Regulation of Natural Killer Cell Ligands by Poxvirus Infection

By

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ABSTRACT

Natural Killer (NK) cells are a major part of the innate immune defence against viral infection and tumours. However, our understanding of how NK cells can be exploited for antiviral and antitumor therapy is still in its infancy. We are interested in the interplay between NK cells and Vaccinia Virus (VACV) since VACV is a basis of vaccine design and has the potential as an oncolytic virus for tumor therapy. Currently, we are focused on how VACV affects the ligands of NK receptors belonging to the NKR-P1 family. This family of C-type lectin-related receptors contains members that can stimulate or inhibit NK cell function.

We first studied the impact of VACV and Ectromelia virus (ECTV) infection on expression of the mouse CLEC2D protein, Clr-b, and Clr-b mediated protection from mouse NK cells. We observed a loss of Clr-b cell surface protein upon infection of murine cell lines and bone marrow derived macrophages with VACV and ECTV. The reduction of Clr-b is more rapid than MHC class I, the prototypic ligand of NK cell inhibitory receptors. Reduction of Clr-b requires an active viral infection but not expression of late viral genes. The loss of Clr-b mRNA appears to be delayed behind loss of Clr-b surface protein. Finally, Clr-b mediated protection from NK cells is lost following VACV infection.

Subsequently, we studied the influence of VACV infection of the expression of human CLEC2D and CLEC2D mediated protection from human NK cells. Human CLEC2D is constitutively expressed on transformed cells and it is believed that cells may need to be stimulated to induce expression. We observed a rapid increase in CLEC2D cell surface protein as detected by 4C7 antibody staining upon VACV infection of human cell lines that gradually decreased over time. The increase of 4C7 staining requires an active infection with the presence of an early viral protein, however, a late viral protein enhances the increase in the 4C7 reactive

protein. We discovered that all cells may express an isoform of the 4C7 reactive protein, and that they all contain an intracellular pool of the 4C7 reactive protein. Although VACV infection causes the upregulation the 4C7 reactive protein, it does not increase surface expression of isoform 1 of CLEC2D which is reported to bind to NKR-P1A (85). Given the lack of increase in isoform 1, NK cells did not recognize infected cells through NKR-P1A.

My results suggest that Clr-b is another mechanism of the missing self recognition system that may act earlier than MHC I recognition in rodents. In contrast, in humans, the function of CLEC2D is still undergoing investigation and my results suggest that intracellular pools of a 4C7 reactive protein may be manipulated by VACV infection. However the function of the CLEC2D isoforms involved in the infection remains an open question. Collectively, these data augment our knowledge in viral evasion and host response to infection. Further studies of this process could facilitate improved vaccine design and cancer therapy that currently uses VACV as a platform.

The thesis is dedicated to my father (Vernon P. Williams) and the memory of my mother (Rose Jasmine Warner-Williams) who taught me patience, dedication and that hard work is the only way to get ahead in this world. Along with my brothers and sisters, my entire family has shown me love and support that helped me through some very long and arduous times.

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List of Abbreviations

Abbreviation	Meaning
Ab	Antibody
ActD	Actinomycin D
ADCC	Antibody Dependent Cell Cytotoxicity
AICL	Activation-Induced C-type Lectin
Amp	Ampicillin
AraC	Cytosine β-D-Arabinofuranoside
ATCC	American Type Culture Collection
BAL	Bronchoalveolar Lavage
BAT3	HLA-B-Associated Transcript 3
BGMK	Buffalo Green Monkey Kidney Cells
BID	BH3 Interacting-Domain Death
BME	Beta-Mercaptoethanol
BMMØ	Bone Marrow Derived Macrophages
CD	Cluster of Differentiation
CEV	Cell-Associated Enveloped Virus
CHS	Contact Hypersensitivity
CHX	Cyclohexamide
CLEC2	C-type Lectin Domain Family 2
CLP	Common Lymphoid Precursor
Clr	C-type Lectin-Related
CMP	Common Myeloid Precursor
CMV	Cytomegalovirus
Cop	Copenhagen
Cr	Chromium
CXCL	Chemokine CXC Ligand
CXCR	Chemokine CXC Receptor
DAPI	4',6-Diamidino-2-Phenylindole
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPX	Deerpox
DR	Death Receptor
ds	Double Stranded
DTH	Delayed Type Hypersensitivity
EBV	Epstein Barr Virus
ECGC	Epigallocatechin-3-Gallate
ECTV	Ectromelia Virus

EDTA	Ethylenediaminetetraacetic Acid
eGFP	Enhanced Green Florescent Protein
ER	Endoplasmic Reticulum
EV	Extracellular Enveloped Virus
FADD	Fas-Associated Death Domain
FBS	Fetal Bovine Serum
GAG	Glycosaminoglycan
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
H60	Histocompatibility Antigen 60
HA	Haemagglutinin
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HI	Heat Inactivated
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSC	Hematopoietic Stem Cell
HSV	Herpes Simplex Virus
IEV	Intracellular Enveloped Virus
IFA	Immunofluorescent Assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cell
ILT	Immunoglobulin-Like Transcripts
iNK	Immature Natural Killer
IRF	Interferon Regulating Factor
ITAM	Immunoreceptor Tyrosine-Based Activating Motif
ITIM	Immunoreceptor Tyrosine-base Inhibitory Motif
IV	Immature Virion
KACL	Keratinocyte-Associated C-type Lectin
kbs	Kilobases
KIR	Killer Immunoglobulin-like receptor
LAK	IL-2 activated NK cells
LAMP	Lysosomal-Associated Membrane Proteins
LB	Lysogeny Broth
LIR	Leukocyte Immunoglobulin-like Receptors
LLT1	Lectin-Like Transcript 1
$LT\alpha_1\beta_1$	Lymphotoxin
LTβR	Lymphotoxin Receptor
M	Molar

Mac-1	Macrophage-1
MALT	Mucosa-Associated Lymphoid Tissue
MCA	Methylcholanthrene
MCMV	Mouse Cytomegalovirus
MEP	Megakaryocyte/Erythrocyte Precursor
MFI	Mean Fluorescence Intensity
mg	Miligrams
MHC I	Major Histocompatibility Complex class I
MIC	MHC class I Chain Related
MIP	Macrophage Inflammatory Protein
mNK	Mature Natural Killer
MOI	Multiplicity of Infection
mRNA	Message Ribonucleic Acid
MULT-1	Murine ULBP-Like Transcript 1
MV	Mature Virion
NA	Neuraminidase
NBF	Neutral Buffered Formaldehyde
NCR	Natural Cytotoxicity Receptor
NK	Natural Killer
NKC	NK Gene Complex
NKP	Natural Killer Precursor
Nkrp1	NK Receptor- p1
NKT	Natural Killer T cell
Ocil	Osteoclast Inhibitory Lectin
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pFA	Paraformaldehyde
PFU	Plaque Forming Units
PI	Post Infection
PTK	Protein Tyrosine Kinase
p4C7	Protein 4C7
RAE-1	Retinoic Acid Early Inducible-1
RAG	Recombination Activating Gene
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RCMV	Rat Cytomegalovirus
RCN	Relative Cell Number
RCTL	Rat C-type Lectin
Rmp1	Resistance to Mousepox 1
RSV	Respiratory Syncytial Virus
RT-PCR	Reverse Transcription Polymerase Chain Reaction

SA	Streptavidin
SE	Standard Error
SLT	Secondary Lymphoid Tissue
STAT	Signal Transducers and Activators of Transcription
TAP	Transporter Associated with Antigen Processing
TfR	Transferrin Receptor
TK-	Thymidine Kinase Negative
TLR	Toll-Like Receptor
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
ULBP	UL16-Binding Proteins
US	Unique Short
V(D)J	Variable, Joining, Joining
VACV or VV	Vaccinia Virus
VZV	Varicella Zoster Virus
WR	Western Reserve
WV	Wrapped Virion
YFP	Yellow Florescent Protein
α-MEM	Alpha Modified Minimum Essential Medium Eagle's

Chapter 1 : Introduction to Natural Killer Cells and their role in viral infections

1. Introduction

In this thesis, I investigate the interaction of NK cells with poxvirus infected cells, focusing on the modulation of particular proteins, that upon infection, influence NK cell response. In order to provide background for these studies, the first part of the introductory chapter is intended to explain the origins of NK cells, the mechanisms by which NK cells perform their functions and the receptor systems that regulate their recognition of an infected cell. I will then provide a review of current literature on NK cell responses in select viral infections and provide background on poxviruses.

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Since discovered in 1969 as a 'non-specific' lymphocyte with the capacity of destroying cancer cells, natural killer (NK) cells are found to be effector cells of the innate immune system that can identify and eliminate mutated, bacterial or virally infected cells without prior sensitization (175). Their name denotes their occurrence in the body and their spontaneous ability to kill lymphomas in non-immunized animals (21). Given this ability, NK cells are increasingly being used, with partial effectiveness, in adoptive immunotherapeutic treatments against solid tumours as reviewed by McDowell *et al* (157). Thus the study of NK cells and their functions may lead to significantly effective therapies in many different fields, including vaccination and tumor therapy.

NK cells are the third largest lymphocyte population in peripheral blood of humans. They are also a distinct subset of a group of innate lymphoid cells (ILCs) that is now known to exist in the innate immune system (66, 253). NK cells make up about 5-20% of human peripheral blood
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mononuclear cells (PBMCs) (224) and approximately 3% of mouse PBMCs (207). They are found in the bone marrow, liver, spleen, thymus, lungs, uterus and to a small extent in secondary lymphoid tissue (SLT) and mucosa-associated lymphoid tissue (MALT) (291). As they travel about the circulatory system, they distinguish healthy cells from unhealthy cells and destroy the latter. NK cells are able to perform this function of recognizing target cells by a repertoire of activating and inhibiting receptors found on their surface that interact with ligands on the targeted cell. They also receive signals from other immune cells, such as Dendritic Cells (DCs) or macrophages that activate them to perform their functions. The actions of the NK cell are regulated by the balance of signals received by the interactions of their receptors with the target's ligands (187) and by cytokines released from infected cells. If the NK cell receives equal or more inhibitory signals from interacting with the target, such as binding of the major histocompatibility complex class I (MHC I), the NK cell will not perform any action against the target cell and will move on. However, if the NK cell receives more activating signals, such as binding viral proteins on the target cell surface, the NK cell will become active and destroy the target cell. Activation of the NK cell can also lead to production and secretion of cytokines. These cytokines, such as interferon gamma (IFN-γ), can affect and regulate many different cells of both the innate and adaptive immune systems and have direct antiviral effects on infected cells. The following sections describe the maturation process and the markers used to identify each stage of NK cell development.

1.1 Development and Maturation

Development of NK cells depends on the activation of particular transcription factors, the acquisition of certain receptors, the use of different cytokines and interactions with stromal cells during the different stages of development. NK cells develop from a pluripotent $CD34^+$

hematopoietic stem cell (HSC) found in the bone marrow. This HSC is termed pluripotent since it differentiates into a common lymphoid precursor (CLP), a common myeloid precursor (CMP) and a megakaryocyte/erythrocyte precursor (MEP) (1, 20). The CLP further branches out to produce progenitors for T, B and NK cells (Figure 1.1) (167, 172, 200). The research described in this thesis involves studies with both mouse and humans, thus the main features of development of both murine and human NK cells will now be further discussed.

1.1.1 Development of Murine NK cells

Development of NK cells is separated into different stages depending on the acquisition of certain receptors, such as Cluster of Differentiation (CD) 122 (Interleukin (IL)-2Rβ), NK1.1 (NKR-P1, in C56BL/6 mice) and CD49b (DX5), along with others receptors required for interacting with the environment (126, 219). The CLP does not express any of these markers nor markers for other cell lineages, therefore it is deemed lineage negative (Lin^-). The CLP develops into a bi-potent T cell/NK (T/NK) cell precursor that is $Lin^-ckit^+flt3^+$ (216, 223). The next stage in development is a pre-NK precursor (pre-NKP) that is found to be $Lin^-CD122^-NK1.1^-CD49b^-NKG2D^+CD244^+$ (291). Subsequently, the NK progenitor (NKP) is $Lin^-CD122^+NK1.1^-CD49b^-NKG2D^+CD244^+$ (204, 291). The NKP leads to the immature NK (iNK) cell that is $CD122^+NK1.1^+CD49b^-NKG2D^+CD244^+$ (204, 291). The final stage of development is the mature NK (mNK) that is $CD122^+NK1.1^+CD49b^+NKG2D^+CD244^+$ and expresses other inhibitory and activating receptors (126, 204).

Many transcription factors and cytokines required for the different stages of development of the NK cell have been identified. Some of these transcription factors are found in Figure 1.1. These transcription factors are activated at different times in order to mature the NK cell and allow it to acquire different receptors, to interact with its environment and to use certain factors

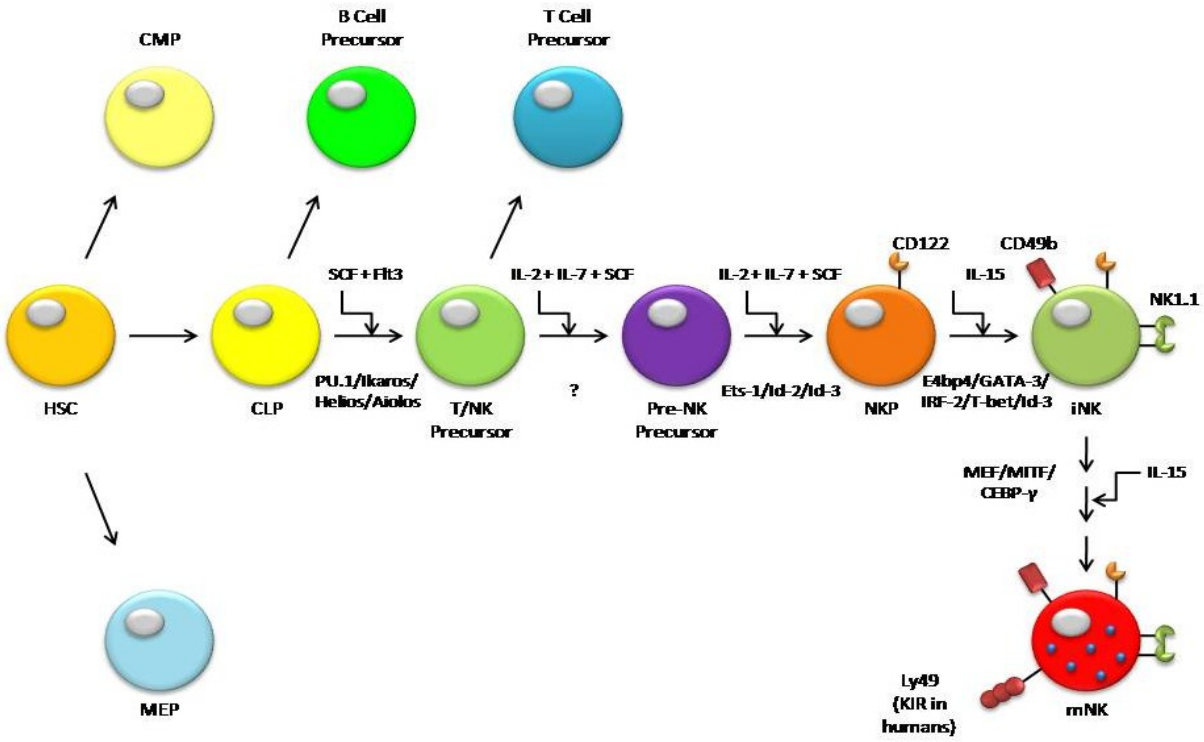

Figure 1.1 *Development of NK Cells*
Progression of the HSC to a mature NK cell. Cytokines required are above the arrows. Transcription factors required are below the arrows.

found in the surroundings. An important receptor that denotes the passage of the pre-NKP to the NKP is the CD122 receptor which complexes with the IL-15Rα and the common γ chain (CD132) to allow the cell to bind to IL-15 as reviewed by Yu *et al* (Figure 1.1) (291). IL-15 promotes the development of NK cells in the bone marrow. Although the NKP stage of development is not dependent on IL-15 (65), IL-15 is required for proliferation and differentiation into phenotypically mature cells (283).

IL-15, however, is critical to the iNK cell. IL-15 is produced from stromal cells in the bone marrow in an Interferon Regulating Factor (IRF) -1 dependent manner (65). iNK cells also interact with bone marrow stromal cells to acquire recognition receptors and to completely differentiate as reviewed by Roth *et at* (220). Once the iNK cell has acquired other ligands, such as CD94/NKG2, the Ly49 receptors, CD43 and the macrophage-1 antigen (Mac-1), it must undergo a series of proliferation steps before it can become fully functional and phenotypically mature (69, 126, 150). Expression of these markers causes proliferation to be reduced and the cell goes to the periphery to become a mature, functional killer (126).

The mNK cell travels to other areas of the body with the aid of chemokine receptors and adhesion molecules where IL-15 is abundant for its survival (108). In these areas, the NK cell can survive for approximately 7-10 days (132, 201, 205). mNK cells now express markers that indicate its transition to maturity such as Mac-1 that demonstrates its capability of producing cytokines and functional competence at cytotoxicity (126). These mature NK cells are now qualified to survey the body for infected and mutated cells.

1.1.2 Human NK cell Development

The development of human NK cells occurs in the bone marrow also from a $CD34^+$ HSC similar to the mouse. As they go through similar stages of maturation, they also require interaction with stromal cells, usage of transcription factors and expression of surface markers such as CD161 (NKR-P1), NKG2A and Killer Immunoglobulin-like receptors (KIRs), which will be discussed shortly. Mature human NK cells have two groups with distinct functions based upon CD56 expression (73, 82, 232). $CD56^{bright}$ NK cells have a high capacity for cytokine production, lower cytotoxic potential, express low levels of CD16 and make up about 10% of the peripheral blood NK cells (291). $CD56^{dim}$ NK cells have greater cytotoxic abilities and express high levels of CD16, but produce little cytokines and they make up approximately 90% of the peripheral blood NK cells (236, 277, 291). The $CD56^{bright}$ NK cells are also thought to be precursors of the $CD56^{dim}$ NK cells (43). Similar mature NK cell groups have also been discovered in mice based upon the markers Mac-1 and CD27 (99). Whichever is the cell phenotype, these mature NK cells go into the periphery where they must interact with other cell types. Here, they must be able to determine self cells versus non-self cells. The methods on how these mature NK cells learn to make this determination are discussed in the following sections.

1.2 NK Cell Education

During development, NK cells acquire surface receptors that are both activating and inhibitory. These receptors determine the actions of NK cells as they survey the environment. Activating receptors cause the NK cell to take action against cells that express ligands to those activating receptors, whether it be foreign (viral or bacterial) or belong to the host. A healthy host cell expresses markers on its surface that identify it as belonging to the host, while an unhealthy host cell can express markers of stress or infection. One such marker that is expressed

on all nucleated cells is MHC I. On a normal basis, MHC I displays endogenous protein fragments on the surface of the cell. However, during an infection, foreign protein can be broken down, loaded onto the MHC I protein and expressed on the surface of the cell. The presence of MHC I on the cell surface identifies the cell as belonging to the host and when displaying endogenous protein, that the cell is healthy.

The healthy host is always expressing proteins that engage stimulatory NK cell receptors. To control the constant activation signals that the NK cell receives, it also must express inhibitory receptors against self molecules, such as MHC I. NK cells must express inhibitory receptors to self molecules, not only to prevent activation against the host, but also to make the activation receptors function appropriately as reviewed by Held *et al* (101).

In MHC I sufficient humans or mice, there are subsets of mature NK cells that lack inhibitory receptors to self molecules. These subsets of NK cells are hyporesponsive to stimulation through their activation receptors and they cannot kill MHC I deficient bone marrow (7, 80, 127). Peripheral NK cells from humans or mice who lack MHC I also have poor activation receptor stimulation. However, they can become responsive when transferred to a MHC I sufficient environment (72, 114). Therefore, inhibitory receptors to self molecules are require to "educate" the NK cell response. Immature NK cells are educated in the bone marrow where they interact with self MHC I molecules. The education must occur through signalling through the inhibitory receptors because mutations within the immunoreceptor tyrosine-base inhibitory motif (ITIM) cause the NK cell to become hyporesponsive, even when the proper self MHC I molecule is present (127, 178).

There are several proposed models to explain how NK cells are educated to become tolerant to self molecules. The disarming model (206, 208) states that activation receptors on immature NK cells are constitutively responsive to stimulus. By acquiring inhibitory receptors to self molecules, the NK cell obtains a balance signal for the activation pathway (Figure 1.2A). Without the inhibitory signals, the NK cell will receive continuous stimulation through its activation receptors and will become hyporesponsive (208, 288), though the mechanisms remain to be determined.

The arming (208) and licensing (127) models both propose that activation receptors on immature NK cells are initially unresponsive to stimulus. By binding inhibitory receptors to self-molecules, the NK cell receives signals that bestow competence to the activation pathways (Figure 1.2B). Without this signal, the NK cell would stay unresponsive and the maturation process would be hindered.

The *cis*-interaction model (42) maintains that just the presence of an unbound inhibitory receptor to MHC I is enough to diminish the signals received from the activation receptors. Ly49 inhibitory receptors to self-MHC I have the ability to bind high affinity MHC I molecules in *cis* (13, 102). In fact, in the presence of the high affinity MHC I molecule, the majority of Ly49A receptors are bound in cis and are not accessible to bind to MHC I molecules on other cells in *trans* (70). By having their self molecule receptor always bound to MHC I, the activation threshold of the NK cell is increased and the cell is tolerant to its stimulatory environment (Figure 1.2C).

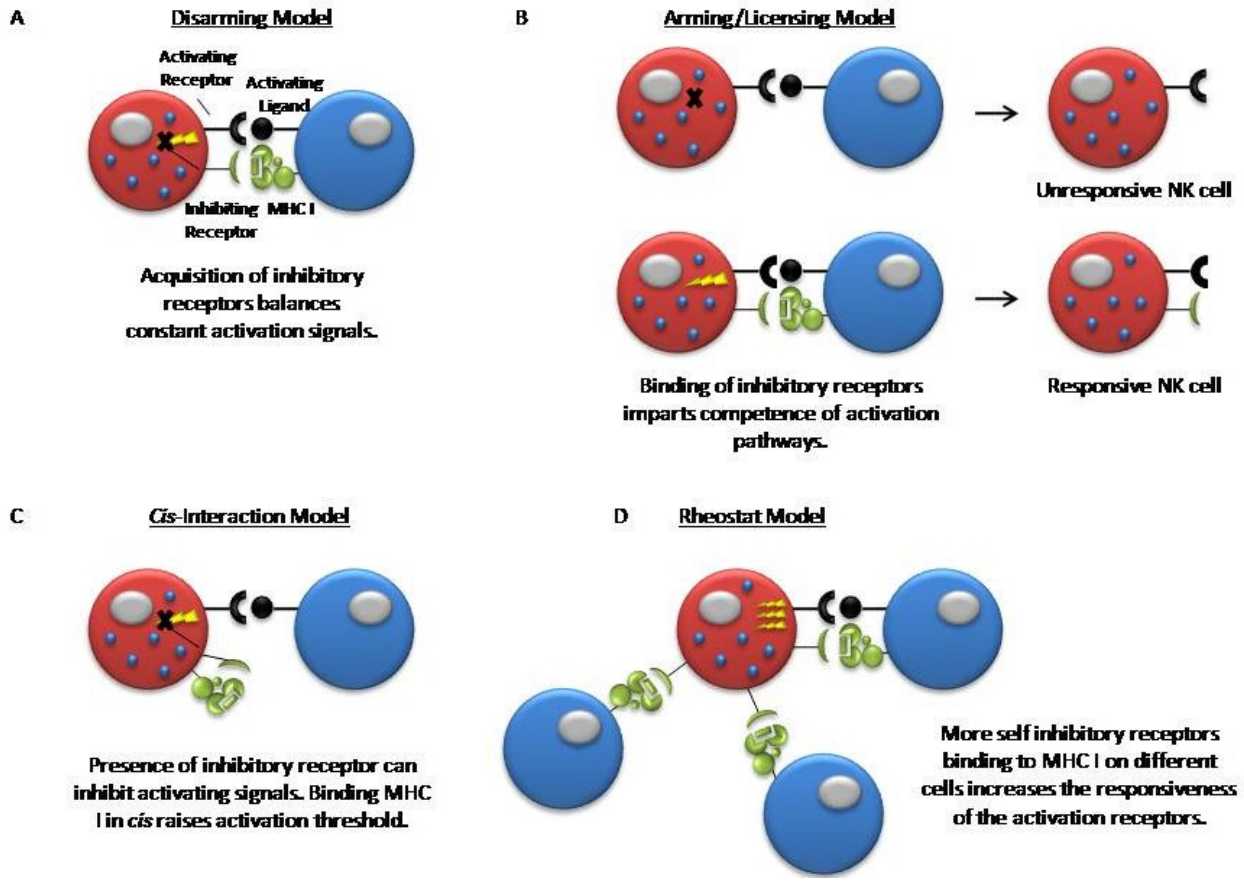

Figure 1.2 *Models of NK Education*
(A) Disarming model, (B) Arming/Licensing model, (C) *Cis*-interaction model and (D) Rheostat model.

The final model, the rheostat model (28, 113), states that the response received from the activation molecules is related to how many self inhibitory receptors the NK cell expresses and the affinity of those receptors to MHC I. As the NK cell expresses more inhibitory receptors to self MHC I, the responsiveness of the activation receptors increase and the interactions with MHC I were required for this response (Figure 1.2D) (113).

These models explain how expression of inhibitory receptors to self molecules are required for proper establishment of the activating pathways. When developing, immature NK cells without inhibitory molecules binding to self MHC I, would be constitutively stimulated, would become anergic and stay in an immature stage. Mature NK cells in the periphery would also become hyporesponsive owing to the constant stimulation. Thus establishing that NK cells become tolerant to self cells by interaction with self MHC I, inhibiting aggression towards self. Once the cell is educated, it can perform in the periphery during times of stress and infection. The next section will discuss the types of functions NK cells carries out during these times.

1.3 NK cell Functions

NK cells function to protect the body from stressed, mutated and infected cells. The NK cell surveys the body using receptors on its surface to distinguish a healthy cell from a sick cell. They also receive signals from other immune cells, such as DCs or macrophages, or from the sick cell itself (79). These signals can come in the form of cytokines released from the immune or sick cells. In response to physical interaction with other cells or with cytokines in the environment, NK cells perform different functions such as cytokine production and direct lysis of a targeted cell. These processes are discussed further in this section.

1.3.1 Cytokines produced by NK cells

NK cells produce numerous different cytokines and chemokines. In response to an infection, IFN-γ, the primary cytokine secreted, has influential effects on infection. IFN-γ is produced mainly by some innate cells, such as NK and NKT cells, and some adaptive cells, such as Th1 $CD4^+$ and $CD8^+$ T cells (231). NK and NKT cells can rapidly generate and secrete IFN-γ when required because they constitutively express IFN-γ message RNA (mRNA), whereas the T cell subpopulations must gain the ability to transcribe the IFN-γ gene over several days following activation (231). IFN-γ is a type II IFN and unlike type I IFNs (IFN-α and IFN-β) that are stimulate in response to virus, it is induced primarily owing to activation with immune cells and inflammatory cytokines (231).

IFN-γ has many roles in controlling the infection. Firstly, IFN-γ can upregulate both MHC I and II antigen presentation, increase the production of MHC I and II subunits, the Transporter Associated with Antigen Processing 1 (TAP1), TAP2, the invariant chain, and the expression and activity of the proteasome inside infected cells (231). Secondly, IFN-γ aids in the activation of macrophages by increasing their phagocytosis, production of proinflammatory cytokines and antimicrobials, such as superoxide radicals, hydrogen peroxide and nitric oxide (231). Thirdly, IFN-γ can polarize the T cell response towards antiviral functions by inhibiting differentiation of IL-4 and IL-17 producing $CD4^+$ T cells and causing them to become pro-inflammatory Th1 cells (180). Fourthly, IFN-γ can cause B cell switch classes to Immunoglobulin (Ig) G (83). Lastly, IFN-γ can exercise direct antiviral activity on infected cells by inducing cellular pathways that interfere with steps of viral replication (231).

IFN-γ is also generated upon bacterial and cancer conditions. It was observed that human patients or mice who had deficiencies in the IFN-γ receptor were highly susceptible to

mycobacterial infections (115, 116, 173). The first observation that IFN-γ was produced and used in cancer came from the use of immunogenic methylcholanthrene (MCA), which can cause fibrosarcomas in BALB/c mice (67). When these MCA tumours were transplanted into other mice, they were rejected following sub-lethal doses of lipopolysaccharides, which could be inhibited with neutralizing monoclonal Abs to IFN-γ (67). It was later shown that IFN-γ insensitive mice that were either deficient in the IFN-γ receptor 1 subunit ($IFN\gamma R^{-/-}$) or in Signal transducers and activators of transcription 1 (STAT1 ($STAT1^{-/-}$)), could develop tumours generated by treatment with MCA, more rapidly and more frequently than wild type mice (117). Thus demonstrating a role for IFN-γ in bacterial and cancer surveillance.

NK cells secrete several cytokines and chemokines other than IFN-γ, in response to cytokines produced by other cells during infection, such as IL-12 from DCs. These cytokines include TNF-α, Granulocyte Macrophage colony stimulating factor (GM-CSF), IL-5, -10, and -13 as examples (5). TNF-α is a proinflammatory cytokine first found to have a role in tumor and bacterial response (3), however it is also known to have a major role in response to infection. Studies have shown that pre-treating cells with TNF-α can inhibit viral replication of Vesicular Stomatitis virus, Encephalo-myocarditis virus and Herpes Simplex virus (8). This treatment can also inhibit any cytopathic effects of these viruses on the infected cells (8). These antiviral effects of TNF-α occur through binding with the TNF receptor 1 (R1) or TNFR2 since removal of both these receptors in mice cause increased amounts of both Vaccinia virus and Ectromelia virus in mice in comparison with mice that have these receptors (10). However, TNF-α antiviral effects may only be seen through interaction with TNFR1 and not TNFR2 (14), and response to infection is specific to the virus (7, 9). The effects of binding TNF-α to these receptors will be discussed further below.

GM-CSF is a hematopoietic growth factor produced not only by NK cells, but also by other immune cells including macrophages and T cells, and other cells such as endothelial cells and fibroblast as reviewed by Shi *et al* (11). The secretion of GM-CSF can have an effect on different immune cells. It is critical for the development and maturation of DCs (11). GM-CSF secretion can enhance cytokine production and antibody dependent and independent cell cytotoxicity of both monocytes and macrophages (4, 6, 13, 15). GM-CSF can increase the cytolytic activity and number of $CD8^+$ lymphokine-activated killer cells (2), though most effects of GM-CSF may work indirectly though interactions with antigen presenting cells (12). Furthermore, studies have shown that GM-CSF causes enhanced antigen presentation of DCs (1). The functions of these specific cytokines discussed in this section and others produced by NK cells, demonstrate the numerous ways that NK cells can aid in controlling infection by inducing effects on many other cell types involved in the response. However, NK cells can also have a direct effects on infected cells by killing them in processes discussed in the following section.

1.3.2 NK cell Mediated Cytotoxicity

As discussed above, during infection, NK cells produce copious amounts of cytokines that aid in the immune response. During this response, Type I IFNs produced from other cells can promote cytotoxic function in NK cells. NK cell lysis of a targeted cell can occur by two main pathways. The main pathway for cytotoxicity is by the degranulation of lytic granules through a synapse formed connecting the NK cell and its target (45, 177). Target cell lysis also can be triggered by death ligands that lead to apoptosis of the target cell. In this project, we detect degranulation of NK cells when incubated with poxvirus infected cells and target cell death by flow cytometry. However, we will introduce both types of cytotoxicity to give a complete picture of NK cell function.

NK cell degranulation occurs when the NK cell binds to a target and receives signals to become activated. This takes place when activation receptors on the NK cell surface bind to their ligands on the target cell and/or there is a loss in inhibition signals going to the NK cell. This binding of receptor to ligand forms the immune synapse to which the lytic granules will be excreted (23). The release of the granules, liberates perforin, granzymes and granulysin onto the surface of the target. This degranulation event can be detected by the surface expression of lysosomal-associated membrane proteins (LAMP), such as CD107a (LAMP-1) or CD107b (LAMP-2), that also serve to protect the NK cell from its own proteolytic enzymes (4, 50).

Inside the granule, perforin is found in a soluble monomer, that upon release through the synapse, anchors to the target cell surface and polymerizes in the presence of calcium to form cylindrical pores (Figure 1.3A) (258). Binding of perforin to the target cell surface is also able to provoke invaginations and formation of internal vesicles in the target cell that stimulate an endocytosis-like event prior to pore formation (Figure 1.3B) (199). On its own in high concentrations, perforin can cause the cell to die by osmotic lysis (258). However, it also serves as a passageway for granzymes and granulysins to enter the target cell and exert their functions.

Granzymes are globular soluble proteins that belong to the serine protease family. Although there are about twelve different granzymes identified and only five that are found in humans, granzymes A and B are the most abundant in the lytic granules and are released as a multimolecular complex (260). These proteases cause apoptosis in both caspase-dependent and -independent manners. Granzyme A cleaves proteins and induces caspase-independent apoptosis by slicing nicks into single stranded Deoxyribonucleic acid (DNA) (Figure 1.3C) (23, 292). Granzymes A and B both also cleave lamins (Figure 1.3D) that are required for nuclear reassembly after mitosis (292). Granzyme B cleaves proteins and causes apoptosis by either

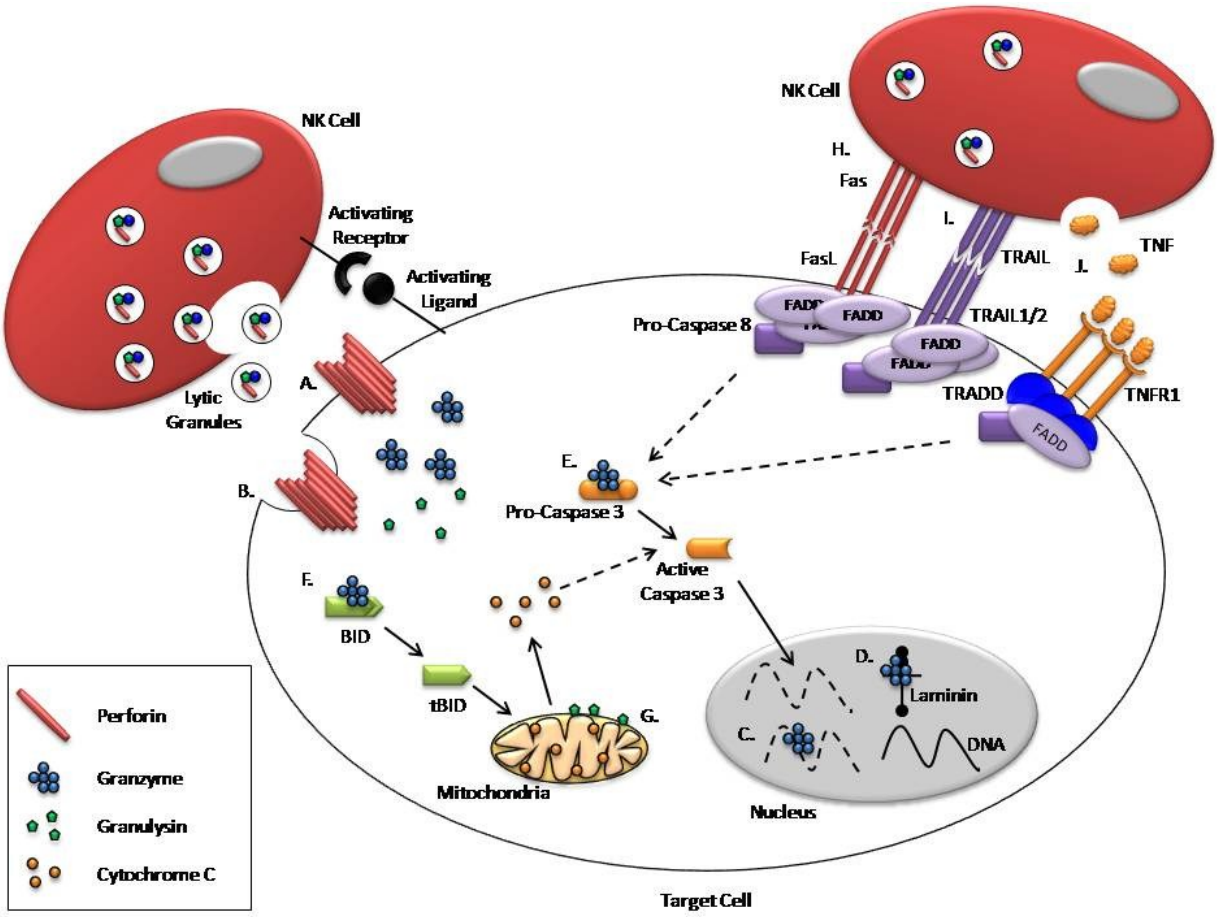

Figure 1.3 *Methods of NK Cytotoxicity*
Upon activation of NK cells, perforin is released into the immune synapse where it will either (A) bind to the target cell membrane and make pores or (B) cause invaginations of the membrane, Granzymes can either (C) cause nicks in the DNA, (D) cleave laminin, (E) activate BID or (F) directly activate Caspase 3. (G) Granulysin can interact with the mitochondria to cause the release of Cytochrome C. (H) Fas on NK cells bind FasL on the target cell, (I) TRAIL on NK cells binds TRAIL1 or TRAIL2 on target cells and (J) TNF from the NK cells bind to TNFR1 on target cells all to cause a cascade activation of Caspase 3 for cell death.

directly activating caspase 3, an executioner caspase, (Figure 1.3E) or by promoting the permeablization of the mitochondrial membrane by cleaving the BH3 interacting-domain death agonist (BID) (Figure 1.3F) (162, 190).

Another enzyme found in the lytic granules is granulysin. Granulysin is a small cationic protein that interacts with the negative charges on the mitochondrial membrane lipids to induce membrane damage, and the release of Cytochrome C (Figure 1.3G) (121, 181). Together, these proteins in the degranulation pathway of cytotoxicity are potent activators of target cell death. Although these are very effective way for the NK cell to kill a target cell, NK cells have also evolved a second pathway to deal a death blow to their target cells.

Death ligands are found on the surface of NK cells. They bind to their receptor on the target cells to cause apoptosis of the target cells. All of these receptor/ligand pairs belong to the TNF or TNF receptor families. The receptors contain a death domain on their cytoplasmic tails that when engaged by their ligand, come into contact with the death domain of an adaptor protein. The adaptor protein recruits and activates caspase 8 which, downstream, activates the executioner caspase 3 to cause apoptosis of the target cell as reviewed by Bonavida (23). One such death ligand is FasL (CD178). FasL is found in trimers on NK cells or in soluble form (23). It binds to the receptor Fas (CD95) on the target cell and associates with Fas-associated death domain (FADD) (Figure 1.3H) (23). Another death ligand is called TNF-related apoptosis-inducing ligand (TRAIL) which binds to either TRAIL1 (Death receptor 4 (DR4)) or TRAIL2 (DR5) on the target cell (Figure 1.3I) (179, 273, 281). The final death ligand is the cytokine TNF. It is produced by a wide range of cells including macrophages, T cells and NK cells, and can cause inflammation, cell activation, migration, proliferation and apoptosis of the target cell (23, 26). TNF is found both on the cell surface and in soluble form in trimers (26). These forms

of TNF bind to different TNF receptors, soluble to TNFR1 and membrane bound to TNFR2, and can cause different outcomes for the target cell (26). TNFR1 is associated with pro-inflammatory pathways and programmed cell death (Figure 1.3J), whereas TNFR2 is associate with tissue repair and angiogenesis (26).

From these different pathways to target cell death, it is understood that NK cells express many different receptors and ligands on their surface that can cause it to become active and kill the targeted cell. Together with the activating receptors, NK cells also have inhibitory receptors on their surface that function to restrain the NK cell from killing randomly. Subsequently, we will now discuss the receptor repertoire of NK cells.

