Modulation of NK Cell Function with Agonistic α-CD137 Antibodies during MCMV Infection

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ABSTRACT

The Tumor Necrosis Factor Receptor Superfamily (TNFR) is responsible in regulating a myriad of physiological function including the regulation of the immune system. Among the members include CD137 (4-1BB), an inducible costimulatory receptor known for its potent activation, proliferation, and survival effects on T cells. Stimulation of NK cells with agonistic α-CD137 antibodies are known to increase IFN-γ production and proliferation in NK cells as well as increase efficacy of anti-tumor responses. However, NK cell death has also been seen in certain circumstances, although the mechanism remains to be determined. In vitro stimulation of NK cells revealed that α-CD137 induced NK cell death occurs through both TNFR1 and TNFR2, although the action of TNF-α and TNF-β remain uncertain. Death was independent of other cytotoxic mechanisms such as granzyme/perforin, Fas-Fas ligand, and TRAIL. During MCMV infection, α-CD137 induces NK cell death during the early phase of infection reducing viral resistance. This causes increased viral proliferation which drives NK cell proliferation, likely through Ly49H-m157 interactions, to high levels by day 4 of infection. The use of α-CD137 as a tumor therapeutic is promising with several applications undergoing clinical trials. However, my results raise concern of other effects including the depletion of NK cells. This may cause a temporary impairment in immune function against pathogenic infections and a compensatory reaction of NK cell proliferation, both of which may cause damage to the host. However, with proper co-stimulation or co-treatments, this impairment may be overcome and prevent adverse effects in patients.

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LIST OF ABBREVIATIONS

ADCC	Antibody dependent cell cytotoxicity
BID	BH3 interacting-domain death agonist
CARP2	caspase 8 and 10 associated RING finger protein 2
CD	cluster of differentiation
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DAP12	DNAX activation protein of 12kDa
DD	Death domain
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ERF	Ets-2 repressor factor
HAART	Highly active anti-retroviral therapy
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
IFNR	Interferon receptor
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
KIR	Killer-cell immunoglobulin-like receptor
LAG-3	Lymphocyte activation gene 3
LCMV	Lymphocytic choriomeningitis virus
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
MIEP	Major immediate early promoter
mTOR	Mechanistic target of rapamycin
Mult1	UL16 binding protein-like transcript 1
NCR	Natural cytotoxicity receptor
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NKT	Natural killer T
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PFU	plaque forming units
PKC	Protein kinase C
qPCR	quantitative polymerase chain reaction
Rae	retinoic acid early inducible proteins
RAG-/-	recombination-activating genes deficient

RIPK	Receptor-interacting serine/threonine protein kinase
RIPK1	receptor-interacting serine/threonine-protein kinase 1
SPF	specific pathogen free
STAT	Signal transducer and activator of transcription
TACE	TNF-α-converting enzyme
TGF-β	Tumor growth factor β
Th1	T helper 1
Th2	T helper 2
TIM3	T-cell immunoglobulin and mucin-domain 3
TNF	Tumor necrosis factor
TNFR	TNF receptor
TNFR1	TNFRSF1a
TNFR2	TNFRSF1b
TRADD	Tumor necrosis factor receptor type 1-associated death domain protein
TRAF	TNF receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
VSV	Vesicular stomatitis virus

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1. INTRODUCTION

1.1 NK cells

Natural Killer (NK) cells are known mainly as effector lymphocytes in the innate immune system. They share commonalities with T cells in that both cells are differentiated from the common lymphoid progenitor and offer resistance against infections and tumors through the action of perforins and granzymes upon activation. However, NK cells do not undergo somatic recombination and rely on germline-encoded receptors. These receptors can be generally divided into activating and inhibitory receptors and include Ly49 (in mice)/KIR (in humans), NKG2, and NCRs. They recognize stress ligands, major histocompatibility complex (MHC) molecules, and MHC related proteins in order to assess the health of a cell. Various tumors and viruses downregulate the expression of MHC I molecules in order to evade surveillance through antigen presentation by T cells. For example, the adenoviral protein E3/19-kDa prevents the expression of MHC I molecules by promoting the retention of the molecules in the endoplasmic reticulum, preventing antigen presentation of viral proteins on infected cells [Cox et al., 1991]. Similarly, MCMV m152 protein retains MHC I molecules in the endoplasmic reticulum (ER)-golgi intermediate compartment with the same result [Ziegler et al., 1997]. A variety of tumors cells also downregulate MHC I expression, and has been linked to increased metastatic ability [Haworth et al., 2014][Isakov et al., 1983][Baetselier et al., 1980]. However, inhibitory Ly49 receptors in the mouse and KIR receptors in humans recognize MHC I molecules [Karlhofer et al., 1992][Moretta et al., 1993]. Lowered MHC I expression on infected and tumorous cells will result in the lack of Ly49 and KIR inhibitory signaling, resulting in the activation of NK cell cytotoxicity. This

recognition of lowered MHC I expression is termed “missing self recognition” [Karre et al., 1985].

While Ly49/KIR recognize MHC I, NKG2 receptors recognize non-classical MHC Ib molecules that present classical MHC I derived peptides: HLA-E in humans and Qa1 in mice. Different NKG2 members can form heterodimers with CD94 in order to survey MHC Ib molecules. NKG2D, however, is an exception and instead recognizes “stress induced ligands” on cells that have been transformed or infected, and activate NK cells. In humans, these stress-induced ligands include MICA and MICB, and UL16 binding proteins (ULBPs) [Waldhauer et al., 2008]. In mice, they are the retinoic acid early inducible proteins (Rae), H60, and UL16 binding protein-like transcript 1 (MULT1) [Cerwenka et al., 2000] [Diefenbach et al., 2000][Carayannopoulos et al., 2002][Diefenbach et al., 2003]. These ligands are widely expressed on various primary tumors and cell lines rendering them susceptible to NK mediated death and also to rejection during tumor transplantation [Diefenbach et al., 2000][Diefenbach et al., 2001][Cerwenka et al., 2001]. These stress ligands are also induced during viral infections. For example, ectromelia mouse poxvirus induced MULT1 expression in C57BL/6 mice and mouse hepatitis virus increased H60, MULT1, and Raet1 in BALB/c mice [Fang et al., 2008].

In addition to self ligands, NK cells are able to recognize pathogen derived ligands as well. Resistance to herpesvirus infections was known to be largely dependent on NK cell activity [Biron et al., 1989][Bukowski et al., 1985]. It was discovered that the resistance of different mouse strains against MCMV was attributable to the *Cmv1* locus, located on chromosome 6 [Scalzo et al., 1990][Scalzo et al., 1992]. This particular area is known as the NK cell gene complex (NKC) and contains a range of NK receptors. Later, it was

discovered that that resistance against MCMV conferred through NK cells was due to the C-type lectin-like receptor Ly49H, which can recognize the viral glycoprotein m157 [Lee et al., 2001][Brown et al., 2001][Arase et al., 2002]. When Ly49H recognizes m157 protein on the surface of infected cells, it interacts with the adaptor protein DAP12, resulting in the phosphorylation of the immunoreceptor tyrosine (ITAM) motif on DAP12 [Smith et al., 1998]. This activates NK cells to increase interferon-γ (IFN-γ) production, cytotoxicity, and other cytokine/chemokine production [Smith et al., 2002]. Ly49H signaling is critical during MCMV infection as IFN-γ production, cell proliferation, and control of viral replication [Sjolin 2002][Fodil-Cornu et al., 2008][SH Lee JEM 2009]. NK cells have a wide range of inhibitory and activating receptors that allow it to assess the health of a cell. These receptors survey target cells for constitutively expressed self-ligands such as MHC class I, stress induced self-ligands, or ligands derived from pathogens such as m157. The balance of all the signals received from these receptors will decide whether to activate or inhibit cytotoxic activity, cytokine production, and proliferation, either killing infected or tumorous cells, or tolerating healthy ones.

1.1.1 NK Cell Function

Cytotoxicity is mainly carried out by cytotoxic molecules stored in secretory lysosomes such as perforin and granzymes. At first, it was thought that perforin was sufficient to induce cytotoxicity of target cells, however, it was shown that perforin alone does not result in the fragmentation of DNA, which is seen in cell mediated cytotoxicity [Duke et al., 1989]. Perforin was then explained as an important protein that aids the entry of granzymes into the cytosol of targets cells by pore formation [Browne 1999]. The most

studied granzyme, granzyme B, is a serine protease and cleaves proteins related to inducing cell death. These include procaspases 3, 6, 7, 8, 9, and 10, which induce caspase dependent cell death [Yang et al., 1998][Darmon et al., 1995] [Duan et al., 1996][Medema et al., 1997][Lippke et al., 1996], or Bcl-2 homology domain3 interacting-domain death agonist (BID) to promote caspase independent mitochondrial permeabilization [Cullen, et al., 2007]. NK cells can also mediate cell death through tumor necrosis factor (TNF) superfamily members. Among these are TNF-α, Fas ligand, and TNF-related apoptosis-inducing ligand (TRAIL) [Zamai et al., 1998][Tartaglia et al., 1993]. The binding of these ligands to their receptors on target cells results in receptor oligomerization and activation of apoptotic pathways through death domains (DD) that are present on the intracellular domains of these receptors.

In addition to cytotoxicity, NK cells are also able to modulate the immune system through the production of cytokines. The most prominent and important cytokine produced by NK cells is IFN-γ. When type I IFNs, interleukins (IL) 2 and 12 bind to their corresponding receptors, interferon receptor (IFNR), IL2R, and IL12R, associated Janus kinases (JAK) are able to phosphorylate tyrosine residues, leading to the recruitment of signal transducer and activator of transcription proteins (STAT) through their SH2 domains. In this case, STAT4 is recruited, leading to the downstream activation of IFN-γ production [Jacobson et al., 1995][Wang et al., 1999][Cho et al., 1996]. IFN-γ is a crucial cytokine with a variety of effects on a range of cell types. NK derived IFN-γ can promote the differentiation of monocytes into macrophages and dendritic cells, which will in turn provide cytokines such as IL12 [Goldszmid et al., 2012]. It is also required for the development of T helper 1 (Th1) cells [Scharton et al., 1993] and for the clearance of various pathogens

including viral, bacterial, protozoal, and fungal [Zhang et al 1997][Gazzinelli et al., 1994][Tripp et al., 1993][Gribaudo et al., 1993][Lucin et al., 1992].

In addition to pathogenic infections, NK cells play a large role in tumor surveillance. As NK cells determine targets for cytotoxicity by detecting activating and inhibitory signals on potential target cells, tumor cells that express stress signals and downregulate MHC molecules become targets for cytotoxicity by NK cells. A significant portion of tumors downregulate MHC expression [Bubenik et al., 2004], which allows them to escape detection by T cells, a significant part of the anti-tumor response by the immune system. Fortunately, NK cells can capitalize on the decreased inhibitory signals caused by the downregulation of MHC molecules and control tumor growth, making them a promising choice as tumor therapies.

1.1.2 Tumor Therapy using NK Cells

Even though NK cells are being studied as a mode of tumor therapy, the tumor microenvironment may pose a problem for NK cells as several evasion mechanisms exist such as inhibition by regulatory cells, cytokines, and modulation of NK receptor ligands [Guillerey et al., 2016]. In the tumor environemt, tumor growth factor beta (TGF-β) has been shown to block the activation of mTOR by IL15, a crucial survival and proliferative cytokine for NK cells [Viel et al., 2016]. This inhibition of mTOR activity decreases the metabolism and proliferation of NK cells, interfering with their ability to combat tumors [Viel et al., 2016]. Membrane bound TGF-β can be expressed by T regulatory cells [Nakamura et al., 2004] which downregulate NKG2D receptors, abrogating NK cytotoxicity against tumors [Ghiringhelli 2005][Ghiringhelli et al., 2006].

In order to maximize NK cell effectiveness, several methods are currently being investigated. One promising approach is adoptive therapy. Ex vivo expansion of autologous NK cells and administration of these expanded cells back into patients have passed phase I clinical trials [Sakamoto et al., 2015], and recent reports show further promise of this approach. Ex vivo stimulation of these cells with IL2, IL15, and IL18 improves NK proliferation and cytotoxicity by increasing NK cell expression of CD25, resulting in the high affinity IL2$\alpha\beta\gamma$ receptor [Leong et al., 2014]. However, once NK cells are transferred into patients, in vivo cytokine concentrations do not seem sufficient to promote significant NK activity against tumors [Parkhurst et al., 2011]. Administration of NK cells in conjunction with activating cytokines have been attempted, but several side effects render this method non-viable. IL2 administration in patients caused severe fluid retention problems [Rosenberg et al., 1985], life threatening vascular leak syndrome, several cardiovascular symptoms, anemia, thrombocytopenia, lymphocytopenia, and eosinophilia in a significant number of patients [Vial et al., 1992].

