells begin to secrete antibodies within the organs that are not observed in the blood before 6 days following infection (Daddario-DiCaprio *et al.*, 2006b; Feldmann *et al.*, 2007; Geisbert *et al.*, 2008). Studies have demonstrated that neutralizing antibodies may aid in protection against viral infection (Kahn *et al.*, 2001; Lefrancois, 1984; Parren *et al.*, 2002; Takada *et al.*, 2007; Takada *et al.*, 2003; Wilson *et al.*, 2000). Hence, a concentrated amount of antibodies within the organs may opsonize not only virally infected cells, but also Ebola viral particles prohibiting further viral infections. The binding of antibodies to the infected cells induces ADCC by the NK cells and stimulates them to degranulate and lyse infected cells.

At the same time, VSVΔG/ZEBOV GP infected cells are secreting cytokines which increase antigen presentation and activate NK cells and other immune cells. All immune cells are then primed and ready to lyse infected cells. Thus, before Ebola can replicate and spread throughout the host, VSVΔG/ZEBOV GP has initiated an appropriate and more specific adaptive immune response against the invading virus.

4.6 Future Work

There are several avenues of research that remain to be addressed in the investigation of the role of NK cells in the *Ebolavirus* therapeutic. Firstly, the mechanism of change in the target cells caused by the VSVΔG/ZEBOV GP virus should be further inspected. A study should examine if there is a correlation between the increase in ZEBOV GP and the decrease in HLA on the target cell surface that specifically targets these cells for NK cell lysis. This could be demonstrated by performing a NK cell

cytotoxicity assay while staining the target cells with fluorescent probes for the ZEBOV GP and HLA. By flow cytometric analysis it could be determined which cell surface markers correlates with target cell death.