1.4 NK cell receptors for Target Cells

Activation receptors on the NK cell bind to a range of ligands on a target cell, which, owing to their positively charged transmembrane domains, are associated with an immunoreceptor tyrosine-based activating motif (ITAM) bearing adaptor molecules. When activated, tyrosines in the ITAM are phosphorylated by Src family protein tyrosine kinases (PTKs). Signalling through the ITAM adaptor molecule initiates activation of the NK cell (Figure 1.4A). On the other hand, inhibitory receptors on the NK cell, bind to mostly MHC I molecules, although other ligands for specific receptors have been found and will be discussed shortly. Unlike the activating receptors, the inhibitory receptors bear a immunoreceptor tyrosine based inhibitory motifs (ITIM) on their cytoplasmic tail. When inhibitory receptors are bound to ligands and co-cluster with activating receptors, the ITIM is phosphorylated by PTKs which lead to a cascade that causes activation of phosphatases that dephosphorylate ITAMs, instigating inhibition of the NK cell functions (Figure 1.4B). The Dynamic Equilibrium hypothesis explains how the balance of activating against inhibitory signals received by the NK cell determine what

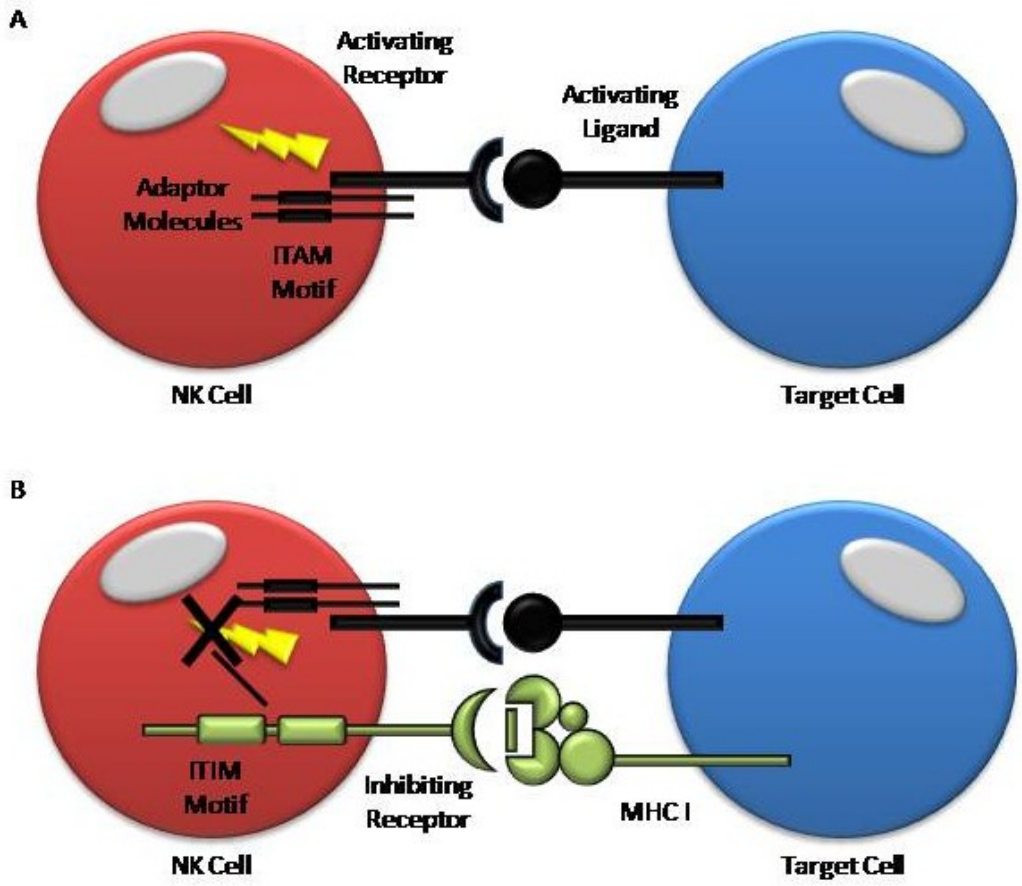

Figure 1.4 *NK Activation versus Inhibition*
(A) Binding of an activating receptor to its ligand on the target cell causes activation signals through the ITAM motifs on adaptor molecules for activation of the NK cell. (B) Binding of inhibitory receptors to MHC I on the target cell causes signals through the ITIM motifs in the cytoplasmic tails of the inhibitory receptors, leading to the inactivation of stimulating signal and inhibition of NK cell function.

course of action the NK cell will take as reviewed by Farnault *et al* (78). As NK cells survey the body, they constantly come into contact with molecules on other cells that can activate them. As a result of expressing inhibition receptors, NK cells also receive stop signals from those same cells. Inhibition signals normally override activation signals. During stress, viral infection or tumour mutations, activation ligands can be increased on the target cell that in turn give enough stimulation to NK cells to overcome the threshold of inhibition. Viral infections have also been seen to down regulate MHC I molecules in order to hide from the adaptive immune response, however, this decrease in MHC I leads to less inhibition signals going to the NK cell and therefore causes its activation. There are several families of both human and/or mouse activating and inhibitory receptors that are highly relevant to this thesis: Killer Immunoglobulin-like receptors (KIRs), Natural Cytotoxicity Receptors (NCRs) and the C-type lectins. These receptors will now be discussed.

1.4.1 NK Activating Receptors

Activating ligands on target cells are expressed on distressed cells. These distress beacons bind to activating receptors on the surface of NK cells to initiate NK cell functions. There are several types of activating receptors on human and mouse NK cells. For example, freshly isolated epithelial and fibroblast cells express MHC class I chain related (MIC) proteins when under stress (91, 296). When expressed under duress, these MIC proteins bind to the NKG2D receptor found on NK cells.

NKG2D is a member of the NKG2 family of receptors is found on both human and mouse NK cells. As discussed above, it binds to stress induced molecules on the target cells, such as MIC A and B and UL16-binding proteins 1-6 (ULBPs) in humans (268). In the mouse, NKG2D binds to retinoic acid early inducible-1 (RAE-1), Histocompatibility antigen 60 (H60)

and murine ULBP-like transcript 1 (MULT-1) (268). These stress molecules can be expressed on the cell surface in response to infection, heat shock, DNA damage and tumorigenesis (184). Unlike the other members of the NKG2 family, NKG2D is the only one that does not couple with CD94, but instead directly associates with the ITAM bearing molecule DAP10 to trigger an activating signal cascade (284).

Both humans and mice NK cells express the immunoglobulin gamma F_c receptor III ($F_c\gamma$RIII), also known as CD16. When cells become opsonised by antibody (Ab), NK cells use CD16 to recognize the Fc portion of the receptor and exert antibody dependent cell cytotoxicity (ADCC) (268). ADCC causes the NK cell to secrete cytokines and perform cytotoxicity on the opsonised cell (34).

The NCRs are members of the immunoglobulin superfamily (Ig-SF) and are strictly found on NK cells. There are three members of the NCRs: NKp30, NKp44 and NKp46. NKp30 is found on all immature and mature NK cells (187). NKp30 binds to human leukocyte antigen (HLA) -B-associated transcript 3 (BAT3) found on DCs and induces cytokine secretion (192). During a human Cytomegalovirus (HCMV) infection, NKp30 binds to HCMV's main tegument protein, pp65, which remarkably suppresses cytotoxicity of this activating receptor, owing to the dissociation of CD3ζ, which aids in signal transduction (10). The second NCR, NKp44, is only found on IL-2 activated NK cells and is considered the first marker specific for activation of NK cell (38, 266). NKp44 binds to the HA of Influenza virus and the HA-neuraminidase (NA) of Sendai virus (11). Binding of NKp44 to these viral proteins causes lysis of the infected cell (11). The final NCR is NKp46 which is expressed on resting and activated mature NK cells and immature NK cells (152). NKp46 binds to the HA of Influenza virus and the HA-NA of Parainfluenza virus to cause lysis of the infected cell (152) and also has endogenous ligands.

All three of the NCRs also recognize and lyse Vaccinia virus (VACV) infected cells (47). Although NKp30 fusion proteins bind to VACV infected cells more readily than fusion proteins for NKp44 and NKp46, all of these proteins can recognize ligands on the surface on VACV infected cells (47). However, exactly what ligand(s) these proteins are binding to are currently unknown.

As was discussed above, NK cells require inhibitory receptors to normally restrict signals from the activating receptors and to cause the activating receptors to be functional. These inhibiting receptors on NK cells will now be discussed.

1.4.2 NK Inhibitory Receptors

Inhibitory receptors are the main factors which prevent NK cells from constantly attacking the body. They provide strong signals that supersede the activation of the NK cell during persistent stimulation of activation ligands present on other cells. In order for the NK cell to become activated one of two events must occur. Either the target cell begins to express more activation ligands that will overcome the inhibition signals, or the target cell losses inhibition signals, such as the loss of MHC I, which causes the cell to only receive activation signals. The ability of NK cells to sense a lack of self MHC I was first described in 1986, suggesting that NK cells inspect cells searching for a deletion or reduction in the expression of self MHC, which would cause killing of that cell (119). They are able to do this with a set of inhibitory receptors specific for MHC I.

The mouse Ly49 family of receptors contains several inhibitory members. The prototypical family members are Ly49A and Ly49C. They contain ITIM motifs in the their cytoplasmic tail that upon binding to their ligands become phosphorylated by kinases to send a

signaling cascade of inhibition. Ly49A binds to the mouse MHC I molecules H-2D(d) or the non classical MHC I molecule H2-M3, to cause inhibition of NK cells (6, 62). Ly49C can bind to the MHC I molecules H-2D(b), H-2K(b) and H-2D(d) to inhibit NK function (249). Ly49C also binds to the mouse Cytomegalovirus (MCMV) viral protein m157, leading to viral evasion (19, 54).

The human analogue to the mouse Ly49 receptors are the KIR molecules which contain both inhibitory and activating receptors. The inhibitory KIR receptors bind to classical MHC I molecules and contain two ITIM motifs in their long cytoplasmic tails. Upon binding to MHC I, the ITIMs are phosphorylated and associate with the tyrosine phosphatase SHP-1 to cause inhibition of activation (36). The inhibitory KIR have two (KIR2DL) or three (KIR3DL) extracellular Ig-domains that bind to either HLA-C or HLA-A/B allotypes respectively (254). Although they are the most studied receptors, the Ly49 and KIR families are not the only family of receptors that can inhibit NK cell function.

The NKG2 family of receptors also contains the inhibitory NKG2A receptor that is found in both humans in mice. NKG2A is found as a heterodimer and couples with CD94, which may aid in stabilizing the dimer on the cell surface (142). In humans, CD94/NKG2A binds to the non-classical MHC I molecule HLA-E (25, 27), whereas in mice CD94/NKG2A binds to Qa-1(b) (263). Binding of HLA-E/Qa-1(b) to CD94/NKG2A causes inhibition of NK cell function. Another lineage of receptors, important to this thesis, are the NKR-P1 family of both activating and inhibitory receptors.

1.4.2.1 NKR-P1 Family of Receptors

The discovery of the NKR-PI family of receptors came about from the endeavour of trying to purify NK cells from the mouse. An antisera, α-NK, was made from immunizing C3H mice with CE thymocytes (90). This antisera was able to diminish NK activity from C57BL/6 and NZB splenocytes against RL1 tumours but did not react with T or B cells (90). The NK alloantigen that bound to the antisera was soon termed NK-1 and was used as a standard to define and purify NK cells (133).

Expression of the NK1.1 epitope was found to be strain specific, only identifying NK cells in CE, C57BL/6, NZB, C58, Ma/My, ST and SJL mice, but not in BALB/c, AKR, CBA, C3H, DBA and 129 mice (90, 134). Blocking this molecule did not alter killing, however, in the presence of the anti-NK1.1 antibody PK136, IL-2 activated NK cells from C57Bl/6 mice, but not BALB/c mice, were induced to lyse Daudi cells that are normally resistant to NK cell lysis (118).

Other NK alloantigens were soon identified (NK2.1, NK3.1, NK4.1, etc) and they were found not only to be genetically related to NK1.1, but also mapped to a similar region of chromosome 6 (37, 234). Within this region, the *Ly49* genes were also found and this section of chromosome 6 was soon called the NK gene complex (NKC) (Figure 1.5) (287, 289). Later, it was discovered that NK1.1 was encoded by the *Nkrp1c* gene, that it was included amongst several genes that belonged to the NK receptor-p1 (Nkrp1) family and that it could function as a NK activator (88, 89, 222).

Subsequently, another NK1.1 antigen, NKR-P1B, was found and seen to inhibit NK cell function (40, 138). Although the *Nkrp1b* gene is said to be cloned from C57BL/6 mice, studies have recently determined that these mice actually express a related *Nkrp1d* transcript that

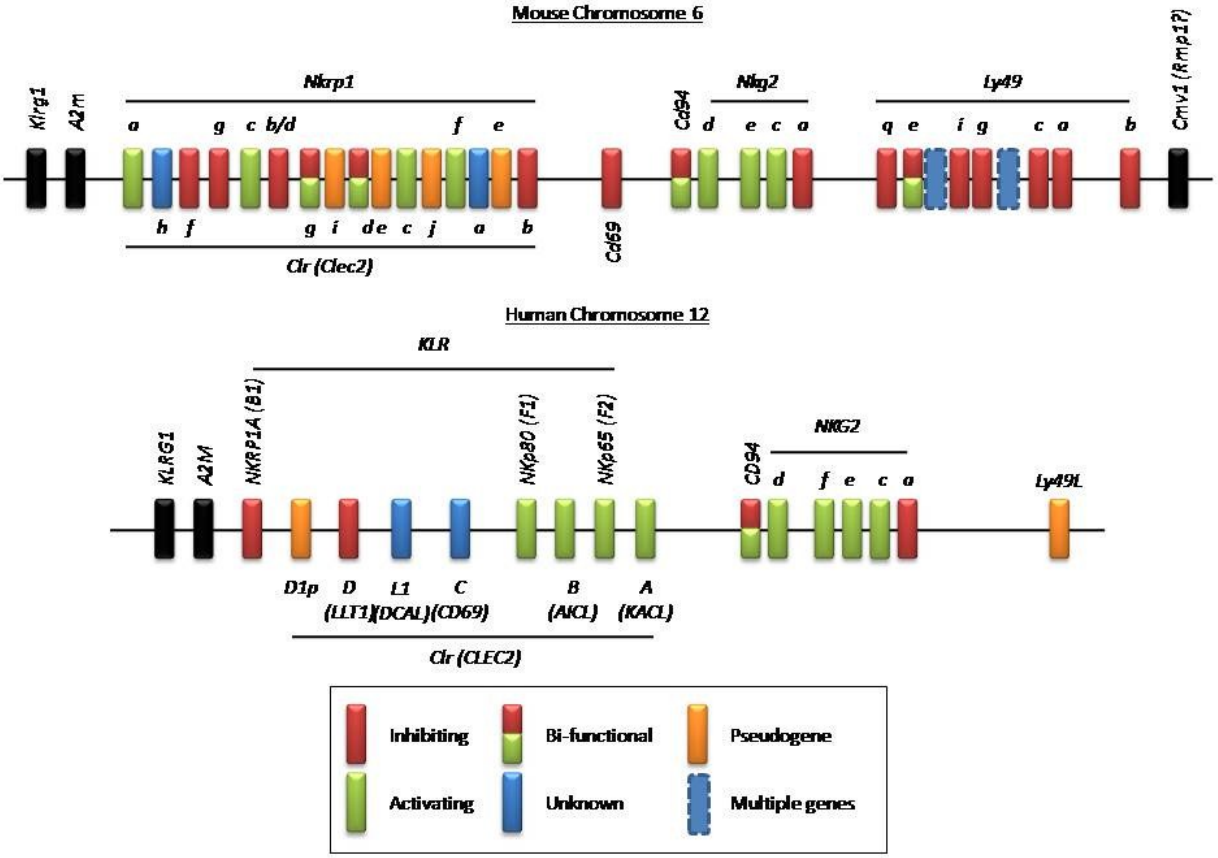

Figure 1.5 *Natural Killer Gene Complex*
The NKC complex for mouse (top) and human (bottom) found on chromosomes 6 and 12, respectively. Genes have been color co-ordinated to indicate function, whereas black genes are outside of the NKC.

encodes a divergent but functionally similar inhibitory receptor, that does not react with the PK136 monoclonal Ab (40, 89, 138). Expression of NKR-P1B seems to be restricted to specific mouse strains including NIH-Swiss, SJL, FVB and CD-1 (39, 40, 138, 146). NKR-P1B has an ITIM in its cytoplasmic tail that associates with SHP-1 when phosphorylated (40, 138). Cross-linking the inhibitory NKR-P1B and the activating NKR-P1C on NK cells demonstrated no killing of the target cells, suggesting inhibitory control over stimulation with NKR-P1C that was not witnessed with stimulation with CD16 (40).

NKP-P1B binds to a C-type lectin-like molecule, that maps to the NKC within the *Nkrp1* genes (Figure 1.5) (39, 109). These molecules were previously identified as playing a role in osteoclastogenesis, and were found to be a family of genes termed the osteoclast inhibitory lectin (Ocil), C-type lectin-related (Clr) or C-type Lectin Domain Family 2 (CLEC2) genes (now known as Clr) (191, 293, 294). Two of the known Clr proteins that have been identified for binding to NKR-P1 are Clr-b and Clr-g. Clr-b binds to the inhibitory receptor NKR-P1B and Clr-g binds to the stimulatory receptor NKR-P1F (39, 109). The 4A6 monoclonal Ab specific for Clr-b determined that Clr-b is expressed broadly on all hematopoietic cells in a manner similar to MHC I (39). The fact that Clr-b is habitually down regulated on tumor cells *in vivo* and *in vitro*, possibly to alert the immune system of an aberrant cell cycle, suggests that it has a role in missing self recognition (39, 203).

This newly identified missing self recognition system is substantiated by several aspects. Firstly, because of its down regulation on tumor lines when compared to normal cells *ex vivo* (39). Secondly, tumour cells expressing high endogenous Clr-b can inhibit their lysis by NK cells, which can be reversed by blocking Clr-b with 4A6 Ab (39). Thirdly, Clr-b expression is high on most hematopoietic cells, low on double positive thymocytes and not observed on

erythrocytes, similar to MHC I (39). Fourthly, Clr-b$^{-/-}$ bone marrow transplanted cells are rejected in C57Bl/6 mice in a NKR-P1B^{hi} NK cell dependent manner similar to MHC I$^{-/-}$ cells (46). Together, these observations demonstrate the idea of another system other than MHC I for NK cells to determine self from non-self.

To this date, only one human NKR-P1 receptor is known that maps to chromosome 12, from the *KLRB1* gene in the human NKC (Figure 1.5) (140). It is a glycosylated, disulfide-linked homodimer, type II membrane protein called NKR-P1A (also known as CD161 or Killer cell lectin-like receptor subfamily B, member 1 (KLRB1)) (140, 269). It is a functional homologue of the mouse NKR-P1B receptor as they are both inhibitory receptors. There may also be a divergent NKR-P1A homolog from the *KLRF1* gene, NKp80, that also maps to the human NKC (Figure 1.5) (214, 267). The amino acid sequence of the human NKR-P1A receptor shows 46% homology to the rat NKR-P1 molecule and 46-47% homology to the three mouse NKR-P1 proteins, NKR-P1A, NKR-P1B and NKR-P1C (140). Human NKR-P1A is found on the majority of CD56^{+} NK cells, although a subset of NK cells are negative (140). NKR-P1A is also found on a variety of subsets of T cells including, CD3^{+} T cells expressing a αβ-T cell receptor (TCR), γδ-TCR, CD4 or CD8 molecules, memory T cell, NKT cells, and Th17 T cells (56, 74, 140, 252). NKR-P1A is also found on resting CD14^{+} peripheral blood monocytes, *in vitro* derived DCs (197) and type 2 innate lymphoid cells (164).

On immature NK cells, NKR-P1A may act as an activating receptor for CXCL8 secretion when cross-linked with monoclonal Ab. In contrast, on mature NK cells NKR-P1A is an inhibitory receptor as it does not induce cytotoxicity against FcR^{+} targets when cross-linked with an α-hNKR-P1A monoclonal Ab (140, 165). When NK clones were incubated with targets, the spontaneous killing of the targets were either partially or completely inhibited when monoclonal

Abs were added (140). This is opposite from the proposed NKR-P1A homolog, NKp80, which is an activating receptor (131). On T cells, NKR-P1A has been shown to be important in migration of γδ-TCR$^+$ T cells, and proliferation of NKT and immature T cells (74, 193, 195, 196, 198). It also seems to play a role in activation and secretion of cytokines in monocytes and DCs (197).

The ligand for NKR-P1A was discovered and was termed Lectin-like transcript 1 (LLT1) (Figure 1.6) (3, 217). The protein was first discovered in 1999 and the transcript is 43-48% homologous to the mouse Clr molecules at the amino acid level (3, 22). Since LLT1 is so similar in sequence and structure to the mouse Clr proteins, it was given a synonymous gene designation, CLEC2D (128). LLT1 also shares high similarity to other gene products in the human NCR: Keratinocyte-associated C-type lectin (KACL (CLEC2A)), Activation-induced C-type lectin (AICL (CLEC2B)) and CD69 (CLEC2C) (Figure 1.5) (22, 96, 242, 269). Like the mouse Clr molecules, the CLEC molecules are genetically linked to their receptors in the NKC: NKp65 binds to KACL, NKp80 binds to AICL, and the binding partner for CD69 is currently unknown (Figure 1.5 and 1.6) (22, 131, 242). LLT1 is also known as human Ocil because a human Ocil was discovered in 2004, however its cDNA encoded a protein identical to LLT1 (107).

LLT1 is only expressed on activated cells, such as toll-like receptor (TLR) activated plasmacytoid DCs and monocyte derived DCs, NK, T and B cells (86, 218), in contrast to Clr-b that is found on all cells. It is also found on glioblastoma cells and respiratory epithelial cells (220, 225). Very recently, the CLEC2D gene was found to have alternative splice transcript variants (85). Isoforms 1 (LLT1), 2 and 4 have transmembrane domains, isoform 3 is a nonsense RNA decay candidate, and isoforms 5 and 6 are soluble forms of isoforms 1 and 4, respectively (85). Of the three isoforms with a transmembrane domain, isoform 1 may be the only one that is

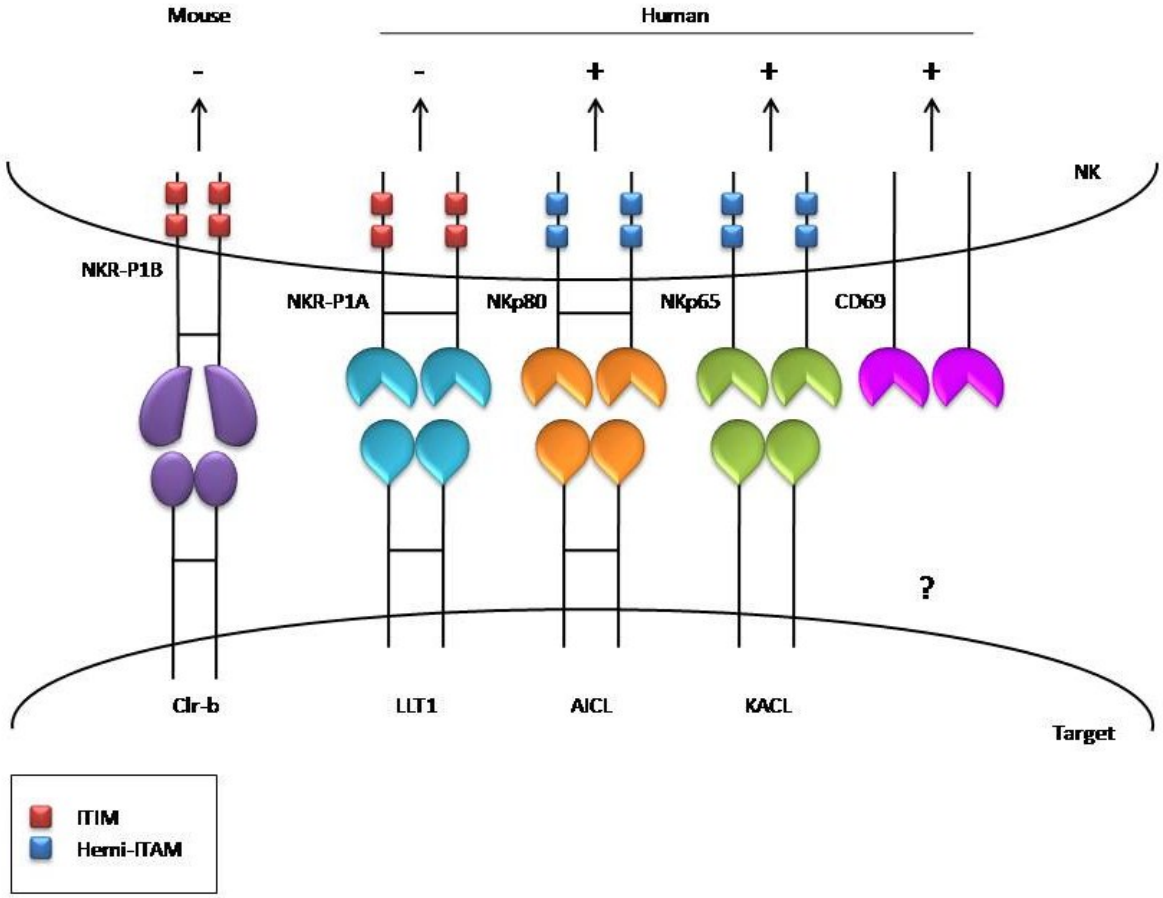

Figure 1.6 *Receptor Ligand Pairs of the NKC*
Receptors from the NK cell (top) encoded in the NKC bind to ligand on target cells (bottom) that are also encoded in the NKC.

expressed at the cell surface and binds to NKR-P1A, whereas isoforms 2 and 4 may remain in the endoplasmic reticulum, based upon their sensitivity to endoglycosidase H activity (85). Isoform 1 is expressed on the NK-92 lymphoma cell line, and when this ligand is bound to NKR-P1A transfected into target cells, these NK-92 cells are activated to secrete IFN-γ (14). However, binding isoform 1 on target cells to NKR-P1A on NK cells, inhibits NK cell cytotoxicity and IFN-γ production (3, 86, 217, 218, 221). This is different from binding isoform 1 on target cells to NKR-P1A on T cells, where IFN-γ production was stimulated when CD3 was engaged as well (3). Thus demonstrating that NKR-P1A may have alternative functions in different cell types.

Knowing that Clr-b has a function in NK cell recognition of self, we anticipated that human CLEC2D would have a similar purpose. Interestingly, isoform 1 of CLEC2D is upregulated on cells exposed to Human Immunodeficiency virus (HIV) and Epstein Barr virus (EBV), or when infected by Respiratory Syncytial virus (RSV) (86, 225). However, expression of CLEC2D has not been described for a poxvirus infection.

1.5 NK Cells Response to Virus

During a infection, macrophages and DCs are known to act quickly to the invasion by phagocytizing the invading microbe. The digestion of the microbe activates these cells and causes them not only to present antigens from the microbe, but also to secrete cytokines and chemokines. These secreted proteins lead to the activation of immune cells and the migration of immune cells, such as NK cells, to the site of infection. NK cells are thought to be important in the response to several infections such as Varicella Zoster virus (VZV), HSV, EBV and HIV-1, etc, as reviewed by Guilmot *et al* (94, 170). This importance may be inferred by the way these viruses seem to have evolved methods to subvert NK cell functions. For instance, crosslinking CD81 on NK cells by the Hepatitis C virus (HCV) envelope protein E2 *in vitro*, leads to

decreased IFN-γ production and cytotoxicity (59). Although E2 does not crosslink CD81 when it is part of a viral particle, HCV virions engage CD81 on NK cells and reduce their cytokine production and anti-viral activity (58, 290). In another example, Influenza virus is known to directly infect human NK cells through clathrin and caveolin dependent routes and kill the infected NK cell (154, 166).

The most widely studied infection mouse model for the role of NK cells in infection is Cytomegalovirus (CMV). While NK cells express different receptors and exert various functions to kill CMV infected cells, the virus has also evolved its own methods to challenge NK cell control over the infection. MCMV encodes several proteins that are known to interfere with the immune response. The m152 glycoprotein down regulates expression of MHC I molecules on the surface of infected cells by obstructing the export of MHC I from the endoplasmic reticulum (ER)-Golgi intermediate compartment/cis-Golgi compartment (295). The m06 glycoprotein binds to MHC I in the ER and redirects it to be degraded in lysosomes (211). The loss of surface MHC I on infected cells causes them not be able to present viral peptide to $CD8^+$ T cells and thus not killed by them, but the loss should make the cell susceptible to NK cell lysis. To protect the infected cell from NK cells, the virus also encodes a third protein that affects MHC I expression. The m04 glycoprotein binds to MHC I in the ER, however this complex is transported to the surface of the cell where it engages inhibitory receptors on NK cells (12, 105, 130). Thus demonstrating how MCMV evades both arms of the immune system by interfering with surface expression of host proteins and expressing decoy proteins.

Another method MCMV uses to evade NK cell function is to down regulate ligands for activating receptors. NKG2D, as discussed in section 1.5.1, binds to stress molecules such as RAE-1, H60 and MULT-1. MCMV encodes for several proteins that inhibit expression of these

stress molecules. Along with down regulating surface MHC I expression, m152 protein reduces surface expression of all RAE-1 proteins, which in turns reduces IFN-γ production from NK cells (135, 148). The m155 protein causes the loss of expression of surface H60 protein from MCMV infected cells by targeting it for degradation in the proteasome (98, 149). The m145 protein reduces expression of MULT-1 on the surface of infected cells (136). The m138 protein, which encodes for a herpes viral Fc receptor, causes MULT-1 to be sent for degradation in the lysosomes and the down regulates surface expression of H60 by two separate methods (144). These data illustrate several proteins that MCMV uses to evade activation of NK cell and thus lysis of the infected cell.

Resistance to MCMV is known to be through the functions of the NK cell in certain mouse strains. In C57BL/6 mice, the activating Ly49H receptor binds to the MCMV glycoprotein m157 which causes activation, expansion and secretion of proinflammatory cytokines from the $Ly49H^+$ NK cells (8, 241). This activation of the $Ly49H^+$ NK cells that specifically recognize MCMV infected cells by expression of m157 leads to the lysis of those infected cells and rapid control of the infection (8, 241). However, in some strains of mice (129/J mice), m157 is a ligand for the inhibitory Ly49I receptor, which aids in immune evasion by the infected cell (54). This dual reaction of the m157 MCMV protein demonstrates how a virus evolved to escape detection but also how the host has evolved to overcome these methods of elusion. In MA/My mice, resistance is exerted through the activating Ly49P receptor that must bind the complex of $H\text{-}2D^k$ MHC I molecule with the MCMV m04 glycoprotein (124). Mice that express both Ly49P and $H\text{-}2D^k$ are resistant to wild type MCMV infection, however, deletion of m04 from the virus, causes the mice to lose their resistance, thus implying that the combination of the host and viral protein are required for Ly49P recognition (124).

NK cells are also now recognized to develop a memory antigen specific response to viral infections. While NK memory was first documented in recombination activating gene (RAG) -1 mice that developed contact hypersensitivity to haptens through hepatic NK cells (176), antigen specific response to viral infections were first discovered during a MCMV infection. As previously discussed the mouse activating Ly49H receptor on NK cells recognizes the MCMV viral protein m157. Due to the recognition of m157, the Ly49H$^+$ NK cell population expands during the acute phase of infection (8, 68, 241). Adoptive transfer of Ly49H$^+$ NK cells into mice lacking Ly49H$^+$ NK cells followed by infection with MCMV causes proliferation, contraction and then establishment of a long lived pool of Ly49H$^+$ NK cells that can be detected up to 70 days post infection (PI) (247). These memory NK cells upon re-stimulation with plate-bound Ab or m157-expressing targets, have enhanced IFN-γ production and degranulation by stimulus with antibody to a stimulatory receptor (247). This memory response seems to be dependent on IL-12 signaling since NK cells that do not express the IL-12 receptor do not proliferate in response to MCMV infection nor are they able to generate memory cells that can protect mice against a MCMV challenge (248). Like memory cells in the adaptive response, these memory NK cells have a distinct surface phenotype. They express higher levels of Ly49H, but no other activating receptor is upregulated, higher expression of KLRG1, CD43 and Ly6C, decreased levels of CD27, and more *Ifng* transcripts in comparison to naive NK cells (247).

The viral strategies of battling host immune defences discussed above in the mouse, are also seen during a HCMV infection. HCMV produces several proteins that abrogate NK cell functions. This virus encodes a MHC I homolog, UL18, that binds to the NK receptor Leukocyte Immunoglobulin-like Receptor (LIR) -1 with very high affinity relative to endogenous MHC I, and prevents LIR-1$^+$ NK cell lysis of the infected cell (15, 55, 202). The HCMV protein UL40

contains an amino acid sequence in its leader peptide that matches to the HLA-E binding peptide (259). Since HLA-E can only bind leader sequences of the classical MHC I and HLA-G, UL40 usurps this function by using it leader peptide to stabilize and upregulate HLA-E expression on the cell surface, independent of TAP, where it binds to CD94/NKG2A and inhibits NK cell activation (143, 259). HCMV also encodes for a protein, UL16, that is able to bind to the stress molecules ULBP1, ULBP2 and MICB to retain them in the ER so that they cannot be recognized by NKG2D which would activate NK cell functions (55, 137, 243, 279). Also, as was previously mentioned, NK cells are inhibited through NKp30 binding to HCMV protein pp65 by the dissociation of CD3ζ (10). These are just a few of many HCMV proteins that can inhibit NK cell recognition.

Similar to MCMV, HCMV also causes an expansion of NK cells that are CD94/NKG2C$^+$ (95). These NK cells expand when incubated with HCMV infected fibroblast but only in the presence of IL-15 and their expansion can be inhibited by blocking CD94 with antibody (95). This expansion is impaired when target cells are infected with a HCMV mutant lacking the US2-11 gene, suggesting that the ligand binding pair of CD94/NKG2C is US2-11 (95). Following this increase in the specific NK population, there is a contraction phase that leaves a fraction of CD94/NKG2C$^+$ NK cells in a long-lived pool with clonal expansion potential (171), demonstrating the development of NK memory in humans as well as in the mouse.

The final CMV strategy comes from the rat CMV (RCMV). RCMV encodes for a protein, rat C-type lectin (RCTL), that resembles rat Clr-b (270, 271). During a RCMV infection, rat Clr-b is rapidly down regulated, while RCTL is quickly expressed on the surface of infected cells (270). Expression of surface RCTL that binds to NKR-P1B on NK cells, leads to inhibition of NK cell lysis (270).

These evasion tactics of CMV had to evolve such that the virus could survive a NK cell attack. As was previously discussed, other viruses are known to have methods of eluding recognition of infected cells by NK cells. Given that it is known that resistance to the mouse causative agent of Mousepox, Ectomelia virus (ECTV), is linked to NK cells receptors encoded within the NKC (76), we decided to investigate if poxviruses have evolved mechanisms to evade NK cell detection via the NKR-P1 system as well. Before reviewing the literature on the role of NK cells in a poxvirus infection, I will provide an overview of poxviruses and their life cycle. I will focus on details that are relevant to the experiments described later in this thesis.

1.6 Poxviruses

Poxviruses are large, brick shaped, enveloped, double stranded (ds) DNA viruses that, unlike most DNA viruses, replicate solely in the cytoplasm (189). The *Poxviridae* family is broken down into two subgroups: *Entomopoxirinae*, poxviruses that infect insects, and *Chordopoxvirinae*, poxviruses that infect vertebrates. Here, the focus is on the latter. There are several genera within the *Chordopoxvirinae* subfamily (Table 1.1) that include virus groups that infect different types of vertebrates, from mice and birds up to larger animals such as humans and camels. Of note, the *Orthopoxvirus* genera contains well known species of viruses such as Variola virus, the causative agent of smallpox, and Vaccinia virus (VACV), the prototypical poxvirus used as a vaccine to eradicate smallpox.

1.6.1 Vaccinia Virus

VACV replicates in the skin of different mammals, though it replicates in the spleen and liver of mice. VACV replicates in many different cell types and depending on the type of cell and strain of the virus, it can replicate to varying titres, which will be discussed further below. This infection can be passed along by touching the infected site. Infection with VACV can cause

Table 1.1 *Chordopoxvirinae* Genus and Species

Genera	Species
Orthopoxvirus	Variola, Vaccinia, Ectromelia, Cowpox, Monkeypox, Rabbitpox, Taterapox, Buffalopox, Camelpox, Volepox, Raccoonpox, Horsepox
Parapoxvirus	Chamois contagious ecthyma, Orf, Pseudocowpox, Bovine papular stomatitis, Squirrel parapoxviurs
Avipoxvirus	Fowlpox, Canarypox, Juncopox, Pigeonpox, Quailpox, Sparrowpox, Starlingpox, Turkeypox
Capripoxvirus	Sheeppox, Goatpox, Lumpy skin disease
Leporipoxvirus	Myxoma, Hare fibroma, Rabbit (Shope) fibroma, Squirrel fibroma
Suipoxvirus	Swinepox
Molluscipoxvirus	Molluscum contagiosum
Yatapoxvirus	Yaba monkey tumor, Tanapox

Modified from Buller *et al*. 1991 and ViralZone (http://viralzone.expasy.org/all_by_species/174.html)

fever, rash, and with lesions that will scab over and heal in approximately 10-14 days (189). In immuno-compromised individuals, there can be serious complications such as progressive vaccinia (necrosis at infection site), eczema vaccinatum (papular, vesicular and pustular infectious rash), ocular vaccinia, etc (189).

VACV has a large genome of 196 kilobases (kbs) that encodes for approximately two hundred different proteins which aid in virus replication and host immune evasion (147). Its genome can be divided into a central region conserved in chordopoxviruses, that encode proteins mostly necessary for replication, and variable terminal regions that encode proteins for virulence and immune evasion, which represent approximately 50% of the virus genes (92). The virion contains a core, which surrounds the dsDNA and enzymes required for transcription of early genes, two lateral bodies, that contains numerous viral proteins and enzymes used for immune regulation (229), and this is all surrounded by one or more membranes which describes the different forms of the VACV virion (Figure 1.7A) (53, 168). The initial form of VACV is the intracellular mature virion (MV) which expresses a single lipid membrane and is thought to be very stable and mediate transmission of the infection between hosts (Figure 1.7B) (103, 104, 168). The MV can be further double wrapped in a modified trans-Golgi or endosomal membrane, termed the wrapped virion (WV) or the intracellular enveloped virus (IEV), and transported on microtubules to the periphery of the cell (Figure 1.7B) (168, 238). The IEV fuses with the cell membrane, and is exposed as a double membrane virus that is attached to the cell membrane termed the cell-associated enveloped virus (CEV), that causes actin polymerization under the cell membrane where the virus is attached (Figure 1.7B) (60, 276). The growing actin tail pushes the virion outward where it is released to infect neighbouring cells and the released virion is designated the extracellular enveloped virus (EV) (Figure 1.7B) (185, 240).

To begin infection, the MV binds to the host cell using four different viral envelope proteins. D8L binds to the glycosaminoglycan (GAG) protein chondroitin sulfate (106), A27L and H3L bind to the GAG protein heparan sulfate (49, 145), and A26L binds to the extracellular matrix protein laminin (48). Twelve other viral proteins, which are components of, or associated with, the entry-fusion complex, aid in the entry of the virus into the cell (168). During infection, the virus enters the host cell in two different pathways depending on the pH of the environment. At a neutral pH, the virus can directly fuse with the host cell membrane, while at a low pH the virus can fuse and enter the cell by the endosomal pathway (256, 257). The two infectious forms of VACV have different outer membranes and thus are thought to have different forms of entry. The MV single membrane contains around twenty-five different viral proteins, while the EV, with its extra membrane surrounding the MV, contains approximately six viral proteins (230). Binding of MVs to the host cell membrane requires at least six of those viral proteins and may be dependent on the strain of VACV (Table 1.2) and the cell type it is entering (17, 265), whereas binding mechanisms for the EV have yet to be described. MVs seem to enter the cell largely by using actin dependent endocytosis (159, 160) and the strain specific route may depend on the MV containing functional viral envelope proteins A25 and A26 which are fusion suppressors (44). EVs may enter through fusion to the cell membrane or following endocytosis (141, 228).