Another approach to tumor therapy is to modulate the immune response through targeting specific molecules by the administration of monoclonal antibodies. One popular approach is immune checkpoint inhibition. “Immune checkpoint” refers to the various mechanisms through which immune cells maintain a state of self-tolerance and prevent autoimmunity. By inhibiting these checkpoints, the immune system is able to increase its function. Immune checkpoint inhibition therapy was initially developed in the context of improving T cell function against tumors through the use of tumor antigens. Utilizing tumor antigens to activate T cells was promising for tumor treatment and the expression of B7 molecules through transfection caused tumor rejection even in resistant melanoma cells.

[Townsend et al., 1993]. However, clinical effectiveness was difficult with this approach, with a reported objective response rate of 2.6% out of 440 patients [Rosenberg et al., 2004]. One suspected cause of the low effectiveness was thought to be cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). In addition to recognition of antigens through the TCR, T cells require costimulatory signals, such as the engagement of its CD28 receptor to B7 ligand on APCs, for activation [Gimmi et al., 1991][Linsley et al.,1991]. Upon activation, T cells also begin expressing the inhibitory molecule CTLA-4. It has similar homology to CD28 and also binds to B7 molecules [Sharma et al., 2015]. Unlike CD28, however, CTLA-4 results in the inhibition of T cell activity [Walunas et al., 1994]. In addition, CTLA-4 has a higher affinity for CD28 than B7 and can inhibit CD28 activation signals and ultimately lead to decreased T cell activity [Sansom, 2000]. This led to the development of blocking antibodies against CTLA-4 in order to inhibit the depression of T cell activity, with clinical trials of ipilimumab showing promising results in metastatic melanoma patients [Hodi et al., 2010][Robert et al., 2011]. The success of this blocking antibody therapy opened up the field of "immune checkpoint" therapy.

Programmed cell death 1 (PD-1) is another immune checkpoint, with its function being the limitation of T cells to prevent autoimmunity during inflammatory responses [Nishimura et al., 1999]. PD-1 is also expressed on T cells upon activation and binds to its ligands PD-L1 [Freeman et al., 2000] and PD-L2 [Ishida et al., 1992]. Various tumor cells, including cells from lung, ovarian, skin, and colon cancers, have upregulated expression of these ligands, which are normally not present on most cells, and are thought to contribute to immune resistance [Dong et al., 2002]. Antibodies against both molecules are undergoing clinical trials with favorable results. Nivolumab, pembrolizumab, and pidilizumab for PD-1,

and BMS-936559, MPDL3280A, and MEDI-4736 for PD-L1 have shown low toxicity during phase I trials with promising signs of tumor regression, with only pidilizumab showing any signs of serious complications [Philips et al., 2015]. These antibodies are currently in phase II and phase III trials. To this date, several immune checkpoints have been identified, among which CTLA-4 and PD-1 are the most studied, but also include others, such as lymphocyte activation gene 3 (LAG-3) and T-cell immunoglobulin and mucin-domain 3 (TIM3) [Huang et al., 2004][Zhu et al., 2005].

Monoclonal antibodies against these key molecules have also been used to increase NK survival and function, and are currently being tested. Although NK cells from healthy donors do not express PD-1, NK cells from patients with multiple myeloma have been shown to upregulate the receptor [Benson et al., 2010]. CT-011, which is a monoclonal antibody against PD-1, can restore NK cell activity against myeloma by blocking PD1-PD-L1 interactions between NK cells and tumor cells [Benson et al., 2010]. In a similar fashion, antibodies against PD-L1 can also increase NK effectiveness against tumors for the same reason, in addition to activating NK cytotoxicity directly through CD16 targeting of antibody coated tumor cells [Boyerinas et al., 2015]. Antibodies against CTLA-4 have also shown promise regarding anti-tumor activity, but the role of NK cells in this case is not entirely clear [Contardi et al., 2005]. Targeting KIRs in this manner is also promising as it is a ubiquitous inhibitory receptor for all cells including healthy and tumorous ones [Morvan et al., 2016]. Phase I trials with the monoclonal antibody against KIR, IPH2101, demonstrated no serious adverse side effects of treatment, although it only had mild effectiveness [Benson et al., 2012][Vey et al., 2012]. The data together suggest a huge potential of monoclonal therapies by targeting specific immune molecules. However, inhibition of immune

suppression is not the only path that can be taken with antibody therapy, and the activation of the immune response is also viable. The TNFR superfamily offers promising targets in this respect.

1.2 TNF and TNFR Superfamily

The Tumor Necrosis Factor Receptor Superfamily has a large role in regulating survival, activation, and death in immune cells, and as such, has been extensively studied for immune modulation. Members include CD40-CD40L, OX40-OX40L, CD137-CD137L, and GITR-GITRL, but the best studied are TNFR1 (TNFRSF1a) and TNFR2 (TNFRSF1b) along with one of its ligand TNF-α. TNF-α is expressed on the surface of cells as a trimer, which can be cleaved by the metalloproteinase TNF-α-converting enzyme (TACE; also called ADAM17) to become soluble [Black et al., 1997]. TNF-α has two receptors, TNFR1 and TNFR2, and bind TNF as trimers as well. Whereas TNFR1 is expressed on most cell types, TNFR2 is expressed mainly on immune cells and endothelia [Faustman et al., 2010]. Membrane bound TNF-α is thought to mainly act through TNFR2 [Grell et al., 1995], but the soluble form can activate both TNFR1 and TNFR2 [MacEwan, 2002], resulting in regulation of pathways involved in survival, activation, and death (Table 1).

1.2.1 TNF and TNFR Function

TNF was initially discovered for its ability to induce death in tumor cells [Carswell, 1975]. This gave promise as a tumor therapy, but human trials revealed many side-effects including fever, chills, fatigue, hypotension, thrombocytopenia, and fluctuations in leukocyte counts [Saks et al., 1992]. This revealed the complexity and breadth of the effects of TNF

Members	Associated TRAFs	Major functions	Expression on NK cells	Functions in NK cells
TNF-R1/2	TRAF1, 2, 3, 5	NF-κB, MAPK, JNK activation, Apoptosis, Inflammation, Necroptosis	TNFR1/2 expressed on NK cells	Stimulate NK effector function, Induce NK cell death
FAS (CD95) with DD	TRAF5	Apoptosis in various cell types, Necroptosis	Both CD95 and CD95L expressed on activated NK cells	Induce NK cell death
4-1BB (CD137) without DD	TRAF1, 2, 3	NF-κB and MAPK activation, Proliferation and survival of T cells	Induced upon activation with IL2, IL15 (mouse), and CD16 stimulation (human)	Induce proliferation and IFN-γ secretion *in vitro*, Enhance NK cell-mediated ADCC *in vivo* against tumors, Impairs cytotoxicity and IFN-γ production in leukemic settings
GITR (CD357) without DD	TRAF1, 2, 3, 4, 5	NF-κB activation, Proliferation and survival of T cells	Induced upon activation with IL2 and IL15 (mouse)	Enhance or inhibit NK cytotoxicity and IFN-γ production (contradictory data in different settings)

Table 1: TNFR members and their characteristics. Selected members of the TNFR superfamily, their associated TRAF molecules, functions, and expression on NK cells. [Croft et al., 2009][Barao et al., 2007][Melero et al., 2013][Xie et al., 2013][Croft et al., 2013][Watts et al., 2005][Brenner et al., 2015][Cheng et al., 2013][Iannello et al., 2009][Shibatomi et al., 2001].

and TNFRs, and is now considered one of the most pleiotropic cytokines, affecting all aspects of the biological system [Sedger et al., 2014]. Regarding the immune system, there are two important effects of TNF and TNFRs: activation and survival, and death. There is always a crucial need for the regulation of life or death pathways during an immune response. Survival, activation, and proliferation must occur to control infections, but the degree of immune activation must be regulated to prevent not only collateral damage to the host, but autoimmune disorders (rheumatoid arthritis, psoriatic arthritis, psoriasis). In a simple view, TNFR1 is linked to both cell death and survival, and TNFR2 is linked to survival and activation. However, the effects are highly dependent on the circumstance.

1.2.2 TNFR Signaling pathway

Even though both TNFR1 and TNFR2 can bind TNF-α, only TNFR1 has an intracellular DD. This DD recruits the tumor necrosis factor receptor type 1-associated death domain protein (TRADD) allowing the formation of complex I by recruiting receptor-interacting serine/threonine-protein kinase 1 (RIPK1), TNFR associated factor (TRAF) 2 or TRAF5, and cellular inhibitor of apoptosis protein (cIAP) 1 or cIAP2 [Brenner et al., 2015]. In contrast, TNFR2 does not have a DD and instead recruits TRAF1 or TRAF2 with cIAP1 and cIAP2 directly [Brenner et al., 2015]. Both can lead to downstream NF-κB activation, but TNFR1 can also lead to cell death. RIPK 1 is the determining factor between death and NF-κB activation depending on its ubiquitylation state. A20 and Cezanne can remove K63-linked polyubiquitin chains whereas caspase 8 and 10 associated RING finger protein 2 (CARP2) can add K48-linked polyubiquitin chains [Brenner et al., 2015]. These processes will prevent NF-κB signaling by causing the degradation of RIPK1 [Enesa et al.,

2008][Wertz et al., 2004][Liao et al., 2008]. CYLD is another protein that can remove K63 and M1-linked polyubiquitin chains from RIPK1 which allows the dissociation of RIPK1 from TNFR1 and assembly of complex IIa: TRADD, FADD, pro-caspase-8, and $FLIP_L$. This leads to apoptosis through the activation of caspase-8 [Wang et al., 2008][Micheau et al, 2003]. If RIPK1 was not ubiquitylated from the beginning, complex IIb will form instead of complex IIa. For example, in situations where cIAPs are depleted and cannot ubiquitylate RIPK1, RIPK1 is able to relocate to the cytosol and forms complex IIb, also known as the ripoptosome: FADD, pro-caspase-8, $FLIP_L$, and RIPK3 [Tenev et al., 2011]. Pro-caspase-8 homodimers will auto-activate, leading to the classical apoptotic pathway. In both complexes, RIPK1 and RIPK3 must be inactivated by caspase-8 [Micheau et al., 2003]. The insufficiency of caspase activity and the lack of RIPK1 and RIPK3 inactivation will lead to the necrosome, comprised of RIPK1 and multiple RIPK3, which recruits MLKL, leading to necroptosis [Oberst et al., 2011]. However, when cIAPs are able to add K63-linked polyubiquitin chains to RIPK1, LUBAC will be recruited. This will be followed by LUBAC further adding M1-linked polyubiquitin chains to RIPK1. This will allow signaling through TAK and the IKK complexes to activate JNK and NF-κB, promoting survival and activation [Brenner et al., 2015].

1.2.3 CD137 (4-1BB)

One member of the TNFR superfamily, CD137 (4-1BB or TNFRSF9), is an inducible co-stimulatory receptor. It can exist as a 30 kDa monomer, a 55 kDa homodimer [Vinay et al., 2006], or a homotrimer [Won et al., 2010], and is expressed on T cells, NK cells, NKT cells, dendritic cells, monocytes, eosinophils, and microglia [Wilcox et al., 2002][Heinisch et

al., 2001][Kienzle et al., 2000]. The expression and aggregation, and consequently signaling, of CD137 is suggested to be assisted by the binding of galectin-9 to its non-ligand binding portion [Madireddi et al., 2014]. The ligand, CD137L, expression is inducible on B cells, dendritic cells, and macrophages [Futagawa et al., 2002][DeBenedette et al., 1997]. Multimerization of CD137 by trimerized ligand binding will bring TRAF2 and TRAF1 adaptor proteins to the intracellular domain [Arch et al., 1998]. TRAF2 molecules can activate downstream pathways such as NF-κB, and MAPK [Brenner et al., 2015]. TRAF1 is crucial for signaling as mice deficient with TRAF1 were unresponsive to α-CD137 stimulation [Sabbagh et al., 2008]. In T cells with TRAF1 deficiency, ERK activation was reduced, leading to higher levels of the apoptotic Bim protein, decreasing cell survival [Sabbagh et al., 2008]. TRAF2 may also be important in signaling as the relative density of TRAF2 molecules after CD137 stimulation is thought to be a potential determinant on the result of CD137 signaling, and activation of the NF-κB pathway induces a higher expression of TRAF1 and TRAF2 [Schwenzer et al., 1999][Arch et al., 1998]. The activation of NF-κB also increases the expression of the anti-apoptotic Bcl-2 family, specifically Bim, bcl-X_L and bfl-1, increasing survival of cells [Lee et al., 2002][Sabbagh et al., 2008].