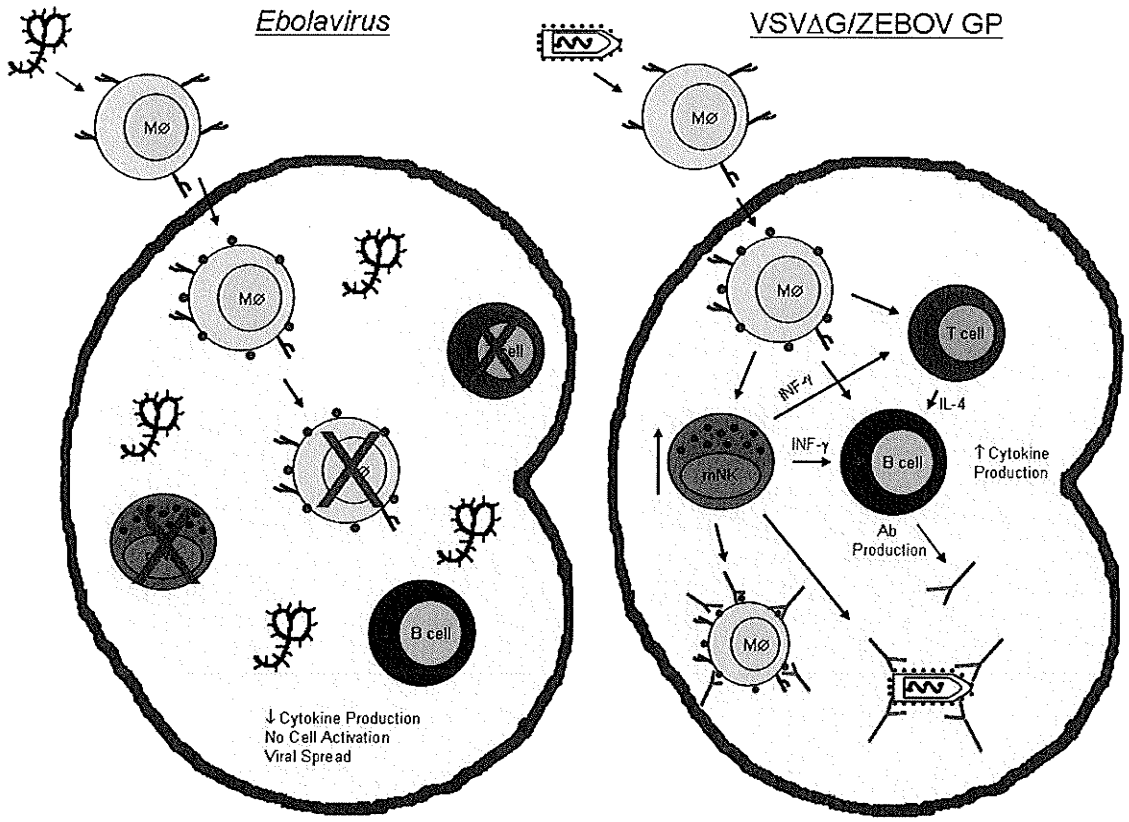

Figure 4.1: Model of VSVΔG/ZEBOV GP virus protection in the lymph nodes.
During an *Ebolavirus* infection, infected macrophages travel to the lymph nodes where the infected cell dies. Loss of the infected cell propagates viral release and a lack of cytokine secretion to stimulate immune function. During the VSVΔG/ZEBOV GP infection, infected macrophages travel to the lymph nodes where it secretes cytokines and stimulates immune cells with viral antigen. The increased cytokine production and antibody secretion allows for immune cell activation and removal of infected cells and viral particles.

Secondly, the specific mechanism of NK cell cytotoxicity should be elucidated. By performing a NK cell cytotoxicity assay, the NK cells could be probed for death

receptors such as FasL and TRAIL. The NK cells could also be probed for lysosomal-associated membrane protein 1 (LAMP1) also known as CD107a. LAMP1 has been described as a marker of $CD8^+$ T cell and NK cell degranulation following stimulation (Alter *et al.*, 2004). Therefore by performing the NK cell cytotoxicity assay and probing for these NK cell markers, one could determine which method of cell lysis the NK cell utilizes to lyse target cells infected with VSVΔG/ZEBOV GP.

Thirdly, one could reveal whether there is a role for the secretion of IFN-β from target cells on the target cells or on the NK cells during the cytotoxicity assay. The target cells secrete IFN-β during the 24 hour infection period. During the infection, there is a decrease in secretion of IFN-β after 18 hours that may be due to in increase in IFN-β receptor on the target cells and the consequent increase in IFN-β uptake. This possibility could be investigated by staining for the IFN-β receptor on the target cells during the infection. In addition, during the cytotoxicity assays performed here, the infectious supernatant is removed from the target cells and new media is added to the target/effector cells during assay. In order to determine the effects of IFN-β during the assay, the target/effector cells could be incubated in the infectious supernatant. The assay could then be monitored for an increase in NK cell cytotoxicity over what is observed when the cells are incubated in new media.

Fourthly, it should be determined if the VSVΔG/ZEBOV GP virus stimulates primary NK cells as only cell lines were used. This study could be executed by incubating the VSVΔG/ZEBOV GP virus with primary NK cells. By flow cytometry, activation markers such as CD69 and NKp44 could be examined for upregulation

following the viral incubation. Primary NK cell activation could also be investigated by ELISA studies of cytokines such as IFN-γ and TNF-α in infectious supernatant.

In animal models, one could determine whether NK cells are activated in different organs following infection with the VSVΔG/ZEBOV GP virus. The animals could be infected with VSVΔG/ZEBOV GP and serial sampling could be performed on the spleen, blood, liver and lymph nodes could be examined to determine if NK cells become activated. Activation of the NK cells could be determined by flow cytometric analysis of both activation markers and intercellular cytokine staining.

Another assay to perform would be the comparison between the cytotoxic activity and cytokine secretion of NK cells following incubation with EBOV or VSVΔG/ZEBOV GP. A difference in the activity of the NK cells after an exposure to either virus would demonstrate that the therapeutic has a differential effect on the NK cells that aid in the survival of the infected individual.