Transcription of poxviral genes occurs in three steps which are temporally controlled by their own promoters (286). Once the virus has bound to the host cell and entered, the lateral bodies diffuse immediately and modify the cellular environment. The core is uncoated and early genes are transcribed and translated. The early genes encodes for products required for host evasion, DNA replication, nucleotide synthesis and intermediate gene transcription (31). Viral DNA replication occurs in viral factories that are made from ER derived membranes, which

Table 1.2 Strains and Entry Pathways of VACV

Species	Entry Method	Reference
Western Reserve (WR)	Endosomal Direct Fusion	(257) (9, 41)
Copenhagen	Direct Fusion	(17)
International Health Department (IHD) -J	Direct Fusion	(264)
IDH-W	Direct Fusion	(44)
Wyeth (Dryvax)	Endosomal	(17)
Elstree (Lister)	Direct Fusion	(17)
Modified Virus Ankara (MVA)	Direct Fusion	(41)

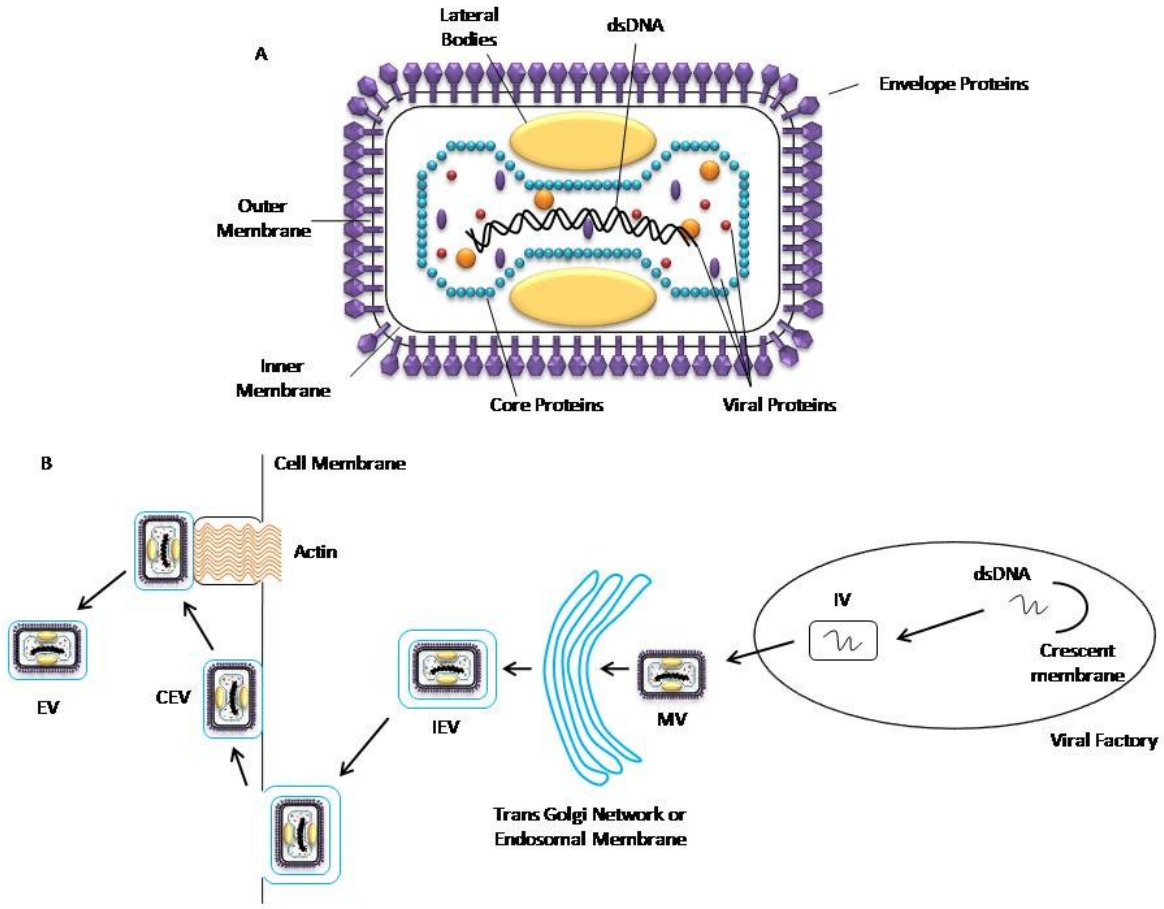

Figure 1.7 *Vaccinia Virus Structure*
(A) Structure of the VACV mature virion. (B) Different forms maturation of VACV virion as it escapes the infected cell. Modified from Condit, *et al*. (2010). Science; 327: 873-6.

vanish during virus assembly (255). The viral DNA is replicated in long concatemers that are cut by a viral endonuclease during packaging (84). Intermediate gene transcription occurs at the same time as DNA replication and encodes for gene products required for late gene transcription (52). Late gene transcription promotes the synthesis of viral structural proteins, which are used to construct the virion (Figure 1.8) (52).

Once all the proteins and viral DNA have been synthesized, the virus begins to assemble within the viral factory. The outer membrane of the virion, derived from the ER (155), is formed as a crescent, that is filled with viroplasm which contains viral core proteins (250). After the crescent is filled with DNA, the membrane is closed by assembling more crescents, to form an immature virion (IV) (147). The IV must go through further morphological and biochemical alterations before it is released from the viral factory as the MV, as reviewed by Liu *et al* (Figure 1.8) (147).

1.6.2 Poxviruses and NK Cells

Cytolytic activities of NK cells are known to be important in defence against poxvirus infections. Infecting mice with VACV either by intraperitoneal or intravenous injection leads to increased cytolytic activity from splenic NK cells (245, 278). Importantly, depleting NK cells in mice leads to an increase in VACV titres and lesion size (35), indicating that they play a role in VACV infections.

Not only are NK activities important in VACV infections, they are also important players in the immune response to the Orthopoxvirus Ectromelia virus (ECTV), the causative agent of mousepox. Some mouse strains, such as C57BL/6 and AKR/J, are resistant to ECTV infection, whereas mouse strains such as DBA/2, A/J and BALB/c are completely susceptible to ECTV

infection (63, 274). Infection of ECTV in predisposed mice leads to lesions on the skin and to death. When infecting mice with different susceptibility to ECTV, resistant C57BL/6 mice have less virus in the spleen in comparison to the susceptible DBA-2 mice (111). NK cell activity is shown to be important when NK cells are depleted from the C57BL/6 mice, that then became vulnerable to ECTV infection and have higher viral titres in their spleen in opposition to mice treated with normal rabbit serum (111). Further studies using C57BL/6 mice with the beige mutation, which leads to impairment of NK cell cytotoxicity (215), showed that these mice are more susceptible to ECTV than wild type mice (111). These studies demonstrate that without NK cells, these mice are more susceptible to infection because they cannot fight the virus.

Although antibody and T cell response are required for recovery from ECTV infection (77), resistance to ECTV has been linked to genes within the NKC (63). The *Rmp1* (Resistance to mousepox 1) gene is the third gene found that controls resistance to ECTV (30), but the first to be mapped the NKC (63). Along with the Ly49, NKR-P1, and NCR receptors, already discussed above, the NKG2 family of receptors is also found within the NKC. NKG2D is an activating receptor that, as discussed in section 1.5.1, binds to stress molecules. Fang *et. al*, found that resistance to ECTV requires NK cell activity but only within the first four days of infection and required both IFN-γ secretion and perforin mediated cytotoxicity (75). This activity of the NK cells is brought about by activation through the NKG2D (75). However, since NK cell cytotoxicity is only reduced but not inhibited by blocking NKG2D in ECTV infected mice, this suggests that NKG2D contributes to, but is not required, for resistance (75). Moreover, NKG2D is not polymorphic, therefore it cannot be the reason for the differences in resistance between different mouse strains to ECTV.

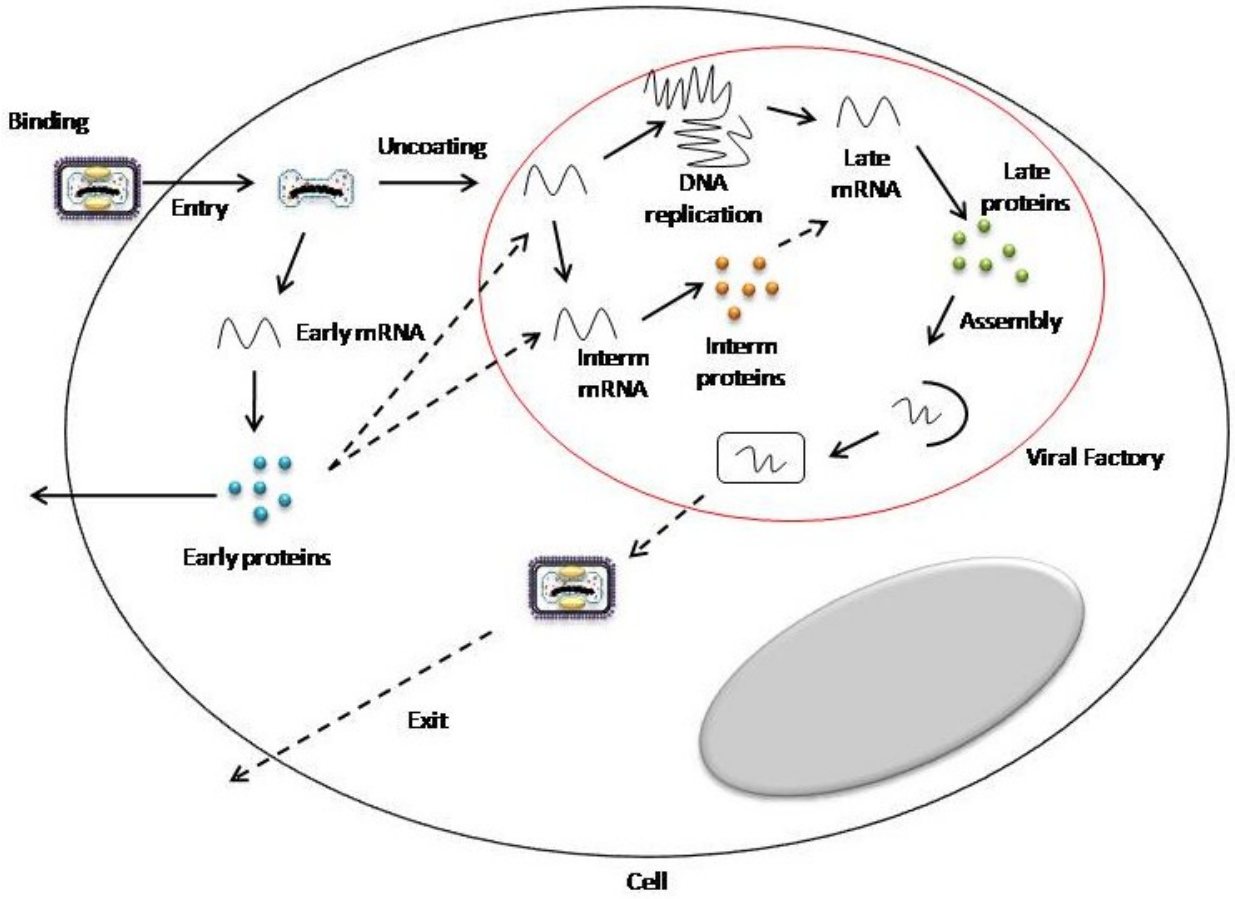

Figure 1.8 *Vaccinia Replication Cycle*
Schematic representation of the replication method of VACV.

It was later discovered that the CD94 molecule is required for the resistance seen in some mouse strains to ECTV (76). The CD94 gene *Klrd1*, is also encoded in the NKC and is required for expression of three NKG2 receptors in humans and mice (NKG2A, -C and -E) by forming heterodimers (125, 187). Mice deficient in CD94 are very susceptible to ECTV but are able to control other viruses such as VACV and lymphocytic choriomeningitis virus (76). Furthermore, expressing a *klrd1* transgene in these mice is able to restore their resistance to ECTV (76). During infection in C57BL/6 mice with ECTV, quantitative polymerase chain reaction (PCR) shows that NKG2E transcripts are increased 16- and 20- fold in the draining lymph nodes on day two and three post infection (76). Correspondingly, increases in the CD94/NKG2E ligand, Qa-1^b, are observed on mouse L cells infected with ECTV (76). When put together in a reporter assay, NK cells that are $CD94^+NKG2E^+NKG2D^+$ increase significantly in GFP expression when incubated with ECTV infected cells, over cells that are $CD94^+NKG2E^+NKG2D^-$ or $CD94^+NKG2C^+NKG2D^+$ (76). This suggests that response to ECTV requires CD94 coupled to NKG2E, which works in synergy with NKG2D. However, there is no polymorphisms in the genes that can explain by C57Bl/6 mice are resistant relative to other mouse strains.

This resistance in mouse to ECTV seen through these receptors, is different than what is known for VACV. As discussed above, NK cell activity is increased in mice infected with VACV, and the loss of NK cells causes an increase in viral titres. NK cell recognition of VACV infected cells is not well understood. In humans, detection of VACV infected cells is through the NCRs: NKp30, NKp44 and NKp46, and requires early VACV gene(s) (47), whereas mice only have NKp46. Although, exactly which ligands for these receptors are being upregulated during the VACV infection is not truly known, except for NKp30, which may be some cellular proteins (47). Recognition of VACV infected cells may be through NKG2A for a subpopulation of NK

cells due to the loss of HLA-E (29). This study also demonstrates that VACV infection does not cause a significant decrease in MHC I expression, nor does blocking MHC I cause a major increase in target cell lysis specific to VACV infection over uninfected cells (47). This small change in MHC I owing to VACV infection coincides with another study that demonstrates that even though there is some loss of HLA-E, there is enough MHC I expression to offer protection through KIR (29). Although a study from our laboratory does demonstrate decreases in MHC I following a longer VACV infection, specifically in HLA C, that can regulate NK cell function through KIR (129). These data may suggests various recognition system may be involved in NK cell response to VACV infection and are likely to be different between the mouse and humans.

1.7 Objectives and Hypothesis

NK cells are important in controlling VACV infections, but it is not well understood how known receptors and receptor/ligand combinations impart resistance. We decided to study the NKR-P1B/Clr-b receptor/ligand pair that is now thought to be another "missing-self" recognition system (39), for its possible contribution to poxvirus resistance. It is known that RCMV infection down regulates Clr-b on infected cells and upregulates a decoy viral protein that binds to NKR-P1B to inhibit NK cell functions (270). It is probable that other viruses have evolved methods to evade NK cell detection through the NKR-P1 receptor including poxviruses.

We want to better understand the mechanisms of poxvirus evasion of our immune systems. Given that there are still poxvirus outbreaks worldwide (169), it is important to study and understand their replication and evasion strategies. Discovering how VACV specifically can manipulate the immune response and immune cells, can lead to advantages in vaccine and oncolytic therapy, seeing that VACV is being used as a biological therapeutic for both vaccinations and cancers (18, 57, 71, 251). Combining this agent with a cell type that naturally

homes to tumour cells would prove to be a incredible method to delivery therapy directly to tumour cells.

To further understand the effects of poxvirus infection on the immune system, we decided to look at both the mouse and human systems of NKR-P1. My research focused on three main objectives. First, to determine if poxvirus infection can affect surface Clr-b expression on mouse cells and how that alteration would influence NK cell functions. Second, to ascertain if poxvirus infection can affect the human homologue of Clr-b, CLEC2D, surface expression on human cells. Third, to establish if any modifications in CLEC2D expression would manipulate NK cell functions. We hypothesized that poxviruses would be able to modulate the surface expression of both Clr-b and CLEC2D on infected cells and that this change in expression would modify NK cell activity towards the infected cells.

Chapter 2 : Methods and Material

2. Methods and Materials

2.1 Overview of Methodology

This project commenced with the determination of what effect poxvirus infection had on the mouse CLEC2D protein, Clr-b. In order to do this, mouse cells were infected with VACV that expressed eGFP on a early/late promoter to determine which cells were infected with VACV. With infection established, we had to ascertain if VACV infection had an effect on Clr-b surface expression. This included determining how infection acted upon Clr-b transcription, at what stage of the infection VACV had effect on Clr-b expression and the effect of the causative agent of Mousepox, ECTV, had on Clr-b protein expression. The final point to resolve, was how the effect of VACV infection on Clr-b, affected NK cell function. This was performed by chromium (^{51}Cr) release assay where mouse cells were infected with VACV and incubated with primary mouse NK cells that expressed the receptor for Clr-b, NKR-P1B.

The next step in this project was to determine the effect of poxvirus infection on the human CLEC2D protein. In order to do this, human cells were infected with eGFP-VACV to clarify which cells were infected. Again, we had to resolve if VACV infection had an effect on CLEC2D expression including when in the infection VACV had this effect, how VACV infection affected CLEC2D transcript and how other poxviruses effected CLEC2D expression. From this information, we sought out to determine how the effect of VACV infection on CLEC2D affected NK cell function. This was performed by a flow cytometric degranulation and killing assay where human cells were infected with VACV and incubated with primary human NK cells that expressed the receptor for CLEC2D, NKR-P1A. The methods for all experiments performed during this thesis will be describe in this chapter.

2.2 Cells and mice

NIH 3T3 continuous mouse fibroblast cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Gibco, Burlington, Ontario) with 10% heat inactivated (HI) calf serum (Invitrogen Gibco). The NIH 3T3 cells were maintained by splitting them 1:10 every 4 days when they were 95% confluent. Briefly, adherent cells were washed once with Phosphate Buffered Saline (PBS). Two millilitres of 0.25% Trypsin-3.8 g/L EDTA (Invitrogen) was added to the cells and the flask was incubated at 37^{o}C, 5% CO_2 until the cells sloughed off after gentle tapping of the flask sides. Cells were cultured in 10 mL of complete media in a 75 cm^2 polystyrene cell culture flask (VWR International, Edmonton, Alberta). C1498 (gifted by Dr. J. Carlyle, University of Toronto), a murine myeloid leukemia cell line, was cultured in DMEM media with 10% HI fetal bovine serum (FBS, Invitrogen Gibco). Briefly, suspension cells were split 1:8 every 4 days when they reached a density of 1-2 x 10^6 cells/mL. Cells were cultured in 10 mL of complete media in a 25 cm^2 cell culture flask (VWR International). 721.221 (221) cells, an EBV transformed B cell line, gifted by Drs. J. Gumperz and P. Parham (Stanford University), and were culture in Iscove's Modified Dulbecco's Medium (Invitrogen Gibco) with 10% HI FBS, 2 mM L-glutamine (Invitrogen Gibco), 10,000 Units/mL penicillin (Invitrogen Gibco) and 10,000 μg/mL streptomycin (Invitrogen Gibco) and maintained as described in C1498 cells, splitting 1:10. KM-H2 cells, a human Hodgkin lymphoma B cell line and K299, a human T cell lymphoma line, were gifted by Dr. R. Ingham (University of Alberta) were cultured in RPMI 1640 (Invitrogen Gibco) supplemented with 10% HI FBS, 2 mM L-glutamine, 10,000 Units/mL penicillin and 10,000 μg/mL streptomycin, and maintained as described in C1498 cells, splitting 1:10. Jurkats, a mouse leukemia T cell line, were obtained from Dr. H.

Ostergaard (University of Alberta) were cultured in RPMI 1640 supplemented with 10% HI FBS, 2 mM L-glutamine, 10,000 Units/mL penicillin and 10,000 µg/mL streptomycin, and maintained as described in C1498 cells, splitting 1:10. NK-92, a human NK lymphoma cell line, was obtained from ATCC and cultured in Alpha Modified Minimum Essential Medium Eagle's (α-MEM, Invitrogen Gibco) supplemented with 12.5% HI horse serum (Invitrogen, Gibco), 12.5% HI Characterized FBS (Hyclone, Logan, Utah), 0.2 mM inositol (Sigma Aldrich, Oakville, Ontario), 0.1 mM Beta-mercaptoethanol (BME, BioShop Canada, Burlington, Ontario), 0.02 mM folic acid (Sigma Aldrich), 2mM L-glutamine, 10,000 Units/mL penicillin and 10,000 µg/mL streptomycin, and maintained as described for C1498 with the addition of 1000 U/ml IL-2 (Tecin, Nutley, NJ), splitting 1:10. 293T (ATCC), a human epithelial kidney cell line containing the SV40 T-antigen, Hela (gifted by Dr. J. Smiley, University of Alberta), a human cervical cancer epithelial cell line, Vero (Dr. J. Smiley), a continuous monkey kidney epithelial cell line, Cos-1 (ATCC), a monkey kidney fibroblast cell line that contains the SV40 T-antigen, and Huh7.5 (gifted by Dr. L. Tyrrell, University of Alberta) a human hepatocarcinoma cell line, were cultured in DMEM supplemented with 10% HI FBS, 2 mM L-glutamine, 10,000 Units/mL penicillin and 10,000 µg/mL streptomycin, and maintained as described for NIH 3T3. Hela, Cos-1 and Huh7.5 cells were split 1:10, Vero cells were spilt 1:8, and 293T cells were split 1:12 every 4 days. HFF-Tel (Dr. J. Smiley), a human foreskin fibroblast cell line, was cultured in DMEM supplemented with 15% FBS, 2 mM L-glutamine, 10,000 Units/mL penicillin and 10,000 µg/mL streptomycin. The HFF-Tel have been transfected with a telomerase plasmid. Thymidine kinase negative (TK-) H143B cells, a human osteosarcoma fibroblast cell line, (ATCC) used for production of VACV, were cultured in DMEM with 10% FBS. Buffalo green monkey kidney (BGMK) cells, a continuous monkey

kidney epithelial cell line, was gifted from Dr. M. Barry (University of Alberta), used for production of ECTV and cultured in DMEM with 10% FBS.

The University of Alberta Animal Welfare and Policy Committee approved all animal procedures. C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). CD-1 mice were purchased from Charles River Laboratories (Wilmington, Massachusetts). Bone marrow-derived macrophages (BMMø) were generated from C57BL/6 mice. Briefly, bone marrow cells were flushed out of the femur and tibia bones with PBS. These cells were washed 3 times with PBS and cultured for 7 to 8 days in RPMI 1640 supplemented with 10% HI FBS, 2 mM L-glutamine, 0.05 mM BME, and 20% supernatants from Chinese hamster ovary cells (kindly provided by Dr. H. Ostergaard) that produce Granulocyte/Macrophage Colony Stimulating Factor (GM-CSF). The purity of the macrophages was determined by staining for F4/80. NK cells were derived from CD-1 splenocytes by harvesting and homogenizing the spleen. Splenocytes were incubated on a nylon wool column for 1 hour to remove adherent cells. The flow through containing the NK cells was cultured in 1000 U/mL IL-2 and the non-adherent fraction removed on day 3. The purity of the NK cells was ~90% as determined by staining for CD3 and NKR-P1B with PK136, and they were used in assays on day 7 or 8.

Human peripheral blood mononuclear cells (PMBCs) was isolated from volunteers. Briefly, blood was mixed with PBS and under laid with Lympholyte H (Cedarlane Laboratories, Burlington, Ontario). Following centrifugation, the layer of PBMCs at the interface was transferred and washed 3 times with cold PBS to keep the macrophages or warm PBS to remove the macrophages.

Primary human NK cells were isolated from PBMCs by negative selection using the Negative Selection Human NK cell Enrichment kit (Stem Cell, Vancouver, British Columbia) as

per manufacturer's direction. Isolated NK cells were then plated on irradiated 221 cells in Iscoves supplemented with 10% HI human serum (Life Technologies, Invitrogen), 500 mM Gentamicin (Invitrogen Gibco) and 1000 Units/mL IL-2 (Tecin). Cells were fed every 2-3 days with new media and IL-2 for 7 days and then split 1:2 every 2-3 days. Primary NK cells were used between 8 and 20 days following isolation. All cells mentioned were cultured at 37^{o}C with 5% CO_2.

2.3 Antibodies and Reagents

All antibodies used in this thesis were titrated for each experiment. The Clr-b specific Ab 4A6 (IgM) was provided by Dr. J. Carlyle, and was purified and labelled with biotin as previously described (39). IgM isotype Ab control was purchased from Cedarlane Laboratories. Streptavidin (SA) -allophycocyanin was purchased from eBioscience (San Diego, California) and SA-phycoerythrin (PE) from Cedarlane Laboratories. The anti–MHC I Ab M142 (rat IgG2a) was kindly provided by Dr. K. Kane (University of Alberta). The IgG2a (51.1) isotype Ab was produced by Dr. D. Burshtyn (University of Alberta) from a hybridoma and purified on protein G sepharose beads (ATCC). Anti-mouse transferrin receptor R.17/217.1.3 Ab (rat IgG2a) was kindly provided by Dr. H. Ostergaard. Rabbit anti-I5L antiserum was provided by Dr. M. Barry (262). Secondary goat anti-rabbit PE was purchased from Cedarlane Laboratories. Anti-mouse NK1.1-PerCP-Cy5.5 Ab and its isotype (IgG2aκ) were purchased from eBioscience. Anti-mouse CD3 PE-Cy5 and anti-mouse F4/80 PE-Cy5 were from eBioscience. Anti-CLEC2D, clone 4C7 (MO1, Mouse IgG1, κ) and clone 2E11 (MO3, Mouse IgG2a, κ) were purchased from Abnova (Walnut, California). Anti-human OCIL/CLEC2D APC, clone M402659 (IgG1) and polyclonal goat anti-human OCIL/CLEC2D (IgG) were purchased from R&D Systems (Burlington, Ontario). Anti-human IgG1, κ (MOPC-21) was acquired from Sigma-Aldrich. The secondary

antibody Goat anti-mouse IgG APC was purchased from Cedarlane Laboratories. Anti-hCLEC2A (KACL, mouse IgG1) was purchased from R&D Systems. Anti-human CD161 (clone B199.2) and its isotype mouse IgG2b were purchased from GeneTex (Irvine, California). Anti-human CD161 Alexa Fluor 647 and its isotype mouse IgG1, κ, Alexa Fluor 647 were purchased from BioLegend (San Diego, California). Anti-human IFN-γ PE-Cy7 (clone 4S.B3) and its isotype mouse IgG1, κ PE-Cy7 were purchased from eBioscience. Mouse anti-human CD107a PE-Cy5 was purchased from BD Pharmingen (Mississauga, Ontario) and its isotype mouse IgG1, κ PE-Cy5 was purchased from eBiosciences. Anti-human CD56 PE, its isotype mouse IgG1, κ PE, Anti-human CD3 eFluor 450 and its isotype IgG2a, κ were purchased from eBiosciences. The secondary antibody Alexa Fluor 647-R-phycoerythrin (Goat anti-mouse IgG1) was purchased from Invitrogen Molecular Probes. The Violet Live/Dead Fixable Dead Cell Stain kit was acquired from Invitrogen Molecular Probes. The ProLong Gold Antifade Reagent with 4',6-diamidino-2-phenylindole (DAPI) was obtained from Life Technologies, Invitrogen. Dimethyl Sulfoxide (DMSO) was bought from Fisher Scientific (Waltham, MA). Brefeldin A solution was purchased from eBioscience. Epigallocatechin-3-gallate (EGCG) was a kindly provided by Dr. L Schang (University of Alberta), was dissolved in DMSO and used at a final concentration of 40 μM. Cyclohexamide (CHX, MP Biomedicals, Solon, OH) stocks were prepared in water and used at a final concentration of 50 μg/mL. Cytosine β-D-arabinofuranoside (AraC, Sigma Aldrich) stocks were prepared in water and used at a final concentration of 50 μg/mL. Actinomycin D (ActD, Sigma-Aldrich) was dissolved at 10 mg/mL and used at a final concentration of 20 mg/mL. The OneStep RT-PCR kit was purchased from Qiagen (Toronto, Ontairo). The Cytofix/Cytoperm^{TM} Fixation/Permeabilization solution kit was bought from BD Biosciences.

2.4 Viruses and Virus Preparation

The viruses used in this project all expressed a fluorescent tag in order to determine which cells were infected by flow cytometry. VACV, strains Western Reserve (WR) and Copenhagen (Cop), both contained a GFP fluorescent tag on a synthetic early/late promoter within the thymidine kinase locus, and were prepared in T150 cm^2 tissue culture flasks by infecting TK-H134B cells with crude virus. The crude virus was made by infecting TK-H134B cells with virus and incubating them for 48 hours at 37^oC with 5% CO_2. Following incubation, cells and supernatant were harvested and spun down. The pelleted cells were freeze thawed three times and sonicated until the suspension was homogenous. This was aliquoted and stored at -80^oC. This crude virus was used to make a semi pure preparation of VACV. The TK-H134B cells were infected with crude virus for approximately 24-48 hours, and harvested before cells began to slough off the bottom of the flask. Cells were harvested by knocking the flask against your hand and the cell suspension was centrifuged. Swelling buffer (see appendix for recipe) was added to the pellet and the tube was put on ice for 30 minutes. Following incubation, cells were sonicated on pulse for 60 seconds, then layered on top of 36% sucrose/1 mM Tris (pH 9). The column was centrifuged at high speeds for 2 hours to obtain a fluffy pellet that was resuspended in plain DMEM. The suspension was vortexed, continuously sonicated for 35 seconds and centrifuged again. The resulting pellet was discarded and the supernatant was diluted 1:1 with 10 mM Tris (pH 9) and centrifuged at high speeds for 1 hour. The resulting pellet was resuspended in plain DMEM, vortexed and sonicated with pulsing for 35 seconds. The suspension was vortexed again, aliquoted and stored at -80^oC.

The semi pure viruses were titred by either x-gal or crystal violet staining. For both types of staining, TK-H134B cells were plated in 6 well plates until the cells were 100% confluent

which was normally the next day. Virus was prepared by serial dilution in duplicates, pipetted onto the monolayer and incubated at 37°C with 5% CO_2 for 48 hours. If x-gal was used for titring, cells were washed and neutral buffered formaldehyde (NBF, see appendix for recipe) was added for 5 minutes at room temperature. The NBF was removed and x-gal stain (see appendix for recipe) was made fresh and added to the cells for 1 hour at 37°C with 5% CO_2. Blue coloured plaques were counted to obtain titre of the virus. If crystal violet was used, cells were washed and crystal violet stain (see appendix for recipe) was added for at least 2 minutes at room temperature. Plaques appeared clear against the purple monolayer.

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:

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

where volume used is equal to the amount of virus used to infect cells in millilitres.

UV inactivated VACV were placed under a UV lamp for 30 minutes and the efficacy of the inactivation was verified by titring on TK-H134B cells and led to $\leq$ 0 PFU/mL.

ECTV, strain Moscow, contained a YFP fluorescent tag and was prepared in T150 cm^2 tissue culture flasks by infecting BGMK cells. Cells were infected with a crude stock for 24-48 hours until the monolayer just starts to lift off. Cells were harvested by knocking the flask against your hand and the cell suspension was centrifuged. Swelling buffer was added to the pellet and the tube was put on ice for 30 minutes. Cells were centrifuged and then the pellet was resuspended in swelling buffer to be dounce homogenized with 100 strokes. The suspension was centrifuged, resuspended in swelling buffer and dounce homogenized with 60 strokes. The

suspension was centrifuged, the supernatant was collected and centrifuged at high speeds for 1 hour. The resulting pellet was resuspended in plain DMEM, vortexed until homogenous and continuously sonicated for 1 minute. The virus stock was then aliquoted and then stored at -80^{o}C. ECTV was titred using the x-gal method on BGMK cells and calculated using the above equation.

Myxoma virus (ΔM127L-mCh), strain Lausanne, was kindly provided by Dr. D. Evans (University of Alberta). It contains a mCherry fluorescent tag on a synthetic early/late promoter and was made as described previously (237).

2.5 Infection

2.5.1 Suspension Cells

Suspension cells were split 1:10 and used 2-3 days following the split while they were still in growth phase. On the day of infection, cells were centrifuged and resuspended in low volume plain media. Cells were then counted and separated into 15 mL conical tubes for uninfected, infected treatments and any other treatment for the experiment. Suspension cells were normally concentrated between $5x10^5$ - $1x10^6$ cells per trial. Cells were concentrated into 500 µL per treatment and infected with the appropriate amount of virus. The tubes were incubated at 37^{o}C with 5% CO_2 for 1 hour with shaking every 15 minutes. Following the hour incubation, cells were plated into 12-well plates in 2 mL per well of complete media and put back into the incubator until the end of the infection. Cells were harvested by pipetting the cells in the well to obtain any cells that may have stuck to the plate and the suspension was put into tubes. Cells were centrifuged for 5 minutes at 1200 x g and processed for the experiment.

2.5.2 Adherent Cells

Adherent cells were split the day prior to the infection such that they would be approximately 95% confluent the next day in 6- or 12- well plates. On the day of infection, medium was removed from the cells and the wells were washed with plain media. Virus was added to the wells in 0.5 mL per well in plain media, and the plates were incubated at 37^{o}C with 5% CO_2 for 1 hour with shaking every 15 minutes. Following incubation, complete media was added up to 2 mL per well and the plates were put back into the incubator until the end of the infection. Cells were harvested by removing the medium and washing the monolayer with PBS. The PBS was discarded and 0.25% trypsin-EDTA was added to the cells until the cells could be easily knocked off the plate. Complete media was added to the wells and the cell suspension was put into tubes. Cells were centrifuged for 5 minutes at 1200 x g and processed for the experiment.

2.6 Flow Cytometry

If working with adherent cells, cells were removed from the plate with 0.25% trypsin-EDTA and washed with cold PBS containing 1 uM Ethylenediaminetetraacetic acid (EDTA) in FACS tubes to keep the cells from sticking to each other. If working with suspension cells, cells were centrifuged to remove media and washed with cold PBS-EDTA. Cold PBS-EDTA was poured out of the tubes and blotted on a kimwipe, which then retained approximately 50-100 µL of PBS-EDTA in the tubes. In most experiments, viability of the cells was tested using a violet live/dead stain that was added at the same time as the primary antibody at a dilution of 1:10. The live/dead staining was first tested by lysising cells with a 0.1% triton-X/PBS solution for 5 minutes. Primary antibody was added and the tubes were incubated at 4^{o}C for 30 minutes. Following incubation, cold PBS containing 1 µM EDTA and 5% HI FBS (now called FACS

buffer) was added to the cells. Tubes were centrifuged at 1200 x g for 5 minutes, and the FACS buffer was removed as above. If required, a secondary antibody, at a dilution of 1:100, was added to the tubes and incubated at 4°C for 30 minutes. Following incubation, cells were washed with FACS buffer, centrifuged at 1200 x g for 5 minutes. FACS buffer was removed and cells were fixed with 4% paraformaldehyde (pFA) overnight at 4°C.

If performing intracellular staining, cells were prepared in FACS tubes as above. One hundred microliters of Cytoperm from the Cytofix/Cytoperm Fixation/Permeabilization solution kit was added to the cells and the tubes were incubated at 4°C for 20 minutes. Following incubation, cells were washed 2 times with 1 mL of 1:10 diluted Cytowash in PBS. Cells were stained as above with 2 exceptions. First, if using the live/dead stain, cells had to be stained with the live/dead stain and washed prior to permeabilization. Second, every wash performed following permeabilization would be performed with diluted Cytowash to maintain pores made by the Cytoperm.

2.7 Chemical Treatments

ActD, AraC and CHX experiments were performed similarly. Cells were centrifuged at 1200 x g for 5 minutes and the pellet was resuspended in low volume of media without additives. Cells were counted and concentrated between $5x10^5$ - $6x10^5$ cells per trial in 500 µL of media. The chemical treatment was add directly to media at the appropriate concentration (see above) and incubated at room temperature for 5 minutes. Following incubation, cells were infected as previously describe (section 2.5.1).

The procedure for EGCG treatments were modified from a protocol provided by Dr. L Schang. Briefly, virus was added to 500 µL of media without additives and either EGCG at a 2x

dilution of 40 μM or DMSO at the same volume was added to the virus. The mixture was incubated at 37°C with 5% CO_2 for 10 minutes. The mixture was then removed from the incubator and place on ice for 12 minutes. While the virus/EGCG mixture was on ice, cells were harvested, counted and concentrated to $5x10^5$ cells per trial in 500 μL of media. Following ice incubation, cells were combined with the virus/EGCG mixture, vortexed and centrifuged at 1200 x g for 5 minutes. Supernatant was removed and cells were processed for flow cytometry.

2.8 Analysis of mRNA

2.8.1 Polymerase Chain Reaction (PCR)

PCR was performed for Clr-b transcript amplification. RNA was isolated from $2.5x10^6$ cells using the RNeasy kit (Qiagen) as per manufacturer's directions. Samples were treated on the columns with DNAse to remove any genomic or viral DNA. RT-PCR reactions were performed with 10–100 ng RNA. All primers were obtained from Integrated DNA Technologies (Coralville, Iowa) and were desalted. Primers specific for Clr-b amplification were 5'-CTC GGT TTT GAC AAC CAG GT-3' and 5'-GAT CCC GTT GTT GTT CAG GGT-3'; for β-actin, 5'-TGT TAC CAA CTG GGA CGA CA-3' and 5'-GGG GTG TTG AAG GTC TCA A-3-; and VACV viral protein D10, 5'-TTC CAG AGT GTT TAT CCA GGG A-3' and 5'-CTC GTT AGA GAT ATT CTT CCG ACA A-3'. The resulting PCR products were analyzed on a 1% agarose gel stained with ethidium bromide.

2.8.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed for CLEC2D transcript amplification. RNA was isolated from 2.5×10^6 cells using the RNeasy kit (Qiagen) as per manufacturer's directions. Samples were treated on the columns with DNAse to remove any genomic or viral DNA. RT-PCR reactions were performed with 1 μg RNA. All primers were obtained from Integrated DNA Technologies.

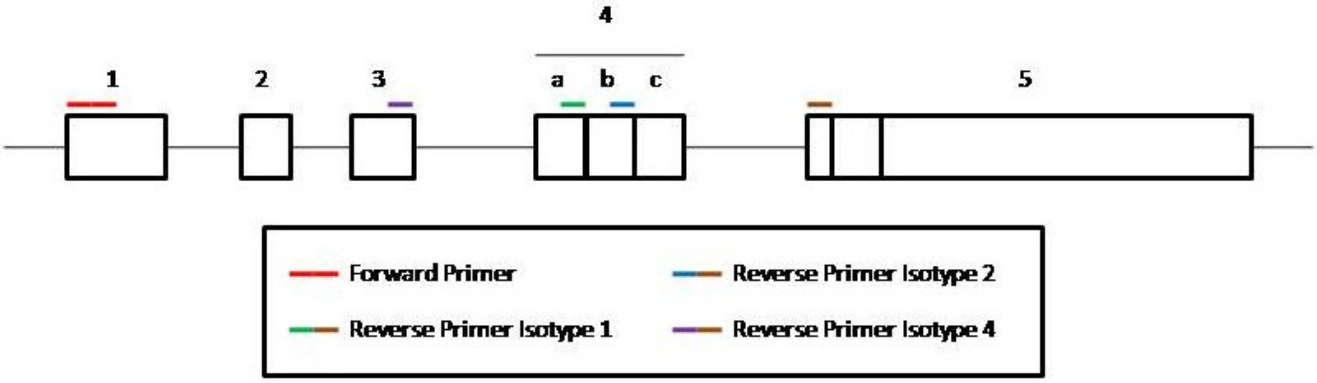

Figure 2.1 *CLEC2D Primer Sets*
Primer sets for each CLEC2D isoform are depicted. Each reverse primer begins in the last exon included in the isoform and ends at the beginning of exon 5.

Primers specific to amplify for CLEC2D isoform 1 were 5'-GAA TTC CGG CAA AAT GCA TG-3' and 5'-AAT TAC TCA TTC TCG GGT AT-3' and based upon the PubMed Accession # AF133299 sequence. Primers specific for detecting the different isoforms of CLEC2D used the same forward primer and different reverse primers and were based upon the PubMed Accession # BC019883 sequence, except for isoform 2, which was based on the sequence provided by Germain *et al* (85) (Figure 2.1). Forward primer was 5′-AGG CAA AAT GCA TGA CAG TA-3′; reverse for isoform 1 was 5'- GAT AGG AAA CTG TCT TGT CC -3'; for isoform 2 was 5'-GAT AGG AAA CCA TGA CAG GT-3'; and for isoform 4 was 5'-GAT AGG AAA CCA TGT CCT GG-3', all based upon Accession # BC019883 on PubMed. For β-actin, 5′-AAG ACC TGT ACG CCA ACA-3′ and 5′-TCC ACA CGG AGG ACT TGC -3′. The resulting RT-PCR products were analyzed on a 1% agarose gel stained with ethidium bromide or SYBR safe.

2.9 Immunofluorescence Assays (IFA)

Fixed suspensions cells were centrifuged onto a microscope slide using a cytospin cassette provided by the Pulmonary Research Group at the University of Alberta. Briefly, a filter pad, to which a circle has been removed from the center, was placed on top of a microscope slide and put into the cytospin loading cassette. One hundred microliters of PBS was placed in the circle and the cassette was centrifuged at 70 x g for 1 minute at 4^{o}C to wet the filter pad. One hundred microliters of a cell suspension (approximately 0.5x10^{6} cells/mL) was placed in the circle and centrifuged at 70 x g for 3 minute at 4^{o}C. The filter paper was removed and the microscope slide was air dried. Adherent cells were grown on poly-D-lysine (Sigma Aldrich) treated coverslips then fixed with 4% pFA. Cells were washed 3x with PBS and incubated with primary Ab for 1 hour at room temperature. Subsequently, cells were washed 3x with PBS and then incubated with the secondary Ab for 1 hour at room temperature in the dark. Cells were

washed 3x with PBS and ~10 µL of ProLong Gold antifade reagent with DAPI was added to the cells in order to stain the cell nuclei. A cover slip was then sealed on the microscope slide with nail polish and visualized by a confocal AIVI Spinning Disc microscope (Quorum Technologies, Guelph, ON) (Faculty of Medicine and Dentistry at the University of Alberta) using a 60x oil lens. Acquired data was analyzed using Fiji ImageJ software for Windows (Version 1.48s, National Institutes of Health, USA).

2.10 293T Transfections

The YFP-LLT1 in a MSCV2.2 plasmid, was provided by Dr. J. Carlyle. Briefly, 80 µL of DH5α competent *E. coli* (Invitrogen) were transfected with 100 ng of the YFP-LLT1 plasmid and put on ice for 30 minutes. The cells were then heat shocked at 42^{o}C for 45 seconds and then put back on ice for 2 minutes. 300 µL of Lysogeny broth (LB) was added to the cells and the mixture was placed in a 37^{o}C shaker for 1 hour. Following incubation, 150 µL of the mixture was plated onto LB plates containing ampicillin (Amp) and incubated at 37^{o}C overnight. The following day, plates were put at 4^{o}C until the afternoon, where a single colony of bacteria was selected and placed into 5 mL of LB-Amp and shaken at 37^{o}C for 12-16 hours. The cultures were removed the subsequent day and used for mini preps using a Qiagen Miniprep kit (Qiagen) and glycerol stocks.

293T cells were split 1:4 the day prior to transfection. On the day of transfection, 2 µg of YFP-LLT1, or as a control, the same amount of water as the YFP-LLT1, was incubated in 90 µL of Opti-MEM (Gibco Life Technologies) for 5 minutes in a cryotube at room temperature. 3 µL of Lipofectamine (Invitrogen) was incubated in 90 µL of Opti-MEM for 5 minutes in another cryotube at room temperature. Following incubation, the DNA mixture (or control water mixture) and Lipofectamine mixtures were combined and incubated at room temperature for 15

minutes. Subsequent to incubation, 720 µL of Opti-MEM was added to the DNA/Lipofectamine combination. Media was removed from 293T cells, that were ~50-60% confluent, and the cells were washed 1x with room temperature Opti-MEM. The DNA/Lipofectamine mixture was then added drop wise directly to the cells. The cells were then incubated at 37^{o}C with 5% CO_2 for 24 hours. After 24 hours incubation, 1 mL of media with additives was added to each well, and the cells were put back into the incubator for another 24 hours. Following incubation, media was removed and cells were removed from the plate using 0.25% trypsin-EDTA. Cells were then washed and prepared for FACS staining as described in section 2.6.