4-1BB signaling is a crucial component in anti-viral resistance as seen in various infection models. In the absence of CD137 or its ligand, dysregulation of cytotoxic CD8+ T cell proliferation, decreased cytokine production, and impaired cytotoxicity were observed in infection models using vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), and influenza [Lin et al., 2009][Kwon et al., 2002]. CD8+ T cell responses were several fold lower during LCMV infections when there was an absence of signaling [Tan et al., 1999]. Furthermore, stimulating CD137 with α-CD137 antibodies increased viral

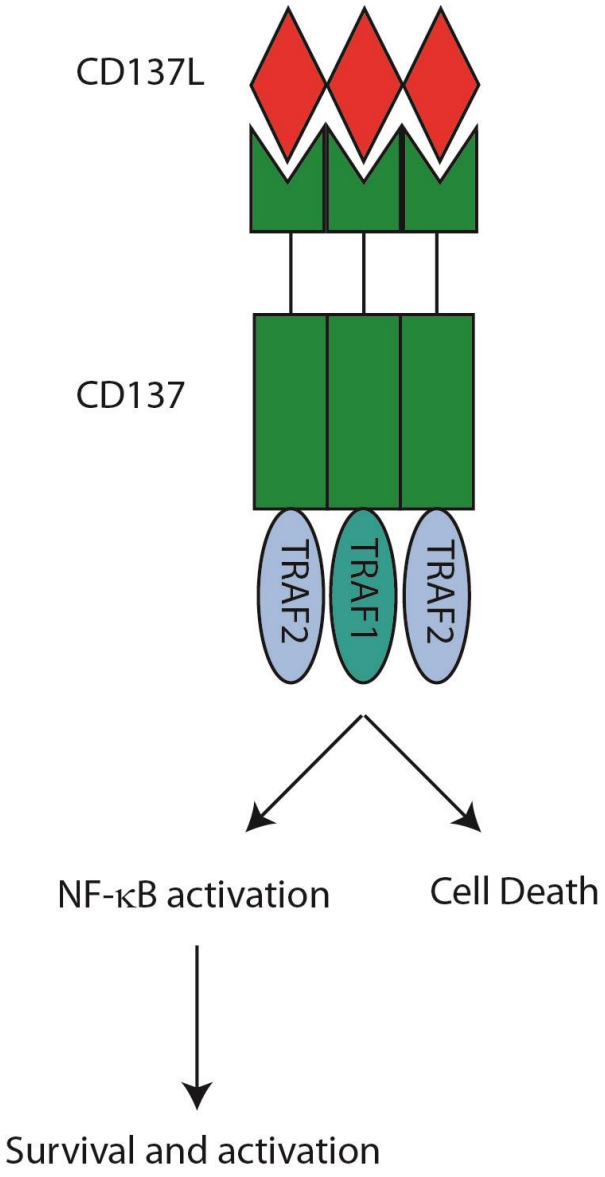

CD137L
CD137
TRAF2
TRAF1
TRAF2
NF-κB activation
Cell Death
Survival and activation

Figure 1: Signaling of CD137 by CD137 ligand. CD137L trimers bind CD137 trimers. TRAF2 and TRAF1 molecules are brought to the intracellular domain of CD137 resulting in either NF-κB activation and thus the activation and survival of cells, or cell death.

resistance to influenza A virus and LCMV by the expansion of CD8+ T cells and also recognition of non-dominant viral antigens [Halstead et al., 2002][Zhang et al., 2007]. In addition, the memory pool of T cells in mice immunized with ovalbumin were greatly increased in size along with the responsiveness and IFN-γ production upon VSV infection [Myers et al., 2006]. α-CD137 treatment has also been seen to increase the rapidity of cardiac and skin grafts in mice, primarily by increasing the proliferation of T cells [Shuford et al., 1997]. This expansion of T cells seems to be correlated to the expression of CD137 on the surface of the cells, increases proliferation, and prevents activation induced cell death even in the absence of CD28 signaling or cytokine activation [Hurtado et al., 1997] [Takahashi et al., 1999]. Although CD137 expression is seen in both CD4+ and CD8+ T cells, they are more pronounced in CD8+ T cells [Takahashi et al., 1999]. Furthermore, CD137 stimulation is much more effective in memory T cells during reactivation than primary activation, which may suggest that although T cell responses rely on CD28 signaling during primary responses, CD137 may be more significant during secondary responses [Bertram et al., 2004] [Lin et al., 2010].

However, in certain conditions, α-CD137 antibodies can induce T cell death. C57BL/6 infected with LCMV Armstrong and subsequently injected with α-CD137 24h later underwent a reduction in T cells, which was dependent on IL10 and TNF-α mediated Fas upregulation and apoptosis, and required the presence of DCs [Zhang et al., 2007][Zhang et al., 2010]. Timing of antibody administration seemed important as mice treated with antibodies long after infection seemed to have positive T cell responses [Zhang et al., 2007]. Thus, it was speculated that α-CD137 stimulated innate immune cells which primed the inflammatory milieu for T cell depletion. Therefore, when the antibody treatment is

administered after T cells have already been activated, less innate immune cells and more CD137 expressing T cells would consume the majority of the antibodies, leading to survival and proliferating signals [Wang et al., 2009]. However, the reason for the different outcomes of CD137 stimulation is not entirely clear, and the reason why the timing of administration produces such different outcomes remains to be determined.

NK cells can also receive benefits through the stimulation of this receptor. CD137 is expressed on NK cells upon IL2 or IL15 stimulation, and stimulation through this receptor further increases responsiveness through the high-affinity IL2R [Wilcox et al., 2002]. However, the full range of consequences of CD137 stimulation on this cell type have not been as thoroughly studied as it has been in T cells, and conflicting data are present in the literature. In vitro studies have demonstrated that CD137 stimulation induces robust proliferation and IFN-γ secretion but not cytotoxicity in NK cells [Wilcox et al., 2002]. Utilizing accessory K562 cells expressing membrane bound IL15 and CD137L, ex vivo expansion of NK cells can be achieved to up to 277 fold in 21 days, and up to 550 fold in 24 days with the further addition of MICA, demonstrating the incredible proliferative signals that are conferred by CD137 signaling [Fujisaki et al., 2009][Gong et al., 2010]. However, other reports show a lack of significant NK response in either cytotoxicity or IFN-γ production [Navabi et al., 2015]. In addition, CD137 stimulation promotes CD8+ T cell expansion through NK cells, suggesting that it also plays a role in regulating and priming the adaptive immune response through NK cells [Wilcox et al., 2002].

Despite its activating effects on NK cells, CD137 stimulation has also been demonstrated to negatively alter homeostasis of the NK population in vivo. Regular weekly injections of α-CD137 decreased NK cell proportions in the spleen but numbers in the liver

and spleen were increased, suggesting that it caused a migration of NK cells from the spleen to other organs [Niu et al., 2007]. The authors suggested that NK trafficking is linked to the expression of CD137 on NK cells. A later study showed that NK cell populations were also diminished in bone marrow chimeras that were treated with α-CD137 independent of the presence of T cells or IFN-γ production [Lee et al., 2009]. This reduction was apparent in the spleen, liver, and bone marrow, which was slightly in contrast to previous results where only splenic NK cell proportions were decreased [Niu et al., 2007]. In addition, further ex vivo stimulation with PMA and ionomycin resulted in increased IFN-γ production and increased annexin-V staining, suggesting that α-CD137 activated cells, but may also cause death [Lee et al., 2009]. The authors suggested it was possible that α-CD137 activates NK cells but may also cause activation induced cell death. However, the increase of NK cells in the liver of wild type mice but decrease in bone marrow chimeras after α-CD137 treatment, combined with results showing that residual NK cells in α-CD137 treated mice express a relatively low amount of CD11b, it is possible that NK cell development is the contributor to the decrease [Choi et al., 2010].

A separate group suggested that NK cell development was regulated by T cells through IFN-γ. In contrast to data from Lee et al that indicate NK cell depletion occurs even in the absence of IFN-γ [Lee et al., 2010], Choi et al demonstrated that NK cells were partially rescued in IFN-γR KO mice, which do not have the receptor for IFN-γ in the spleen [Choi et al., 2010]. Furthermore, there was a complete rescue of diminished NK cells in the bone marrow, suggesting an important role of CD137 in regulating NK cell development [Choi et al., 2010]. These bone marrow NK cells did not express CD137 suggesting that the decline in NK populations is not reliant on NK cells. CD137+/+ T cells, but not CD137-/- T cells,

were able to diminish bone marrow NK cells when transferred into CD137-/- RAG-/- mice [Choi et al., 2010]. Thus, the authors suggested that NK cell populations were controlled by IFN-γ through CD137 expressing T cells. However, this only demonstrates that T cells can diminish NK cell populations, but it is possible that other cell types may contribute to the decreased NK populations through other mechanisms. The hypothesis that CD137 may play a role in regulating NK cell development is further backed by studies in CD137 deficient mice. These mice had decreased proportions of NK and Natural killer T (NKT) cells and decreased IFN-γ production and cytotoxicity [Vinay et al., 2004]. The decrease in cytotoxicity against YAC-1 cells in vitro and RMA-s in vivo was proposed to be the secondary effect of a decreased NK cell proportion among splenocytes, but not due to the actual decrease in the function of individual NK cells [Vinay et al., 2004]. The data together indicates a strong role of CD137 in the activation and function of NK cells, and possibly in the homeostasis of NK cells. However, the exact effects, mechanisms, and the degree of CD137 stimulation on NK cell death or development are unclear.

1.2.4 Agonistic Antibodies as Tumor Therapies

Agonistic α-CD137 antibody therapy for treating tumors was demonstrated initially against established tumors in mice. When antibodies were administered in mice against P815 mastocytoma and the poorly immunogenic Ag104A sarcoma, the large established tumors were eradicated along with the induction of CD4+ and CD8+ T cells [Melero et al., 1997]. Further studies showed that immunization with tumor antigens and treatment with α-CD137 antibodies managed to break “immunological ignorance” of other poorly immunogenic tumors including C3, TC-1, and B16-F10 [Wilcox et al., 2002]. This provided important

evidence for the role of CD137 signaling in tumor resistance. Further evidence was seen when CD137L expression on tumors increased T cell cytotoxic responses which was further amplified with the addition of CD28-B7 signaling [Guinn et al., 1999].

Although α-CD137 treatment started due to its promising effects on T cell activity, NK cells are also modulated greatly in tumor immunity. α-CD137 treatment in mice inoculated with P815 cells also showed that NK cells were an important mediator of tumor resistance through a regulatory function [Melero et al., 1998]. In a B16-F10 melanoma model, tumor resistance was found to be dependent on cytotoxic T cell action that was primed through NK cells, and the absence of either cell type decreased tumor resistance [Xu et al., 2004]. In vivo α-CD137 administration along with tumor antigens resulted in a polarization into a type I response through a decrease in IFN-γ and increase in IL4 production in response to a tumor antigen, suggesting an important role of CD137 and NK cells in directing proper immune responses [Ito et al, 2004]. Evidently, NK cells are able to direct and promote proper immune responses against T cells through immunoregulation.

NK cells are also able to act directly against tumors as well, mainly through the enhancement of antibody dependent cell cytotoxicity (ADCC). NK cells that have come into contact with trastuzumab, a monoclonal antibody treatment against breast cancer, was able to clear tumors more effectively upon treatment with α-CD137 [Kohrt et al., 2012]. Patients that have received trastuzumab had increased levels of CD137 expression on their NK cells, suggesting potential for α-CD137 administration [Kohrt et al., 2012]. The effectiveness of Rituximab (CD20), which is used to treat B cell lymphomas, was increased by α-CD137 treatment through the enhancement of NK cell activity [Kohrt et al., 2012]. Rituximab induced CD137 expression on NK cells through Fc-FcR binding, and increased

degranulation and cytotoxicity in both murine and human xenotransplant models [Kohrt et al., 2011]. Cetuximab, a treatment for head and neck cancer and colorectal cancer, also benefits from α-CD137 treatment. Following α-CD137 administration, several murine tumor xenograft models showed improved clearance and survival [Kohrt et al., 2014]. This was mainly mediated through increased ADCC by NK cells, but an increase in tumor specific T cells was also observed.