Lastly, one could examine the Ebola post-exposure response to the VSVΔG/ZEBOV GP virus in mice deficient in NK cells in comparison to mice that have NK cells. This experiment would determine the importance of NK cells in the post-exposure response since if the NK deficient mice die from the Ebola infection while the mice with NK cells do not, this would suggest an essential requirement of NK cells for the VSVΔG/ZEBOV GP virus response.

4.7 Concluding Remarks

In the present study, a NK cell mediated cytotoxicity assay for the VSVΔG/ZEBOV GP virus was established. By utilizing this assay, the following conclusions may be drawn:

1. Target cells infected with the VSVΔG/ZEBOV GP virus increase the expression of ZEBOV GP on the cell surface.
2. Target cells infected with the VSVΔG/ZEBOV GP virus decrease the expression of HLA on the cell surface.
3. Infecting target cells with the VSVΔG/ZEBOV GP virus leads to the secretion of IFN-β in some cell types.
4. NK cell lyse VSVΔG/ZEBOV GP infected target cells more frequently than uninfected cells.
5. Although NK cells are not readily infected by VSVΔG/ZEBOV GP, the VSVΔG/ZEBOV GP virus can directly stimulate NK cells to secret IFN-γ.

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6 Appendix

Appendix I

Primer Seqences for all Ebola strains 1

Name	Sequence	Use
Zaire Kikwit Primers:		
EBOVGP95140rXhoI	AATCTCGAGACAATGGGTGTTACAGGAATATTGC	Cloning
EBOVGP952171fNheI	AATGCTAGCTTACTAAAAGACAAATTTGCATATACA	Cloning
GP1,2Fwd01	CAGGTCCGGTGTCCCACCAAAGG	Sequencing
GP1,2Rev01	CCTTTGGTGGGACACCGGACCTG	Sequencing
GP1,2Fwd02	CGCTGAAGGTGTCGTTGC	Sequencing
GP1,2Rev02	GCAACGACACCTTCAGCG	Sequencing
GP1,2Fwd03	CTGCTCCAGCTGAATGAGAC	Sequencing
GP1,2Rev03	GTGAATCTTGATTACCGTTGGACG	Sequencing
GP1,2Fwd04	GTTCAAGTGCACAGTCAAGG	Sequencing
GP1,2Rev04	CCTTGACTGTGCACTTGAAC	Sequencing
GP1,2Fwd05	GAGCAAGAGCACTGACTTCC	Sequencing
GP1,2Rev05	GGAAGTCAGTGCTCTTGCAC	Sequencing
GP1,2Fwd06	GGATGAAGGTGCTGCAATCG	Sequencing
GP1,2Rev06	CGATTGCAGCACCTTCATCC	Sequencing
GP1,2Fwd07	CGGACTGCTGTATCGAACC	Sequencing
GP1,2Rev07	GGTTCGATACAGCAGTCCG	Sequencing
EbolaGP1KspIr	GACCCGCGGTTACTATCTTCGAGTTCTTCTCCCG	Sequencing
EBOgp 540-562f	CCGGTGCCGGTATGTGCACAAAG	Sequencing
EBOgp 585-881f	GACCTACGTCCAACTTGAATCAAG	Sequencing
EBOgp 1098-1103f	GGTCAGAGTCCGGCGC	Sequencing
EBOGP2Sac2r	GACCCGCGGTTACTAAAAGACAAATTTGC	Sequencing
EBO May GP2 1963f	GCTGTATCGAACCACATGATTG	Sequencing