2.11 Cytotoxicity Assays

2.11.1 Chromium (^{51}Cr) Release Assays

C1498 target cells were infected with VACV (multiplicity of infection (MOI) of 10 pfu/cell) for 18 hours, and an aliquot was removed to assess the expression of Clr-b. The cells ($5x10^5$) were labelled with approximately 10 µCi [^{51}Cr] (sodium chromate; NEN) for 1 hour at 37°C in 5% CO_2. The target cells were washed and the viable cell count determined using trypan blue exclusion and pre-incubated with either 40 mg/mL 4A6 or isotype control Ab for 10 minutes prior to the addition of NK cells. Target cells were plated at 2500 live cells per well in V-bottom microtiter plates in triplicate. The spontaneous release was determined in the presence of the Abs. The NK cells were washed, diluted to the appropriate concentration in assay media, and added to the labelled target cells. The plates were incubated at 37°C in 5% CO_2 for 4 hours. The released ^{51}Cr was quantified for 25 µL supernatant in 100 µL of scintillent Optiphase supermix (PerkinElmer) and analyzed in a Wallac 1450 Microbeta Trilux Liquid Scintillation and Luminescence Counter (PerkinElmer). The percent lysis was calculated by the following equation for measured for each targets:

$$100 \times \frac{\text{(specific } ^{51}\text{Cr release - spontaneous release)}}{\text{(maximum release - spontaneous release)}}$$

The spontaneous release was not increased in the infected cells relative to the uninfected controls. Experiments were only included if the maximum release was three fold higher than the spontaneous release.

2.11.2 Degranulation, IFN-γ and Killing Assays

For these combined assays, $2x10^5$ NK cells were utilized as a base number to add as many target cells as required. 221 cells were harvested and infected as previously describe (section 2.5.1). Following infection target cells were washed in plain media and concentrated to correct numbers in 100 µL of killing media (see appendix for recipe). Target cells were plated in a 96-well V-bottom microtiter plates in triplicate. Ten microliters of CD107a PE-Cy5 and 10 µg/mL of anti-CD161 or IgG2b Abs were added to the appropriate wells. Primary NK cells were concentrated to suitable numbers in 100 µL of killing media. NK cells were then added to the target cells at a 3:1 ratio (also titrated at 1:3). The plate was covered and centrifuged at 300 x g for 3 minutes at room temperature. The plate was then incubated at 37°C with 5% CO_2 for 1 hour. Following incubation, 1 µL of a 1:1000 dilution of Brefeldin A solution was added to target/NK mixtures and the plate was put back into the incubator for another 2 hours. Subsequent to incubation, the plate was centrifuged at 1200 x g for 5 minutes. Media was removed, cells were resuspended in cold PBS-EDTA and transferred to FACS tubes where cells were stained as described previously (section 2.6). When assaying for IFN-γ production, 5 µL of anti-human IFN-γ PE-Cy7 was added after the above assay was completed and the cells were permeablilized.

2.12 Statistics

The statistical significance between conditions was calculated using a Student *t* test, considered significant at a 95% confidence limit, and noted as $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$.

Chapter 3 : Poxvirus Infection Associated Down Regulation of C-Type Lectin-Related-b Prevents NK Cell Inhibition by NK Receptor Protein 1B

The data presented in this chapter has been published in The Journal of Immunology (2012). All the experiments were performed by myself, with the exception of the time course in Figure 3.3, performed by Evan Wilson, and the PCR in Figure 3.4, performed by Chelsea L. Davidson.

A version of this chapter has been published. Kinola J N Williams, Evan Wilson, Chelsea L Davidson, Oscar A Aguilar, Li Fu, James R Carlyle and Deborah N Burshtyn. 2012. Poxvirus Infection Associated Down Regulation of C-Type Lectin-Related-b Prevents NK Cell Inhibition by NK Receptor Protein 1B. J Immunol; 188: 4980-91.

3. Poxvirus Modulation of Clr-b

3.1 Introduction

NK cells can be triggered by the increase of stimulatory ligands, the decrease of inhibitory ligands, or a combination of both mechanisms. The first and best described of these receptor systems is the inhibition of NK cells by classical and non-classical MHC class I proteins. This recognition system that senses missing self is believed to have evolved to allow NK cells to detect virus infected cells, because many viruses interfere with normal expression of MHC I proteins to evade T cell responses (163). The receptor systems for MHC I have undergone considerable selective pressure and have diverged significantly between species, and this divergence is exemplified by the differences between humans and rodents. Viruses have evolved several mechanisms to evade these systems of detection by NK cells, including expression of MHC I mimics and providing peptides for HLA-E (212, 233, 275).

Humans and mice also share the NKR-P1 family of receptors that recognize endogenous proteins, but the ligands for the NKR-P1 receptors are unrelated to MHC I and are not directly involved in Ag presentation (161). Instead, NKR-P1 receptors interact with C-type lectin-related proteins encoded within the NKC (39, 109). The best understood receptor is NKR-P1B (also known as NKR-P1D in C57BL/6 mice), which recognizes Clr-b. Clr-b is broadly expressed on normal cells, but frequently reduced on transformed cell lines that are susceptible to NK killing (39). Understanding of the role of the NKR-P1 receptor system in defence against viral infection is still emerging; however, a rat CMV (RCMV) isolate provides a clear example of its importance. RCMV infection causes down regulation of Clr-b on rat cells, and consequently, RCMV possesses a Clr-b decoy homolog (RCTL) that engages rat NKR-P1B to protect RCMV infected cells from NK attack (270). The mechanism of Clr-b down regulation during RCMV

infection remains unknown, but other studies have shown that Clr-b is down regulated following cellular stress, suggesting cells may have a host pathway to alert NK cells by deliberately down regulating Clr-b during infection (81).

Poxviruses are interesting candidates to potentially regulate the NKR-P1 and Clr families during NK cell-mediated detection of infection. Poxviruses use a wide variety of immune evasion strategies, including interference with cytokines that impact the NK cell response (24, 209). The immune response in mice to both the mouse pathogen ECTV and the prototypic poxvirus VACV involves NK cells (35, 64, 75, 182). A very recent study indicates that NK cells even develop memory for VACV (87). Various activating receptors on NK cells such as CD94/NKG2E and NKG2D are required for NK cell defence against ECTV in mouse (75, 76), and recognition of VACV infection by human NK cells involves NKG2A (29) and perhaps the loss of HLA-E and classical MHC I molecules (29, 129). Moreover, certain strains of mice, particularly C57BL/6, are resistant to VACV and ECTV infection (35, 226, 227, 274), and depletion of NK cells converts resistant mice to a susceptible phenotype (35). The strain dependent resistance to ECTV maps to the NKC (63). Although VACV causes some reduction of mouse classical MHC I molecules (33), no evidence has emerged to suggest classical MHC I and Ly49 receptors are involved in resistance.

Given the potential for the NKR-P1 and Clr system of receptors and ligands to be involved in the response to poxvirus infection and the ability of poxviruses to interfere with protein expression through destabilization of cellular transcripts (32, 93, 285), we tested the hypothesis that Clr-b is modulated during poxvirus infection. This chapter will discuss the results of this study as published (282).

3.2 Results

3.2.1 Infection with VACV reduces Clr-b cell surface expression

To assess the effect of VACV infection on Clr-b expression, we infected NIH 3T3 and C1498 cells with VACV at a MOI of 10 for 12 hours. Cells were then analyzed by flow cytometry for surface Clr-b using biotinylated 4A6 (39) and SA-allophycocyanin. We observed a substantial loss of Clr-b surface expression for both cell lines (Figure 3.1A and B). In contrast, exposure of the cells to UV-treated virus had no effect on the level of Clr-b. To determine if the loss of Clr-b corresponded to infection of the cells and whether the loss was occurring on cells that were still alive and able to replicate the virus, we performed a similar experiment with C1498 cells and a virus expressing enhanced GFP (eGFP-VACV). In this case, the cells were also treated with a fixable live/dead stain. We observe a good correlation between our live gate by forward and side scatter analysis and the ability to exclude the live/dead cell stain (Figure 3.1C). Moreover, the loss of Clr-b is evident within the live cells and within the infected cells expressing eGFP (Figure 3.1D).

We next assessed how the dose of virus affects the amount of Clr-b that is lost using the eGFP-VACV virus to simultaneously monitor the infected and uninfected cell subsets. The comparison of Clr-b loss with the amount of eGFP expression is illustrated in the dot plots depicted in Figure 3.2A for NIH 3T3 cells and Figure 3.2C for C1498 cells. A dose-dependent response was observed for both cell subsets, with significant reductions for both the $eGFP^{hi}$ and $eGFP^{lo}$ populations of cells, particularly at higher MOI (Figure 3.2E and F), corresponding to the doses in which most of the cells are also eGFP positive (Figure 3.2). Notably, for the NIH 3T3 cells, the $eGFP^{lo}$ subset often has low Clr-b expression similar to the $eGFP^{hi}$ subset at each dose, whereas for the C1498 cells, a reduction in Clr-b is more evident on the $eGFP^{hi}$ cells. However,

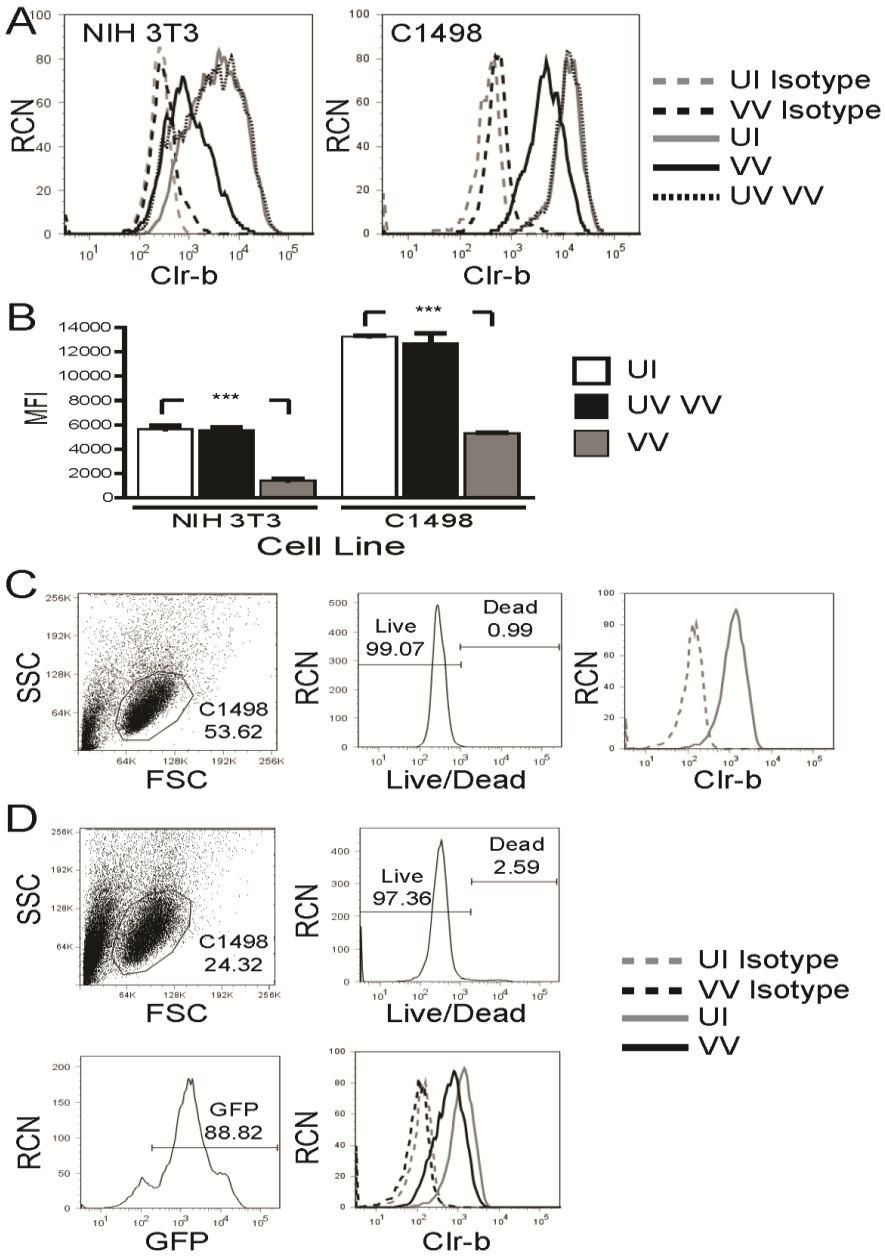

Figure 3.1 *Clr-b down regulation by VACV*
NIH 3T3 and C1498 cells were incubated in media or infected at a MOI of 10 with VACV or an equivalent amount of UV inactivated VACV for 12 hours. (A) Clr-b surface staining with the Ab 4A6 shown relative to isotype control Ab was determined by flow cytometry. (B) The average mean fluorescence intensity (MFI) for three experiments done in triplicate is plotted for each condition. The error bars represent the SE. (C and D) C1498 cells were infected at a MOI of 10 for 12 hours prior to staining for Clr-b and with a fixable live/dead cell stain. (C) illustrates the staining on the uninfected control cells, whereas (D) shows the gating strategy, eGFP expression, and Clr-b staining for the infected live cells. The p values were calculated using an unpaired two-tailed t test. The results are an average of the three experiments, each done in triplicate. ***p = 0.0005. RCN, Relative cell number

by these methods, we cannot distinguish if the eGFP^{lo} cells with lower Clr-b are actually infected, are more resistant to infection by VACV, or if soluble factors released from the infected cells may drive down Clr-b expression on uninfected cells.

3.2.2 The rate of Clr-b loss from the cell surface is greater than that of MHC I

To further characterize the reduction of Clr-b expression, we performed a time course analysis using C1498 cells to compare the changes in Clr-b relative to other cell-surface proteins. We also evaluated the effects of these treatments on the expression of MHC I surface proteins, the ligands of the Ly49 receptors, and the highly stable transferrin receptor (TfR) that recycles through the endosomal pathway. We infected the cells with eGFP-VACV at a MOI of 10 to ensure all of the cells were infected. For comparison with the normal rate of Clr-b cell-surface turnover, we also treated the cells with 50 mg/mL cyclohexamide (CHX) to block protein synthesis. CHX treatment caused a detectable loss of Clr-b at 4 hours, illustrating that Clr-b is normally quite rapidly turned over at the cell surface under steady state conditions (Figure 3.3A). The virus induced down regulation was slightly slower, but both treatments promoted a significant reduction of Clr-b expression by 12 hours (Figure 3.3A and B). We observed less VACV mediated downregulation of MHC I, only obvious in the histograms after 12 hours of infection (Figure 3.3A), whereas the effects of CHX on MHC I expression were more obvious and more rapid. In some experiments, VACV had little effect on MHC I levels at any time point (Figure 3.3B). However, as expected, surface TfR expression remained relatively unaffected by VACV infection or CHX treatment. These results show that VACV does not alter the expression of each cell surface protein to the same extent, and, of particular interest in this study, the rate of Clr-b loss is closer to treatment with a protein synthesis inhibitor than is the loss MHC I, as

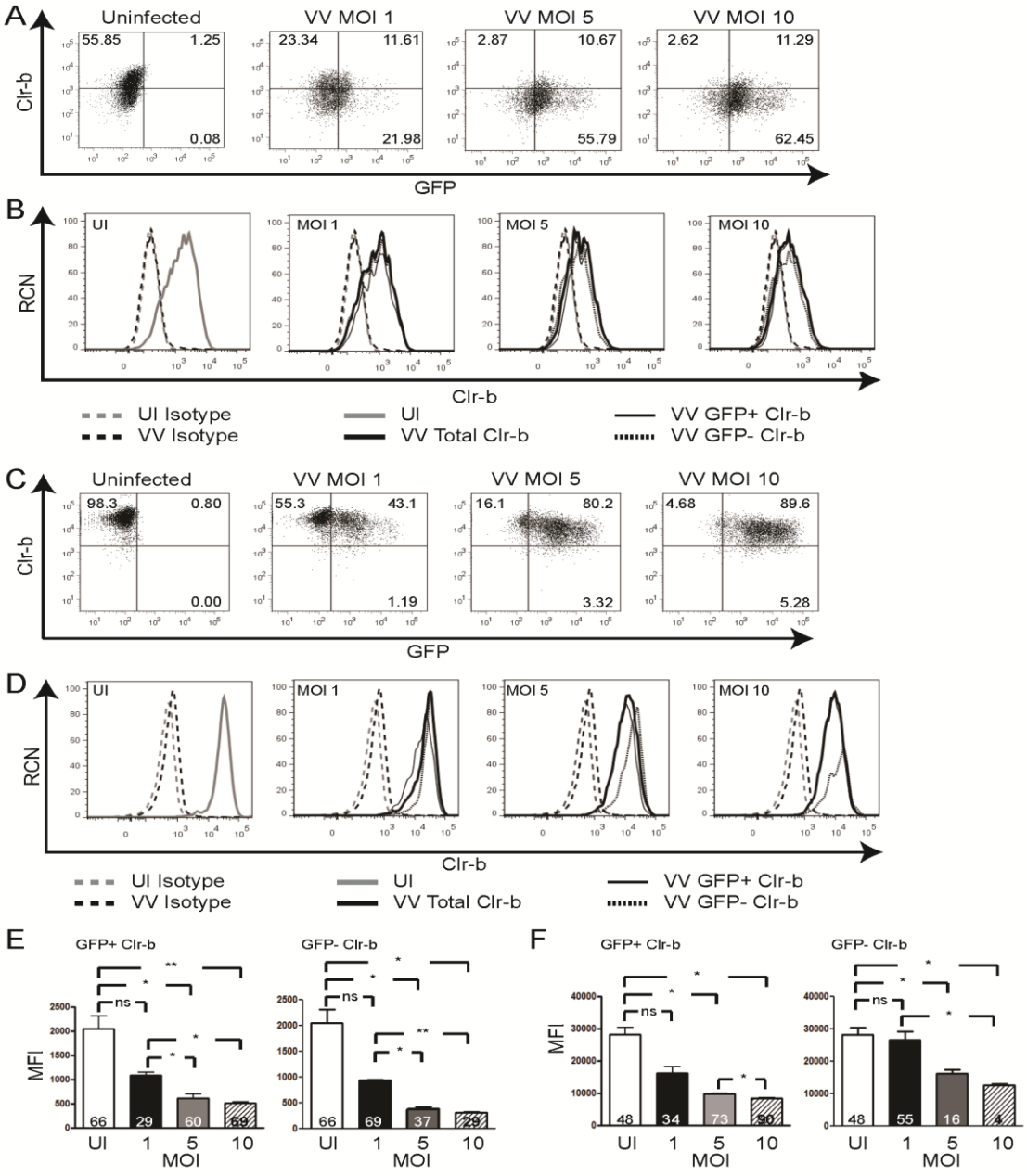

Figure 3.2 *Clr-b loss is correlated with increasing does of virus*
Clr-b loss is correlated with increasing dose of virus. NIH 3T3 cells (A, B, E) and C1498 cells (C, D, F) were infected at the indicated MOI with eGFP-VACV for 12 hours and analyzed by flow cytometry for eGFP and Clr-b expression. (A) and (C) show the relative expression of Clr-b and eGFP in representative samples from one experiment. The quadrants are set to the uninfected cells and isotype control for Clr-b. (B) and (D) depict the histogram profiles for Clr-b expression on the gated subsets for eGFP expression as indicated by the legend below the panels. (E) and (F) are the average MFI for triplicate samples on the same day. The numbers within the bars indicate the percentage of the live cells found within the gate. The error bars indicate the SE. The p values shown above the panels were calculated using an unpaired two-tailed t test. The results are representative of three experiments. *p = 0.05, **p = 0.005.

depicted in Figure 3.3B. This suggests the two are modulated by different mechanisms, and, perhaps, Clr-b loss could be detected by NK cells prior to loss of MHC I.

3.2.3 Clr-b transcript is reduced during infection

Infection with VACV could cause Clr-b to be reduced through a variety of mechanisms including internalization, degradation, or decreased production. It is well established that poxviruses can interfere with cellular transcripts, leading to reductions in host proteins (16, 32, 93, 186). Therefore, we compared the rate of Clr-b protein loss to the amount of mRNA. As a positive control to detect loss of Clr-b mRNA, we treated the cells with 20 mg/mL of Actinomycin D (ActD) to inhibit transcription elongation. The rate of loss of Clr-b from the cell surface is quite similar on VACV infected cells compared with cells treated with ActD (Figure 3.4A). Using 50 ng of RNA, there is a clear loss of Clr-b message at the 12 hour time point and some loss by 8 hours (Figure 3.4C). In contrast, there was little change in the detection of the abundant and stable message for β-actin. We could also detect a corresponding increase in message of the viral gene D10. Therefore, as expected, VACV infection of C1498 cells causes a reduction in Clr-b message, but in comparison with treatment with ActD, the kinetics of the loss of Clr-b mRNA appear to be delayed relative to the loss of Clr-b cell surface protein.

3.2.4 Early infection is sufficient for Clr-b reduction

To better delineate the viral processes involved in Clr-b down regulation, we repressed late gene expression by inhibiting viral DNA replication using cytosine β-D-arabinofuranoside (AraC). AraC treatment eliminated the appearance of cells with high expression of eGFP in both cell types (Figure 3.5A). This is likely due to AraC's effect on replication of the viral genome, preventing greater production of eGFP that in this recombinant is under the control of a synthetic early and late promoter (97). It is also interesting that by this measure, the infection seems to

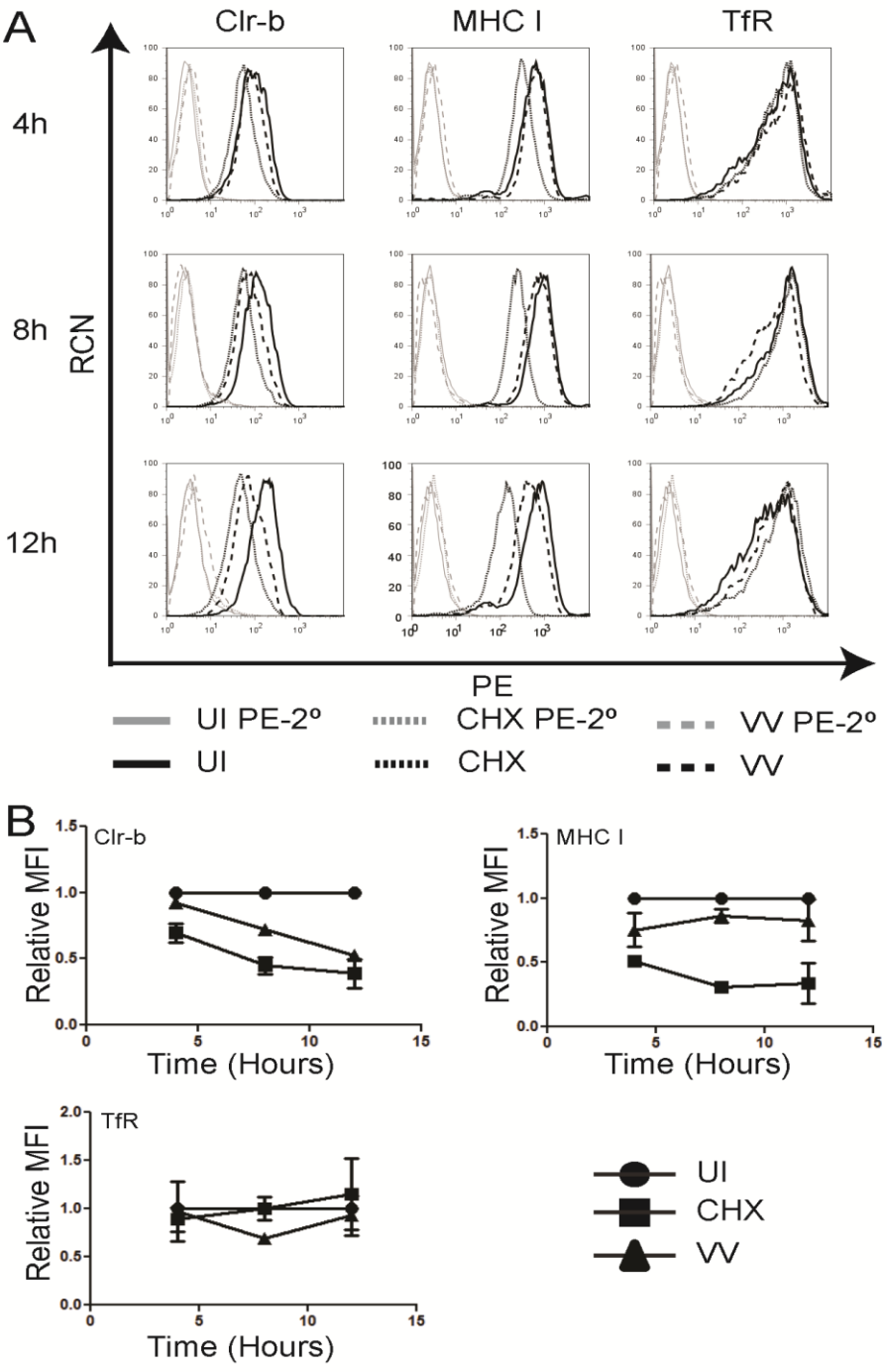

Figure 3.3 *Time course analysis of Clr-b loss following infection*
(A) C1498 cells were infected with a MOI 10 of eGFP-VACV or treated with 50 mg/mL cyclohexamide (CHX). The cells were surface stained for Clr-b, MHC I, or transferrin receptor (TfR) at the specified time points and analyzed by flow cytometry. (B) The relative loss of each protein shown by decay curves that were plotted by calculating the ratio of the MFI of infected to uninfected (UI) at each time point for the treatments. The results correspond to the average of three independent experiments. The error bars indicate the SE.

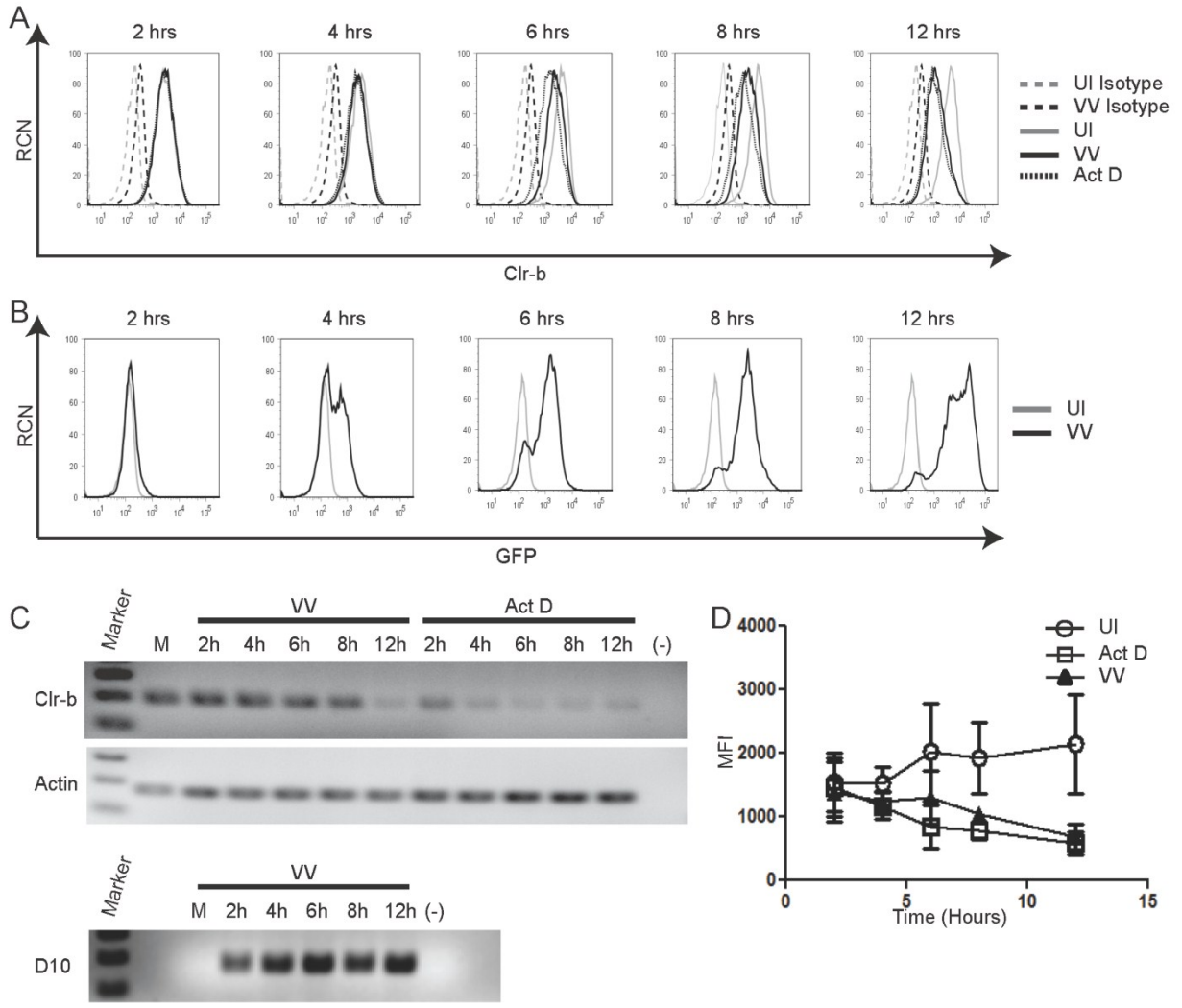

Figure 3.4 *Kinetics of changes in Clr-b surface protein expression and steady-state mRNA levels* (A) C1498 cells were incubated in media alone or 20 mg/mL actinomycin D or infected with eGFP-VACV at a MOI of 10 for the indicated periods. At each time point, C1498 cells were harvested and stained with anti-Clr-b. (B) The expression of eGFP is depicted for the same samples as in (A). (C) RT-PCR analysis was performed using 50 ng of RNA/sample. (D) The average MFI of Clr-b expression for three independent experiments is shown. The error bars represent the SE. UI, Uninfected.

progress better in the NIH 3T3 cells than in the C1498 cells, although we did not observe this in all experiments (Figure 3.2 and D). AraC treatment alone led to a slight reduction of Clr-b at 12 hours, but this was not statistically significant (Figure 3.5C). AraC did not prevent eGFP-VACV mediated Clr-b downregulation (Figure 3.5B and C). The results suggest that neither DNA replication nor late gene expression are required for the observed VACV mediated reduction of Clr-b.

3.2.5 Clr-b reduction occurs with normal macrophages and ECTV

Clr-b is often reduced in transformed cells; therefore, we wanted to ensure that the effects we observed upon infection were not dependent on a transformed state of C1498 cells or a high passage number of NIH 3T3 cells. To do this, we generated GM-CSF stimulated bone marrow derived macrophages (BMMØ) from C57BL/6 mice. Clr-b expression on these cells peaks at about day 7, and although these cells do not have uniform expression of Clr-b, the majority can be infected by eGFP-VACV. The expression is clearly down regulated within the infected (i.e., $eGFP^+$) cells (Figure 3.6). Curiously, it appears that the remaining uninfected cells have low Clr-b expression as well, suggesting the Clr-b high cell subset or cell type may be preferentially infected.

We next asked whether a more physiologically relevant poxvirus caused a similar change in Clr-b expression by using the bona fide mouse pathogen ECTV. We optimized infection of our cells with ECTV using intracellular staining for the viral protein I5L. We found good expression of I5L in the cell lines and weak but detectable expression in the BMMØ at 18 hours using a MOI of 5 (Figure 3.7A and B).We also observe a pronounced loss of Clr-b expression in all three cell types upon ECTV infection (Figure 3.7C and D). Together, these results indicate that both VACV and ECTV infection can promote Clr-b down modulation on primary cells.

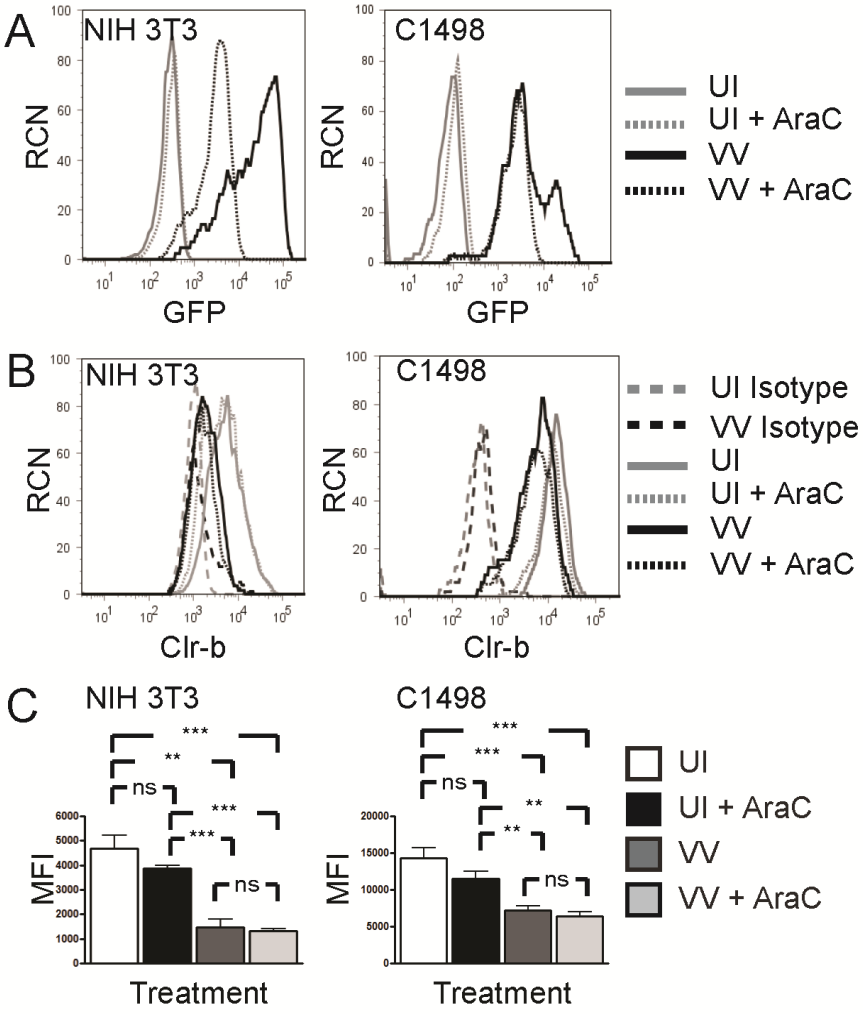

Figure 3.5 *Clr-b loss occurs in the presence of an inhibitor of late gene expression*
NIH 3T3 or C1498 cells were incubated with media or infected with a MOI of 10 of eGFP-VACV for 12 hours in the presence or absence of 50 µg/mL AraC. The expression of eGFP (A) or Clr-b (B) is shown for representative infected and uninfected cells relative to isotype control. (C) shows average MFI for three experiments each done with triplicate samples. The error bars indicate the SE. The p values shown above the panels were calculated using an unpaired two-tailed t test. **p = 0.005, ***p = 0.0005. UI, Uninfected.

3.2.6 VACV infection leads to the loss of functional recognition of Clr-b

To determine the functional consequences of VACV mediated Clr-b down regulation, we assessed the effect of VACV infection on Clr-b mediated protection from IL-2 activated NK cells (LAK). To perform these experiments, we used NK cells derived from CD-1 mice that have high expression of NKR-P1B, as shown in this study by staining with the NK1.1 Ab (Figure 3.8A). We used C1498 cells as the target cells because their expression of Clr-b is sufficient to protect cells from NK cells with NKR-P1B (Figure 3.8B and C). As expected, masking Clr-b on uninfected targets with the anti-Clr-b Ab 4A6 (IgM) caused an increase in lysis compared with target cells incubated with an isotype control Ab (Figure 3.8C), demonstrating Clr-b mediated protection of the target cells. The protection through Clr-b interaction with NKR-P1B was completely abrogated by 18 hours of infection with VACV, as there is no difference in the lysis between isotype control and anti-Clr-b monoclonal Ab treatment of the target cells (Figure 3.8C). At this time point, the reduction in Clr-b was quite pronounced (Figure 3.8B), even though the cells were still intact. However, the lysis of the infected targets was slightly less than mock-treated cells, suggesting that the virus might also interfere with NK mediated cytotoxicity through another mechanism. Notably, similar results were also observed after 12 hours VACV infection; however, at this intermediate time point, we only observed a partial reduction in Clr-b mediated protection and a blunting of the overall lysis (Figure 3.9).

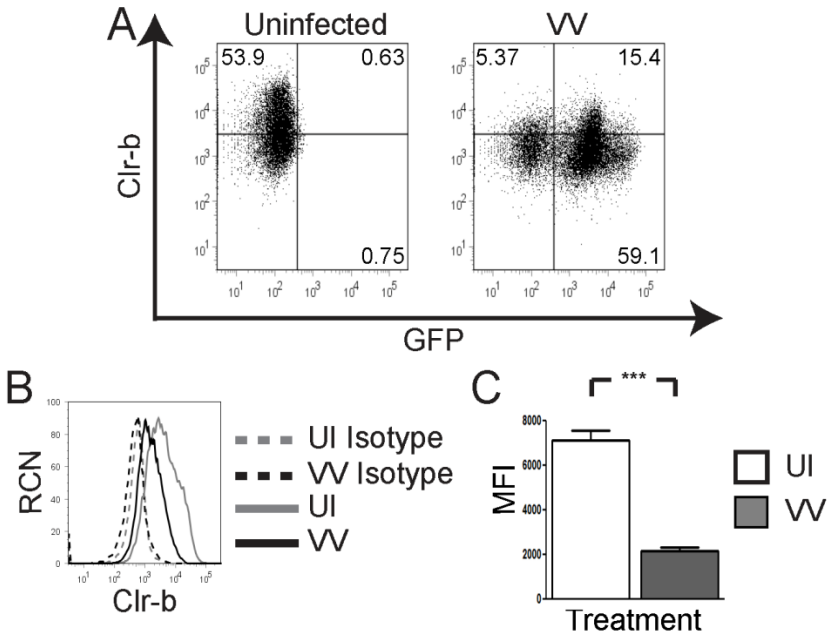

Figure 3.6 *Clr-b down regulation on primary BMMØ*
Day 7 BMMØ were infected with eGFP-VACV at a MOI of 10 for 12 hours and analyzed by flow cytometry. (A) The expression of Clr-b and eGFP. The quadrants were set using the uninfected cells (eGFP) stained with control Ab. (B) The profile for Clr-b staining is shown in the $eGFP^+$ gate for the infected cells relative to uninfected (UI) cells. The staining is indicated in the legend. The results are representative of three experiments. (C) The average MFI for Clr-b expression within the $eGFP^+$ cells for three experiments done with triplicate samples. The background staining is subtracted from the values. The error bars represent the SE, and the p values were calculated using an unpaired two-tailed t test where ***p = 0.0005.

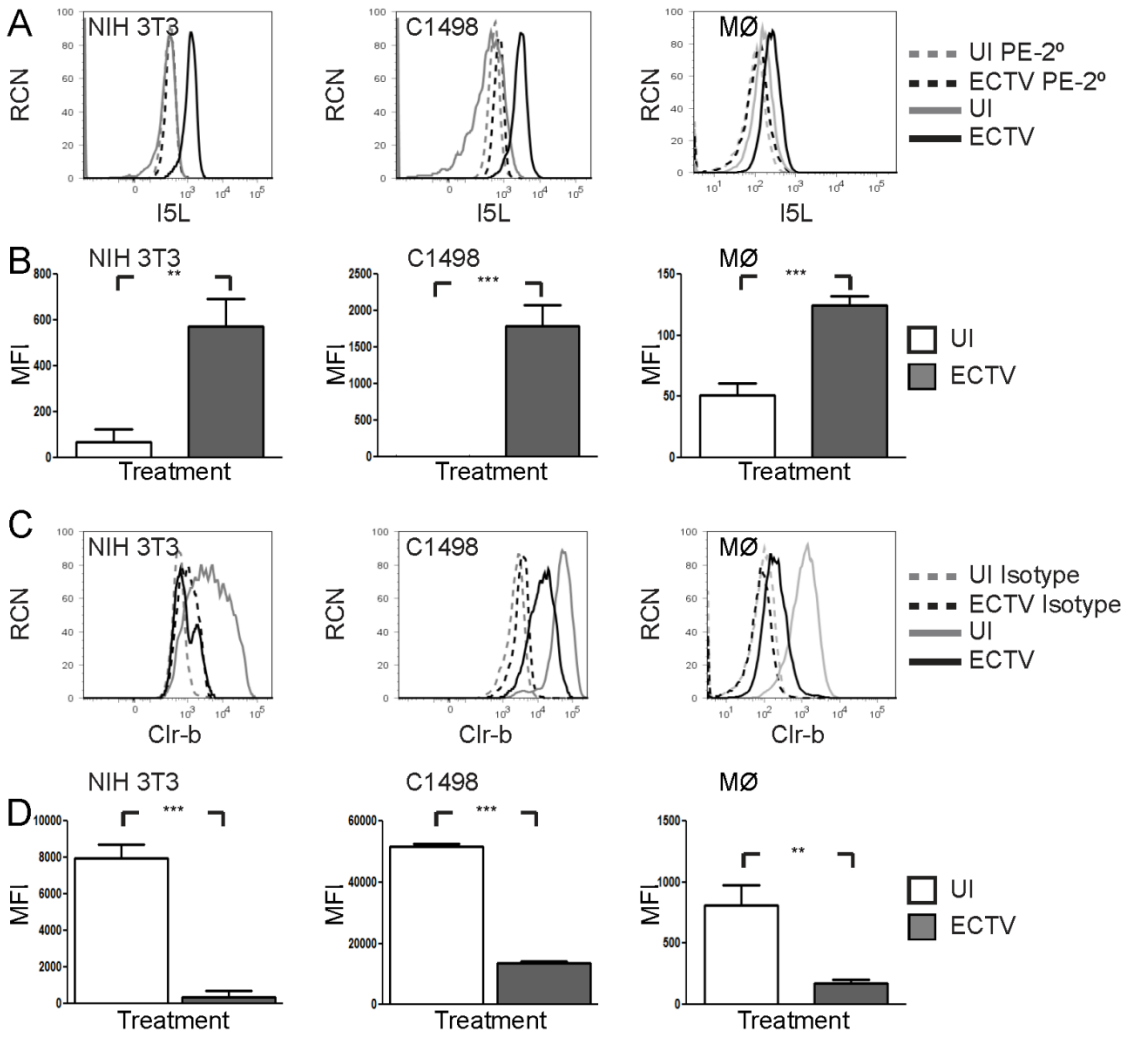

Figure 3.7 *Clr-b down regulation by ECTV*
Cells were infected with ECTV at a MOI of 5 for 18 hours for NIH 3T3 cells and BMMØ and 24 hours for C1498 cells. (A) The cells were analyzed for expression of I5L by intracellular staining. The background was established by staining with secondary alone and staining on uninfected (UI) cells. (B) The background-corrected average MFI is plotted for I5L expression of triplicate samples. (C) The cells were analyzed for surface expression of Clr-b as before. (D) The background-corrected average MFI is plotted for Clr-b expression of triplicate samples. For (B) and (D), the p values were calculated using an unpaired two-tailed t test, and the error bars represent the SE. The results represent the average of three experiments. **p = 0.005, ***p = 0.0005.