Currently, several clones of monoclonal antibodies against CD137 have promise and are in clinical trials. Urelumab, by Bristol-Myers Squibb, passed its phase I trial but phase II trials revealed hepatic toxicity at high doses. Triweekly administration of more than 1 mg/kg resulted in adverse effects that were dose dependent [Segal et al., 2017]. These effects included transaminitis, fatigue, and nausea being the most common. However, a dose of 0.1 mg/kg triweekly was determined to be safe and continue to be seen as a possible monotherapy or as a combination therapy. Promising results from Pfizer was obtained with the monoclonal antibody PF-05082566, where no correlation between dose and frequency or severity of adverse effects were observed, and all trials were terminated only due to disease progression [Segal et al., 2014]. Treatments were administered once every four weeks and were even seen to have disease stabilizing effects in patients.

Combined therapies may be an effective way to synergistically increase the effectiveness of individual treatments. Treatment of α-CD137 antibodies increased the effectiveness of rituximab therapy (α-CD20) against B cell lymphomas by directly increasing the cytotoxicity of NK cells through ADCC [Kohrt et al., 2011]. α-CD137 treatment also increased the effectiveness of cetuximab treatment against murine xenograft models including head and neck cancer [weng et al., 2014], and trastuzumab against

xenograft models of human breast cancers in the same manner [kohrt et al., 2012]. An appealing combination comes from combining α-CD137 treatment with "checkpoint inhibitors". The tumor microenvironment can act to suppress the immune system. One consequence is the upregulation of immune "checkpoint" receptors such as PD-1 and CTLA-4. By combining blocking antibodies against these checkpoint molecules and agonistic antibodies against α-CD137, the reversal of immune cell suppression by checkpoint inhibitors can be combined with immune stimulation by CD137 in order to effectively control tumors. Blocking CTLA-4 and activating CD137 in this manner has proven to be effective in some murine tumors [Curran et al., 2011]. Treatments have also been reported to alleviate each other's adverse effects; α-CD137 alleviates the autoimmune-like syndromes of α-CTLA-4 treatment, and α-CTLA-4 decreases the influx of cell infiltration into the liver, reducing liver pathology [Kocak et al., 2006]. Similarly, targeting both the blocking of PD-1 and stimulation of CD137 resulted in promising results in CT26 and B16-F10 grafts [Shindo et al., 2015][Chen et al., 2015], but interestingly at high doses of greater than 1 mg/kg, α-PD-1 treatment seemed to exacerbate the toxicity of α-CD137 [Chen et al., 2015]. Human clinical trials are currently recruiting and underway for urelumab (α-CD137) and nivolumab (α-PD-1) combination therapy (NCT02253992), for solid tumors and B cell non hodgkins lymphoma, and urelumab (α-CD137) and pembrolizumab (α-PD-1) combination therapy (NCT02179918) for solid tumors [Bartkowiak et al., 2015].

Currently, the knowledge regarding the role that CD137 plays in NK cell function and homeostasis is limited. As more CD137 related therapies against tumors are being tested, it is critical to understand the full range of effects it may have on the immune system. Most of the research regarding CD137 is related to its effects on T cells, but it is becoming more

apparent that other cell types, such as NK cells, may also be affected in significant ways. Further understanding of the effects and mechanisms of CD137 stimulation in NK cells will not only allow the minimization of detrimental side effects and maximization of treatment effectiveness to the benefit of the host, it will also provide a greater understanding of the survival and death pathways through TNFRs in NK cells.

1.3 Cytomegalovirus

Cytomegalovirus (CMV) is part of the β-herpesvirinae superfamily of the herpesviridae family. They are enveloped with a lipid bilayer and have an icosahedral capsid [Chen et al., 1999][Fields et al., 2007]. Inside the capsid, the human cytomegalovirus (HCMV) contains double stranded DNA of 235 kb that codes for over 200 genes, making it one of the largest among herpesviruses. HMCV can be transmitted through body fluids such as saliva, sexual contact, placental transfers, breastfeeding, and blood and organs transfers [Slobedman et al., 1999]. As with most herpesviruses, their life cycle consists of a productive, latent, and reactivation phase [Jenkins et al., 2004]. In the productive phase, the virus undergoes replication and production of new infectious viral particles [Fields et al., 2007]. Affected cells involved in dissemination of the virus are thought to be monocytes, macrophages, lymphocytes, dendritic cells, and endothelial cells [Taylor-Wiedemanet et al., 1991][Soderberg et al., 1993][Schrier et al., 1985][Sénéchal et al., 2004][Grefte et al., 1993]. In an immunocompetent host, the infection is rarely serious, and seroprevalence in the developed world ranges from 30% – 90% [Staras et al., 2006]. Although primary infections are mostly asymptomatic, a small proportion of infections may show symptoms of

mononucleosis: fever, myalgia, lymphadenopathy, and hepatomegaly [Gandhi et al., 2004][Sissons et al., 2002].

After the acute phase of the infection, CMV is able to establish latency in which the virus remains dormant with a lack of new viral particle production. This latency is established in myeloid lineage cells such monocytes, and undergo limited viral gene expression [Stanier et al., 1989] [Taylor-Wiedeman et al., 1991]. This latency is thought to be due to the suppression of the major immediate-early promoter (MIEP) of CMV by cellular proteins. The Ets-2 repressor factor (ERF) binds to the MIEP and recruits histone deacetylases to silence immediate early genes [Wright et al., 2005]. However, chromatin remodeling can cause the acetylation of histones, releasing repression of MIEP, in cases such as the differentiation of monocytes into dendritic cells [Reeves et al., 2005]. One of the key factors affecting reactivation is thought to be TNF-α, as TNF-α induced activation of protein kinase C (PKC) and NF-κB results in the transcription of the immediate early genes of CMV [Stein et al., 1993][Prosch et al., 1995].

The most serious cases however, are seen in immunocompromised individuals or newborns, or during inflammation, infections by other pathogens, and stress [Prosch et al., 2000][Kutza et al., 1998]. Congenital CMV infections can lead to morbidity, mortality, deafness, and neurodevelopmental abnormalities [Fowler et al 2006][Ross et al., 2005]. This is a serious concern as congenital infections from seropositive mothers during pregnancy is approximately 0.64% and seronegative mothers 1-4% [Kenneson et al., 2007], among which 10 – 15% of infections are symptomatic with a mortality rate of 30% [Stagno et al., 1986]. HCMV infections during human immunodeficiency virus (HIV) also poses a serious concern. HCMV takes advantage of the immunodeficiency posed by HIV and previously affected

almost half of all HIV infected patients whom had a median survival of 4-9 months, making it one of the most important opportunistic pathogens [Gerard et al., 1997]. The development of highly active anti-retroviral therapy (HAART), a combination of at least three different classes of drugs, improved HIV outcome in addition to HCMV related disease [S'Sullivan et al., 1999]. However, even with improved treatments, HCMV remains an important factor that accelerates and determines mortality among HIV patients [Griffiths, 2006] [Sabin et al., 2000] [Webster et al., 1989].

Patients who have received organ transplants are also at risk of reactivation or primary infection by HCMV, with up to 50% patients exhibiting symptoms of infection [Patel et al., 1997][Fishman et al., 1998]. Risk factors include seropositivity of donors and recipients, immunosuppression regimen, age, and genetic factors [Hoffman et al., 2010][Humar et al., 2009][Azebedo et al., 2015]. Mild symptoms of the infection, termed "CMV syndrome", include fever, anorexia, malaise, with the rare case of mononucleosis, lymphadenopathy, and splenomegaly [Sia et al., 2000]. More serious effects of HCMV infection are due to opportunistic superinfections and allograft rejection. HCMV is thought to pave the way for further infections, and superinfections with fungi and bacteria have been seen in the respiratory system and liver of transplant patients [Paya et al., 1993][Rubin et al., 1990]. Seropositive graft survival of kidneys is known to be decreased in HCMV seronegative recipients with a decline of approximately 25% [Schnitzler et al., 1997][Sawyer et al., 1993]. In heart transplants, complications by ventricular dysfunction and atherosclerosis are thought to be due to chronic rejection of the organs [McNamara et al., 1996][Grattan et al., 1989]. HCMV ultimately results in lower patient outcome after transplants. Patients who have undergone liver transplants were observed to have higher mortality rates with up to double

the risk of death and graft loss at years 1, 3, and 5 [de Otero et al., 1998][Rosen et al., 1998], whereas patients who have undergone heart transplants had a survival rate of 32% by year 5 [Grattan et al., 1989].The most commonly studied animal model of CMV is the murine cytomegalovirus (MCMV) as it shares many commonalities with HCMV. What is important to note is the relevance of MCMV to NK cell study. Depletion of NK cells with depleting α-NK1.1 antibodies in MCMV resistant C57BL/6 mice caused infections to be lethal [Scalzo et al., 1992]. It was discovered that the *Cmv1* locus was a crucial factor in determining susceptibility to MCMV infection [Scalzo et al., 1990][Scalzo et al., 1992]. Further study of *Cmv1* revealed that resistance against MCMV was conferred through NK cells, largely due to the c-type lectin-like receptor Ly49H, which can recognize the viral glycoprotein m157 [Lee et al., 2001][Brown et al., 2001][Daniels et al., 2001][Arase et al., 2002].

The role of NK cells is critical in controlling acute MCMV infections, and susceptible mice with insufficient NK function exhibit lethality. Ly49H is a crucial NK receptor against MCMV and is a large factor in determining NK cell expansion during MCMV [Lee et al., 2009]. Without this receptor, NK cells are unable to proliferate in order to control infections. This is due to the lack of Ly49H recognition of the m157 glycoprotein of MCMV, hence the absence of sufficient activating signals, preventing the proliferation of NK cells and control of viral replication. Therefore, the MCMV infection model provides an effective way to study NK cell function in vivo. MCMV models have been a popular choice to study various aspects of NK function including cytotoxicity and proliferation [Dokun et al., 2001], IFN-γ production [Sumaria et al., 2009], and even immunoregulatory functions to control the adaptive response in a viral setting [Lee et al., 2009]. The role and function of NK cells in this model are well studied and can provide many insights into the effects of different drugs

and treatments, and what effects it will have on NK cell function and overall host outcome. Examining the effects of agonistic α-CD137 monoclonal antibodies on NK cells during MCMV infection will provide a deeper understanding of its effects on NK cell function and the side effects it may incur when used in clinical settings as an anti-tumor therapy. This may be important in preventing undesired and possibly serious adverse health effects in patients undergoing therapy.

2. HYPOTHESIS

Agonistic α-CD137 antibody treatment can modulate NK cells to alter their effectiveness during MCMV infection.

STATEMENT OF OBJECTIVES

To test my hypothesis, I have set four objectives to assess NK cell function after α-CD137 stimulation of NK cells.

I. Investigate the in vitro effects of α-CD137 stimulation on NK cells

II. Determine the mechanism of α-CD137 stimulation on NK cells

III. Investigate the effects of in vivo α-CD137 treatment in mice

IV. Investigate the effects of in vivo α-CD137 treatment during MCMV infection in mice

3. MATERIALS AND METHODS

3.1 Mice

C57BL/6 mice were purchased from Charles River and bred in a specific pathogen free (SPF) housing facility. C57BL/6-Prf1tm1Sdz/J (Prf1 KO) and B6.MRL-Faslpr/J (B6 lpr) mice were purchased from the Jackson Laboratory and bred in the University of Ottawa SPF facility. Prf1 KO mice are homozygous for a targeted mutation that disrupts exon 3 of the perforin gene, rendering all mRNA non-functional. B6 lpr mice have a lymphoproliferative disorder that is caused by spontaneous mutation at exon 2, which gives a premature termination sequence, rendering the protein unable to bind its ligand. All procedures followed the guidelines of and were approved by the University of Ottawa Animal Ethics Committee and the Canadian Council on Animal Care. Mice utilized were between 7-12 weeks of age. B6.129-Tnfrsf1<atm1Mak>/J (TNFR2-deficient), B6.129S2-Tnfrsf1<btm1Mwm>/J (TNFR1-deficient), and B6.129S-Tnfrsf1<atm1Imx>Tnfrsf1b<tm1Imx>/J (TNFR1/TNFR2 KO) mice were purchased from Jackson Laboratory. These TNFR mutant mice have targeted mutations in the TNFR genes that render the proteins unable to bind TNF.