Name	Sequence	Use
Ebov95101R	TGTGGATGACAGTTTGTCAC	Sequencing
Ebov95221R	TGGCACGTCAGTTGCCACTC	Sequencing
Ebov95281R	TTCATAATTGACCACCTTTG	Sequencing
Ebov951661R	CTGTCTCAACCCACAGATTA	Sequencing
Ebov951721R	GCGTAGCTCGGTTGTGGCTC	Sequencing
Ebov1616f	GGCTCCCTTAAATGTATCTCCCCGACTACGTG	Site Directed Mutagenesis
Ebov1647r	CCGAGGGAATTTACATAGAGGGGCTGATGCAC	Site Directed Mutagenesis
Sudan Boniface Primers:		
SudanSt.B120FB	AATCGTCTCCTCGAGCACCATGGAGGGTCTTAGCCTACT	Cloning
SudanSt.B2149R	AATCGTCTCGCTAGCTCAACAAAGCAGCTTGCAGAC	Cloning
EBOV Sudan 220f	CTTTGGGTGTTGTGACCAACAG	Sequencing
EBOV Sudan 330f	GAGGGGAGCGGAGTATCTACTG	Sequencing
EBOV Sudan 430f	GGGCTGAAAATTGCTACAATCTT	Sequencing
EBOV Sudan 581f	CAAGGATGGAGCTTTCTTCCTCT	Sequencing
EBOV Sudan 671f	GATATTGGCTAAACCAAAGGAA	Sequencing
EBOV Sudan760f	CCACATCCTACTTGGAGTACGA	Sequencing
EBOV Sudan870f	CAGTTCCTTTTCCAGCTGAATG	Sequencing
EBOV Sudan1090f	GAGAACTACAAAGGGAAGAAT	Sequencing
EBOV Sudan1230f	GAAGAGTAGATGTGAATACTC	Sequencing
EBOV Sudan 1440f	CAACAACACCACCGAGAAACT	Sequencing
EBOV Sudan1620f	GACAAGTTAACACCAGGGCCAC	Sequencing
EBOV Sudan 1740f	GCATATACACTGAAGGCCTTAT	Sequencing
EBOV Sudan 1878f	GGAAGGCCATAGATTTCCTTCTG	Sequencing
EBOV Sudan2000f	CATGATTTCATCGACAACCCTT	Sequencing
EBOV Sudan 219r	GCATGGAAAAGGCCTTTTGAAA	Sequencing
EBOV Sudan 379r	CCCCAACGCTTTGTCGCAGATGG	Sequencing
EBOV Sudan 652r	TACCCCCTCAGCAAAATTGACTC	Sequencing
EBOV Sudan 852r	CCAGAAGAACAAAAGTATTATTG	Sequencing
EBOV Sudan 1076r	CATCGTCTTCTGTCTCGTTGAGCG	Sequencing

Name	Sequence	Use
EBOV Sudan1316r	TGGAGATCTGCATGTTGTTACCGT	Sequencing
EBOV Sudan 1432r	GGTGGTCATCACTGGTAGTTTTG	Sequencing
EBOV Sudan1559r	GACCGTTGCTTGTGGACTCTTGTG	Sequencing
EBOV Sudan1799r	GTTGTCTGAGTCCACAGACTAAGG	Sequencing
EBOV Sudan 1958r	CAGTGATGTTTTTGGTCCAATC	Sequencing
Sudan Gulu Primers:		
SudanGulu75FBsm	AATCGTCTCCTCGAGCACCATGGGGGGTCTTAGCCTAC	Cloning
SudanGulu2104RB	AATCGTCTCGCTAGCTCAGCAAAGCAGCTTGCAAAC	Cloning
SEBOV Gulu 99R	GCATGGAAAAGGCCTTTTGGAA	Sequencing
SEBOV Gulu 260R	CCCCAACGCTTTGTTGCAGATGG	Sequencing
SEBOV Gulu 910F	GAAGAGCTGTCTTTCGAAGCTT	Sequencing
SEBOV Gulu1081F	ACATTGCCGTCTCAGAATTCGA	Sequencing
SEBOV Gulu 1192R	TCTGCATATGGTTGCCGTTAGT	Sequencing
SEBOV Gulu 1211F	GACCGAGCTCCAGCCAAATCCC	Sequencing
SEBOV Gulu 1320R	TTCCTCGGTGGCCATCACTGAT	Sequencing
SEBOV Gulu1401F	GGTTAAAACTGTCCTGCCACAG	Sequencing
SEBOV Gulu1462R	CTGTTACTGTTGAAGTTATTAG	Sequencing
SEBOV Gulu 1620R	TTCCGCACCCGGTCCAAAGTAC	Sequencing
SEBOV Gulu 1861R	CAGTGATGTTTTTTGTCCAATC	Sequencing
SEBOV Gulu 1760F	GGAAGGCCATAGATTTCCTTCTG	Sequencing
SEBOV Gulu 1882F	CATGATTTCATCGACAACCAAT	Sequencing
SEBOV Gulu 2002R	GAGCAATAATTGCAATAATAAT	Sequencing