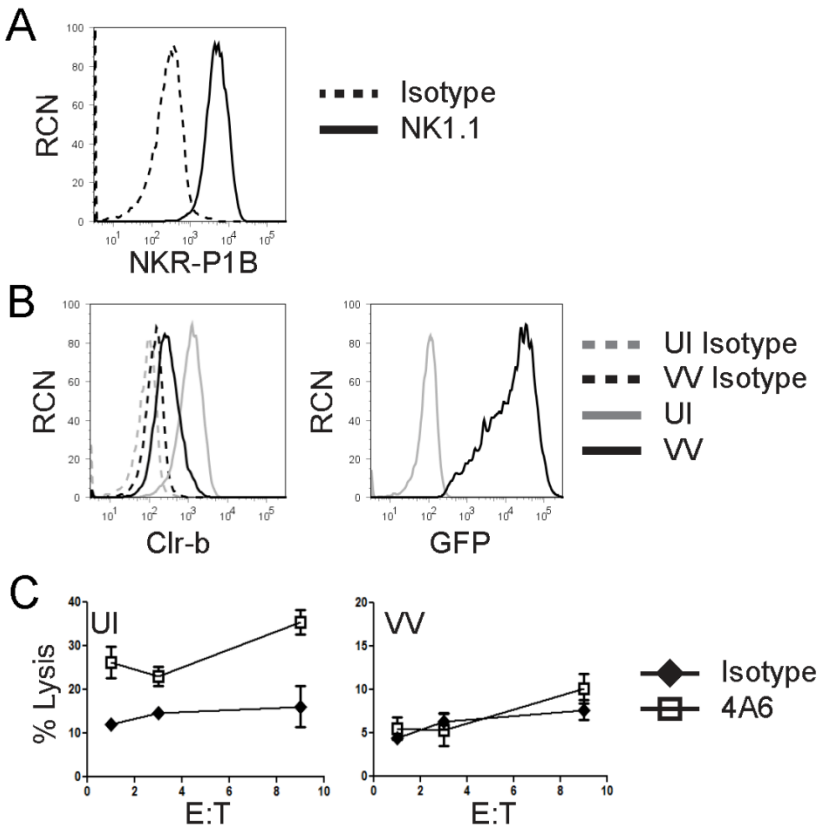

Figure 3.8 *Functional recognition of Clr-b following VACV infection*
(A) Day 7 NK cells from CD-1 mice analyzed for NKR-P1B expression by staining with NK1.1. (B) C1498 cells were infected with VACV at a MOI 10 for 18 hours and analyzed for loss of Clr-b (left panel) and GFP expression (right panel) by flow cytometry. (C) Mock-treated and infected C1498 cells shown in (B) were labeled with [^{51}Cr] and used as targets in a cytotoxicity assay with the NK cells shown in (A). The assay was performed in the presence of 4A6 (40 mg/mL) or isotype control Ab (40 mg/mL). The spontaneous lysis was determined in the presence of the Abs. The error bars represent the SE. The results are representative of three experiments. UI, Uninfected.

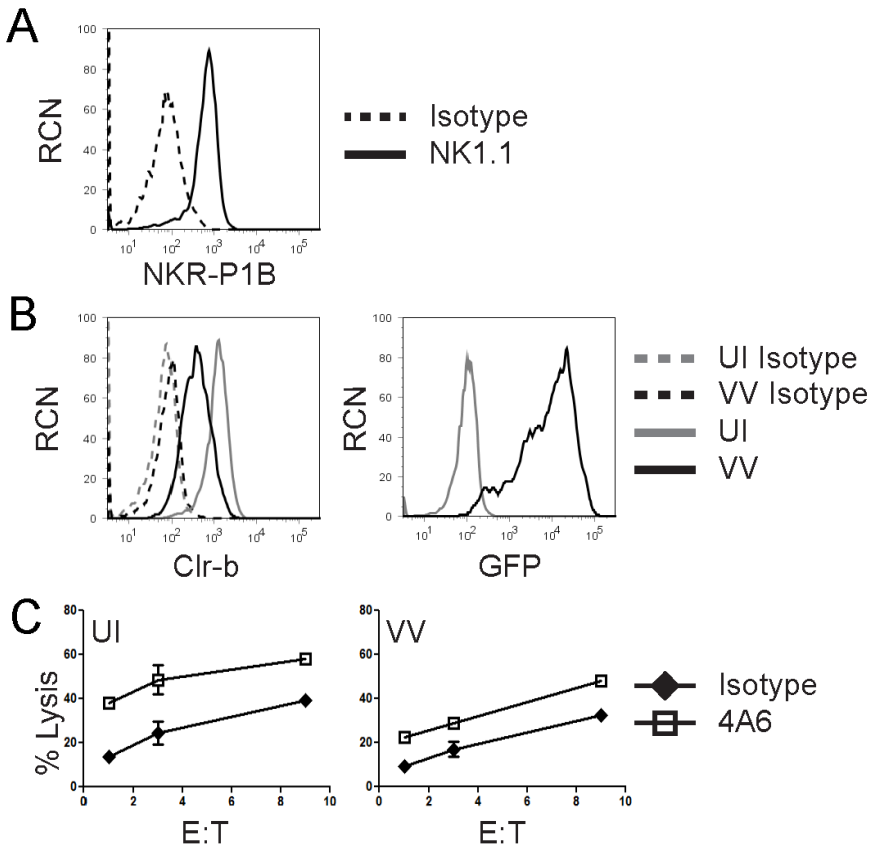

Figure 3.9 *Functional recognition of Clr-b following 12 hour VACV infection*
(A) Day 8 NK cells from CD-1 mice analyzed for NKR-P1B expression by staining with NK1.1. (B) C1498 cells were infected with VACV at a MOI 10 for 12 hours and analyzed for loss of Clr-b (left panel) and GFP expression (right panel) by flow cytometry. (C) Mock treated and infected C1498 cells shown in Panel B were labeled with ^{51}Cr and used as targets in a cytotoxicity assay with the NK cells shown in A. The assay was performed in the presence of 4A6 (40 mg/mL) or isotype control antibody (40 mg/mL). The spontaneous lysis was determined in the presence of the antibodies. The error bars represent the standard error.

3.3 Summary

We have demonstrated in this study that Clr-b expression is down regulated from the surface of mouse cells following infection with two different orthopoxviruses. We observed that the loss was relatively rapid in comparison with MHC I. The loss was most pronounced within the $eGFP^{hi}$ cells, but it is also worth noting that in some experiments, a subset of Clr-b low cells remained uninfected or less productively infected (negative/low for eGFP), particularly within the BMMØ.

The precise mechanism of how Clr-b is lost from the cell surface remains unknown, but we have established that it requires an active infection and not expression of late viral genes. The mRNA for Clr-b became only weakly detectable in our assay by 12 hours, suggesting that viral interference with host cell mRNA likely contributes to a proportion of the Clr-b loss, particularly at later time points. However, the reduction of Clr-b message at early time points was less than observed for treatment with ActD, an inhibitor of transcription (and DNA replication), whereas the loss of surface protein between the two treatments was remarkably similar. This disparity suggests that mechanisms in addition to virus mediated suppression of Clr-b mRNA are involved in the observed reduction of Clr-b surface protein. There are at least two possible scenarios by which virus infection could lead to decreased Clr-b: one possibility is that the host cells innately sense infection and/or respond by down regulating Clr-b surface protein (either specifically or by proxy) to alert NK cells of an infection, and a second is that the virus may encode proteins that target Clr-b (and/or Clr/Clec2) function. Poxviruses are known to employ several strategies to block expression of host proteins that are involved in activation of the immune response (5, 61, 210), and there are indications that Clr-b might be involved in immune activation, making it a target for the virus. For mouse cells, the loss of Clr-b appears to dominate when an APC is

actively infected, as we observe a decrease of Clr-b expression on infected macrophages. Poxviruses might interfere with Clr-b protein trafficking and turnover, as they encode a wealth of proteins that manipulate the ubiquitin pathway (235).

We observed a reduction of Clr-b within the time frame it takes for VACV to complete its replication in culture (33), suggesting that the ability of NK cells to detect the loss would be in time for NK mediated antiviral effects to contain the virus and/or recruit other immune effector cells. In contrast, the reduction of classical MHC I molecules we observe is quite modest and similar to that previously reported (33). In terms of NK cells sensing the loss of Clr-b, we found that infection of target cells does prevent Clr-b from mediating protection of infected cells. However, we did not observe any significant increase in the lysis of the infected target cells compared with uninfected controls. In single experiments performed at an earlier time point, we observed an intermediate effect in which Clr-b was reduced but not gone, and there remained some protection through Clr-b, albeit to a lesser extent than the uninfected targets (Figure 3.9).

The reduction of Clr-b during infection by poxviruses opens the possibility that NKR-P1 receptors influence the NK response to poxviruses in different strains of mice. Although many strains of mice exhibit variegated expression of the NKR-P1 receptors, we examined NK cells from CD-1 mice because they uniformly express NKR-P1B; however, there are no reports, to our knowledge, regarding the relative susceptibility of CD-1 mice to ECTV or VACV. It is also worth noting that C57BL/6 mice, which are resistant to these viruses, express an activating NKR-P1 receptor on all NK cells. In conclusion, the findings presented in this study extend NK-mediated missing-self recognition of Clr-b to poxviruses, but future studies are needed to elucidate the mechanism(s) of Clr (Clec2) regulation and the relative role of the NKR-P1 (Klrb1) receptor system in the NK cell response to poxviruses *in vivo*.

Chapter 4 : Poxvirus Modulation of an Anti-CLEC2D Antibody Reactive Protein

4. Poxvirus Modulation of an Anti-CLEC2D Antibody Reactive Protein

4.1 Introduction

The human homologue of mouse Clr-b, Lectin-like transcript 1 (LLT1), is expressed on activated T cells, B cells, NK cells, toll-like receptor (TLR) activated plasmacytoid DCs and TLR-activated monocyte-derived DCs but not on resting cells (218). The first functional role of LLT1 described that binding antibody to LLT1 on NK cells causes NK cells to produce IFN-γ, but not induce cytotoxicity (156). Later, the receptor pair, NKR-P1A (also known as CD161) was discovered and was found to cause inhibition of cytokine production and cytotoxicity of NK cells when bound to LLT1 on target cells (3, 217).

The *CLEC2D* gene, that encodes for LLT1, is in found in the NK gene complex in close proximity to the *KLRB1* gene that encodes for its receptor, NKR-P1A (191, 217). The *CLEC2D* gene gives rise to six isoforms: three which have transmembrane domains (isoforms 1, 2 and 4), one which produces a nonsense RNA decay candidate (isoform 3) and two which are soluble (isoforms 5 and 6) (85). LLT1 is isoform 1 (will now be known as isoform 1) and is the only isoform that binds to NKR-P1A (85).

Unlike Clr-b, CLEC2D isoform 1 is not found constitutively expressed on cells, but expression is induced by TLR stimulating molecules, such as lipopolysaccharides (LPS) and CpG oligodeoxynucleotides, cytokines or virus (86). It is also expressed on a variety of tumor lines. Both Epstein-Barr virus (EBV) and human immunodeficiency virus (HIV) are able to induce surface expression of isoform 1 on peripheral B cells when PBMCs are exposed to virus (86). Isoform 1 can also be induced on the surface of respiratory epithelium cells when infected by Respiratory Syncytial virus (RSV) (225). Therefore, just as VACV was able to modulate

expression of Clr-b during infection (282), we set out to determine if expression of CLEC2D as a whole could be modified by VACV infection and if its modification could alter the function of NK cells.

We considered it a possibility that CLEC2D could decrease similar to Clr-b. Therefore, we initially studied the effect of VACV infection in transformed cell lines that express LLT1. On the other hand, CLEC2D could increase similar to what is seen in the HIV, EBV and RSV infections, although general disruption of host proteins by viral proteins made this unlikely. This chapter describes my investigation of the modulation of CLEC2D during poxvirus infections and the surprising findings with a CLEC2D "specific" antibody.

4.2 Results

4.2.1 Live VACV infection increases CLEC2D surface expression

We first wanted to determine the effects of VACV infection on CLEC2D. To do this, we incubated 721.221 (221) cells with media or with VACV (strain Western Reserve (WR)) that expresses GFP (eGFP-VACV) for 12 hours at a MOI of 10. The cells were harvested, stained with 4C7, that binds to isoforms 1, 2 and 4 (85), and analyzed by flow cytometry. Following 12 hours of infection, most of the 221 cells expressed GFP while the uninfected cells showed no expression for GFP (Figure 4.1A). Uninfected cells expressed low levels of CLEC2D on the surface of the cells that was significantly enhance by VACV infection (Figure 4.1A and B). These data suggest that VACV induces CLEC2D on 221 cells.

Given the somewhat surprising increase, we considered the possibility that 4C7 may cross-react with a viral protein. To ensure that this was not the case, we tested a fibroblast cell line, NIH 3T3, because it was a mouse cell line that cannot express CLEC2D. We incubated NIH

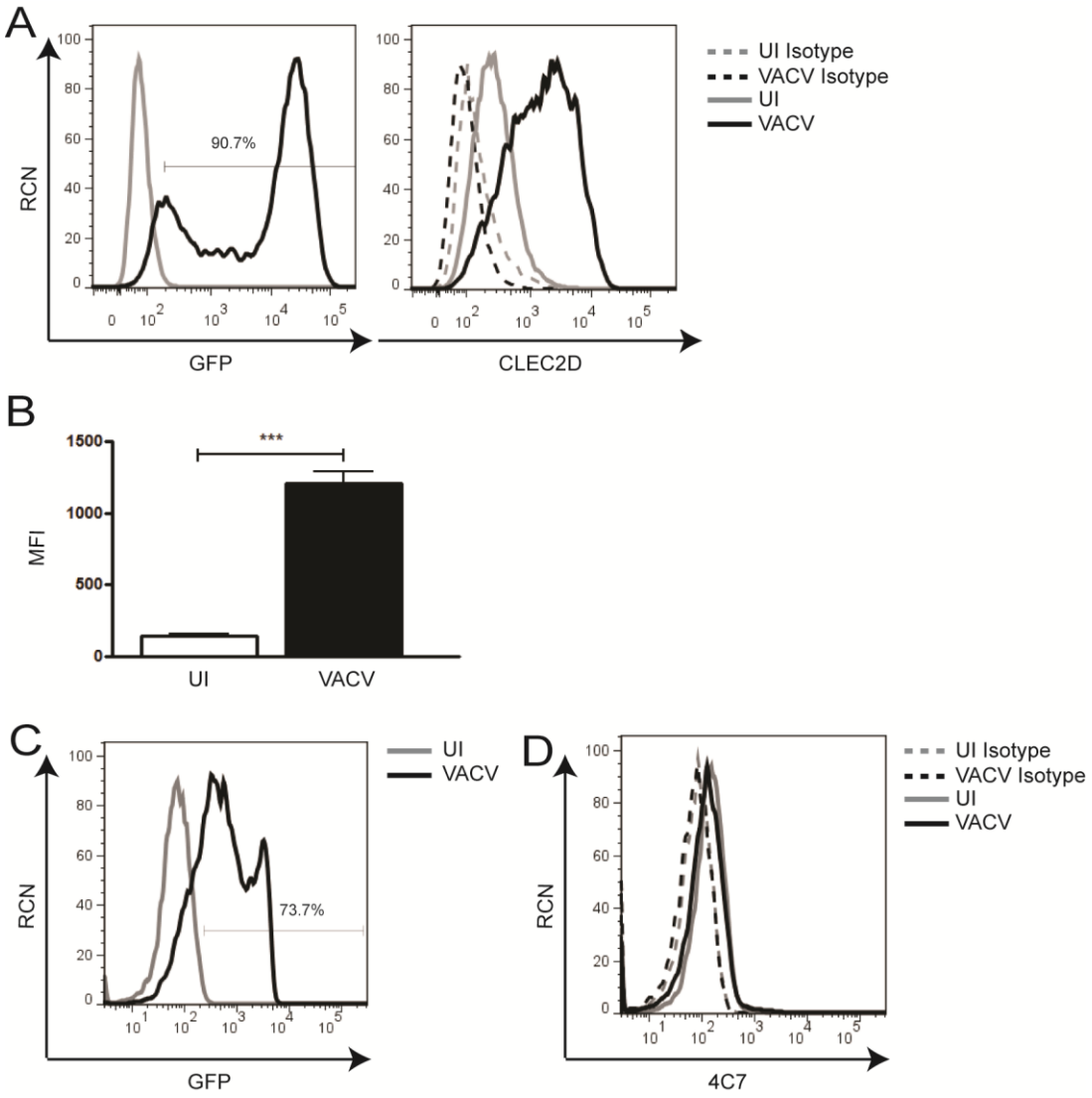

Figure 4.1 *VACV infection causes upregulation of a 4C7 reactive protein*
221 cells were infected with eGFP- VACV at a MOI of 10 for 12 hours and stained with 4C7 for flow analysis. (A) GFP and surface 4C7 staining. (B) MFI of surface CLEC2D following infection. NIH 3T3 cells were infected with eGFP-VACV at a MOI of 5 for 4 hours. (C) GFP expression and (D) 4C7 staining on NIH 3T3 cells. n = 3 experiments each done in triplicate, all data points included. ***$p < 0.0001$.

3T3 with media or eGFP-VACV for 4 hours at a MOI of 5. Cells were then stained with 4C7 and analyzed by flow cytometry. Uninfected cells did not express GFP, whereas infected cells showed ~74% expression (Figure 4.1C). Even though the NIH 3T3 cells were able to be infected by VACV, seen by the GFP expression, the background staining with 4C7 did not change with VACV infection (Figure 4.1D). Given that there was no increase in staining with 4C7 on the infected NIH 3T3 cells, this suggests that 4C7 does not detect a viral protein. Therefore, I proceeded with a systematic dissection of the steps in infection leading to the increase in 4C7 staining.

4.2.2 Time course analysis of VACV induced 4C7 reactivity

To follow the kinetics and dose response of expression detected by 4C7 following VACV infection, we incubated 221 cells with media or with eGFP-VACV at MOIs of 1, 5 and 10 and harvested samples at 2, 4 and 8 hours post infection (PI). Cells were then stained with 4C7 and analyzed by flow cytometry. At 2 hours PI, uninfected cells did not express GFP, however GFP was observed for all MOIs and correlated well with the MOI (Figure 4.2A). As time progressed through the infection, all infected cells increased in GFP expression until by 8 hours most cells were expressing GFP (Figure 4.2A). Uninfected cells had stable levels of 4C7 signal on the surface of the cells for the duration of the time course (Figure 4.2B). Cells infected with eGFP-VACV had significantly higher amounts of 4C7 staining on the surface of the cells than uninfected cells by 2 hours PI that increased with the amount of virus that was added to the cell (Figure 4.2B). The time point of peak 4C7 protein detection depended on the dose (Figure 4.2B).

Given that 4C7 protein staining increased overtime and other viruses and cause an increase in isoform 1 mRNA (225), we simultaneously looked at CLEC2D mRNA over the time course of this infection. In uninfected cells, CLEC2D mRNA expression was relatively constant

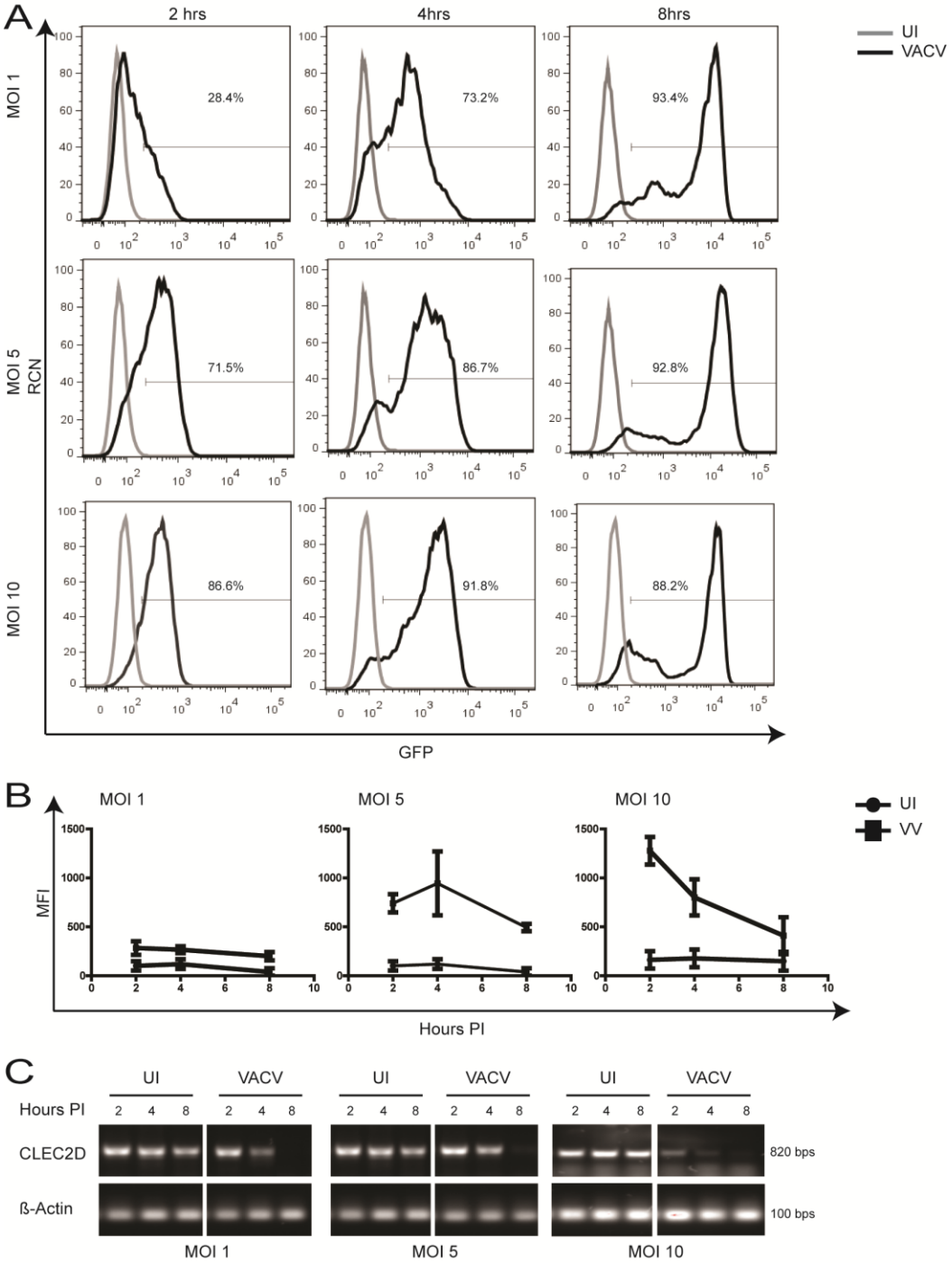

Figure 4.2 *4C7 surface expression is lost over time*
221 cells were infected with eGFP-VACV at various MOIs and for the indicated times, and stained with 4C7 for flow analysis. (A) GFP expression of infected 221 cells over time. (B) Surface expression of CLEC2D on 221 cells over time based upon geometric means of 4C7 staining. (C) Representative RT-PCR of mRNA extracted from 221 cells following infection over time. n = 3 experiments each done in triplicate, all data points included.

(Figure 4.2C). In VACV infected cells, we did not detect an increase in mRNA levels that would correspond to the induction or peak of surface 4C7 staining, but it did decrease over time to undetectable levels even at a MOI of 1 (Figure 4.2C). This loss of message is consistent with VACV destroying host cellular transcripts between 3-6 hours PI (183), and might explain why after the initial increase, the surface expression of the 4C7 reactive protein almost returns to baseline amounts in these experiments. These data together suggest that VACV infection causes an increase in CLEC2D surface expression that is independent of new transcription and is lost over time as the mRNA is lost.

4.2.3 Protein synthesis is not required for 4C7 reactive protein upregulation

The apparent increase in the 4C7 reactive protein surface expression occurs without an increase in transcript. Therefore, we considered that the 4C7 reactive protein could be regulated post-transcriptionally by enhanced mobilization of a pool of protein stored within the cell. To determine if new protein synthesis was required in order to cause the increase in 4C7 staining, we treated 221 cells with cyclohexamide (CHX) for 5 minutes and then incubated them with media or eGFP-VACV at a MOI of 5 for 2 hours. Uninfected cells did not express any GFP, however, all infected untreated cells expressed GFP (Figure 4.3A). Treating cells with CHX for 5 minutes before infection caused a decrease in the GFP expression indicating that viral protein expression was inhibited (Figure 4.3A). Some GFP expression was observed in these CHX treated cells, however this could be due to GFP protein from the viral preparation that the cells have taken up. Given that with the 5 minute pre-treatment some GFP was still detected, we also treated the cells for 1 hour with CHX prior to a 4 hour infection. Similar results were observed where uninfected cells did not express GFP, infected untreated cells had high GFP expression, and infected CHX treated cells had low expression of GFP, likely due to contamination in the

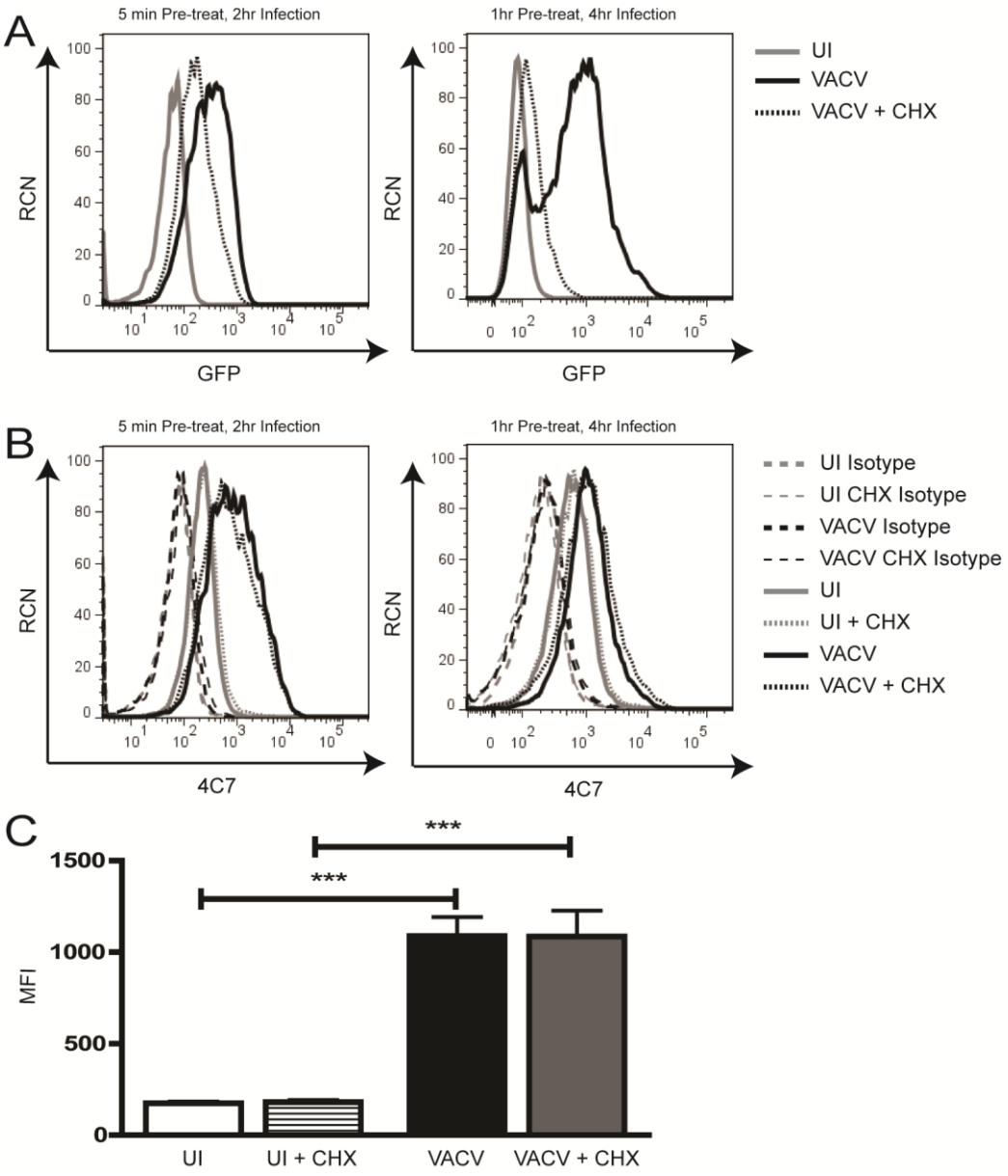

Figure 4.3 *CHX treatment does not prevent upregulation of 4C7 signal*
221 cells were pre-treated with cyclohexamide (CHX) then infected with eGFP-VACV at a MOI of 5 for 2 hours. (A) GFP expression of 221 cells with indicated treatment. (B) Surface 4C7 staining on 221 cells with indicated treatment. (C) MFI of surface 4C7 staining on 221 cells following 5 minute pre-treatment and 2 hour infection. n = 3 experiments each done in triplicate, all data points included. 1 hour pre-treatments were performed twice. **p < 0.001 and ***p < 0.0001.

virus preparation with GFP protein (Figure 4.3A). Uninfected cells, whether treated with CHX or not, had negligible surface 4C7 staining, while VACV infected untreated cells had a significant increase in surface 4C7 staining (Figure 4.3B and C). Treating cells with CHX before infection, whether it was 5 minutes or 1 hour, did not cause a change in the surface 4C7 staining from untreated VACV infected cells (Figure 4.3B and C). This suggests that the increase in surface 4C7 staining does not require new protein synthesis of either a viral or host protein.

4.2.4 4C7 detects an intracellular pool of protein in 221 cells

Since the 221 cells were treated with CHX and were still able to increase their surface 4C7 staining, we suspected that the cells have a pool of the 4C7 reactive protein that could be rapidly mobilized to the cell surface. Subsequently, we exposed the 221 cells to media or eGFP-VACV at a MOI of 5 for 5 minutes and examined them at early time points. Uninfected cells had no expression of GFP and very little to no 4C7 staining on the surface of the cells (Figure 4.4A and B). There was some GFP expression on the infected cells (Figure 4.4A), however, that most likely comes from the semi-pure virus that we add to the cells containing some carry-over GFP from the virus preparation. Amazingly, infected cells had a significant increase in 4C7 staining on the surface of the cells within 5 minutes of exposure to virus (Figure 4.4A and B). Given that surface expression occurs rapidly and no new protein is required for the expression (see Figure 4.3), we permeabilized the 221 cells and stained them with 4C7. Untreated cells have nominal surface 4C7 staining, whereas permeabilized cells have very high staining with 4C7 (Figure 4.4C). This suggested that there may be an intracellular pool of a 4C7 reactive protein in 221 cells that is able to be mobilized rapidly during a VACV infection. This idea was confirmed by confocal microscopy that illustrated cytoplasmic staining using 4C7 that was not seen using the

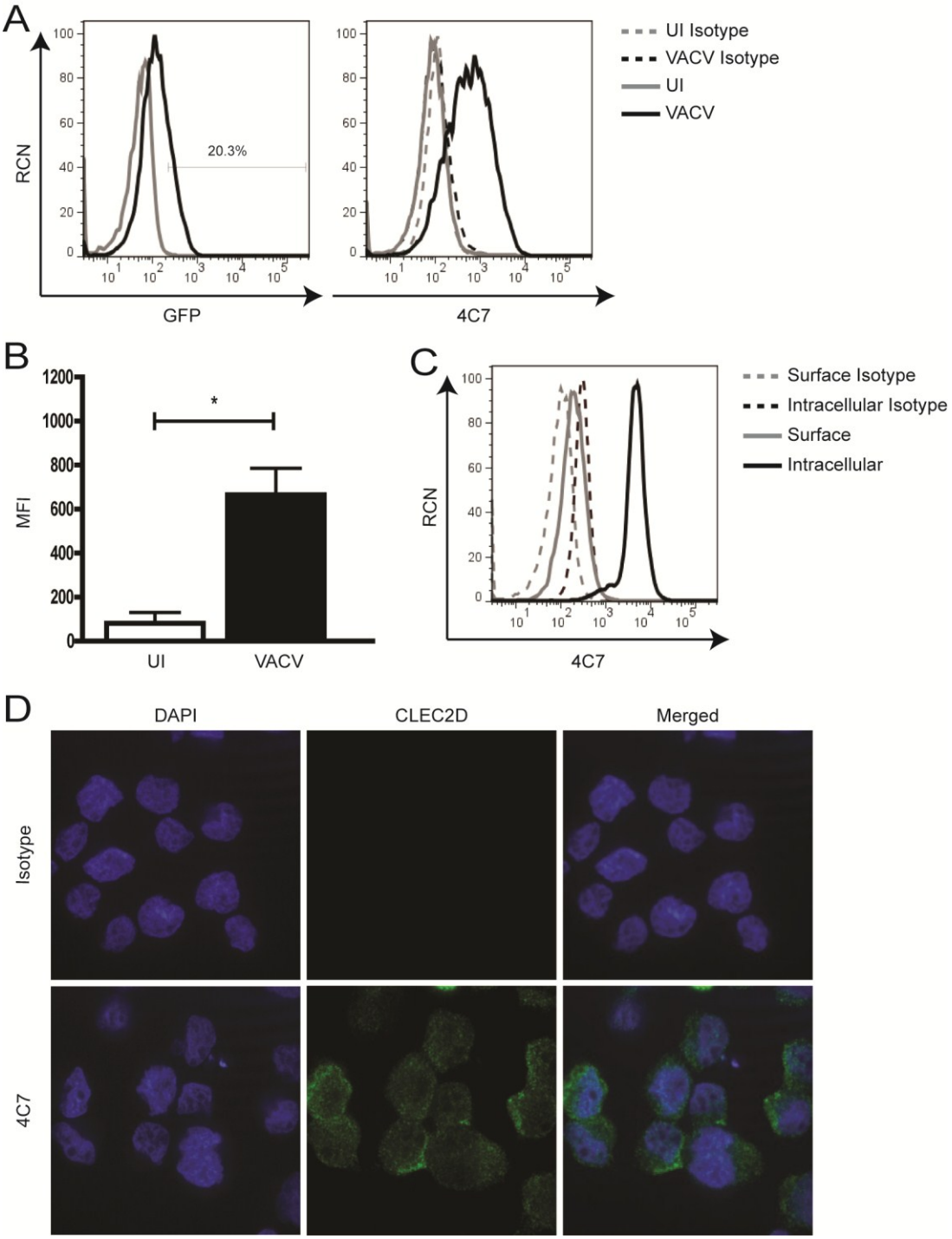

Figure 4.4 *221 cells have an intracellular pool of 4C7 reactive protein*
221 cells were infected with eGFP-VACV for 5 minutes at a MOI of 5. (A) GFP and surface 4C7 staining of 221 cells following infection. (B) MFI of surface 4C7 staining following 5 minute infection. (C) Surface and intracellular 4C7 staining on uninfected cells. (D) Confocal microscopy of 221 intracellular 4C7 staining (60x magnification). n = 3 experiments each done in triplicate, all data points included. *$p < 0.01$.

isotype control (Figure 4.4D). These data suggests that 221 cells contain an intracellular pool of CLEC2D, barring any cross reactivity of 4C7 with another unknown protein.

4.2.5 Live viral attachment is required for surface 4C7 signal

Seeing that exposure to the virus was causing rapid changes in surface 4C7 staining, we wanted to determine what stage of infection was required to cause the upregulation. The first step of infection is viral binding to the cell surface. Therefore, we decided to inhibit binding of the virus to see the effects on surface 4C7 staining. We treated eGFP-VACV with Epigallocatchin gallate (EGCG) which is a green tea catchin that inhibits viral binding to heparan sulfate or to sialic acids (51). The virus was treated with media, DMSO or EGCG for 10 minutes at 37^{o}C and then 12 minutes on ice. The virus was treated with DMSO because EGCG is dissolved in it, hence we had to test if the DMSO itself had an effect on CLEC2D. The virus was then added to cells for 5 minutes at a MOI of 5. Uninfected cells did not express GFP (Figure 4.5A). 221 cells infected with virus treated with media or DMSO produced a slight signal for GFP, as observed previously at 2 hours in the presence of CHX, which was probably due to the GFP protein in the virus preparation that was taken up by the cells (Figure 4.5A). Cells infected with virus treated with EGCG had less GFP signal than untreated or DMSO treated cells likely due to the fact that less virus could bind to the cell due to the treatment (Figure 4.5A). Since the EGCG was dissolved in DMSO, we first compared surface 4C7 staining with media and DMSO treated virus. Uninfected cells had no change in the little surface 4C7 staining with the DMSO treatment (Figure 4.5B). Infected cells, whether treated with media or DMSO treated virus had a major increase in surface 4C7 staining, but no difference between the 2 treatments was observed (Figure 4.5B). On uninfected cells, EGCG had a small but statistically significant increase in surface 4C7 staining over DMSO treated uninfected cells (Figure 4.5B and C). DMSO treated

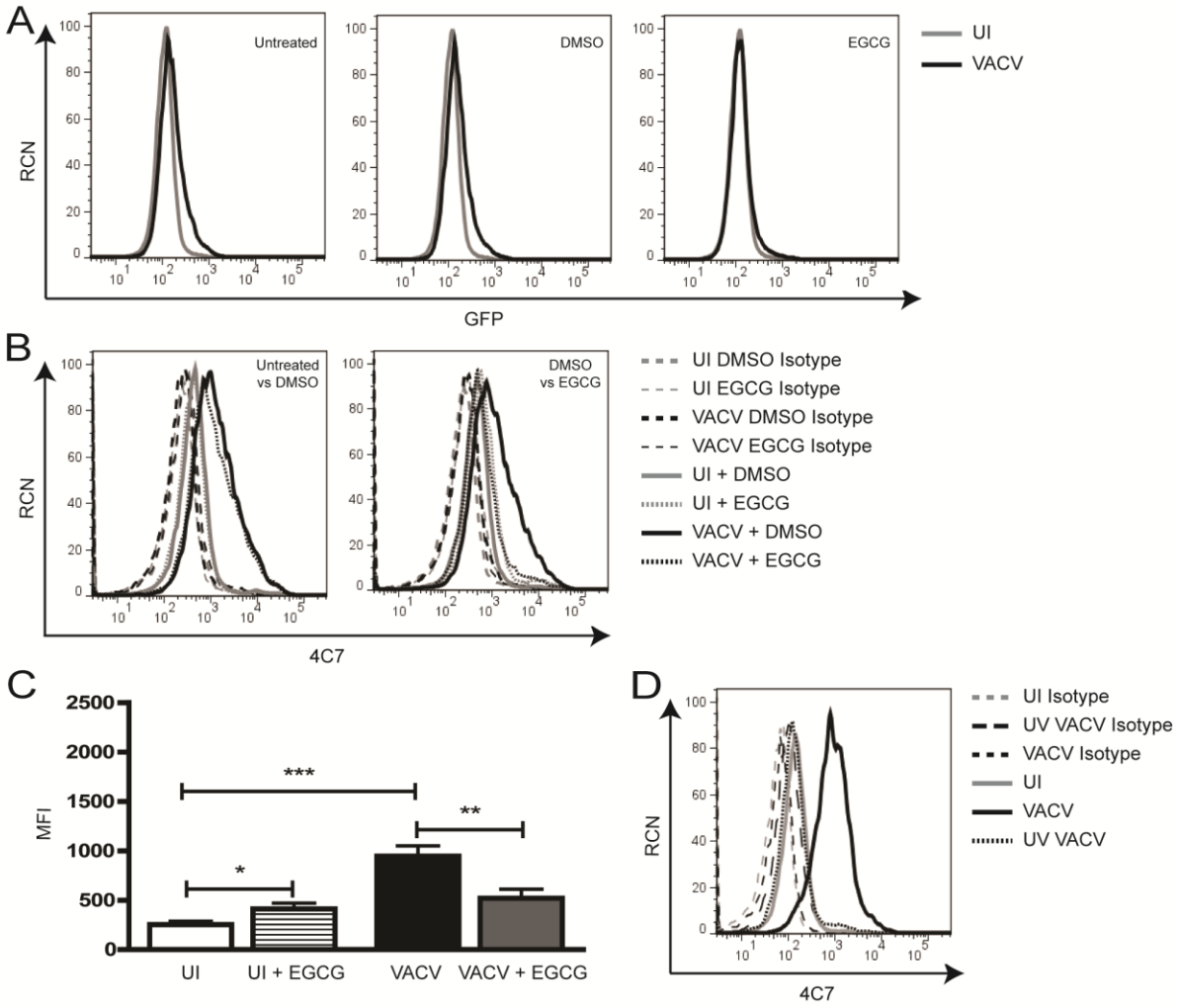

Figure 4.5 *Live viral binding is required for upregulation of the 4C7 reactive protein*
eGFP-VACV was pre-treated with media, DMSO or EGCG for 10 minutes at 37°C and 12 minutes on ice. 221 cells were then infected with treated eGFP-VACV at a MOI of 5 for 5 minutes. (A) GFP expression on 221 cells infected with eGFP-VACV treated with media (untreated), DMSO or EGCG. (B) Surface 4C7 staining on 221 cells infected with eGFP-VACV treated with media, DMSO or EGCG. (C) MFI of surface 4C7 staining on 221 cells with DMSO or EGCG treated eGFP-VACV. n = 3 experiments each done in triplicate, all data points included. (D) 4C7 staining on 221 cells treated with media, UV inactivated VACV or eGFP-VACV for 12 hours at a MOI of 5. n = 1 *$p < 0.01$, **$p < 0.001$ and ***$p < 0.0001$.

virus caused a larger increase in surface 4C7 staining whereas EGCG treated virus had similar levels of 4C7 staining to uninfected cells treated with EGCG (Figure 4.5B and C). This data suggests that EGCG has a small affect on surface 4C7 staining and inhibits the virus from binding to the cells preventing the increase of surface 4C7 staining observed due to VACV infection.