3.2 Preparation of Agonistic anti-CD137 antibodies

Monoclonal agonistic α-CD137 antibodies were purchased from Bio X Cell (clone: 3H3, rat IgG2a) and hybridoma (3H3, rat IgG2a) were also received from Dr. Tania Watts from the University of Toronto and Dr. Robert Mittler from Emory University. Hybridoma were cultured in 10 ml of DMEM media (Gibco) containing 10 FBS until cells were sufficiently proliferating. Cells were then transferred to large culture flasks in 150 ml per

flask and left until media turned yellow and cell death was observed. Media was then centrifuged for 1 hour at 10,000 rpm at 4ºC. Supernatant was filtered through a vacuum filter (Thermo Scientific) and stored at -20 ºC for a maximum of one month or proceeded onto antibody purification.

Supernatant containing antibodies were mixed in a 1:1 ratio with borate buffer (pH 8.0) and run through protein G columns (Exalpha Biological) multiple times to capture antibodies through the Fc portion. The column was then washed with borate buffer and eluted with 25 ml of citric acid buffer (pH 2.5) in order to retrieve bound antibodies. The elution product was mixed with 5 ml of Tris-HCl (pH 9) and either HCl or NaOH was added to bring the solution to approximately 7.0 pH. The antibody concentration was measured with a Nanodrop spectrophotometer (Thermo Scientific) at an absorbance of 280 nm. This process was repeated until a significant decrease in antibody retrieval was observed. The final products were combined and brought to a final concentration of 1 mg/ml and stored at 4ºC. F(ab)2' digestion was performed with pepsin (Thermo Scientific) for 10 hours in a 37ºC shaking water bath. Digested product was washed and run through a protein G column. Flow-through was collected and concentration was measured with a Nanodrop spectrophotometer.

3.3 MCMV

The Smith strain of MCMV (ATCC® VR-1399™) was purchased from the American Type Culture Collection (ATCC). BALB/c mice were infected with 5E3 plaque forming units (pfu) of MCMV and salivary glands were harvested 21 days p.i. in Media 199 (Gibco). The glands were washed, homogenized at 1500 rpm for 10 seconds with a Polytron homogenizer (PT 1600E), and centrifuged at 2500 rpm for 15 minutes at 4ºC. The

supernatant was aliquoted into 1.5 ml volumes, and stored in liquid nitrogen as a master stock. Working stocks were prepared by aliquoting 30 μl of the master stock and storing at -80ºC. One vial from the working stock was serially diluted by a factor of 10 in DMEM containing 2% FBS and plaque assays were carried out to determine the pfu.

3.4 MCMV Infection with α-CD137 stimulation

4 hours prior to MCMV infection, mice were treated with 200 μg of α-CD137 antibodies diluted in PBS intraperitoneally. Mice were then infected with 20,000 pfu of MCMV diluted in MCMV injection buffer (Media 199 with 5% FBS) intraperitoneally. On day 0, 1.5, and 4 of infection, organs were harvested and weighed. Leukocytes were isolated for further analysis.

3.5 Poly(I:C)

Polyinosinic-polycytidylic acid sodium salt was purchased from SIGMA-ALDRICH. Poly(I:C) and was stored at -20ºC and thawed at room temperature (RT) for use. For poly(I:C) injections, 200 μg of α-CD137 antibodies were injected per mouse intraperitoneally 4 hours prior to an intraperitoneal injection with 200 μg of poly(I:C) diluted in 200 μl of PBS. Organs were taken from mice on day 0 or 2, weighed, and processed for further analysis.

3.6 Cell Isolation

3.6.1 Splenocyte Isolation

Spleens were taken from B6 mice, ground, and filtered through 75 μm nylon cell strainers (Fisherbrand) in 5 ml of HyClone™ RPMI 1640 media (GE Healthcare

Lifesciences). Splenocytes were then treated with ACK red blood lysis buffer for 30 seconds and washed in media. Cells were washed by centrifugation for 10 minutes at 1200 rpm and filtered again to obtain a single cell suspension in media for further analysis.

3.6.2 Liver Lymphocyte Isolation

Livers were taken from mice, ground, and filtered through 75 um filters in RPMI media. After 3 washes with media for 10 minutes each at 1800 rpm, cells were resuspended in 5 ml of 40 % percoll solution (GE Healthcare) diluted in PBS and carefully layered by pipette onto a 2 ml 70% percoll solution diluted in PBS. Gradient centrifugation was performed for 25 minutes at 2500 rpm with no breaks at RT. The top layer of plasma was removed by vacuum pipetting and the middle layer containing leukocytes was pipetted into fresh media. Cells were then washed, counted, and resuspended in media for further analysis.

3.6.3 PBMC Isolation

Blood was harvested with sodium heparin to prevent coagulation. Blood was then treated with ACK red blood cell lysis buffer until the solution began to clear in color. RPMI was added immediately and washed. This was repeated until sufficient red blood cell lysis has occurred and cells were then resuspended in media for further analysis.

3.7 MCMV Plaque Assay

Mouse embryonic fibroblasts were plated onto 150 mm plates for 3 days until confluence and split into two plates using Trypsin. 3 days later, they were plated onto 24 well plates until confluence. Spleens and livers from MCMV-infected mice were homogenized with the MagNA Lyser homogenizer (Roche) at 7000 rpm for 10-15 seconds

in 2% DMEM containing 2% FBS, and pipetted onto 24 well plates containing confluent MEF cells for 1 hour with intermittent shaking every 20 minutes, followed by washing with 2% DMEM and a 3 day incubation. Cells were then fixed with 10% formalin and stained with crystal violet for 10 minutes each. Plates were gently washed with water and let dry. Plaques were counted under the microscope and calculated for pfu/g of organ.

3.8 Cell Staining and Flow Cytometry

α-NK1.1 (PK136), α-CD3 (17A2 and 145-2C11), α-CD27 (LG.7F9), α-CD11b (M1/70), α-Ly49H (3D10), α-TCR-β (H57-597), α-IFN-γ (XMG1.2), α-CD25 (PC61.5), α-CD49b (DX5), from eBioscience, α-BrdU (3D4) from BD Biosciences, mouse IgG1 kappa (MOPC-21), rat IgG2b kappa (RTK4530) from BioLegend, Fixable Far Red Live/Dead from Invitrogen. Cells were washed in PBS (2% FBS), incubated with surface antibody cocktail for 25 minutes, and fixed with 2% PFA. For intracellular granzyme and IFN-γ staining, 96 well plates were coated with a-NK1.1, α-Ly49H, or isotype control overnight for 16 hours at 4ºC. Cells were cultured in coated wells or with free IL2 + IL12 for 1 hour in RP-10 media (RPMI-1640, 10% FBS, 1X penicillin/streptomycin, 1% L-Glutamine, 10 mM HEPES, 50 μM β-mercaptoethanol). Brefeldin A was at a concentration of 5 μg/ml and the cells were stained after 4 hours. Cells were stained and analyzed by flow cytometry. Cells were acquired using LSR Fortessa (BD Biosciences) and FACS Cyan ADP (Beckman Coulter), and analyzed using Kaluza software v2 (Beckman Coulter).

3.9 RNA Isolation

1 ml of TRIzol (Life Technologies) was added to splenocytes. 200 μl of chloroform was added after 5 minutes and samples were shaken. After 2 minutes, samples were

centrifuged at 12000 rpm for 15 minutes at 4ºC. The aqueous phase containing RNA was transferred to 500 µl of isopropyl alcohol and incubated at RT for 10 minutes. Samples were centrifuged at 12000 rpm for 10 minutes and supernatant was discarded. RNA was washed with 1 ml of 70% ethanol. RNA was left at RT to partially dry and resuspended in RNase free water.

3.10 cDNA Synthesis

iScript gDNA Clear cDNA Synthesis Kit (BIO-RAD) was used to synthesize cDNA from RNA isolates. iScript DNase and iScript DNase buffer were added to RNA isolates suspended in RNase free water. Samples were run in a thermocycler to digest DNA for 5 minutes at 25ºC and inactivate DNase for 5 minutes at 75ºC. iScript Reverse Transcription Supermix was added to samples and incubated in the thermocycler; 5 minutes at 25ºC for priming, 20 minutes at 46ºC for reverse transcription, and 1 minute at 95ºC for inactivation of reverse transcription.

3.11 qPCR

cDNA was added to PCR strips along with FastStart Universal SYBR Green Master (Roche), forward and reverse primers, and distilled water. 50 cycles were run at the following conditions: 1 minutes at 95ºC, 20 seconds at 95ºC, 30 seconds at 57ºC, 20 seconds at 72ºC. All samples were normalized to β-actin mRNA levels.

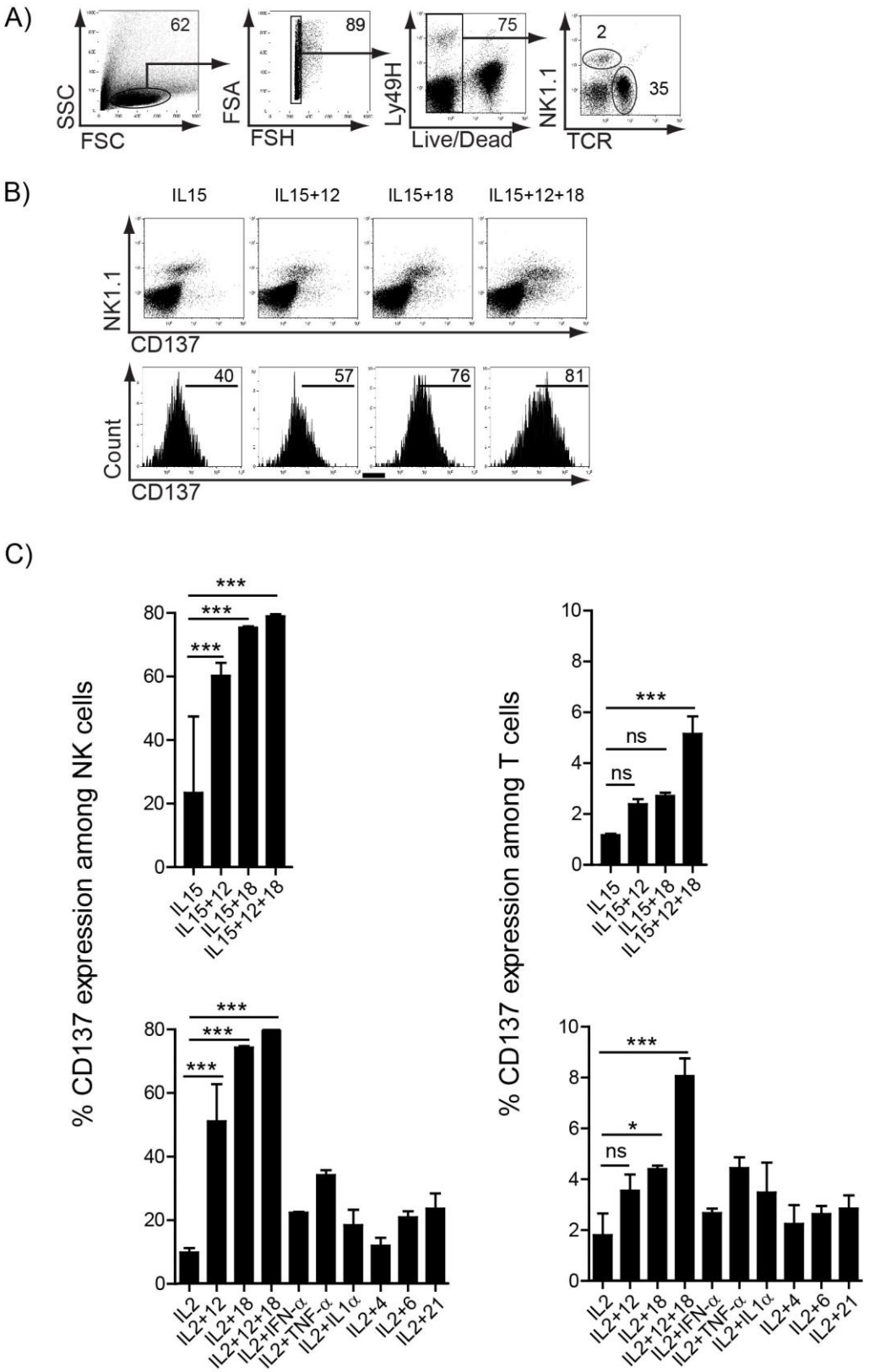
4. RESULTS

4.1 CD137 Expression on NK cells

4.1.1 CD137 expression on NK cells is induced upon cytokine activation with IL2, 15, 12, and 18

It is known that IL2 and IL15 induce CD137 expression on NK cells [Wilcox et al., 2002]. But whether other cytokines can also induce CD137 expression is not known. To investigate alternative means of CD137 expression in NK cells, different cytokines that play a role in NK activation, as well as those seen during MCMV infection, were examined: IL12, IL18, IFN-α, TNF-α, IL1α, IL4, IL6, and IL21.