Appendix 2

Ebola Glycoprotein Nucleotide Sequences 1

Nucleotide sequence of *Zaire ebolavirus* Kikwit glycoprotein open reading frame (2031bps) from Genbank (Reference # AY354458). Note nuclceotide 1289 change (found in green) found in Winnipeg virus stock that differed from Genbank (not changed). Site directed point mutation (found in red) of nucleotides 1631 from c → t to make this sequence match the sequence of the Winnipeg virus stock strain. Also found in blue is 8A (nucleotide 880) sequence for GP_2 back editing.

atgggtgtta caggaatatt gcagttacct cgtgatcgat tcaagaggac atcattcttt
ctttgggtaa ttatcctttt ccaaagaaca ttttccatcc cacttggagt catccacaat
agcacattac aggttagtga ggtcgacaaa ctggtttgcc gtgacaaact gtcatccaca
aatcaattga gatcagttgg actgaatctc gaagggaatg gagtggcaac tgacgtgcca
tctgcaacta aaagatgggg cttcaggtcc ggtgtcccac caaaggtggt caattatgaa
gctggtgaat gggctgaaaa ctgctacaat cttgaaatca aaaaacctga cgggagtgag
tgtctaccag cagcgccaga cgggattcgg ggcttccccc ggtgccggta tgtgcacaaa
gtatcaggaa cgggaccgtg tgccggagac tttgccttcc acaaagaggg tgctttcttc
ctgtatgacc gacttgcttc cacagttatc taccgaggaa cgactttcgc tgaaggtgtc
gttgcatttc tgatactgcc ccaagctaag aaggacttct tcagctcaca ccccttgaga
gagccggtca atgcaacgga ggacccgtct agtggctact attctaccac aattagatat
caagctaccg gttttggaac caatgagaca gagtatttgt tcgaggttga caatttgacc
tacgtccaac ttgaatcaag attcacacca cagtttctgc tccagctgaa tgagacaata
tatacaagtg ggaaaaggag caataccacg ggaaaactaa tttggaaggt caaccccgaa
attgatacaa caatcgggga gtgggccttc tgggaaacta aaaaaaacct cactagaaaa
attcgcagtg aagagttgtc tttcacagct gtatcaaaca gagccaaaaa catcagtggt
cagagtccgg cgcgaacttc ttccgaccca gggaccaaca caacaactga agaccacaaa
atcatggctt cagaaaattc ctctgcaatg gttcaagtgc acagtcaagg aagggaagct
gcagtgtcgc atctgacaac ccttgccaca atctccacga gtcctcaacc ccccacaacc
aaaccaggtc cggacaacag cacccacaat acacccgtgt ataaacttga catctctgag
gcaactcaag ttgaacaaca tcaccgcaga acagacaacg acagcacagc ctccgacact
ccccccgcca cgaccgcagc cggacccct a aaagcagaga acaccaacac gagcaagggt
accgacctcc tggaccccgc caccacaaca agtccccaaa accacagcga gaccgctggc
aacaacaaca ctcatcacca agataccgga gaagagagtg ccagcagcgg gaagctaggc
ttaattacca atactattgc tggagtcgca ggactgatca caggcgggag gagagctcga
agagaagcaa ttgtcaatgc tcaacccaaa tgcaacccta atttacatta ctggactact
caggatgaag gtgctgcaat cggactggcc tggataccat atttcgggcc agcagccgag
ggaatttaca tagaggggct gatgcacaat caagatggtt taatctgtgg gttgagacag
ctggccaacg agacgactca agctcttcaa ctgttcctga gagccacaac cgagctacgc
acctttcaa tcctcaaccg taaggcaatt gatttcttgc tgcagcgatg gggcggcaca
tgccacattt tgggaccgga ctgctgtatc gaaccacatg attggaccaa gaacataaca
gacaaaattg atcagattat tcatgatttt gttgataaaa cccttccgga ccaggggac
aatgacaatt ggtggacagg atggagacaa tggataccgg caggtattgg agttacaggc
gttataattg cagttatcgc tttattctgt atatgcaaat ttgtctttta g