To test if viral entry was sufficient to increase 4C7 staining, we used a UV inactivated VACV. The UV inactivation was verified by titring by a plaque assay. 221 cells were incubated with media, UV inactivated VACV or eGFP-VACV for 12 hours at a MOI of 5 at 37^{o}C with 5% CO_2. Following incubation, cells were harvested and stained with 4C7 for flow cytometry. Uninfected cells expressed a small amount of 4C7 surface staining that increased significantly with infection with eGFP-VACV (Figure 4.5D). When cells were treated with UV inactivated VACV, there was no change in 4C7 staining over uninfected cells (Figure 4.5D). Data shown in Figure 4.5D at a MOI of 5, was also performed at a MOI of 10 with similar results (n = 3, with each experiment done in triplicate). These data together suggest that a replication competent virus must bind to the cell in order to cause upregulation of surface 4C7 staining.

4.2.6 A late VACV protein is required for surface 4C7 signal

To explain how it is possible that the effect on 4C7 staining is independent of new protein synthesis but sensitive to UV inactivation, we hypothesized that a virion associated protein, that is directly sensitive to UV or not released into the cell following UV treatment (see Figure 4.5), is responsible for the increase in surface expression. Most virion associated proteins are made late in the infection and the production of this protein late in infection could be important to sustain expression of the 4C7 reactive protein. Therefore, we tested if Cystosine β-D-arabinofuranoside (AraC), that inhibits DNA replication and viral replication, would prevent this

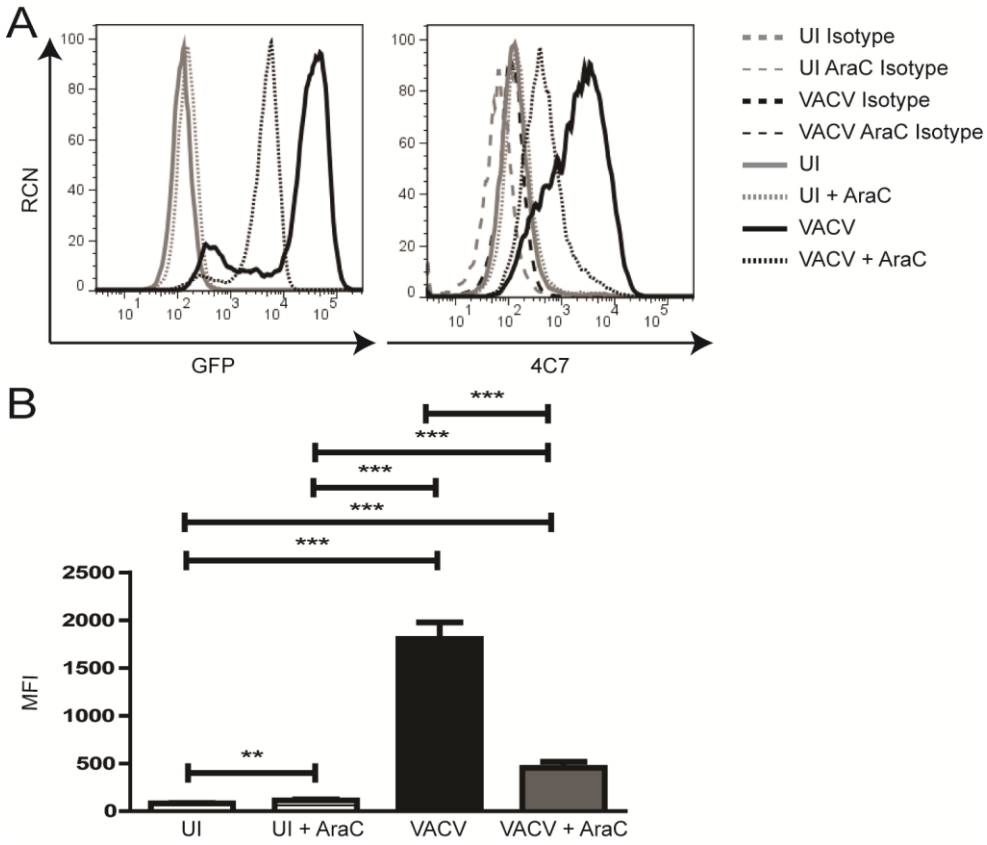

Figure 4.6 *A late viral protein is required for 4C7 staining*
221 cells were pre-treated with AraC for 5 minutes and infected with eGFP-VACV for 12 hours at a MOI of 5. (A) GFP expression and surface 4C7 staining of 221 cells following infection. (B) MFI of surface 4C7 staining treated with AraC. n = 3 experiments each done in triplicate, all data points included. ***$p < 0.0001$.

protein(s) from causing the increase in 4C7 staining later in the infection. We treated 221 cells with AraC for 5 minutes, then incubated them with media or eGFP-VACV at a MOI of 5 for 12 hours. Uninfected cells did not express any GFP and minimal surface 4C7 staining whether treated with AraC or not (Figure 4.6A and B). Infected cells had a significant increase in GFP, while infected AraC treated cells had detectable but reduced GFP expression, given that the late gene promoter activity was inhibited (Figure 4.6A). Infected cells had a significant increase in surface 4C7 staining (Figure 4.6A and B). Infected AraC treated cells had a significant increase in 4C7 staining over uninfected cells, however it was significantly lower than infected untreated cells (Figure 4.6A and B). This suggests that a late gene contributes to the increase in 4C7 staining during the VACV infection. However, fitting with a model that a virion associated protein triggers the early increase in 4C7 staining, AraC has no effect on 4C7 staining out to 8 hours of infection (Eaton and Burshtyn, unpublished observation).

4.2.7 Increase in 4C7 staining is specific to VACV-Western Reserve

To determine if the increase in 4C7 staining observed in poxviruses is a general phenomenon of poxviruses or unique to VACV or orthopoxviruses, we infected 221 cells with another poxvirus and another strain of VACV. We utilized eGFP-VACV (strain WR) and Myxoma, a *Leporipoxvirus* that causes myxomatosis in rabbits and expresses mCherry. We also used a recombinant eGFP expressing VACV-Copenhagen, another less virulent strain of VACV. VACV-WR and Myxoma virus had high expression of GFP and mCherry respectively. The eGFP-VACV-Copenhagen, was unable to infect 221 cells with much efficiency as seen by the low GFP expression (Figure 4.7A). As previously observed, eGFP-VACV-WR caused a significant increase in surface 4C7 staining (Figure 4.7B). EGFP-VACV-Copenhagen

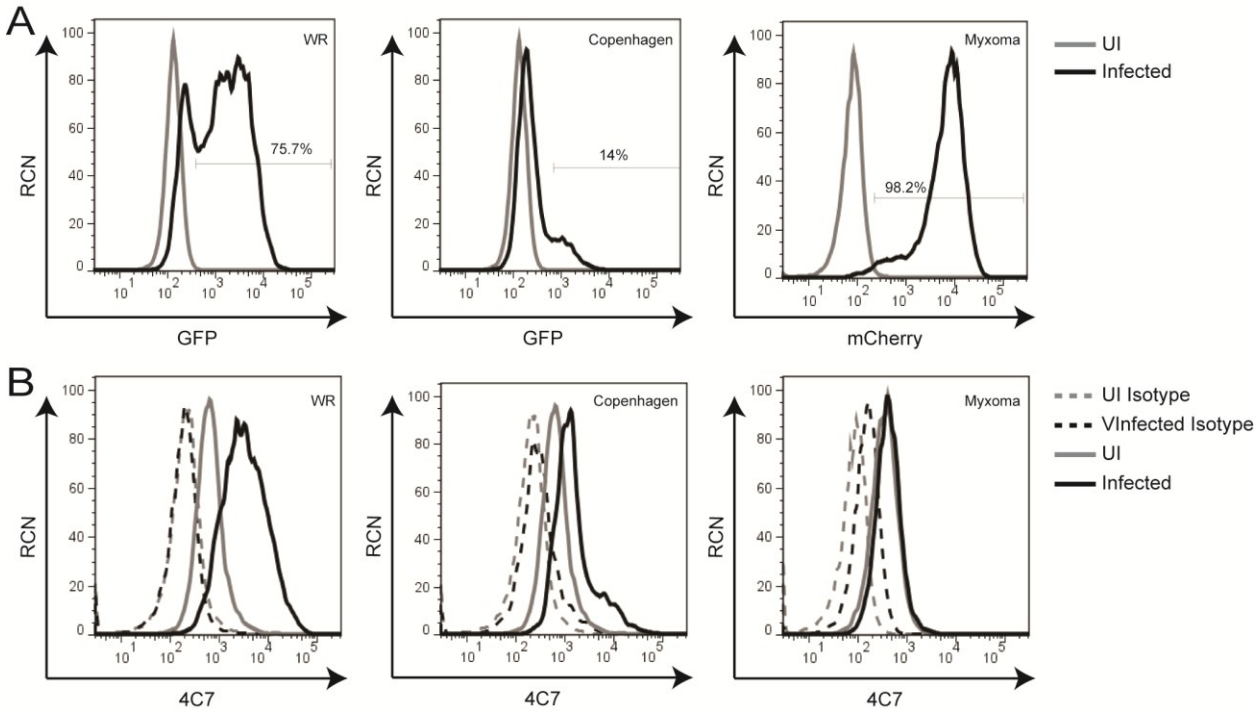

Figure 4.7 *221 cells are not permissive to all strains of VACV*
221 Cells were infected with different strains of VACV and Myxoma virus for 4 hours. (A) Fluorescent expression for 221 cells incubated with media, eGFP-VACV-WR (MOI 10), eGFP-VACV-Copenhagen (MOI 10) or mCherry-Myxoma (MOI 5). (B) Histograms of surface 4C7 staining on 221 cells following infection with indicated viruses. n = 3 experiments each done in triplicate, all data points included. *$p < 0.01$, **$p < 0.001$ and ***$p < 0.0001$

demonstrated a small increase in 4C7 staining, however this expression is based upon the small proportion of cells that were infected (Figure 4.7B). Unlike eGFP-VACV-WR, mCherry-Myxoma virus was unable to cause an increase in surface 4C7 staining (Figure 4.7B). This deficiency of 4C7 reactive protein upregulation from the mCherry-Myxoma infection is likely due to this poxvirus not being able to cause this effect. Given that eGFP-VACV-Copenhagen was unable to infect 221 cells effectively, we explored the 4C7 reactive protein regulation using a more infectable cell line, Hela, that we found did have 4C7 staining PI, and will be explained in the following chapter.

All the viruses were able to infect Hela cells to a high degree evidenced by the high expression of GFP and mCherry (Figure 4.8A). Uninfected Hela cells did not stain with 4C7 (Figure 4.8B and C). EGFP-VACV-WR was able to cause a significant increase in 4C7 staining over uninfected cells (Figure 4.8B and C). EGFP-VACV-Copenhagen was not able to cause an increase in 4C7 staining over uninfected cells and had significantly less 4C7 staining than the eGFP-VACV-WR infection (Figure 4.8B and C). MCherry-Myxoma virus was able to cause a small by statistically significant increase in 4C7 staining over uninfected cells (Figure 4.8B and C).

Subsequently, we discovered that the Hela cells we used in these experiments had been infected with mycoplasma, whereas the 221 cells were not infected. Infected mycoplasma free Hela cells showed high expression for GFP following 2 hours of infection (Figure 4.8D). However, there was no increase in 4C7 staining over uninfected cells (Figure 4.8D), even though these cells still stained with 4C7 intracellularly (Figure 4.8E). This lack of upregulation in the mycoplasma free cells suggest that the Hela cells had to be pre-stressed by infection before they gained the ability to translocate the 4C7 reactive protein to the cell surface following eGFP-

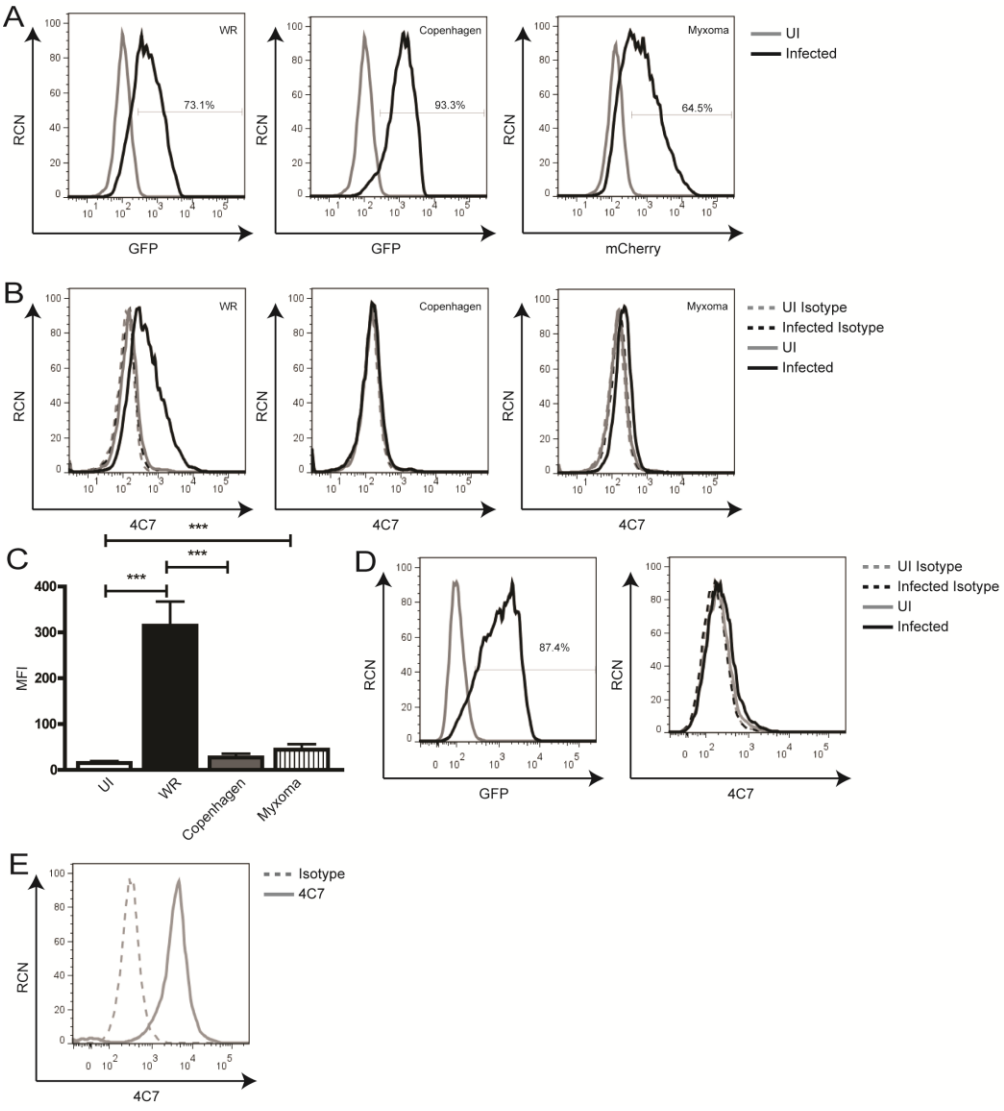

Figure 4.8 *Increase in 4C7 staining is specific to VACV-Western Reserve*
Hela cells were infected with different poxviruses for 4 hours. (A) Fluorescent expression for Hela cells incubated with media, eGFP-VACV-WR, eGFP-VACV-Copenhagen or mCherry-Myxoma (MOI 5). (B) Surface 4C7 staining on Hela cells infected with different poxviruses. (C) MFI for surface 4C7 staining on Hela cells. (D) GFP and surface 4C7 staining on mycoplasma free Hela cells infected with eGFP-VACV-WR for 2 hours at a MOI of 5. (E) Intracellular 4C7 staining in mycoplasma free Hela cells. n = 3 experiments each done in triplicate, all data points included, for WR and Copenhagen. n = 2 experiments each done in triplicate, all data points included, for Myxoma. ***$p < 0.0001$

VACV infection. Although, since the 221 cells were mycoplasma free, they do not require the same mechanism for expressing the 4C7 reactive protein after infection with eGFP-VACV, indicating that there may be a separate mechanism for 4C7 reactive protein expression in different cells. Altogether, these data suggest that different poxviruses are capable of causing an increase in the 4C7 reactive protein, although the Copenhagen strain of VACV lacks this ability. This also implies that the ability to cause this increase may be dependent on the cell type, seeing that Myxoma virus was able to cause this effect in Hela cells but not in 221 cells and that Myxoma virus was able to produce more mCherry in 221 cells than Hela cells at this time point.

4.3 Summary

This chapter investigated the effects of VACV on the surface expression of CLEC2D. Previous studies have shown that other viruses can upregulated CLEC2D isoform 1 transcription and expression on the surface of the cell (86, 218, 225). Here, we have demonstrated that VACV is able to cause an increase in surface expression of a 4C7 reactive protein following infection in two tumor cell lines. Surprisingly, the upregulation does not involve transcription or translation of CLEC2D. Other poxviruses tested had the ability to cause this reaction, though in the VACV species we only tried WR and Copenhagen, and only the WR strain was able to accomplish this feat.

A dose dependent response of 4C7 staining to the viral infection was observed, where over time, the upregulation observed decreased back to uninfected levels. This change in surface 4C7 staining could be due to different possibilities such as the initial translocation of the protein from the intracellular pool and then loss because of protein turn over, or the loss of host message during the infection. Inhibiting viral binding to the cells prevents this increase as well. The best model that fits the data is that the virus has to bind and release a protein into the cell to bring

about the observed upregulation of the 4C7 reactive protein. Such a protein would be contained in the virion, likely in the lateral bodies, and released following uncoating of the virus. The UV inactivation either prevents the release, the virus does not enter the cell or the protein itself is UV labile. The protein, may also be a late viral protein because blocking DNA replication caused the upregulation not to be as substantial later in the infection.

We have also discovered a previously unknown intracellular pool 4C7 reactive protein in 221 and Hela cells. This pool was recognized because exposing the cells to virus caused a very rapid increase in the surface expression of a protein that bound to the 4C7 antibody that was not dependent on new protein being made from the cell.

The discovery that VACV-WR may upregulate CLEC2D on the surface of the cell begets the subsequent questions. Is this a strategy of the virus to evade NK cells? Does the presence of increased CLEC2D on the cell surface following a poxvirus infection, cause an change in NK cell function? These questions will be investigated in the next chapter.

Chapter 5 : Investigation of the Molecule Detected by the 4C7 Antibody Upon Infection with Vaccinia Virus

All the experiments were performed by myself, with the exception of the monocyte profiles in Figure 5.3, performed by Felipe Barboza and some cell line infections in Figures 5.10 and 5.11, performed by Supraja Rengan.

5. Investigation of the Molecule Detected by the 4C7 Antibody Upon Infection with Vaccinia Virus

5.1 Introduction

The results of the previous chapter raised many questions about the protein recognized by 4C7 that was upregulated during VACV infection. To test if causing the infected cell to externalize this protein was a strategy of the virus to evade recognition by the NK cells, I assessed the effects on stimulation of primary NK cells that express the NKR-P1A receptor. Interestingly, in the course of this study, we discovered a note worthy regulation of NKR-P1A on cultured NK cells that we will discuss shortly. The 4C7 antibody can recognize all isoforms of CLEC2D, even the isoforms that are "normally" retained in the cell, and KACL. Therefore, I tested the hypothesis that one of the isoforms normally retained in the cell was being exported to the cell surface during VACV infection and detected by the 4C7 antibody. Furthermore, we wanted to determine if the effect seen during VACV infection was particular to highly transformed cells such as 221 and Hela cells, or if this effect occurred in other cells types. To investigate this question, we would expose different cell lines to VACV to determine if the upregulation of the 4C7 reactive protein was also observed. This chapter will discuss these and other findings.

5.2 Results

5.2.1 VACV does not enhance protection through the NKR-P1A receptor

Now that it is known that VACV infection can cause an increase in a surface 4C7 reactive protein on two cell lines (221 and Hela), that we sought to uncover if the increase can inhibit NK cell function through NKR-P1A. *Ex vivo* NK cells have many inhibitory receptors for MHC I, that could conceal any effects of NKR-P1A inhibition if there are any. Therefore, 221 cells are a

suitable target cell line to use since they are MHC I deficient and hence any inhibition seen would not be through the conventional KIR, CD94/NKG2A or LIR-1 receptors. We incubated 221 cells with media or eGFP-VACV at a MOI of 10 for 1 hour since this time point was the peak of surface 4C7 staining on 221 at this MOI and there was a high amount of infection (see Figure 4.2). Following infection, the 221 cells were plated in a 96-well plate with either IgG2 or anti-CD161 antibodies which should bind to NKR-P1A and block binding of NKR-P1A on NK cells to CLEC2D on target cells. Although we did not have a positive control for the blocking of this particular antibody in our hands, it has been shown to work in previous studies (218). Primary NK cells were isolated from PBMCs, cultured in IL-2 and incubated with 221 cells at a 3:1 effector to target (E:T) ratio, which is ideal to use as there is the ability to observe an increase or decrease of any occurrences (Figure 5.1A). This E:T ratio was titrated to determine the best ratio to use for these experiments. Cells were incubated together for 3 hours to allow enough time for detection of NK cell degranulation, and is a time point at which the infected target cells are mostly alive in the absence of NK cells based on live/dead cell staining. Following incubation, the cells were harvested, washed and stained with antibodies for analysis by flow cytometry. We used a flow cytometry based assay that provides maximum information about the viability of the target cells as well as the response of the NK cells using a marker of degranulation. In addition, it allows examination of the NK cell response relative to their NKR-P1A expression.

NK cells and 221 cells were separated for analysis based on CD56 expression, as only the NK cells express CD56 (Figure 5.1B). The cells were also stained with a live/dead cell stain and the live $CD56^-$ 221 cells were further analyzed for infection and 4C7 staining (Figure 5.1C). In the experiment shown, ~73% of the infected cells expressed GFP following 1 hour of

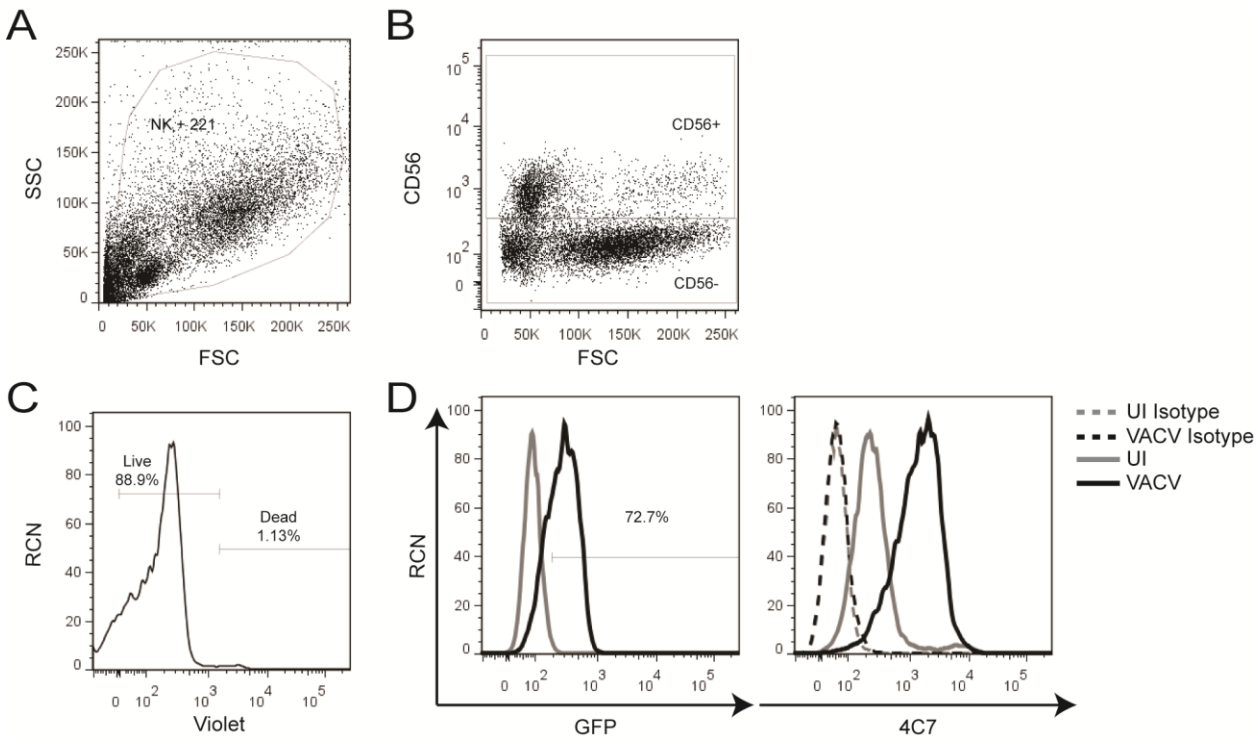

Figure 5.1 *Controls for NK degranulation assay*
(A) 221 cells and primary NK cells were incubated together and (B) separated by expression of CD56. (C) $CD56^-$ cells were stained with a live dead cell stain and (D) expression of GFP and surface 4C7 staining on live cells were analyzed by flow cytometry. n = 3 experiments each done in triplicate.

infection (Figure 5.1D). The uninfected cells has low levels of 4C7 staining on the surface of the cell, but the infection caused a significant increase in 4C7 staining (Figure 5.1D).

We first examined the actual death of the target cells during interaction with NK cells. The $CD56^-$ 221 cells were examined for a live/dead cell stain following infection and incubation with the primary NK cell. We expected a decrease in infected cell death relative to uninfected cells that would be blocked by anti-CD161. With the addition of anti-CD161 monoclonal Ab, we expected to see an increase in target cell death since NKR-P1A would not be able to bind to CLEC2D. However, there was little to no decrease in target cell death after infection and blocking NKR-P1A did not cause an increase in cell death (Figure 5.2A). In 2 of the 3 donors utilized, infection of the 221 cells caused a slight decrease in target cell death, but the decrease did not reach statistical significance (Figure 5.2A). The presence of the antibody to block NKR-P1A did cause a slight increase in cell death, though it also did not reach statistical significance (Figure 5.2A). When looking at the average of the 3 donors, blocking NKR-P1A with anti-CD161 monoclonal Ab did not significantly increase the percentage of cell death (Figure 5.2B). Also, VACV infection did not drastically decrease the percentage of cell death suggesting that the increase in "CLEC2D" is not sufficient to protect the cells (Figure 5.2B). However, since NKR-P1A expression is not uniform on the NK cells, it is possible that only cells with high NKR-P1A expression are inhibited and therefore, we cannot see the full effect since we cannot gate on target cell death based on NKR-P1A expression.

In the course of choosing donors for this assay, we wanted NK cells with high expression of NKR-P1A. We obtained blood from a panel of volunteers and isolated their NK cells to look at surface NKR-P1A expression. *Ex vivo*, all donor's express high amount of NKR-P1A on their

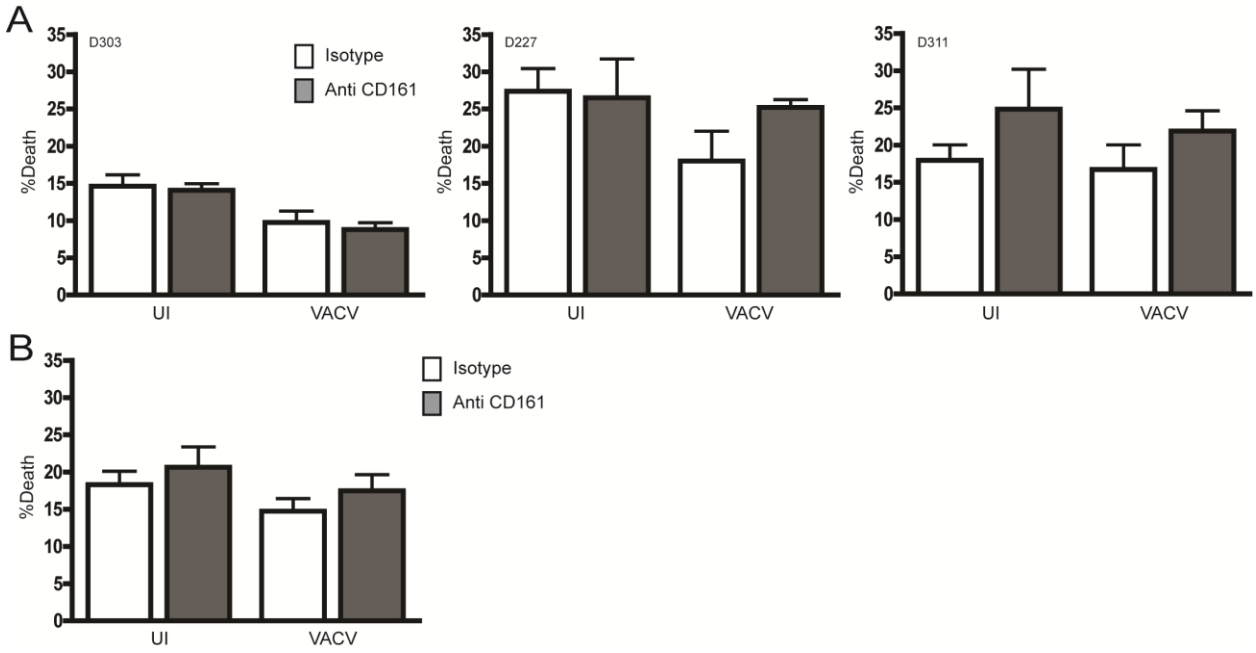

Figure 5.2 *221 cells are not protected from primary NK cell lysis at early time points after infection with VACV*

221 cells were stained with a live dead cell. (A) Percentage of target cell death following incubation with primary NK cells at a 3:1 E:T ratio from different donors with and without blocking NKR-P1A receptor with anti-CD161. (B) Average percentage of target cell death from all the donors. n = 2 experiments each done in triplicate, except for D227 n = 1 done in triplicate.

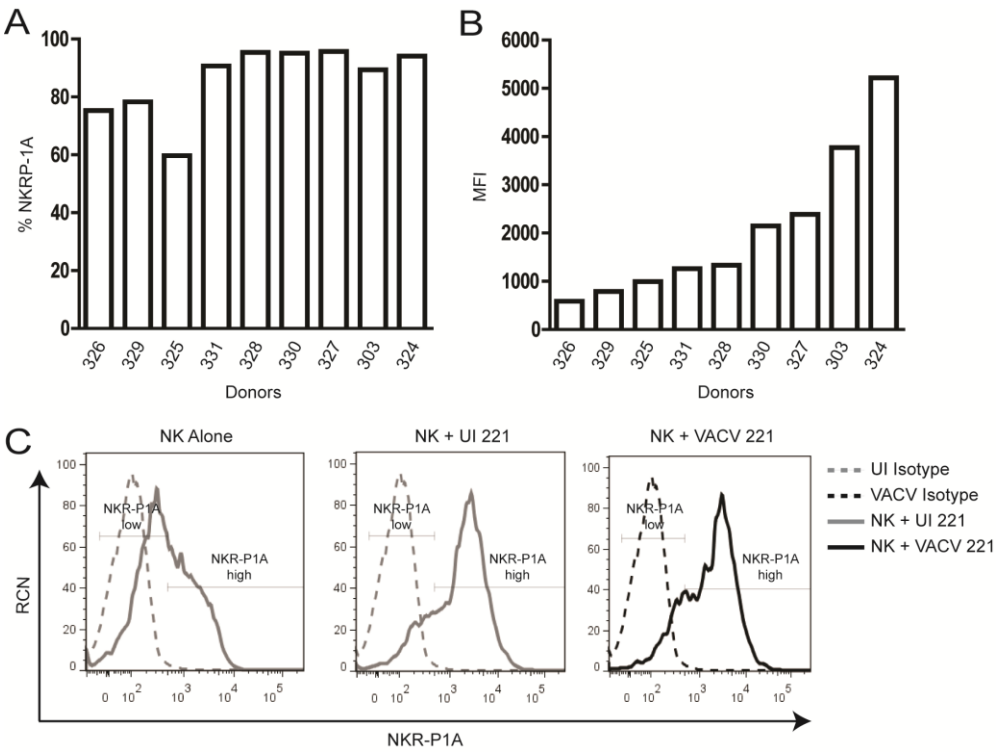

Figure 5.3 *NKR-P1A is induced with incubation with target cells*
Primary NK cells were isolated from PBMCs. *Ex vivo* NK cells were stained for (A) percentage of NKR-P1A and (B) MFI of NKR-P1A on the surface on primary NK cells. Primary NK cell were cultured in IL-2 and then incubated with uninfected or VACV infected 221 cells. (C) Representative histograms of NKRP-1A staining on primary IL-2 stimulated NK cells with or without target cells. D303 n = 3 experiments each done in triplicate, D227 n = 1 experiment done in triplicate, D311 n = 2 experiments each done in triplicate, D323 n = 2 experiments each done in triplicate. All points included.

NK cells (Figure 5.3A). However, the amount of NKR-P1A on the cells varied from donor to donor (Figure 5.3B). Of the 4 donor's NK cells that were cultured in IL-2 for at least 8 days, we noticed that the percentage of NKR-P1A slightly decreased (Figure 5.3C). Interestingly, during incubation with target cells for the degranulation assay, surface expression of NKR-P1A increased (Figure 5.4A and B). This observation occurred in every donor used no matter the initial expression pattern of NKR-P1A and regardless if the targets were infected or not. Given that there was an increase in both CD107a and NKR-P1A when the NK cells were incubated with target cells, we investigated if these two molecules could be located in the same vesicles. To do this, we adhered primary NK cells to a microscope slide using a cytospin, permeabilized the cells and stained them for both CD107a and NKR-P1A for visualization by confocal microscopy. All cells were stained with DAPI to envision the nucleus of the cell (Figure 5.5). Isotype controls did not give any background staining on the NK cells (Figure 5.5). CD107a and NKR-P1A staining was observed to be separate in the cells (Figure 5.5). Looking at the merged panels, it is clear that there is no overlap in staining for CD107a and NKR-P1A (Figure 5.5). The staining for NKR-P1A also demonstrates that not all NK cells have the same concentration of NKR-P1A, similar to what was seen in Figure 5.3, that different donor have different amounts of NKR-P1A on the cell surface. This suggests that both the receptor and the ligand may share the ability to be stored and rapidly externalized.

Returning to the effects of VACV upregulation of the 4C7 reactive protein on NK cells, to determine the effects of the VACV infection of target cells specifically on the NKR-P1A^{+} cells, the CD56^{+} NK cells were separated into NKR-P1A^{low} and NKR-P1A^{high} fractions. The NKR-P1A^{high} cells constitutively displayed some CD107a on the surface of the cells (Figure

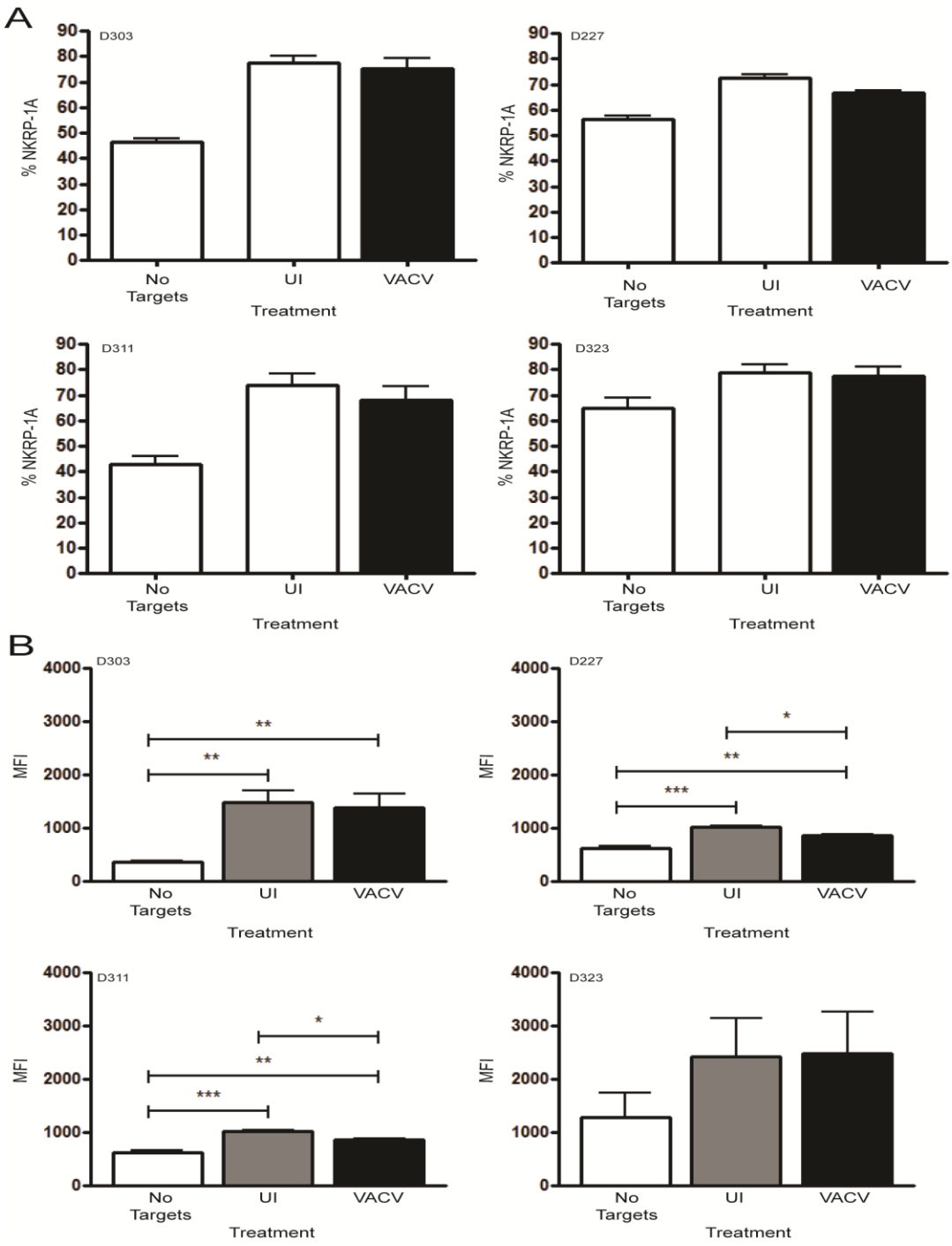

Figure 5.4 *NKR-P1A expression increases on primary NK cells with addition of target cells*
Primary NK cell were cultured in IL-2 and then incubated with uninfected or VACV infected 221 cells. (A) Percent NKR-P1A on 4 donors with or without target cells. (B) MFI of NKR-P1A on 4 donors with or without target cells. D303 n = 3 experiments each done in triplicate, D227 n = 1 experiment done in triplicate, D311 n = 2 experiments each done in triplicate, D323 n = 2 experiments each done in triplicate. All points included. *$p < 0.01$, **$p < 0.001$ and ***$p < 0.0001$

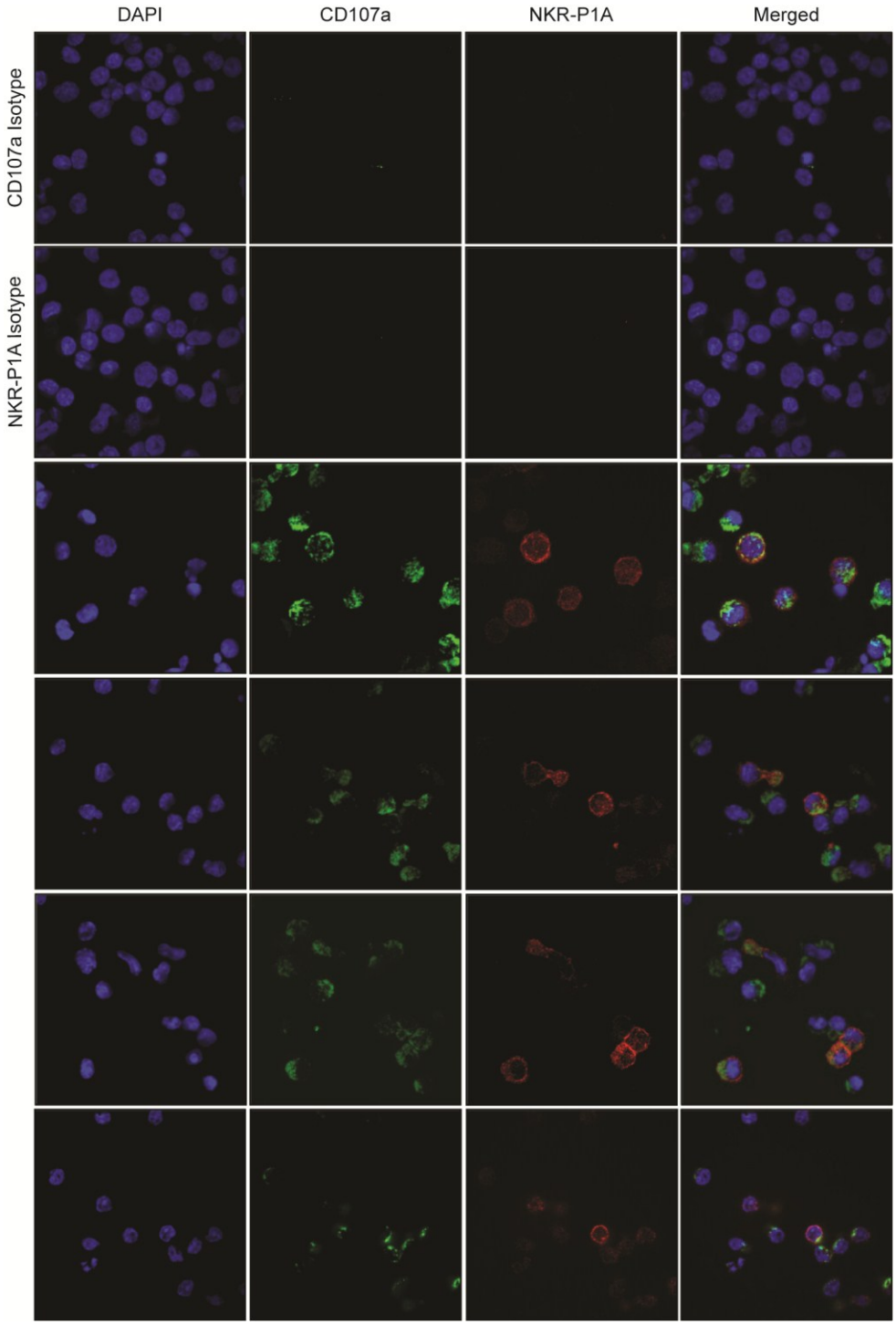

Figure 5.5 *NKR-P1A and CD107a do not co-localize*
Primary NK cells (D303) were stained with antibodies against CD107a and NKR-P1A to determine if they co-localize together. Representative staining is illustrated. n = 3 experiments.