Splenocytes were cultured with IL12, IL18, IFN-α, TNF-α, IL1α, IL4, IL6, and IL21 (10 ng/ml) in combination with IL2 or IL15 (10 ng/ml) for 24 hours, and CD137 expression on NK cells was measured by flow cytometry. IL2 and IL15 were added to ensure NK cell survival throughout the 24-hour period. Cells were gated on singlet cells, live cells, and then NK1.1+TCR- NK cells (Figure 1A). NK cells were then analyzed for CD137 expression (Figure 1B). IL12 or IL18, in combination with IL2 or IL15, induced strong expression of CD137 on NK cells (Figure 1B, C). Either IL2 and IL15 in combination with IL12+18 had an additive effect producing the highest expression of CD137 on NK cells (Figure 1C). The other cytokines, IFN-α, TNF-α, IL1, IL4, IL6, and IL21, did not have significant effects on CD137 expression on NK cells (Figure 1C). T cells increase their expression of CD137 slightly upon stimulation with IL15+12+18 or IL2+12+18, and very slightly with the remaining cytokines (Figure 1C). The increase in the expression, however, are minor (< 10% of population) and higher expression is likely to create a significant change in the function of the T cell population.


A)
62
SSC
FSC
89
FSA
FSH
75
Ly49H
Live/Dead
2
35
NK1.1
TCR
B)
IL15
IL15+12
IL15+18
IL15+12+18
NK1.1
CD137
40
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IL15
IL15+12
IL15+18
IL15+12+18
IL2
IL2+12
IL2+18
IL2+12+18
IL2+IFN-α
IL2+TNF-α
IL2+IL1α
IL2+4
IL2+6
IL2+21

Figure 2: CD137 Expression on NK cells is induced upon cytokine activation with IL2, 15, 12, and 18. A) Splenocytes were gated on singlet cells, live cells, and based on NK1.1 and TCR. B) Representative dot plots and histograms for the expression of CD137 on NK cells after stimulation with IL2, IL15, IL12, IL18, IFN-α, TNF-α, IL1α, IL4, IL6, and IL21. All cytokines were combined with either IL2 or IL15 to ensure NK cell survival. C) Percentage of CD137 expressing NK cells upon different cytokine stimulations. One-way ANOVA and Tukey's post hoc test was performed. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data is representative of two independent experiments.

4.1.2 CD137 expression on NK cells is dependent on cytokine concentration

A titration assay was done with IL2, IL15, IL12 and IL18 to assess NK cell dose dependency for CD137 expression. Splenocytes were cultured with increasing concentrations of IL2, 15, 12 or 18 alone for 24 hours. Cells were gated on singlet cells, live cells, and NK1.1+TCR- NK cells (Figure 2A). NK cells were then analyzed for CD137 expression by flow cytometry (Figure 2B). All four cytokines induced CD137 expression in a dose dependent manner, with IL2 and IL15 being the strongest inducers (Figure 2C). Although IL12 and IL18 were not as effective in inducing CD137 expression on NK cells, the combination of IL12 and IL18 was able to increase CD137 expression on NK cells to a greater level than either alone, suggesting a synergistic effect among the cytokines (Figure 2C). Thus, IL2 and IL15 induced CD137 expression was significantly increased upon the addition of IL12 and 18 (Figure 1C). The data suggests that during infection, NK cells will upregulate CD137 expression to a maximum level in response to a combination of cytokines that include IL2, IL15, IL12, and IL18.

4.2 NK cells have increased IFN-γ production on day 1 and day 2 post α-CD137 stimulation

To characterize NK cell function after stimulation with agonistic α-CD137 antibodies, 96 well plates were coated with PBS, isotype rat IgG, α-NK1.1, or α-Ly49H to stimulate NK cells among total splenocytes, and α-CD137 antibodies were added to the media (Figure 3A). Cells were then added with media and stimulated in antibody coated wells with IL15 (10 ng/ml), or in uncoated wells with IL2+12 (10 ng/ml). Flow data was analyzed by gating

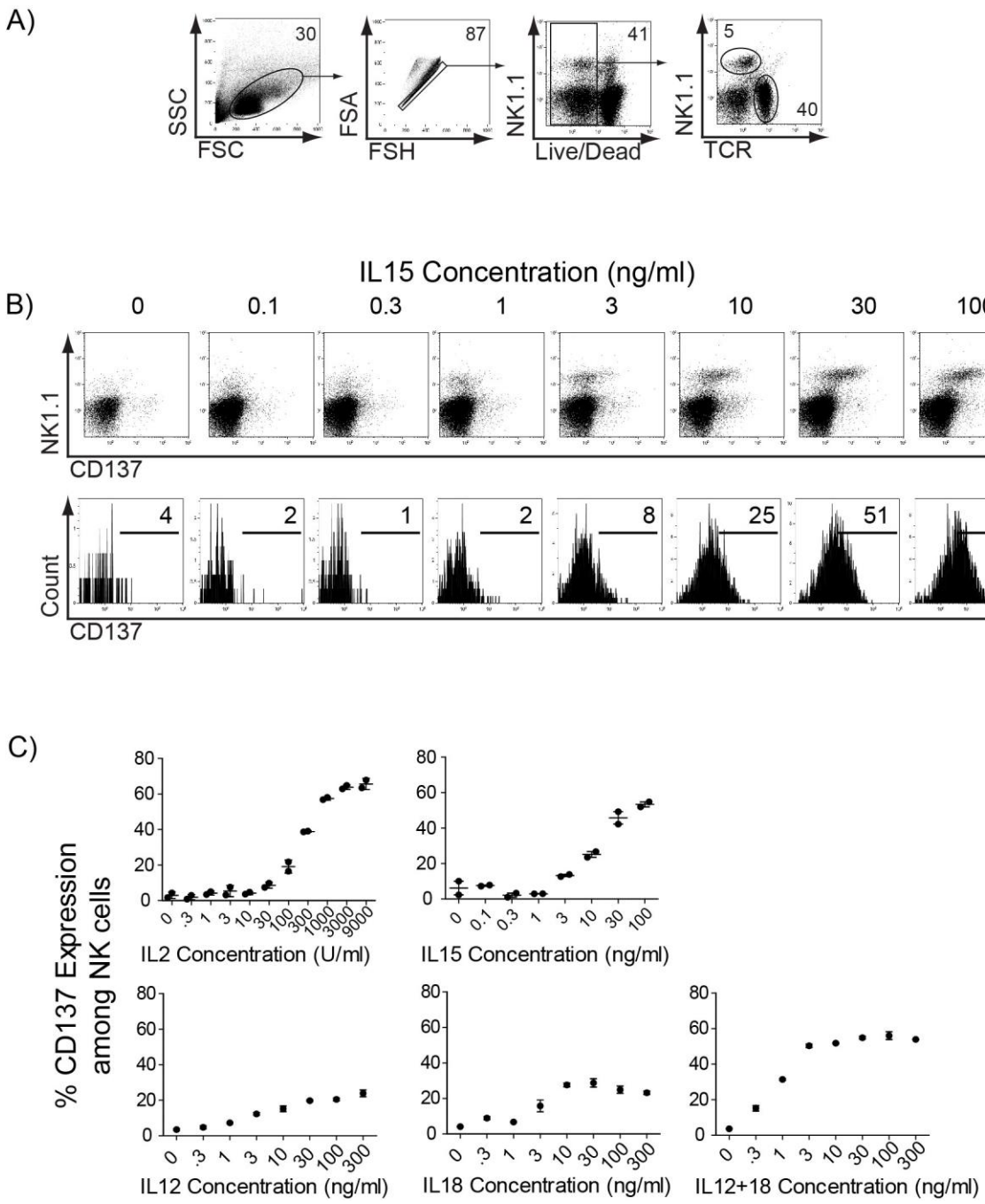
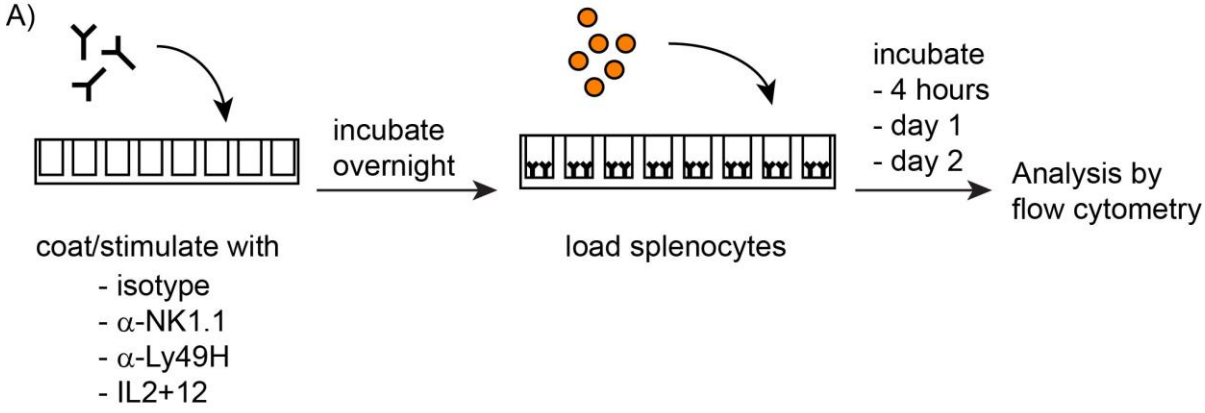
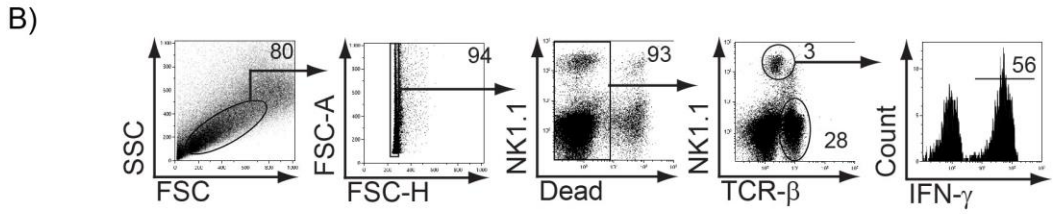

A)
30
SSC
FSC
87
FSA
FSH
41
NK1.1
Live/Dead
5
40
NK1.1
TCR
B)
IL15 Concentration (ng/ml)
0
0.1
0.3
1
3
10
30
100
NK1.1
CD137
4
2
1
2
8
25
51
51
Count
CD137
C)
% CD137 Expression among NK cells
IL2 Concentration (U/ml)
IL15 Concentration (ng/ml)
IL12 Concentration (ng/ml)
IL18 Concentration (ng/ml)
IL12+18 Concentration (ng/ml)

Figure 3: CD137 Expression on NK cells is dependent on cytokine concentration. Total splenocytes were cultured in 96 well plates with a combination of IL2, 15, 12, and 18 for 24 hours. Cells were then stained and analyzed by flow cytometry. A) Splenocytes were gated on singlet cells, live cells, and based on NK1.1+TCR- NK cells. B) Representative dot plots and histograms of CD137 expression on NK cells after 1 day of stimulation with IL15. C) Percentage of CD137 expressing NK cells after stimulation with indicated cytokines for 1 day. Unlike Figure 1, cells were cultured with only one cytokine; IL12, 18, and 12+18 were not combined with IL2 or 15. Data is representative of two independent experiments.

on singlets, live cells, and then NK1.1+TCR- NK cells after 4 hours, 1 day, or 2 days of stimulation (Figure 3A). These cells were then analyzed for IFN-γ positive populations (Figure 3B). The results showed that short term α-CD137 stimulation of 4 hours had no effect on IFN-γ of NK cells (Figure 3C). Cells stimulated with isotype and α-CD137 antibodies had nearly no signs of cytokine production, whereas cells stimulated with α-NK1.1, α-Ly49H, or IL2+IL12 exhibited an increase in IFN-γ production, but there was no change upon stimulation with α-CD137 (Figure 3C left). Cells treated with α-Ly49H and IL2+12 had elevated levels of IFN-γ in the isotype rat IgG and α-CD137 treated groups compared to the untreated group (Figure 3C left). Although there was an apparent increase, there was no difference between the isotype rat IgG treated group and the α-CD137 treated group (Figure 3C left), suggesting that α-CD137 stimulation had no effect on NK function. The apparent increase compared to the untreated groups can be explained due to the Fc portion of the antibodies that are present in both rat IgG and α-CD137, which may bind and activate Fc receptors on NK cells increasing IFN-γ production. Despite the lack of NK cell response to α-CD137 stimulation at 4 hours, other groups have shown that α-CD137 treatment increases the proliferation and IFN-γ production of NK cells by day 3 [Wilcox et al., 2002]. Since CD137 expression is known to increase upon activation with IL2 or IL15, it may be that the expression of CD137 was insufficient at 4-hours for α-CD137 stimulation to have significant effects on NK cell function.