Nucleotide sequence of *Sudan ebolavirus* Boniface glycoprotein open reading frame (2362bps) from Genbank (Reference # U28134). Note nucleotide changes (found in green) found in Winnipeg pCAGGS Sudan Boniface plasmid stock that differed from Genbank (not changed). Also found in blue is 8A (nucleotide 940) sequence for GP_2 back editing.

atggagggtc ttagcctact ccaattgccc agagataaat ttcgaaaaag ctctttcttt
gtttgggtca tcatcttatt tcaaaaggcc ttttccatgc ctttgggtgt tgtgaccaac
agcactttag aagtaacaga gattgaccag ctagtctgca aggatcatct tgcatccact
gaccagctga aatcagttgg tctcaacctc gaggggagcg gagtatctac tgatatccca
tctgcgacaa agcgttgggg cttcagatct ggtgtgcctc ccaaggtggt cagctatgaa
gcaggagaat gggctgaaaa ttgctacaat cttgaaataa agaagccgga cgggagcgaa
tgcttacccc caccgccgga tggtgtcaga ggctttccaa ggtgccgcta tgttcacaaa
gcccaaggaa ccgggccctg cccgggtgac tatgcctttc acaaggatgg agctttcttc
ctctatgaca ggctggcttc aactgtaatt tacagaggag tcaattttgc tgaggggtga
atgcattct tgatattggc taaaccaaag gaaacgttcc ttcaatcacc ccccattcga
gaggcagtaa actacactga aaatacatca agttactatg ccacatccta cttggagtac
gaaatcgaaa attttggtgc tcaacactcc acgacccttt tcaaaattaa caataatact
tttgttcttc tggacaggcc ccacacgcct cagttccttt tccagctgaa tgataccatt
cacttcacc aacagttgag caacacaact gggaaactaa tttggacact agatgctaat
atcaatgctg atattggtga atgggctttt tgggaaaata aaaaaaatct ctccgaacaa
ctacgtggag aagagctgtc tttcgaaact ttatcgctca acgagacaga agacgatgat
gcgacatcgt cgagaactac aaagggaaga atctccgacc gggccaccag gaagtattcg
gacctggttc caaaggattc ccctgggatg gtttcattgc acgtaccaga aggggaaaca
acattgccgt ctcagaattc gacagaaggt cgaagagtag atgtgaatac tcaggaaact
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ccagagaata taacaacagc ggttaaaact gtttgccac aagagtccac aagcaacggt
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tgtaggatcc tgggaccaga ttgttgcatt gagccacatg attggaccaa aaacatcact
gataaaatca accaaatcat ccatgatttc atcgacaacc ctttacccaa tcaggataat
gatgataatt ggtggacggg ctggagacag tggatccctg caggaatagg cattactgga
attattattg caatcattgc tcttctttgc gtctgcaagc tgctttgttg a

Nucleotide sequence of *Sudan ebolavirus* Gulu glycoprotein open reading frame (2031bps) from Genbank (Reference # AY316199). Note nucleotide changes (found in green) found in Winnipeg virus stock that differed from Genbank (not changed). Also found in blue is 8A (nucleotide 880) sequence for GP_2 back editing.