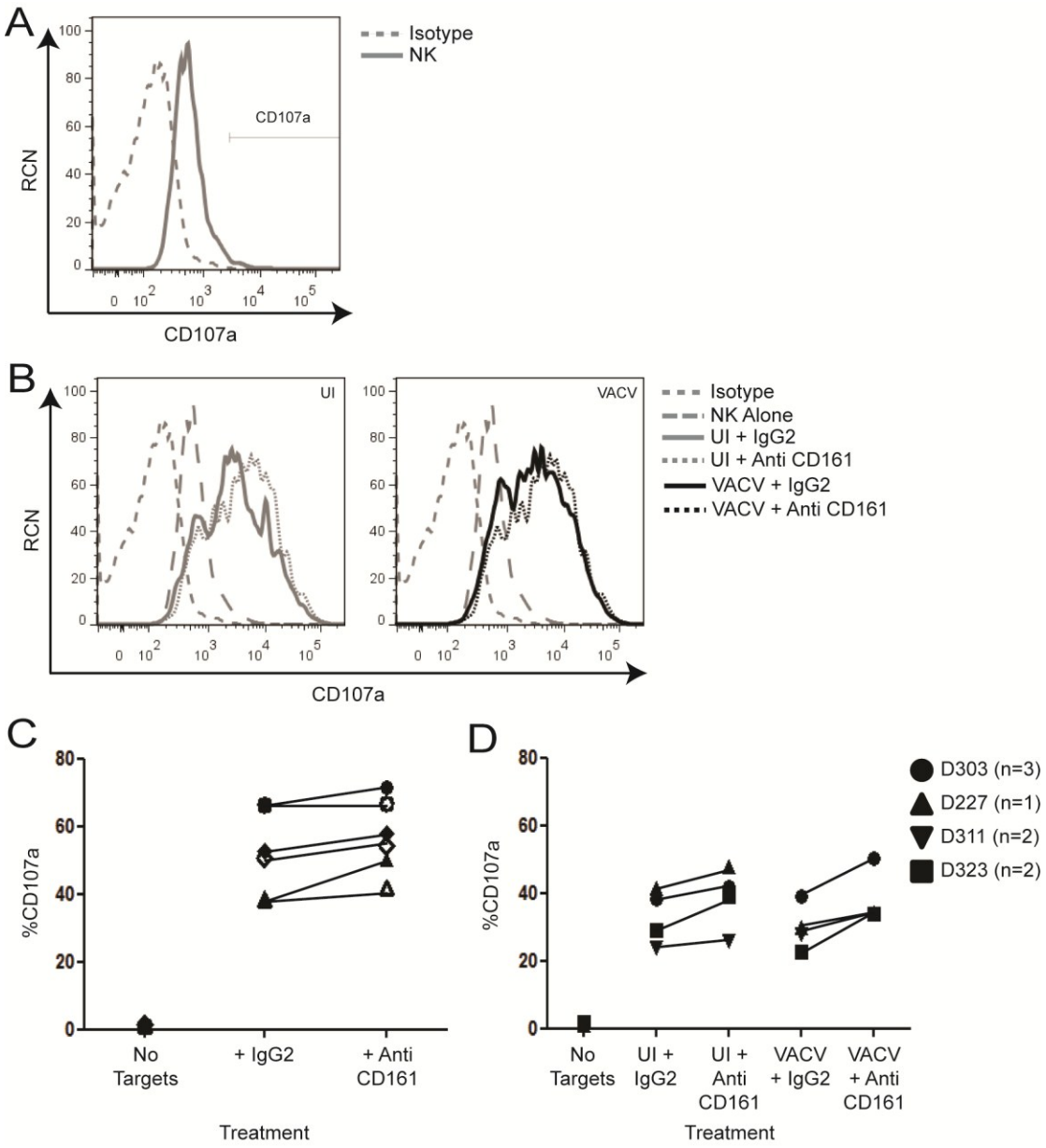

Figure 5.6 *Degranulation is increased with target cell addition but not with infection*
IL-2 stimulated primary NK cells were incubated uninfected and infected 221 cells. (A) Surface CD107a expression of NKR-P1A^{high} NK cells incubated with 221 cells. (B) Representative histograms of CD107a staining on the NKR-P1A^{high} NK cells incubated with target cells. (C) Average percentage of CD107a on primary NK cells incubated with 221 cells from donor 303 for 3 experiments done in triplicate. Shapes indicated a different experiment. Uninfected cells are in open shapes and infected are cells in black shapes with each shape corresponding to a single experiment. (D) Average percentage of CD107a on primary NK cells incubated with 221 cells from 4 donors. D303 n = 3 experiments each done in triplicate, D227 n = 1 experiment done in triplicate, D311 n = 2 experiments each done in triplicate, D323 n = 2 experiments each done in triplicate. All points included.

5.6A). When incubated with infected or uninfected target cells, the NKR-P1A^{high} cells all increased the amount of CD107a on the surface of the cells (Figure 5.6B). To block the interaction of CLEC2D and NKR-P1A, we added either an anti-CD161 monoclonal Ab or and isotype control, IgG2. Adding IgG2 or anti-CD161 antibody to the cells did not seem to affect the increase in CD107a on NK cells whether they were incubated with uninfected or infected cells (Figure 5.6B). Looking at the percent of CD107a on primary NK cells from a single donor (D303), it is clear that the addition of target cells increases NK cell degranulation (Figure 5.6C). Infection of the target cells did not cause the expected decrease in CD107a even with the significant increase in surface 4C7 staining on the target cells (Figure 5.6C). Blocking NKR-P1A binding to CLEC2D did cause a slight increase in CD107a, however it is not significantly higher on infected target cells as would be expected for the increase in "CLEC2D" observed (Figure 5.6C). These trends were also demonstrated in other donors as well. Blocking NKR-P1A on NK cells did cause an increase in the percent of cells expressing CD107a, however infection neither decreased the amount of CD107a due to the increase in "CLEC2D" nor did it cause more CD107a to be expressed when the receptor was blocked (Figure 5.6D).

A more sensitive function that may be affected by action on VACV infected cells is cytokine production. A fraction of the NK cells produce IFN-γ in response to 221 cells and blocking the interaction of NKR-P1A on NK cells and CLEC2D on target cells caused a slight increase in this cytokine production (Figure 5.7). In contrast to induction of CD107a, IFN-γ production was significantly decreased when interacting with VACV infected cells compared to controls (Figure 5.7). However, blocking NKR-P1A when target cells were infected did not cause the expected increase of cytokine production over isotype treated cells (Figure 5.7). Another observation was that within the NKR-P1A^{+} NK cells, a smaller percent of the cells were

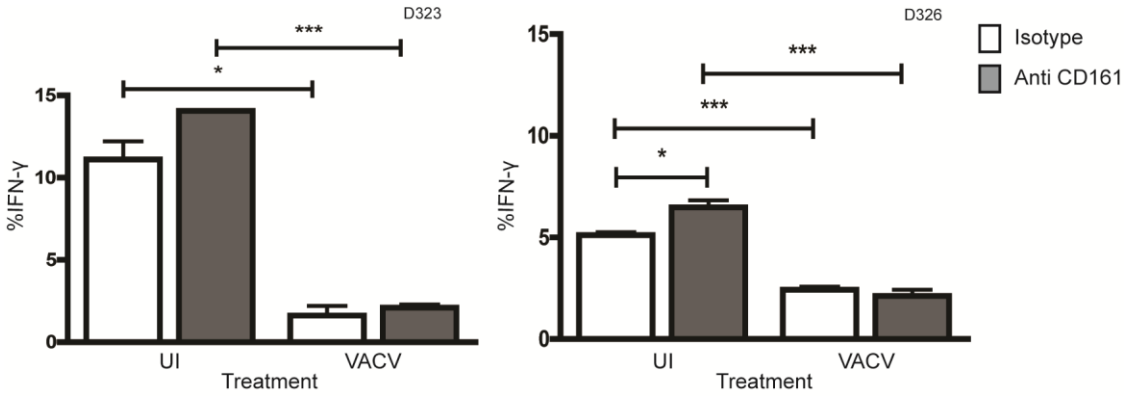

Figure 5.7 *VACV infection causes decrease in IFN-γ production*
Primary NK cells from different donors were incubated with 221 cells and an intracellular stain was performed for IFN-γ on $CD56^{+}$NKR-$P1A^{+}$ cells. Percentage of NK cells expressing IFN-γ was graphed. n = a single experiment performed in triplicates on 2 donors on two separate days. *p > 0.01 and ***p > 0.0001.

induced to make IFN-γ than to degranulate. These data demonstrate that even though VACV infection causes an increase in surface 4C7 staining, it does not inhibit NK cells degranulation nor lysis of target cells. Yet even though degranulation is not affected, IFN-γ production was affected by infection, demonstrating that VACV infection causes changes in different functions of NK cells, but this effect is unlikely to be through interaction with NKR-P1A.

5.2.2 VACV infection upregulates other forms of CLEC2D

CLEC2D has several isoforms and only isoform 1 (aka LLT1) binds to NKR-P1A (85). Therefore, since we did not observe protection of the 221 cells through the upregulated CLEC2D and NKR-P1A, we speculated that the protein detected on the cell surface may be an isoform of CLEC2D that does not engage NKR-P1A or a different protein altogether. In order to assess which isoforms were present in 221 cells, which are reported to have isoforms 1, 2 and 4 (85), and other potential target cells that were thought to be negative based upon the literature (85), we designed primers against the 3 transmembrane isoforms (1, 2 and 4) and performed RT-PCR on 4 different human cell lines including 221 cells before and following 2 hours infection with eGFP-VACV at a MOI of 5. 221 cells express message for isoform 1 that decreases with infection (Figure 5.8A). 293T cells show detectable expression of isoform 1 before infection, but none after infection, whereas the other cell lines do not show any expression for isoform 1 (Figure 5.8A). Only the 221 cell line have detectable isoform 2 transcript, that is still present following infection with eGFP-VACV (Figure 5.8A). All of the cell lines show expression isoform 4 mRNA before and after infection (Figure 5.8A). Surprisingly, there is an unexplainable band at 1000 bps that is seen in all cell lines, indicating that our primers pick up a larger protein from the cells that is similar to isoform 4 (Figure 5.8A). This data suggests that all these cell lines may have the ability to express at least one form of CLEC2D.

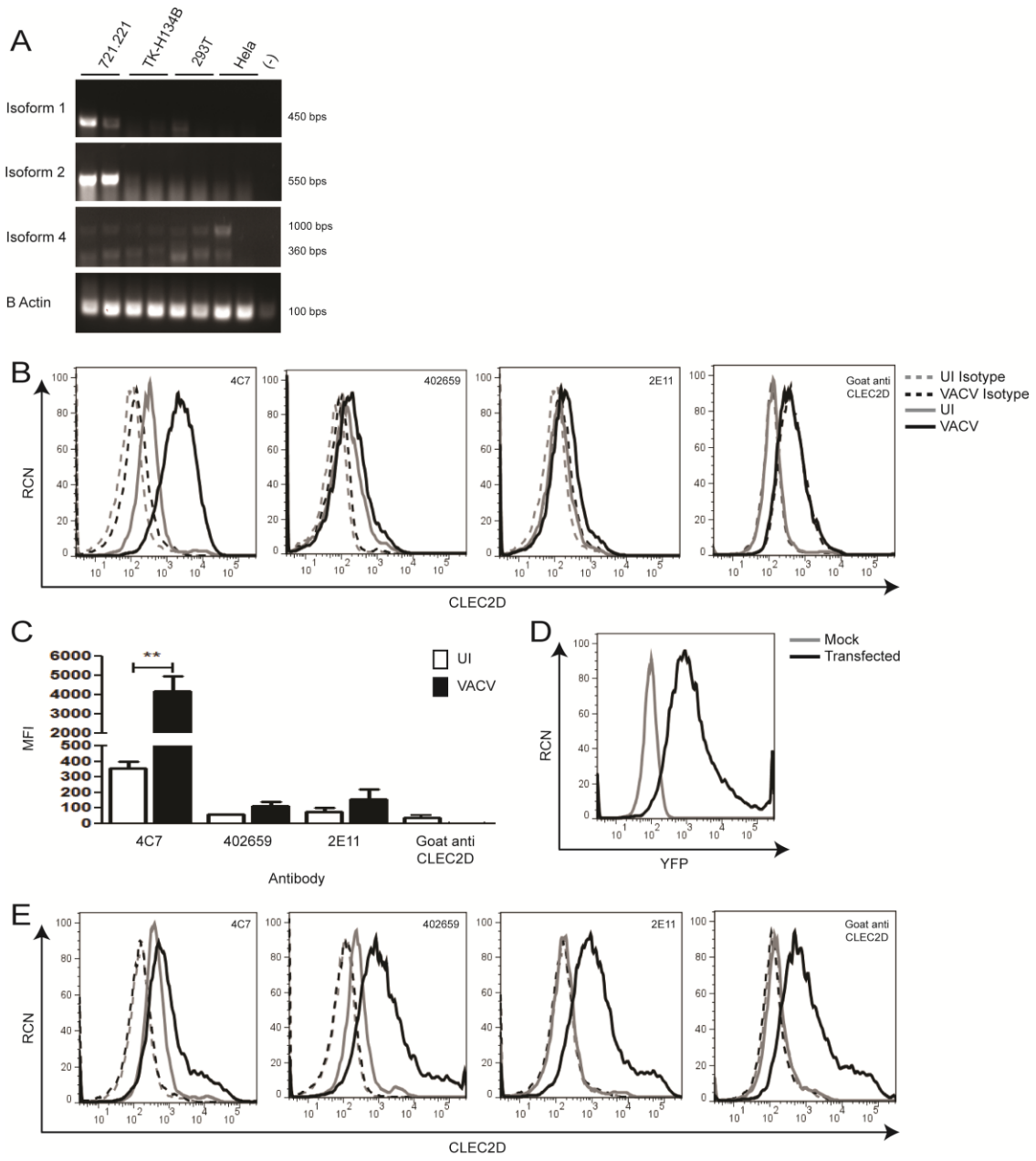

Figure 5.8 *VACV infection does not cause increase in isoform 1*
(A) RT-PCR of the different isoforms of CLEC2D in several different cell lines, with uninfected samples paired with VACV infected samples for each cell line (2 hour infection at a MOI of 5). (B) Surface staining of 221 cells with different CLEC2D antibodies. (C) MFI of surface CLEC2D expression on 221 cells with different CLEC2D antibodies. 293T cells were transfected with water or a YFP-LLT1 plasmid for 48 hours. (D) Representative histograph of YFP expression from 292T cells. (E) Surface staining of 293T cells transfected with YFP-LLT1 with different CLEC2D antibodies. n = 3 experiments in triplicates, All points included. n = 1 for 293T transfections. **p > 0.001.

To determine which isoform was being upregulated during the VACV infection, we incubated 221 cells with media or eGFP-VACV for 4 hours at a MOI of 5. Cells were then harvested and stained with different CLEC2D antibodies for analysis by flow cytometry. Uninfected cells had little to no surface expression of CLEC2D (Figure 5.8B). As we observed before with the 4C7 antibody that can recognize isoforms 1, 2 and 4, there was a significant increase in 4C7 staining following VACV infection (Figure 5.7B and C). With the antibody 402659, which is reported to only recognize isoform 1 (LLT1) (85), there was a small increase in isoform 1 above uninfected cells (Figure 5.7B and C). The 2E11 antibody also demonstrated a small increase in LLT1 above uninfected cells, suggesting it too may only recognize isoform 1 since its profile was similar to 402659 (Figure 5.8B and C). The goat anti CLEC2D antibody, which we determined should recognize isoforms 1, 2 and 4 based upon the peptide sequence the antibody was made against, did not demonstrate and surface staining for CLEC2D before or after infection (Figure 8B and C), suggesting that the protein may not be an isoform of CLEC2D.

We next needed to ensure that these antibodies could recognize isoform 1. To do this, we transfected 293T cells, that are reported to be CLEC2D negative (85), with a YFP tagged LLT1 (isoform 1) plasmid. 48 hours following transfection, the mock treated 293T cells did not express any YFP, whereas the transfected 293T cells expressed high amounts of YFP (Figure 5.8D). We then surface stained the mock treated and transfected cells with the different antibodies against CLEC2D. Unlike the 2E11 and goat anti CLEC2D antibodies, the 4C7 and 402659 antibodies detected low amounts of isoform 1 on the surface of mock treated cells (Figure 5.8E). All the antibodies were able to detect high amounts of isoform 1 on the transfected cell surface, similar to the YFP profile, although the 4C7 antibody histogram showed a little less staining than the rest (Figure 5.8E). This data indicates that all of these antibodies can detect isoform 1.

We also performed intracellular stain on 221 cells. We were able to determine that 221 cells do have high staining for isoform 1 by the 402659 and 2E11 Abs, however, 4C7 staining is higher (Figure 5.9A). These data suggest that even though 221 cells have message for isoform 1 and contain isoform 1 protein, during the VACV infection, isoform 1 may only be increased on the surface in small amounts that are not enough to increase inhibition of NK cells through NKR-P1A. This data also suggests that isoforms 2 and 4 may translocate to the cell surface during infection. The other antibody that we expected to recognize isoforms 2 or 4 did not detect staining on infected cells even though we proved that it could see isoform 1. This suggest that the 4C7 reactive protein may be something other than CLEC2D.

4C7 is also reported to bind to KACL (85), and therefore we tested if VACV was increasing KACL. To do this, we incubated 221 cells for 2 hours with eGFP-VACV at a MOI of 5 and subsequently stained the cells for analysis by flow cytometry. The 221 cells had surface 4C7 staining that increased significantly with infection as seen previously (Figure 5.9B). However, 221 cells did not have surface expression of KACL and the lack of expression continued following infection (Figure 5.9B). This indicates that it is likely an isoform of CLEC2D, or another unknown protein, that increases during VACV infection not KACL.

5.2.3 VACV causes an increase in 4C7 staining in other cell lines

To date, CLEC2D is only found in a small subset of activated immune cell and lung epithelial cells. However, we have observed surface expression of CLEC2D in Hela cells, a cervix epithelial cell line (see Figure 4.8) by 4C7 staining, albeit only when contaminated by mycoplasma,. Therefore we wanted to determine if other cell lines expressed CLEC2D by 4C7 staining and if this expression could be altered by VACV infection. We obtained 10 human cells

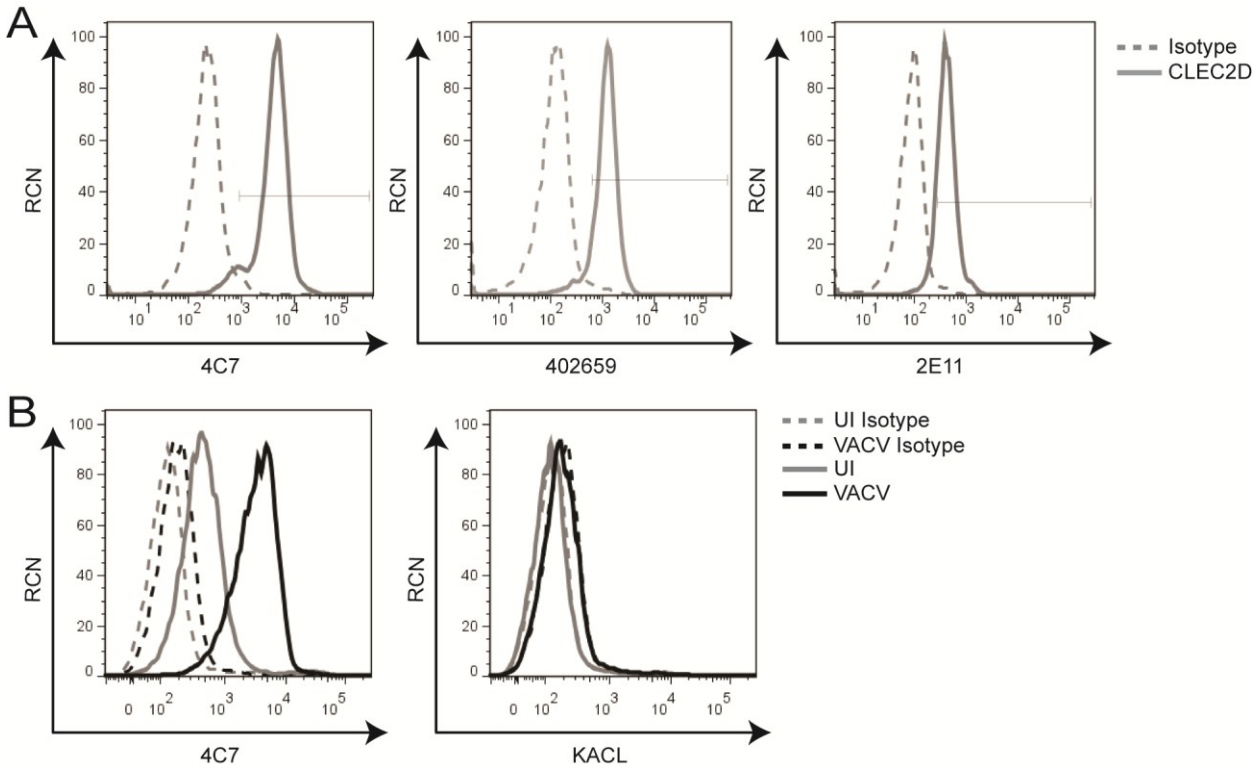

Figure 5.9 *Recognition of CLEC2D antibodies*
(A) Intracellular staining of 221 cells with different CLEC2D antibodies. (B) Surface staining of 221 cells incubated with media or eGFP-VACV (MOI of 5) for 2 hours with 4C7 and KACL antibodies. n = 3 experiments.

lines, 2 non-human primate cell lines and a mouse cell line to use as a negative control. Seeing as we observed high GFP expression and the peak of 4C7 staining with a MOI of 5 at 4 hours in 221 cells, we incubated these cell lines in media or with eGFP-VACV at a MOI of 5 for 4 hours. The cells were then harvested and then stained with 4C7. All the uninfected cell lines had some level of 4C7 staining on the surface of the cells, whereas the infected cells all had a significant increase in 4C7 staining over uninfected cells (Figure 5.10A). The infected 221, HFF-Tel and Huh7.5 cell lines displayed increases in 4C7 staining, however, the changes were not statistically significant at the 95% confidence limit (Figure 5.8A). In the 221 cells, this is contrary to what is normally seen, however in these experiments, this is due to a large error bar, not to little change in the surface 4C7 staining. Both non-human primate cell lines also demonstrated a significant increase in 4C7 staining over uninfected cells (Figure 5.10B) suggesting that 4C7 can react on the non-human primate version of the 4C7 reactive protein and that the phenomenon of VACV induced upregulation extends to these cells. The mouse cell line did not demonstrate any staining with 4C7 with or without infection (Figure 5.8B), confirming that this antibody does not cross react with the mouse homologue Clr-b and that this antibody does not detect a viral protein. We also performed RT-PCR on all the uninfected cell lines to determine if these cell lines had transcript for the different isoforms of CLEC2D. We were able to detect transcript for isoforms 1 and 2 in the lymphocyte cell lines except for Jurkats and K299 cells (Figure 10C). We were unable to detect any of the isoform in the other cell lines, including the monkey and mouse cell lines, although message for β-actin was very faint (Figure 10C). This mRNA data is contrary to the upregulation of the 4C7 reactive protein expression seen in the infection in all the cell line. However, this could be due to lower amounts of CLEC2D in the other cell lines or that the message and protein is made following stimulation.

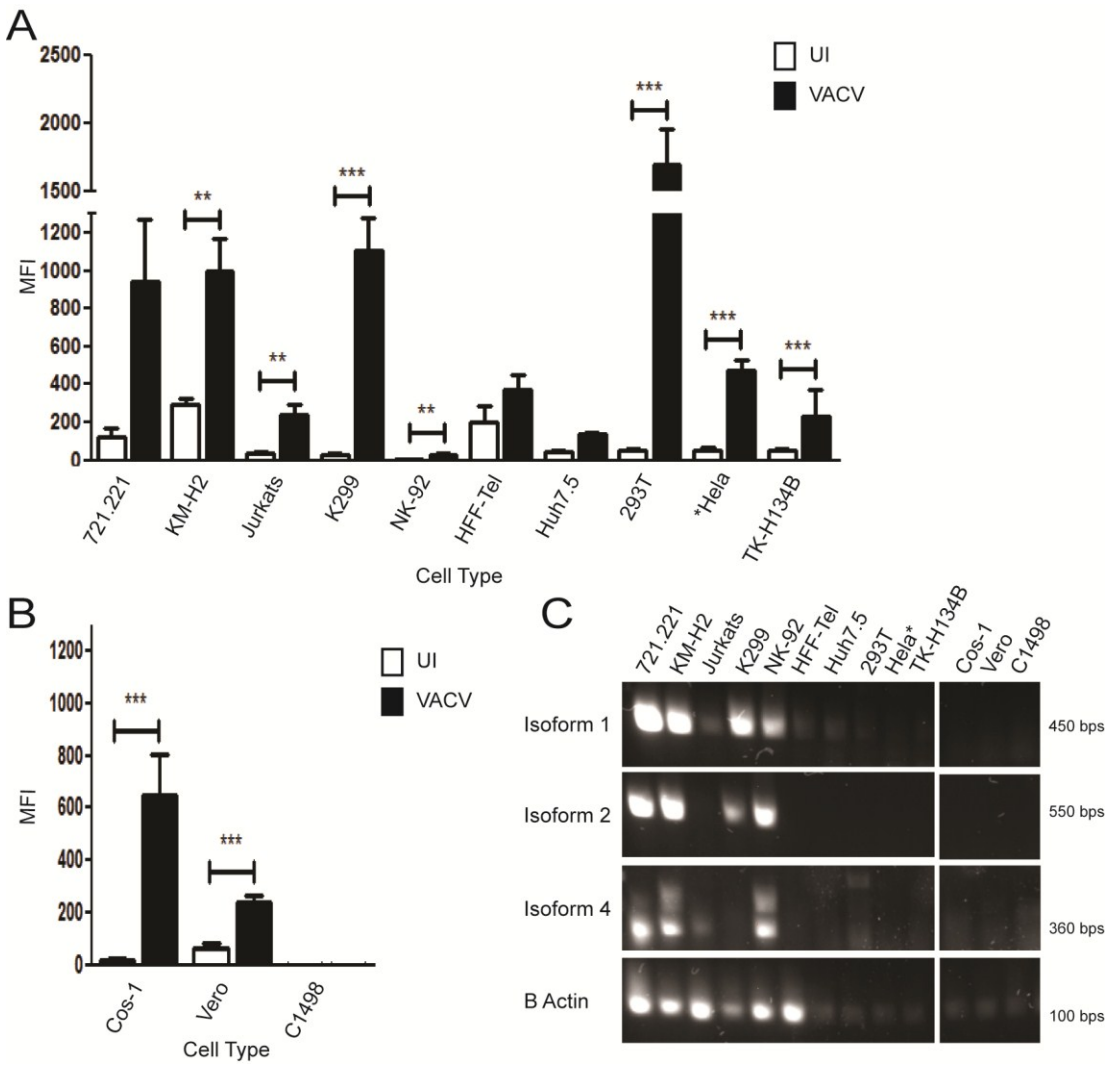

Figure 5.10 *VACV infection causes increase in 4C7 staining in many cell lines*
Cell lines were infected with eGFP-VACV at a MOI of 5 for 4 hours. (A) MFI of surface 4C7 staining of several different human cell lines following infection. (B) MFI of surface 4C7 staining of non-human primate and mouse cell lines following infection. (C) RT-PCR of CLEC2D isoforms in different cell lines. n = 3 experiments in triplicates, all points included. **p > 0.001 and ***p > 0.0001. Hela cells used here are mycoplamsa contaminated.

Since the 221 cells were shown to have an intracellular pool of the 4C7 reactive protein (see Figure 4.4), we went to investigate if these human and non-human primate cell lines also contained an intracellular pool that could be rapidly mobilized to the cell surface during VACV infection as was observed for 221 cells. All the cell lines were incubated with media or eGFP-VACV at a MOI of 5 for 5 minutes. The cells were then harvested then stained with 4C7. The uninfected human cell lines all displayed some level of 4C7 staining (Figure 5.11A). The VACV infected human cells mostly all exhibited a rapid upregulation of the 4C7 reactive protein over uninfected cells during the 5 minutes of exposure to VACV (Figure 5.11A). Interestingly, the highest increases in 4C7 staining was observed in the lymphocyte cell lines, whereas the other cell types had little to no change following viral exposure, indicating that this rapid upregulation may be cell specific. The non-human primate cell lines showed not a large, but a significant increase of surface 4C7 staining over uninfected cells, whereas the mouse cell line remained negative as expected (Figure 5.11B). This indicated to us that these human and non-human primate cell lines may have an intracellular pool of the 4C7 reactive protein. To test this hypothesis, we performed intracellular staining on HFF-Tel, Hela and TK-H134B cell lines. All of these cell lines showed little to no surface 4C7 staining (Figure 5.11C). When permeabilized, all these cell lines exhibited high 4C7 staining (Figure 5.11C and D). The pattern of the 4C7 staining in HFF-Tel and 293T cells we also examined by confocal microscopy. Each cell line did not stain with the IgG1 isotype control, however, they did show punctate cytoplasmic staining with 4C7 (Figure 5.11E). It is possible that different cell types have an intracellular pool of the 4C7 reactive protein, however the tendency of the different cell types to move this protein to the cell surface seems to be cell dependent. It is also possible that we are observing staining of one

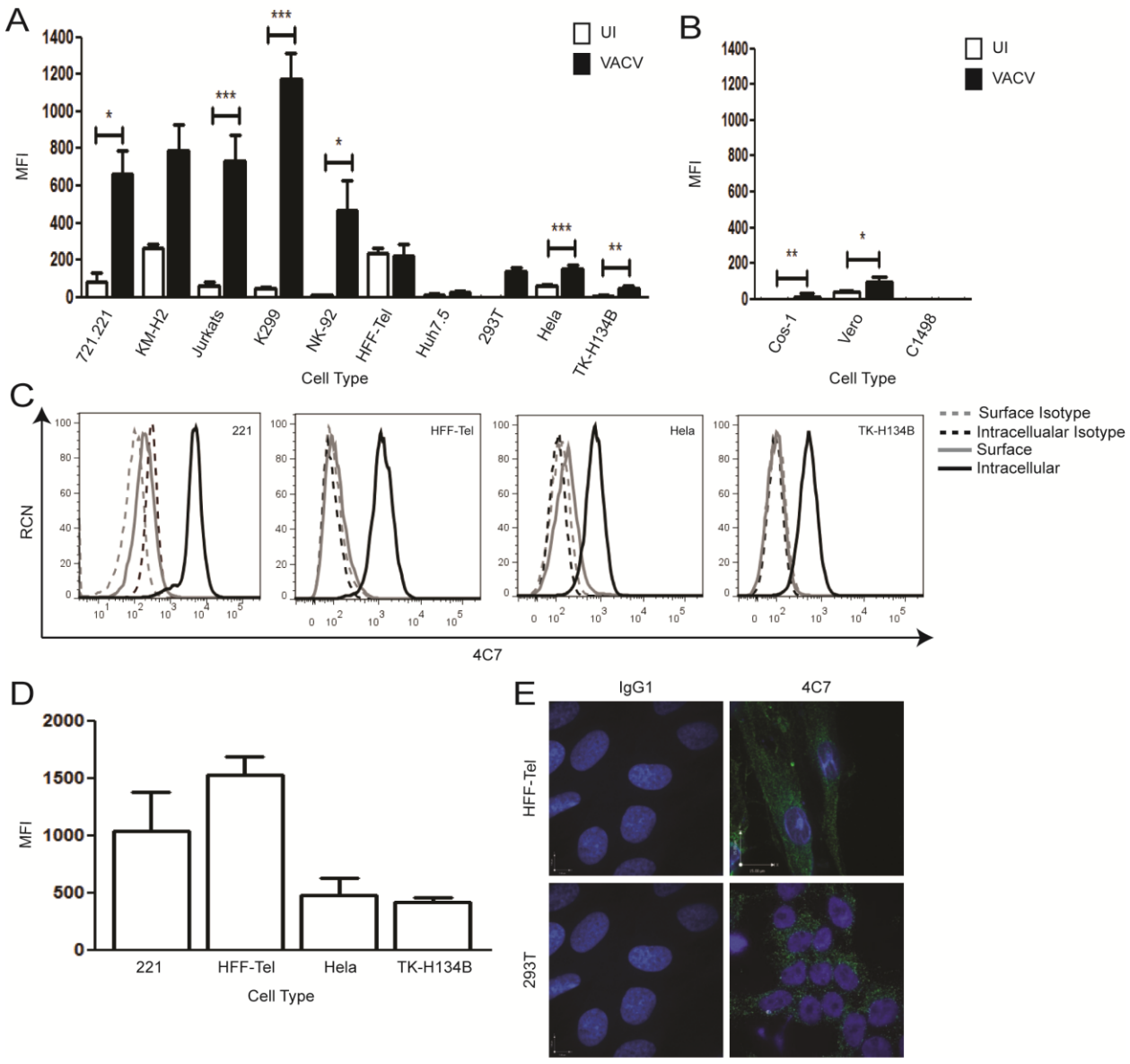

Figure 5.11 *Many cell types have intracellular pools of the 4C7 reactive protein*
(A) MFI of surface 4C7 staining on human cell lines following a 5 minute infection with eGFP-VACV at a MOI of 5. (B) MFI of surface 4C7 staining on non-human primate and mouse cell lines following a 5 minute infection with eGFP-VACV at a MOI of 5. (C) Surface and intracellular 4C7 staining in difference uninfected human cell lines. (D) MFI of intracellular 4C7 staining for different uninfected human cell lines. (E) Confocal microscopy of intracellular 4C7 staining (60x magnification). n = 3 experiments in triplicates, all points included. *p > 0.01, **p > 0.001 and ***p > 0.0001. Hela cells used here are mycoplasma contaminated.

isoform over another which may behave differently during infection, however, reagents to these other isoforms are unavailable. These data demonstrate that different cell types can express the 4C7 reactive protein on the cell surface which is upregulated when infected with VACV and that many cell types have intracellular pools of this protein.

5.3 Summary

This chapter investigated the interaction of VACV induced "CLEC2D" on NKR-P1A$^+$ NK cell functions. We first observed that although infection was able to slightly decrease the amount of target cell death, blocking the interaction of NKR-P1A with CLEC2D by antibody to the receptor did not cause a significant increase in target cell death, suggesting that the increase in 4C7 staining is not CLEC2D isoform 1. We then observed that the addition of target cells, infected or not, to NK cells caused an increase in degranulation by NK cells by CD107a surface staining as expected given the amount of lysis. Whatever protein we detected with the 4C7 antibody does not inhibit of NK cell degranulation. However, VACV infection of target cells did instigate lower levels of IFN-γ production in NK cells. These data indicate that while VACV infection has an effect on a protein detected by 4C7, this protein does not interact with NKR-P1A, suggesting that isoform 1 of CLEC2D was not being externalized by VACV infection.

By infecting 221 cells with VACV and staining the cells with Abs against the different forms of CLEC2D, we determined that VACV infection was not upregulating isoform 1 but most likely isoforms 2 or 4 or another protein that reacts with 4C7. Further investigation with an Ab that should detect isoforms 2 and 4 point towards a protein distinct from CLEC2D. This upregulation was also observed in many different cell lines. Like 221 cells, other lymphocytes were able to rapidly upregulated the 4C7 reactive protein during VACV infection, while other

cell types were not as capable in this ability. However, they still have an intracellular pool of a 4C7 reactive protein that is mobilized to the cell surface later infection.

Interestingly, incubating target cells, infected or not, with NK cells caused an increase in NK surface expression of NKR-P1A. Although there was an increase in both CD107a and NKR-P1A during incubation with target cells, these molecules are not located in the same vesicles within the NK cell, suggesting that NKR-P1A is dynamically regulated.

The data presented in this chapter opened up many more questions than it answered. What is the protein that is detected by 4C7 and is it related to the CLEC family? Given that this protein is not binding to NKR-P1A, is there a receptor for it? These questions and more will be discussed in the next chapter.

Chapter 6 : Discussion

6. Discussion

In this thesis we studied the effects of poxvirus infection on the CLEC2D protein in both mice and humans. Furthermore, we investigated how these effects of poxviruses infected target cells affected NK cell functions. Studies had demonstrated that the mouse CLEC2D protein, Clr-b, is used as a missing self recognition system that allows NK cells to recognize infected cells without the use of MHC I (39). Humans also possess a CLEC2D protein that is homologous to Clr-b with respect to receptor interaction, but different with respect to expression pattern. Therefore we set out to determine how these two proteins were affected by poxvirus infection and how NK cells would recognize them through this additional missing self recognition system. This next section will discuss the results from the numerous experiments performed during this project and determine the relevance of the studies.

6.1 Poxvirus Down Regulation of Clr-b Prevents NK cell Inhibition

6.1.1 Summary

In these studies, we found that VACV and ECTV infection is able to cause the loss of surface Clr-b in different cell types, including BMMØ. This loss not only occurs in cells that express GFP and are therefore definitely infected, but also occurs in neighbouring cells that either do not express GFP, or that GFP expression is below detection. This loss in cell surface Clr-b protein occurs more rapidly than the loss of surface MHC I, and take place before Clr-b message is degraded by viral proteins. Only early infection with VACV is required for Clr-b to be removed from the cell surface. Finally, VACV infection causes a loss in Clr-b which in turn causes NK cells to lose their inhibition and lyse infected cells. Taken together, these findings support the hypothesis of host regulation of Clr-b during infection which in turn causes NK cells to lose their inhibition and lyse the infected cell.