When NK cells were stimulated for 1 or 2 days, there was a significant increase in IFN-γ production in the α-CD137 treated group (Figure 3C middle and right). The increased production of IFN-γ in cells treated with rat IgG+IL2+12 compared to IL2+12 alone is a


A)
incubate
overnight
incubate
- 4 hours
- day 1
- day 2
Analysis by
flow cytometry
coat/stimulate with
- isotype
- α-NK1.1
- α-Ly49H
- IL2+12
load splenocytes


B)
SSC
FSC
80
FSC-A
FSC-H
94
NK1.1
Dead
93
NK1.1
TCR-β
3
28
Count
IFN-γ
56

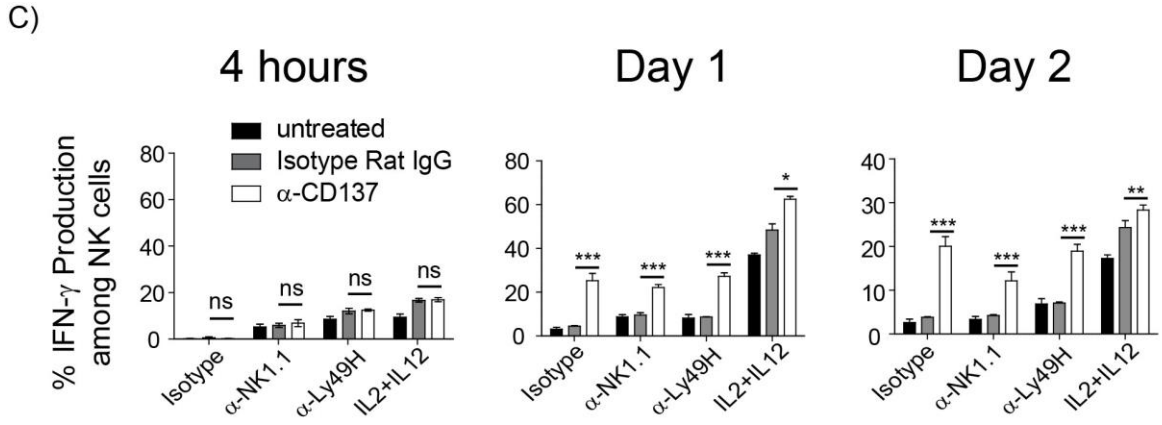

C)
4 hours
Day 1
Day 2
untreated
Isotype Rat IgG
α-CD137
% IFN-γ Production
among NK cells
ns
***
*
**
Isotype
α-NK1.1
α-Ly49H
IL2+IL12

Figure 4: NK cells have increased IFN-γ production on day 1 and day 2 post α-CD137 stimulation. A) Splenocytes were cultured in 96 well plates and stimulated with isotype rat IgG, α-NK1.1, α-Ly49H antibodies (plates coated at a concentration of 10 μg/ml) or IL2+12 (10 ng/ml each) for 4 hours, 1 day, or 2 days, in addition to no treatment, isotype Rat IgG or α-CD137 antibody stimulation in solution (10 μg/ml). B) Splenocytes were gated on singlet cells, live cells, and NK1.1+TCR- NK cell. Cells were then gated for IFN-γ expression. C) Percentage of IFN-γ expression among NK cells. One-way ANOVA and Tukey's post hoc test was performed. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data is representative of two independent experiments.

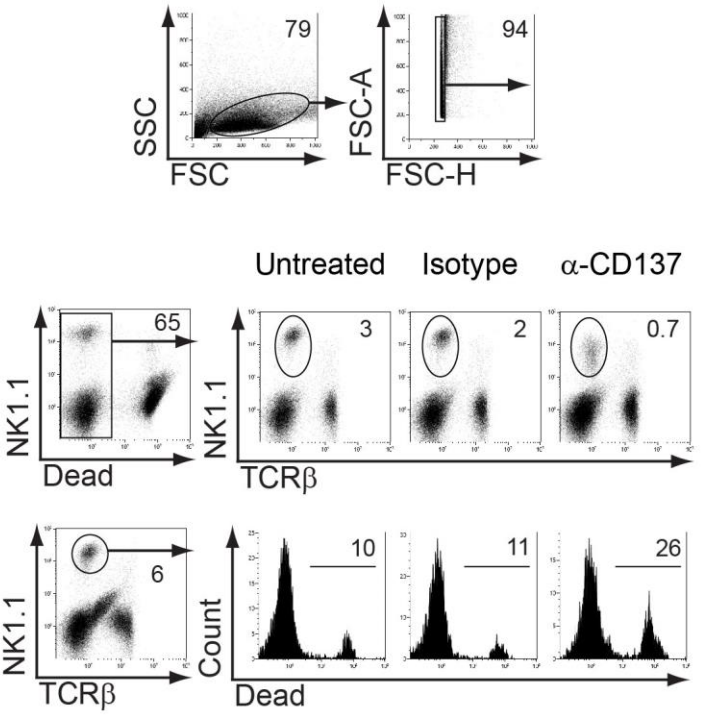
result of the activation of NK cells by IL2 and 12. The two cytokines together have potent IFN-γ inducing effects through the action of STAT1 and 4 [Miyagi et al., 2007][Wang et al., 2000]. This explains why the treatment with IL2+12 in conjunction with α-CD137 was the highest producer of IFN-γ overall. α-CD137 alone or combined with α-NK1.1, and α-Ly49H, although not as high, increased IFN-γ production by a factor of over 2 compared to both untreated and isotype rat IgG control groups (Figure 3C middle and right). Thus, it appears that NK cells require an activation duration that is longer than 4 hours in order to upregulate the expression of CD137 for α-CD137 stimulation to have significant effects on its function.

4.3 α-CD137 mediated NK cell death

4.3.1 α-CD137 stimulation induces NK cell death on day 2 in vitro

I have shown that short term stimulation with α-CD137 yields no effects on NK cells, and that modulation of NK cell function by α-CD137 occurs only by day 1 and 2. Despite an increase in apparent function through increased IFN-γ production, NK cells also seemed to undergo cell death. Cells were cultured with 10 ng/ml of IL15 in 96 well plates with 10 μg/ml of α-CD137 antibodies for 2 days and were then analyzed by flow cytometry. Cells were gated on singlets, and percentage of live NK cells among total splenocytes and proportion of death among NK1.1+TCR- cells were measured (Figure 4A). Despite the increase in IFN-γ production and proliferation reported by Wilcox et al after long term CD137 stimulation, NK proportions were contradictorily decreased and death in NK cell proportions were increased by day 2 (Figure 2A, B). NK percentages were decreased by half while NK cell death was increased by a factor of 2 (Figure 4A, B).

A)


79
SSC
FSC
94
FSC-A
FSC-H
Untreated
Isotype
α-CD137
65
NK1.1
Dead
3
2
0.7
NK1.1
TCRβ
6
NK1.1
TCRβ
10
11
26
Count
Dead

B)

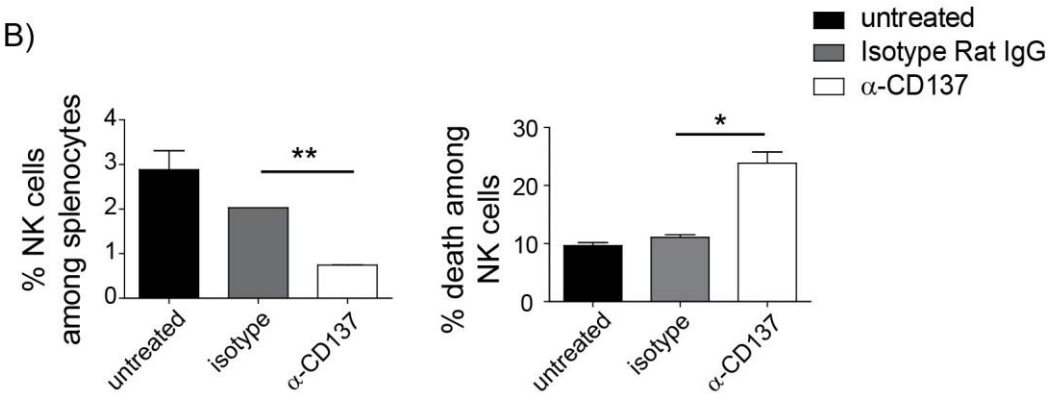
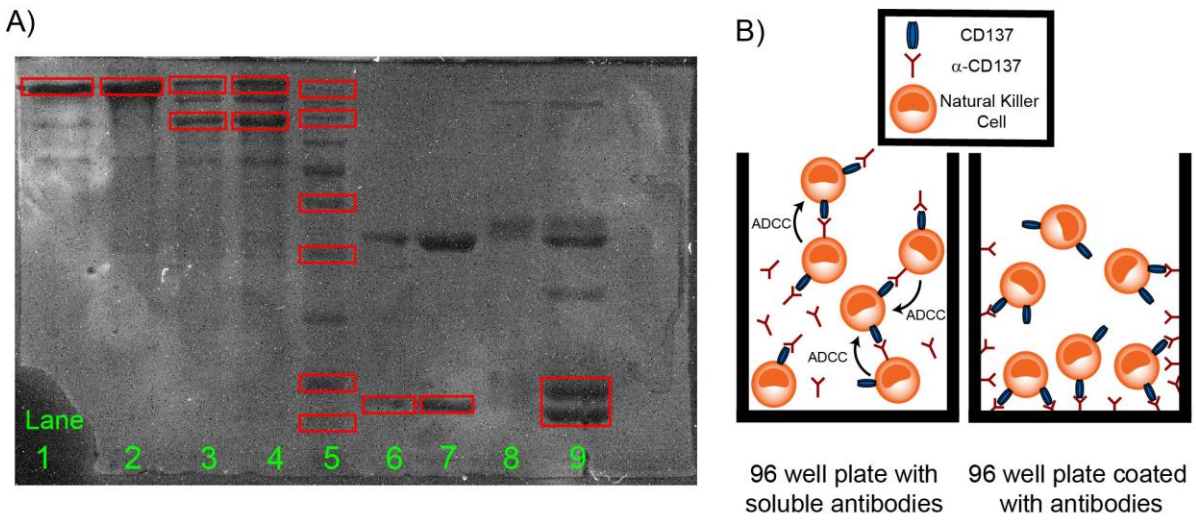

untreated
Isotype Rat IgG
α-CD137
% NK cells among splenocytes
**
untreated
isotype
α-CD137
% death among NK cells
*
untreated
isotype
α-CD137

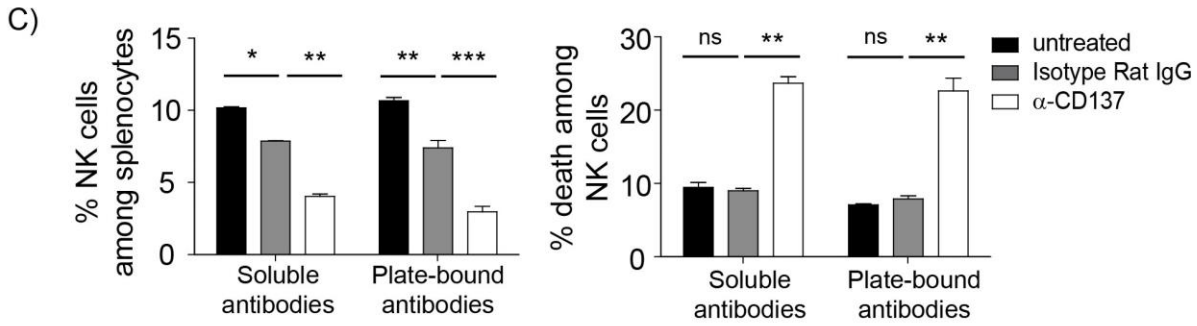
Figure 5: α-CD137 stimulation induces NK cell death on day 2 in vitro. A) Representative plots for NK cell proportion among splenocytes and percentage of death among NK cells. Cells were gated on singlet cells, live cells, and NK1.1+TCR- NK cells (middle row) and NK1.1+TCR- NK cells, and dead cells (bottom row). B) NK1.1+TCR- NK cell proportion among total splenocytes after 2 days of stimulation with α-CD137 (10 μg/ml) and percentage of death among NK cells. One-way ANOVA and Tukey's post hoc test was performed. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data is representative of two independent experiments.