atgggggtc ttagcctact ccaattgccc agggacaaat ttcggaaaag ctctttcttt
gtttgggtca tcatcttatt ccaaaaggcc ttttccatgc ctttgggtgt tgtgactaac
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tctgcaacaa agcgttgggg cttcagatct ggtgttcctc ccaaggtggt cagctatgaa
gcgggagaat gggctgaaaa ttgctacaat cttgaaataa agaagccgga cgggagcgaa
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tgcaggatcc tgggaccaga ttgttgcatt gagccacatg attggacaaa aaacatcact
gataaaatca accaaatcat ccatgatttc atcgacaacc ccttacctaa tcaggataat
attattattg caattattgc tcttctttgc gaaagcaagc tgctttgctg a

Appendix 3

Recipes 1

DMEM-10:

- Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich, Oakville, Ontario, Canada, Cat# D5796)
- 10% heat inactivated fetal bovine serum (FBS, Multicell Technologies, Warwick, Rhode Island, USA, Cat# 08550)
- 2 mM L-glutamine (Invitrogen Gibco, Burlington, Ontario, Canada, Cat# 25030-081)
- 10,000 units/mL penicillin and 10,000 μg/mL streptomycin (Invitrogen Gibco, Cat# 15140-122),

αMEM-12:

- Alpha Modified Minimum Essential Medium Eagle containing sodium bicarbonate (αMEM, Sigma-Aldrich, Cat# M4526)
- 12.5% heat inactivated FBS
- 12.5% heat inactivated horse serum (Invitrogen Gibco, Cat# 16050-122)
- 0.2 mM Folic Acid (Sigma-Aldrich, Cat# F875-25G)
- 0.2 mM myo-Inositol (Sigma-Aldrich, Cat# 17508-100G)
- 0.1 mM 2-mercaptoethanol (βME, Sigma-Aldrich, Cat# M7522-100ML)
- 10,000 units/mL penicillin, 10,000 μg/mL streptomycin
- 2 mM L-glutamine

RPMI-10:

- RPMI medium 1640 containing 4.5 g/L D-glucose, 2.393 g/L HEPES buffer, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 0.1100 g/L sodium pyruvate (Invitrogen Gibco, Cat# 03-0078DJ)
- 10% heat inactivated FBS
- 10,000 units/mL penicillin and 10,000 μg/mL streptomycin

TSS Buffer:

- 85 mL LB broth
- 10 g polyethylene glycol
- 5 mL DMSO
- 50 mM $MgCl_2$

DMEM-2:

- DMEM
- 2% heat inactivated FBS
- 2 mM L-glutamine

10% Resolving Gel:

- 4.8 mL of sterile water
- 2.5 mL of 40% acrylamide/bis solution (Bio-Rad Laboratories Inc., Hercules, California, USA, Cat# 161-0148)
- 2.5 mL of 1.5 M TRIS-HCl ph 8.8
- 100 µL of 10% SDS (Fisher Scientific, Ottawa, Ontario, Canada, Cat# FL-05-0904)
- 100 µL of freshly made 10% ammonium persulfate (APS, Sigma Aldrich, Cat# A3678-100G)
- 4 µL tetramethylethylenediamine (TEMED, Fisher Scientific, Cat# BP150-20).

4% Stacking Gel:

- 3.15 mL of sterile water
- 0.5 mL of 40% acrylamide/bis solution
- 1.25 mL of 1.0 M TRIS-HCl
- 50 µL of 10% SDS
- 50 µL of 10% APS
- 8 µL TEMED.

Anode Buffer:

- 75 mL of 0.67 M Boric acid
- 200 mL of methanol
- 725 mL of distilled water

Cathode Buffer:

- 75 mL of 0.67 M Boric acid
- 50 mL of methanol
- 875 mL of distilled water