6.1.2 Implications of Results

Potential Mechanisms of Clr-b Down Regulation:

We observed that Clr-b was down regulated with VACV infection and that the infection had to be active in order to cause this down regulation. This observation of decreased Clr-b is known to occur in tumor cell lines (39), however it has only been reported for one other instance that a virus, RCMV, could also cause this loss (270). The loss of Clr-b on the surface of infected cells is probably a function of host surveillance. We observed here that VACV infection slowly decreases MHC I over 12 hours of infection, however, Clr-b down regulation was much more rapid by 8 hours PI. It is possible that cells use both systems of recognition to alert the immune system of infection. Clr-b would be more sensitive to infection, at least to VACV infection, than MHC I, as it is lost first. The host would be able to recognize earlier in the infection that it has been infected and start an immune response before MHC I is down regulated and has its own effects on the immune system. In response to chemical or physical insults, the cellular stress pathways are shown to cause down regulation of Clr-b surface protein. This down regulation is dependent on the ubiquitin/proteasomal pathway or a ubiquitin-dependent process affected by the proteasomal inhibitors MG132 or lactacystin (81). Therefore, it is possible that in response to cellular stress from infection or sensing viral DNA in the cytoplasm, cells internalize and/or degrade Clr-b protein faster through cell-intrinsic mechanisms.

VACV also encodes proteins that could specifically interfere with Clr/Clec2 function, possibly through direct association and trafficking by the Clr proteins or through virally encoded proteins that drive ubiquitination (235). However, removing an inhibitory receptor from the cell surface could be detrimental to the virus infection. It is more likely that the loss of Clr-b is part

of the host response to infection and that the virus evolved to hinder host recognition by deploying it own decoy on the cell surface, especially since we observed less lysis of infected cells than uninfected cells. It is not understood if the loss of Clr-b is due to host reaction to the virus or due to the virus attempting to evade the immune system, especially since surface protein is lost before Clr-b message is lost. Studies to determine which is responsible will have to be performed.

VACV Infection Causes Bystander Effect in Uninfected Cells:

Interestingly, the loss of Clr-b was not only observed in cells that were obviously infected by GFP expression, but cells within the same tube of infection that did not express GFP also lost surface Clr-b expression indicating that either they were infected and GFP was below detection levels, or that there is some bystander effect from something possibly secreted by the infected cells. This bystander effect is known in cancer therapy where non-irradiated cells reaction to signals produced by neighbouring irradiated cells and act as if they themselves were irradiated (100). Therefore it is possible that the infected cells either produce some product of infection, or by physically touching their neighbour send signals that they are infected and as a group to decrease their expression of Clr-b. Viruses such as HIV are also known to have bystander effects on uninfected cells. HIV causes the death of naive T cells possibly by non-infectious HIV virions that can bind to the cell's T cell receptor and lead to death of both naive $CD4^+$ and $CD8^+$ T cells without infecting them (TCH failure) (123). Therefore, there is also a possibility that there are viral particles within our stock that are non-infectious, but can still cause an effect on Clr-b expression. However, since UV inactivated virus was unable to cause down regulation of Clr-b, but is supposed to still be able to bind to the cell, this scenario is

unlikely. Further testing would be needed to delineate the mechanism of Clr-b down regulation during this virus infection.

VACV Infection Leads to Loss of NKR-P1B Recognition:

The loss of Clr-b due to VACV infection was also demonstrated here to have effects on NK cell function through the NKR-P1B receptor since blocking NKR-P1B interaction with Clr-b on uninfected cells with an anti-Clr-b Ab was able to cause in increase in target cell death. During infection, Clr-b was lost on the surface of infected cells and there was no difference in cell lysis when using the anti-Clr-b Ab or an isotype control. However, unexpectedly, less infected cells were killed by NKR-P1B$^+$ NK cells than uninfected cells, suggesting that the virus may have other unknown effects on cytolysis, such as upregulating a decoy inhibitory ligand, as is seen in RCMV (270), by reducing ligands for activating receptors or by preventing the perforin/Granzyme B death pathway (244). For example, it is also possible that later in the infection, VACV may interfere with proteins that NK cells require for efficient lysis of a target, such as CD43, a sialophorin that is known to regulate NK cell chemokine synthesis, cytolysis and tyrosine kinase activation (2, 174). Whereas VACV is known to negatively regulate T cell adhesion and activation through CD43 (153), regulation of CD43 on NK cells during a viral infection is unknown. Another possibility of VACV interference is the HA protein, encoded by both VACV and ECTV, that binds to the activating receptors NKp30 and NKp46 on human NK cells, which seems to counter intuitively result in an overall reduction in target cell lysis (112). Coincidentally, the VACV protein NI limits mouse NK cell responses *in vivo* (110), and the SP1-2 protein encoded by VACV and ECTC can prevent Fas-mediated apoptosis and NK cell activation *in vivo*, respectively (122, 158). Thus VACV infections have the ability inhibit NK cell lysis of infected cells through several means. Studies to elucidate what proteins have the

inhibitory effects on NK cells observed in these experiments should be carried out. Knowing the protein(s) that could possibly succeed in inhibiting the removal of infected cells would be beneficial in improving treatments using VACV for vaccination or cancer treatment.

6.1.3 Model of Poxvirus Effects on Mouse Clr-b

Our proposed model of what is occurring during a poxvirus infection of mouse cells takes into account that the mechanism of how Clr-b is down regulated is currently unknown. However, an outline of the activation of NK cells can be projected. The virus first binds to the target cell, enters, early viral mRNA is made, viral proteins are produced and the virus uncoats its DNA (Figure 6.1A). Overtime, Clr-b is lost from the surface of the cell either by host removal due to cell stress (81), or by viral elimination early in the infection since early infection is sufficient for loss of Clr-b (Figure 6.1A). Either way, host mRNA, for Clr-b and other proteins, is degraded by viral proteins later in the infections, since VACV is known to degrade host mRNA (213) (Figure 6.1A). During the infection, due to viral stress, the cell may begin expressing stress molecules on the surface of the cell that can bind to NKG2D (Figure 6.1B). Viral proteins that are stimulatory in nature may also be exposed to the surface (Figure 6.1B). NK cells, due to the loss of inhibition from Clr-b, and the expression of stress molecules that can bind to NKG2D and/or CD94/NKG2E, are activated to degranulate and lyse the infected cell (Figure 6.1B). Although our data demonstrates some inhibition of NK cell lysis of the infected cells through unknown methods, this model only demonstrates the activation of NK cells. The inhibition observed in our experiments may be due to possible inhibitory viral decoy molecules that we do not detect with antibody or possibly the virus HA protein that can inhibit NK cells through NKp46 (112), being expressed on the cell surface and hindering NK cell functions. The inhibition could also be due to VACV proteins inhibiting the apoptotic pathway (244). Studies for the inhibition of NK cells

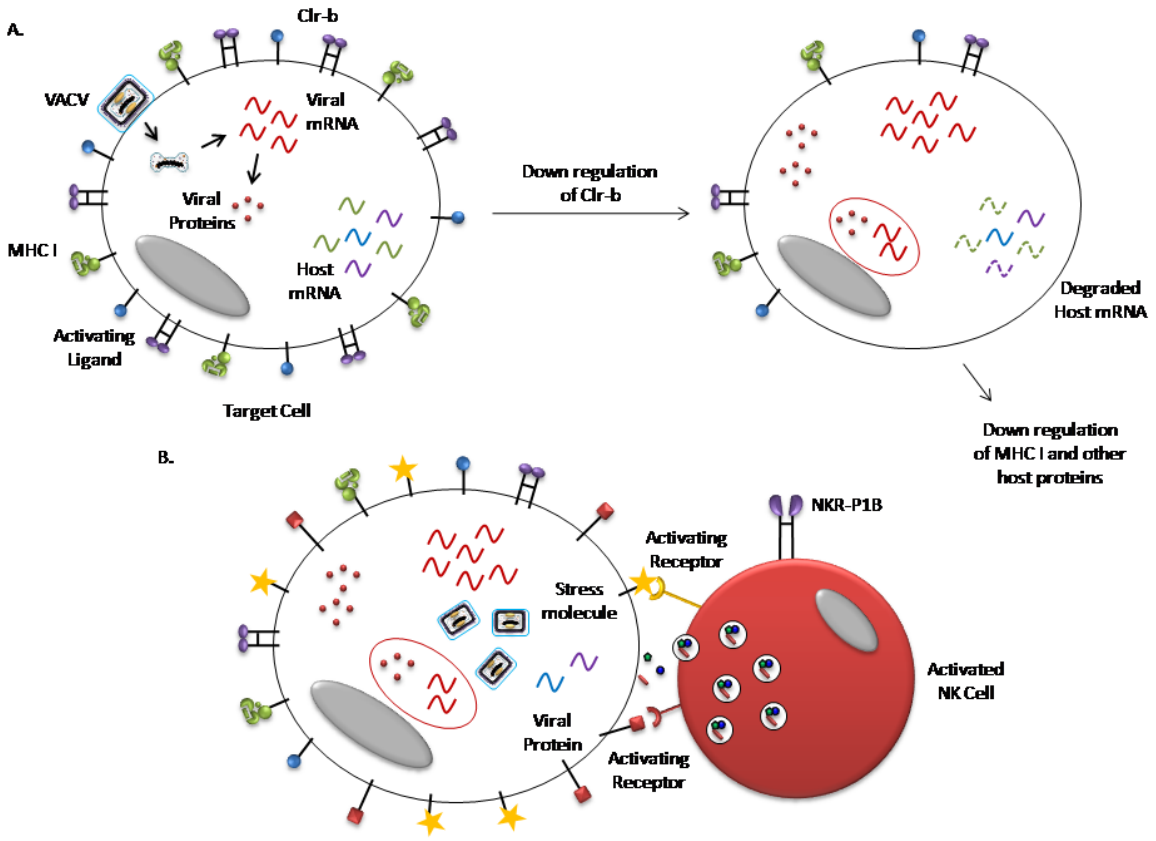

Figure 6.1 *Model of poxvirus effects on mouse Clr-b*
(A) VACV infects cells inducing production of viral mRNA and proteins. Clr-b is down regulated from the cell surface followed by degradation of host transcripts and loss of other host proteins. (B) Viral proteins and stress molecules are expressed at the cell surface that can stimulate NK cells.

during this virus infection will be discussed next.

6.1.4 Future Directions

Given that there are low amounts of target cell lysis following infection of mouse cells with VACV but still activation of the NK cell to degranulate, there are some more experiments that could be performed in order to determine what relevance the loss of Clr-b has in a mouse infection. First, it would have to be establish if the loss of Clr-b is due to a host response or due to a viral action, although it is more likely a host response. This could possibly be carried out by infecting cells and performing a pull down assay to determine if any proteins, be it host or viral are interacting with Clr-b. Another study that should be performed is to determine how the loss of Clr-b during a poxvirus infection in the mouse affects the resistance of the mouse to infection. As previously discussed, resistance to ECTV is mapped to the NKC and that resistance is thought to involve NKG2D and CD94/NKG2E (75, 76), whereas resistance to VACV involves NKG2A (29). Here we would focus on the Clr-b/NKR-P1B receptor pair affecting the resistance of a mouse strain known to be resistant to ECTV. Recently, a NKR-P1B knockout mouse in the C57Bl/6 background has been made (203) and a Clr-b knockout mouse is available (120). We could compare the infections of the wild type C57Bl/6 mice to either of the knockout mice and observe if the loss of the receptor/ligand interaction during infection decreases the mouse's resistance. These experiments would allow us a better understanding of how the host uses this additional system of self recognition and how the immune system reacts to it.

6.2 Poxvirus Regulation of a 4C7 Reactive Protein in Human Cells

6.2.1 Summary

In the second part of the thesis, we discovered that VACV infection appeared to cause an apparent significant increase in CLEC2D expression using the antibody 4C7. The infection had

to be an active infection since a UV inactivated virus that could still bind, but not cause infection, had no effect on 4C7 staining. This increase in the 4C7 reactive protein occurred over time, the peak of expression depended on the MOI, and the subsequent decrease coincided with the loss of CLEC2D message. When determining the effects of the infection, it was observed that the blocking the virus from physically binding to the cells inhibited the upregulation, no new protein was required, and although early infection could cause the increase, DNA replication or a late protein was a factor in sustaining this upregulation at 12 hours PI. Interestingly, increases in 4C7 staining could be seen within minutes of the cells being incubated with the virus. That being said, we discovered that many types of cells contain an intracellular pool of a protein(s) detected by 4C7 that can be rapidly transferred to the surface of the cell during infection. We also demonstrated that other poxviruses could also cause an increase in 4C7 staining, but not as strong as VACV. However, it depended on the strain of VACV that was used to cause the change in surface expression.

In the course of this investigation, we found that most human NK cells express high percentages of NKR-P1A, however, the amount of the receptor on the cells varies between donors, as seen before (140). Although expression is high on the NK cells, this expression decreases overtime in culture, but increases when stimulated with target cells. When NKR-P1A$^+$ NK cells are incubated with VACV infected target cells, although they have increased amounts of the 4C7 reactive protein on their cell surface, this does not protect them from cytolysis by NK cells, nor does NK cells degranulation become inhibited due to infection. Although IFN-γ secretion is greatly decreased when NK cells are incubated with VACV infected cells, the method of inhibition does not seem to be through NKR-P1A.

We were able to detect different isoforms of CLEC2D in different cell lines, where not all cells expressed each of the isoforms, but 221 cells that were our target in the assays with NK cells, had message for the three CLEC2D proteins that have transmembrane domains as seen previously (85). During VACV infection, it was determined that 221 cells were not upregulating much isoform 1, which is the only one known to bind to NKR-P1A, however, it does have protein for isoform 1 inside the cells, which is not surprising since they have isoform 1 message, detected by staining the cells with antibody specific against isoform 1. Also, VACV infection does not appear to upregulate another CLEC protein, KACL (CLEC2A), which the 4C7 antibody is able to recognize, which is not surprising seeing as KACL mostly restricted to keratinocytes, a type of cell in the epidermis. We attempted to utilize another antibody that should recognize isoforms 1, 2 and 4, but there was no affect on surface staining following infection suggesting that it may be another unknown protein that the 4C7 antibody recognizes on the cell surface following infection. Finally, cell types, other than activated white blood cells, have an intracellular pool of the 4C7 reactive protein that can be expressed on the surface of the cells. Together, these data suggest that VACV does not upregulated isoform 1 during infection, however other isoforms or an unknown protein may be expressed on the cell surface. However, these other proteins do not seem to interact with NKR-P1A.

6.2.2 Implications of Results

Poxvirus May Upregulate Different Isoforms of Human CLEC2D or an Unknown Protein:

Opposite to what we saw for Clr-b, we observed that VACV infection causes the human CLEC2D protein, as detected by the 4C7 antibody, to be upregulated on cells. This upregulation depended on the strain of VACV, though other poxviruses also cause the induction but not to the

same degree. Similar to Clr-b, an active, early infection was required for the event to occur, however, late protein(s) are also used to sustain this event at later time points. Interestingly, VACV infection did not upregulate much of isoform 1. Using antibodies specifically against isoform 1, we determined that isoform 1 was not being upregulated during the VACV infection. This is contrary to what is reported in the HIV and EBV, where using antibodies specifically against isoform 1, upregulation of isoform 1 was detected (86). Whereas in the RSV infection, isoform 1 transcripts were increased and protein could be detected on the cell surface using the 4C7 antibody, which also depended on production of type I IFNs (225). As VACV infections decrease cellular transcripts (213), the lack isoform 1 on the cell surface is not completely surprising. However, the appearance of a rapidly expressed protein following VACV infection was surprising and will be discussed shortly.

Since we used the 4C7 antibody, that recognizes isoform 1, 2 and 4, the increase of protein on the cell surface could indicating that other isoforms of CLEC2D (Figure 6.2), or another protein that is recognized by the 4C7 antibody are amplified after infection. We were able to detect message for all three isoforms in 221 cell, suggesting that they are capable in making the transcripts for all three isoforms and possibly in producing protein from them. The function of these different isoforms is currently unknown. It is known that many different genes can be alternately spliced to form different isoforms of a protein and that these proteins could have different functions in the body. An example of this is seen in activation of c-Jun N-terminal kinase (JNK) by TRAIL receptors, where the short isoform of JNK1 sends anti-apoptotic signals and long isoforms send pro-apoptotic signals (151). In this study, we were not able to ascertain what the function of the different isoforms of CLEC2D are, but other studies report that they do not interact with nor send any signals through NKR-P1A (85). This coincides with our data, that

Figure 6.2 *Human CLEC2D Isoforms*
Human CLEC2D isoforms where start codons (ATG) and stop codons (TAG or TGA) are shown. The red boxes indicate location of the transmembrane domain. (Modified from (85)).

NKR-P1A$^+$ cells were not stimulated through interaction with the 4C7 reactive protein. The only effect VACV infection of target cells had on the NK cells was to inhibit IFN-γ production. This could be due to the fact that more stimulation is required for IFN-γ production than NK degranulation (261).

A possibility for cells containing different isoforms which may have various functions within the cells, would be to inhibit the expression of one another. Seeing as isoform 1 is an inhibitory ligand, during an infection, the host would want to activate NK cell function by removing any inhibition signal from the cell surface. The other CLEC2D isoforms could function to hold isoform 1 within the cell and/or go to the surface themselves to activate other immune cells. As we have no concrete proof that other CLEC2D isoforms are expressed on the cell surface during VACV infection, more studies will have to be performed to determine if they can be expressed.

Interestingly, Deerpox encodes a protein, DPV-W848_83-142, that is highly similar to isoform 1 in the receptor binding region (Figure 6.3A) and could possibly be used as a viral decoy during a Deerpox infection. VACV, on the other hand encodes for several C-type lectins that could also be used as a decoy or possibly to interfere with NK activity in VACV's natural host. For example, VACV protein A40R, is found in the EV and is found early in the plasma membrane following VACV infection (239, 280). Although A40R has limited similarity to isoform 1 (Figure 6.1B), it may be possible that, similar to VACV A33R, it could interact with a host ligand due to the protein conformation that could be similar to isoform 1 (246). Further studies to understand the difference in NK function inhibition during VACV infection and the possibility of a viral decoy are required.

A.

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LLT1   53   VAALSAIRANCHQEPSVCLQAACPESWIGFQRKCFYFSDDTKNWTSSQRFCDSQDADLAQ  112
            +  L A +++  QEP++     CP+ WIGF  KCFYFS+D+KNWT    FC S  A L +
DPV    68   IIILMAFKSDT-QEPTI-KYVTCPKGWIGFGYKCFYFSEDSKNWTFGNTFCTSLGATLVK  125

LLT1   113  VESFQELNFLLRYKGPSDHWIGLSRE-QGQPWKWINGTEWTRQFPILGAGECAYLNDKGA  171
            VE+ +ELNFL RYK  SDHWIGL+RE    PWKW + + +   F I G GECAYLND
DPV    126  VETEEELNFLKRYKDSSDHWIGLNRESSNHPWKWADNSNYNSSFVITGTGECAYLNDIRI  185

LLT1   172  SSARHYTERKWICSKSDIHV  191
            SS+R Y  RKWICSK+  ++
DPV    186  SSSRVYANRKWICSKTYTNI  205
```

B.

```
LLT1   48   IVCGMVAA-------LSAIRANCHQEPSV-------CLQAACPESWIGFQRKCFYFSDDT  93
            ++CG++         L  +       PS+        ++  CP  WI +  KC + S D
A40R   15   VICGLIVGIIFTATLLKVVERKLVHTPSIDKTIKDAYIREDCPTDWISYNNKCIHLSTDR  74

LLT1   94   KNWTSSQRFCDS--QDADLAQVESFQELNFLLRYKGPSDHWIGLSR--EQGQPWKWI---  146
            K W   +  C +   ++DL ++E+  EL+FL   +    +W+G S    Q  P+ +I
A40R   75   KTWEEGRNACKALNPNSDLIKIETPNELSFLRSIR--RGYWVGESEILNQTTPYNFIAKN  132

LLT1   147  ---NGTE  150
               NGT+
A40R   133  ATKNGTK  139
```

Figure 6.3 *Protein alignment of Isoform 1 and Poxvirus C-type ligands*
(A) Alignment of human CLEC2D isoform 1 and Deerpox (DPX) protein, DPV-W848_83-142. Proteins have 50% similarity. (B) Alignment of human CLEC2D isoform 1 and VACV C-type ligand, A40R. Proteins have 25% similarity. Isoform 1 (LLT1) accession # = AAF22159. DPV-W848_83-142 accession # = YP_ 227519. A40R accession # = YP_233047.

It is also possible that during the incubation, NK cells became infected with VACV by associating with infected cells carrying residual virus. VACV also has many proteins that could interfere with the production of IFN-γ in NK cells as well (188). However, since we can determine that the NK cells were not infected during this incubation (Figure 6.4) as it takes 8-20 hours before new virus is produced, this situation is not likely. Nonetheless, VACV infection is upregulating protein(s) that react with 4C7 but not with NKR-P1A.

Rapid Upregulation of a 4C7 Reactive Protein:

Another possibility is that the 4C7 antibody is recognizing another unknown protein. Previous studies on CLEC2D utilizing the 4C7 antibody to detect CLEC2D found that 4C7 also recognizes the related KACL protein, but not AICL (86, 218). However, a study to determine if that was all 4C7 was binding has not been performed. Hence, there possibly is another protein that is detected by 4C7 that is found in an intracellular pool and/or is transferred to the cell surface during VACV infection. Since we utilized a second antibody that should recognize isoforms 1, 2 and 4, and it did not detect any upregulation following VACV infection, this suggests that it is another unknown protein that is being expressed during infection. This finding is novel that a host protein is rapidly mobilized to the cell surface during VACV infection, especially since VACV has the tendency to degrade host mRNA.

The 4C7 reactive protein is most likely a host protein that is rapidly mobilized at the time that VACV enters the cell, seeing that we could not detect 4C7 staining on a mouse cell that cannot express CLEC2D and therefore it is less likely to be a viral protein. It is also possible that this unknown protein could be a host protein carried with from the virus during viral replication. However, since we could not detect signal with the 4C7 antibody following infection of

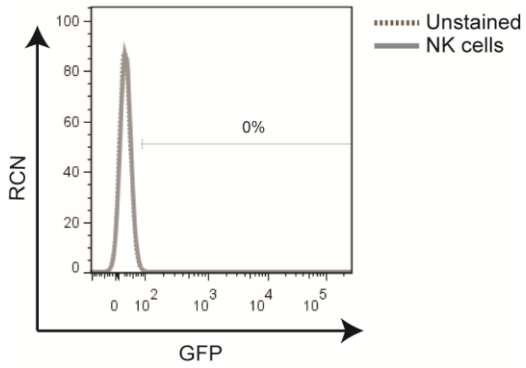

Figure 6.4 *Incubation with infected target cells does not infect NK cells*
221 cells were incubated with media or eGFP-VACV at a MOI of 10 for 1 hour, then placed with primary IL-2 activated NK cells for 3 hours. Figure depicts GFP expression for $CD56^{+}$ NK cells following incubation with target cells. n = 3 experiments each done in triplicate.

mouse cells with the virus, this is unlikely. The reason for expressing the 4C7 reactive protein following VACV infection remains a mystery, however we can speculate on this. One possibility is that when the virus binds and enters the cell, this protein is mobilized to the surface to mark the cell as infected, and perhaps aiding in blocking superinfection. Late in infection, VACV viral proteins found on the cell membrane inhibit fusion of subsequent virions (272). A more recent paper has demonstrated that superinfection can be prevented early in the infection by inhibiting fusion of the virus with the cell membrane by an unknown method (139). It is possible that once entering the cell and releasing proteins in the lateral bodies, this causes the 4C7 reactive protein to also be translocated to the cell surface to aids in hindering superinfection with other proteins from lateral bodies, or that it is just a side effect of movement of proteins to the cell surface. If this is so, what other are proteins are being exported to the cell surface with the 4C7 reactive protein that we have not detected with this or other antibodies?

The fact that only the one strains of VACV can cause the upregulation of the 4C7 reactive protein can be a matter of cell entry methods. Where WR can enter the cell by both direct fusion and the endocytic route, Copenhagen can only enter by direct fusion as discussed previously. On the other hand Myxoma was also able to cause some upregulation of the 4C7 reactive protein, but not the extent of WR. It has been found that Myxoma can also enter the cell by the endosomal route, but the pathway that it uses is slightly different from the pathway utilized by VACV (265). Therefore, studies to determine the differences in the route of infection and the proteins used for superinfection exclusion should be performed. Additionally, studies to determine exactly what protein 4C7 is detecting during VACV infection and its function will have to be performed.

NKR-P1A Expression Increases when Cultured with Target Cells:

It is known that NKR-P1A is constitutively expressed on peripheral blood monocytes and on *in vitro* derived DC, and that this expression was upregulated when the cells were cultured in GM-CSF (197). It is also reported that NK cells upregulate NKR-P1A when cultured in IL-12 but not in IL-2, however, this upregulation occurs over a period of days and required new protein to be translated (194). Here, we demonstrated that following at least a week of culture in IL-2, NKR-P1A expression was increased when NK cells were incubated with target cells. This upregulation occurred within 3 hours of being with the target cells, and since our confocal data can detect NKR-P1A within the cells (Figure 5.5), it is unlikely that this is *de novo* production. Induction of NKR-P1A with target cells, unlike with IL-12 which is thought to be an inflammatory response (194), may be a response of the NK cell to upregulate inhibitory receptors in order to inhibit itself from killing randomly. Studies to determine the function of this upregulation of NKR-P1A with target cells should be performed.

All Cells Contain an Intracellular Pool of the 4C7 Reactive Protein

In these studies, we observed an intracellular pool of protein that may be CLEC2D or a protein that binds to the 4C7 antibody. It was reported previously that B cells lines have message for the three CLEC2D proteins that have transmembrane domains, however, only isoform 1 was detected on the surface of the cell (85). This intracellular pool of 4C7 reactive protein was not just observed in 221 cells, that are a B cell line, but also detected in many other cell types including fibroblasts and epithelial cells. Since the function of the different isoforms and the 4C7 reactive protein is unknown, the reason to hold these preformed proteins inside the cell is mysterious. It was observed that the cells have the ability to rapidly migrate the 4C7 reactive

Table 6.1 Summary of 4C7 Reactive Protein Upregulation and CLEC2D Transcript

Cell Line	Cell Type	Type of cell Line	5 minute upregulation	4 hour upregulation	PCR Transcript		
					1	2	4
721.221	B cell	Transformed	√	√	√	√	√
KM-H2	B cell	Cancer	√	√	√	√	√
Jurkat	T cell	Cancer	√	√	x	x	√
K299	T cell	Cancer	√	√	√	√	x
NK-92	NK cell	Cancer	√	√	√	√	√
HFF-Tel	Human Forskin Fibroblast	Transformed	x	x	√	x	x
Huh7.5	Hepatocyte	Cancer	x	x	√	x	X
293T	Kidney Epithelial	Transformed	x	√	√	x	√
Hela	Cervical Carcinoma	Cancer	√	√	x	x	√
TK-H134B	Fibroblast	Cancer	√	√	x	x	√

protein to the surface of the cells during infection that was mostly seen in lymphocytes, where other cells could do it, but not to the same extent. Unlike when using 4C7, a different antibody, that should detect all three isoforms, did not detect any protein on the cell surface during VACV infection. Hence, it is possible that the rapidly expressed protein is not any isoform of CLEC2D. However, since we do not have a positive control to verify that is antibody could recognize isoforms 2 and 4, we cannot truly rule it out either. Nonetheless, most of these cells also had transcripts for at least 1 isoform of CLEC2D (Table 6.1), and hence may have the ability to express it during infection. Therefore, the function and identity of this pool may be different in different cells since they could not all rapidly upregulate this protein during infection. Experiments to determine of what this pool of 4C7 reactive protein is, should be carried out.

6.2.3 Human Model of Poxvirus Regulation of a 4C7 Reactive Protein

The proposed model for the effects of poxvirus infection on the 4C7 reactive protein is slightly more complicated than the mouse model. As we are not positive of the identity of the 4C7 reactive protein, we will address this protein as protein 4C7 (p4C7). A resting cell expresses a normal compliment of host activating and inhibitory ligand on it cell surface. As seen on the 221 cells, it may also express minimal amount of p4C7 and isoform 1 on the surface (Figure 6.5A). Inside the cell, there is a pool of p4C7. When the virus binds and/or enters the cells, the is a rapid upregulation of p4C7 on the cell surface which may include little to no isoform 1 (Figure 6.5B). Overtime, there will be a turnover of cell surface proteins and since viral proteins produced can degrade host mRNA including CLEC2D mRNA, the pool of p4C7 is reduced and there is little left to put back on the cell surface (Figure 6.5C). Depending on when the NK cell comes into contact with the infected cell, it can encounter inhibition from viral proteins or viral decoys, along with MHC I if it is early in the infection. Here, the NK cell will receive mostly

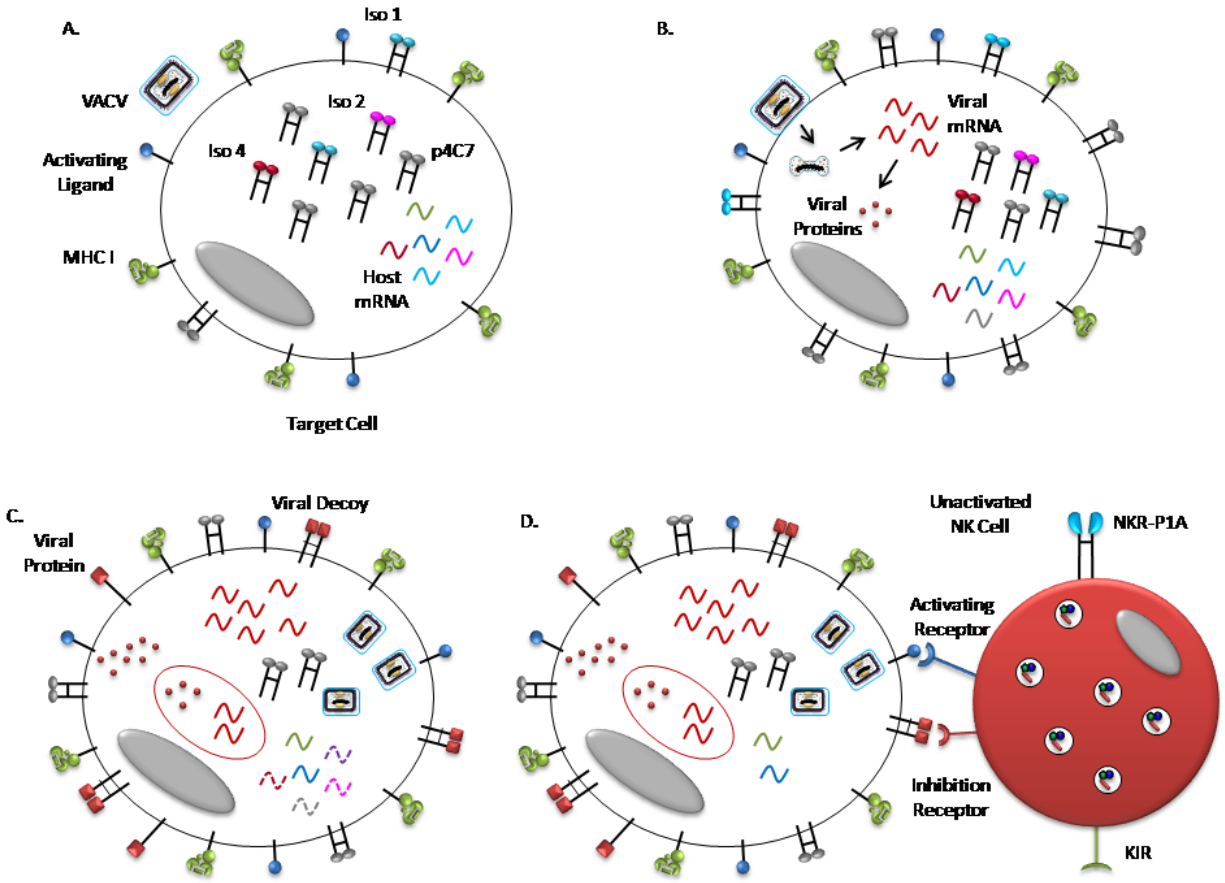

Figure 6.5 *Human model of poxvirus modulation of p4C7*
(A) An uninfected cell has little p4C7 on the cell surface, but contains an intracellular pool of p4C7 and CLEC2D. (B) VACV infects cells causing an upregulation of p4C7 on the cell surface, while viral mRNA and proteins are produced. (C) Over time, protein turnover occurs and viral proteins degrade CLEC2D mRNA and other host proteins, therefore there is little p4C7 left to go to the cell surface. (D) Viral proteins and/or CLEC2D decoys are expressed at the cell surface that can possibly inhibit NK cell function.

inhibitory signals from the infected cell and it will remain inactive (Figure 6.5D). In this model, p4C7 does not interact with NKR-P1A, given that we saw no NK function through NKR-P1A.

Looking more closely at the early events of translocation of p4C7, we have determined the steps required for this to occur. Firstly, a replicating virus must bind and enter the cell as blocking virus binding and the addition of a UV virus does not cause expression of p4C7 (Figure 6.6A). However, there is a possibility that the UV virus had been treated very harshly and could not bind to the cell, and therefore could not cause upregulation of p4C7. By titring the UV treatment of the virus, we could determine if this was the case. When the virus enters the cell, the intracellular pool of p4C7 is rapidly mobilized to the cell surface (Figure 6.6B). This is known because inhibiting *de novo* synthesis of protein does not inhibit the movement of p4C7, and we have visualized pools of p4C7 in numerous types of cells. As the infection progresses, protein turnover causes a decrease of p4C7 on the cell surface, while the virus degrades host mRNA, leaving less amounts of p4C7 on the cell surface. However, expression of a late viral protein is required to sustain p4C7 on the cell surface (Figure 6.6C). The function of p4C7 is currently unknown. Studies to determine what the protein is and what its function is should be performed.

6.2.4 Future Directions

There are many open questions about poxvirus regulation of the 4C7 reactive protein, beginning first with what the 4C7 Ab detecting during the VACV infection. It is possible that 4C7 is detecting isoforms 2 and 4. Although we have used a goat antisera that should detect these isoforms based on the peptide immunogen used to generate the peptide, we do not have a positive control that it actually does bind to them. In order to determine if these other isoforms

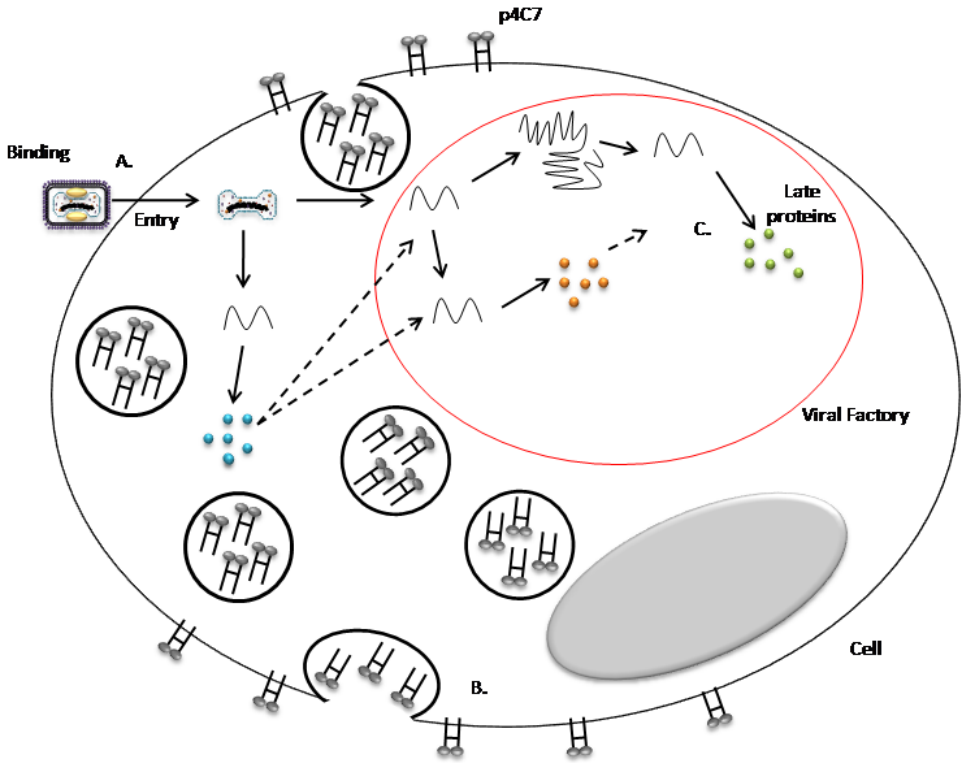

Figure 6.6 *Translocation of p4C7 during VACV infection*
(A) Virus binds and enters the cells that contains intracellular pools of p4C7. (B) Sensing of the virus causes the readymade p4C7 protein to translocate to the cell surface. (C) Translation of late viral proteins allow for continued expression of p4C7 late in the infection.

are being upregulated, constructs for isoforms 2 and 4 should be made and transfected into 293T cells. Staining these transfected cells with the other antibody that should see all three isoforms, would ensure its reactivity. We also have to obtain antibodies specifically against isoforms 2 and 4, then perform the transfections and infection again to determine what surface protein(s) is induced on different cell types during VACV infection. If isoforms 2/4 are not mobilized during infection, then we should determine what exactly does 4C7 bind to by performing an immunoprecipitation assay following infection of cells, and mass spectrometry to isolate proteins binding to 4C7.

If VACV infection is upregulating isoforms 2/4, then we would need to determine what the function of these isoforms are. To be able to do this, we would want to know the receptor(s) and the cell types that these isoforms bind to. This could be done by generating soluble tagged isoform 2 and 4 protein and screening many cell types by flow cytometry to determine if the protein binds to any cells. If the protein does bind to a cell type, a pull down assay and mass spectrometry can be performed to establish a receptor. Determining the function of the different isoforms could also explain why different viral infections cause upregulation of one isoform over the other.

The next question that arises from this project is if this action of 4C7 reactive protein upregulation is due to the viral evasion or to host surveillance or just a product of viral entry. Knowing the identity of the protein(s) being detected by 4C7 would really help to determine what direction to pursue. One way to see if this is a function of the virus, we can use the finding that different strains of VACV have differences in its ability to increase the 4C7 reactive protein. In this thesis, we saw that VACV strain Copenhagen was unable to cause the upregulation of 4C7 reactive protein that strain WR was able to produce. Therefore, we could use the differences

between the viruses genomes and uncover any dissimilarities in the genes and proteins they encode. Once resolved, we could generate the protein(s) that are unlike in the two strains. To verify if they cause the upregulation of 4C7 reactive protein in cells we can transfect cells with the protein(s) and infect with Copenhagen to see if we see an increase in 4C7 staining. Another way to do this would be to generate a recombinant Copenhagen virus that encodes for the missing protein(s) to see if during infection the 4C7 reactive protein is upregulated. To understand why this protein would help the host establish an immune response, we need to identify the function of the isoforms and/or 4C7 reactive protein and what immune cells they activate or inhibit if any.

A final question that arose from this project, was in what vesicles is the unknown protein located in. To uncover the location, confocal microscopy can be performed by staining different cell lines with 4C7 and antibodies against each isoform of CLEC2D to determine the composition of the intracellular pool. Different cell lines can be used here because it is possible that different cells express separate vesicles for additional 4C7 reactive proteins, that may also be separate from the ones that hold the intracellular CLEC2D isoforms. Once that is established, cells can be stained for different compartments in the cell such as the ER, Golgi, and various endosomes. The location of the intracellular pool may also explain why the 4C7 reactive protein is expressed so rapidly following incubation with VACV in certain cells and not in others.

6.3 Concluding Remarks

The studies presented in this thesis were intended to bring comprehension to host interactions with poxvirus infections. Here, it was demonstrated that the mouse Clr-b molecule is down regulated during a poxvirus infection, leading to the activation of NK cells. It was then observed that VACV infection causes an upregulation of a protein that reacts with the 4C7 Ab in

human cells. This is the first demonstration of a rapid surface upregulation of a host protein following poxvirus infection. However, as we could not identify the protein, we cannot determine its effect on NK cells, but we did rule out an interaction with the known CLEC2D receptor, NKR-P1A. Identifying the molecule 4C7 binds to will be necessary to understand the biological significance of this phenomenon. Investigating how viruses interact with a host, enables us to not only build upon our understanding of host immunity and/or viral evasion methods, but will hopefully improve on therapies for both vaccination and cancers that use VACV as a platform.

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Appendix

Recipes

Swelling Buffer:

10 mL	1M Tris pH8
2 mL	1M $MgCl_2$

Bring to 1L with water

X-Gal Stain:

9.5 mL	PBS
100 µL	500 mM K Ferricyanide
100 µL	500 mM K Ferrocyanide
200 µL	100 mM $MgCl_2$
100 µL	50 mG/mL X-gal

Neutral Buffered Formaldehyde (NBF):

700 mL	Distilled Water
110 mL	37% Formaldehyde
100 mL	PBS

Bring to pH 7.4 using Na_2CO_2

Bring to 1L with distilled water

Crystal Violet Stain:

2%	Crystal Violet w/v
2 parts	Ethanol
1 part	37% Formaldehyde
7 parts	Water