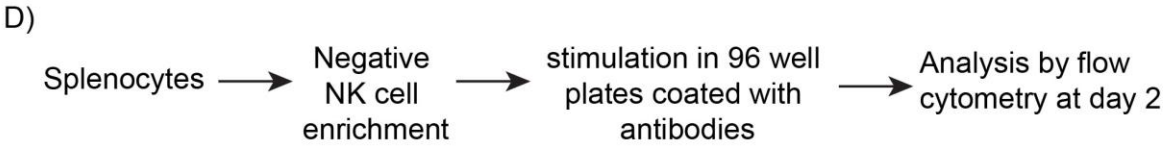
4.3.2 α-CD137 induced NK cell death is independent of ADCC and intrinsic to NK cells

To identify the cause of NK cell death, I first investigated two possibilities. Two common mechanisms by which antibodies can induce death of a cell are through ADCC and complement dependent cytotoxicity (CDC). In ADCC, cells coated by antibodies will be recognized by NK cells through FcRγRIII (CD16) and induce the release of granzymes and perforin, which mediate death on target cells [Murphy et al., 2012]. Complement proteins will also recognize cells bound by antibodies, eventually forming membrane attack complexes (MAC), which puncture the cell membrane, leading to cell death [Murphy et al., 2012]. However, cell death through complement activation and formation of MAC is not likely as complement proteins from samples have been washed away during splenocyte preparation and those present in fetal bovine serum (FBS) have been inactivated during FBS preparation by heat inactivation at 56ºC. Therefore, I investigated if ADCC was responsible for NK cell death through α-CD137.

A convincing method to test the action of α-CD137 antibodies in vitro would be to utilize pepsin to cleave the Fc portion from the F(ab)2' portion of α-CD137 antibodies, preventing the binding of Fc receptors to α-CD137, ultimately excluding effects that would be facilitated by Fc regions. I have successfully digested α-CD137 antibodies using the pepsin digestion method (Figure 5A). A polyacrylamide gel run with the digests showed successful digestions. Lane 1 and 2 show the full intact antibodies and lanes 3 and 4 show the digested products, indicating partial digestions. Full digestion of antibodies by pepsin is known to be difficult to achieve. Lane 4 shows the F(ab)2' portion that is under 135 kDa.


A)
Lane
1 2 3 4 5 6 7 8 9
B)
CD137
α-CD137
Natural Killer Cell
ADCC
ADCC
ADCC
96 well plate with soluble antibodies
96 well plate coated with antibodies


C)
% NK cells among splenocytes
15
10
5
0
* ** ** ***
Soluble antibodies
Plate-bound antibodies
% death among NK cells
30
20
10
0
ns ** ns **
Soluble antibodies
Plate-bound antibodies
untreated
Isotype Rat IgG
α-CD137


D)
Splenocytes
Negative NK cell enrichment
stimulation in 96 well plates coated with antibodies
Analysis by flow cytometry at day 2

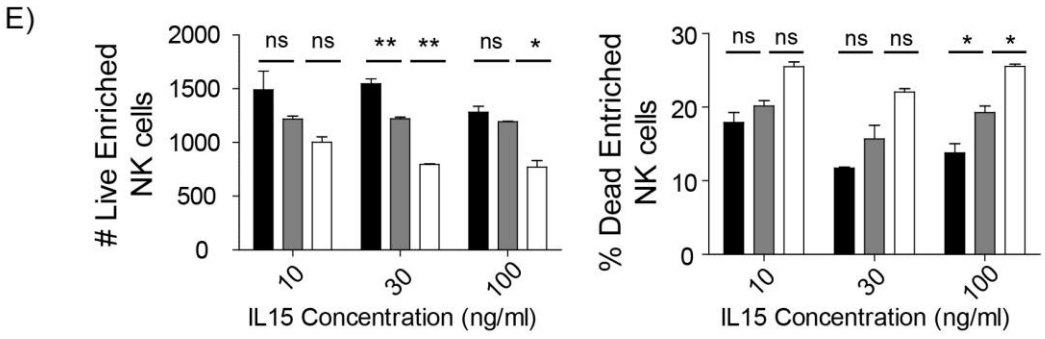

E)
Live Enriched NK cells
2000
1500
1000
500
0
ns ns ** ** ns *
10
30
100
IL15 Concentration (ng/ml)
% Dead Entriched NK cells
30
20
10
0
ns ns ns ns * *
10
30
100
IL15 Concentration (ng/ml)

Figure 6: α-CD137 induced NK cell death is independent of ADCC and intrinsic to NK cells. A) Gel of pepsin antibody digestion. Lane1-2: full antibody, Lane 3-4 pepsin digestion, Lane 5 Ladder (Red Boxes from top to bottom 245, 180, 63, 48, 25, 20 kDa), Lane 6-9 are duplicates of lanes 1-4 treated with β-mercaptoethanol. B) Illustration depicting the possibility of fratricidal NK cell death through α-CD137 mediated ADCC. C) Percentage of NK cells and percentage of death among NK cells among total splenocytes after a 2 day treatment with soluble or plate bound α-CD137 antibodies (plates coated at a concentration of 10 μg/ml). D) Splenocytes were enriched negatively for NK cells for a purity of ~70%. Purified NK cells were then stimulated in 96 well plates coated with α-CD137 antibodies and 10, 30, 100 ng/ml of IL15 for 2 days. E) Number of live NK cells and dead NK cells after stimulation with α-CD137 antibodies for 2 days. One-way ANOVA and Tukey's post hoc test was performed. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data is representative of one independent experiment.

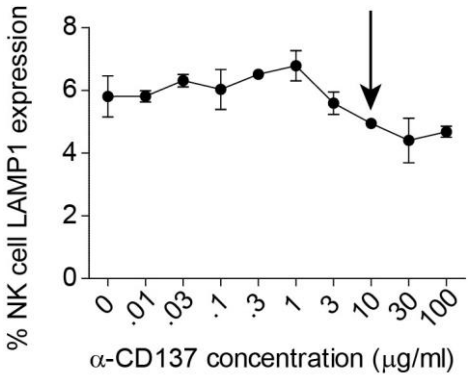
Lane 6 and lane 9 have the same input, but further treated with β-mercaptoethanol to break down the antibodies into heavy and light chains. Lane 9 shows 2 bands near 25 kDa: a heavy chain that has been digested by pepsin and the light chain, whereas lane 6 only has the light chain. Lanes 3 and 8 show digestion, but the amount of antibodies used was less than lane 4 and 9, leading to weaker bands. Although the digestion was successful, attempts to separate and purify the F(ab)2' portions from the Fc and full antibodies were unsuccessful. Protein G binds antibodies through the Fc portion. However, when the pass-through of the digest was captured, there were no F(ab)2' portions to be found. Measuring protein concentration with a nanodrop yielded no protein. Therefore, I was unable to test the effects of F(ab)2' portions of α-CD137 antibodies on NK cells.

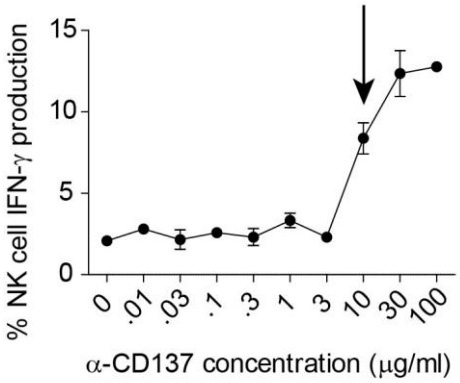
As α-CD137 F(ab)2' purification was unsuccessful, I examined the possibility of NK cell death through fratricidal ADCC mediated through α-CD137 antibodies. In coated wells, as the antibodies are bound to the walls of the wells, they are not free to mediate cell death through ADCC (Figure 5B). Therefore, any effects seen in such situations can be suggested to solely be through the stimulation of the CD137 receptor. A comparison between cells cultured in plates coated with α-CD137 antibodies and cells cultured with soluble α-CD137 revealed no difference in the amount of cell death among NK cells, indicating that ADCC does not play a role (Figure 5C). Not only is α-CD137 induced death independent of ADCC, it also seems intrinsic to NK cells, requiring no other cell types. Splenocytes were harvested from wild type mice and NK cells were enriched through negative NK cell enrichment kits to a purity of ~70% (Figure 5D). These cells were then incubated in α-CD137 coated 96 well culture plates with IL15 (3, 10, 100 ng/ml) and analyzed by flow cytometry on day 2 (Figure 5 D). Numbers of live NK cells, as well as the percentage of death among NK cells, was

measured. The results showed that the decrease in the NK cell population as well as NK cell death still occurred in the enriched NK cell culture (Figure 5E). The effects were less significant when cells were cultured at 10 ng/ml of IL15, but remained consistent at 30 and 100 ng/ml when compared to the effects seen in total splenocytes cultured at 10 ng/ml. It is possible that this discrepancy is due to other cell types contributing to the survival and death of NK cells through the production of cytokines such as IL15. Macrophages and dendritic cells are able to produce IL15, a survival and proliferation signal for NK cells, possibly allowing better survival of NK cells when cultured together.

4.3.3 α-CD137 induced NK cell activation and death have identical thresholds

Examination of NK function and death has surprisingly shown that α-CD137 stimulation can lead not only to activation but also death. To determine if these effects are dose dependent and if a balance between activation and death can be achieved through the careful administration of a specific concentration of antibodies, a titration assay was carried out to measure both the activation and death in NK cells. Splenocytes from wild type mice were cultured in 96 well plates with 10 ng/ml of IL15 in α-CD137 coated wells for 2 days. Cells were then stained and analyzed for LAMP1, IFN-γ, and proportion of death among NK cells. An increase in IFN-γ production, and increase in NK cell death all occurred starting with wells coated at a concentration of 3 μg/ml of α-CD137 (Figure 6). There does not seem to be a concentration where α-CD137 can promote activation while avoiding death. This indicates that α-CD137 induced activation and death occur at identical thresholds, suggesting that the activation and death both occur through the same CD137 signaling


% NK cell LAMP1 expression
8
6
4
2
0
0
.01
.03
.1
.3
1
3
10
30
100
α-CD137 concentration (μg/ml)


% NK cell IFN-γ production
15
10
5
0
0
.01
.03
.1
.3
1
3
10
30
100
α-CD137 concentration (μg/ml)

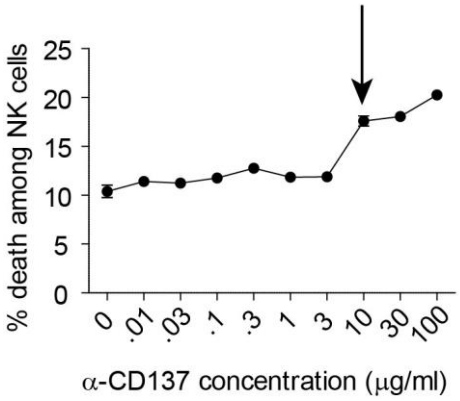

% death among NK cells
25
20
15
10
5
0
0
.01
.03
.1
.3
1
3
10
30
100
α-CD137 concentration (μg/ml)

Figure 7: α-CD137 induced NK cell activation and death have identical thresholds. Splenocytes from B6 mice were cultured in IL15 (10 ng/ml) in 96 well plates coated with α-CD137 (plates coated at a concentration of 10 μg/ml). Cells were stained and analyzed for LAMP1 (top), IFN-γ (middle), and cell death among NK cells (bottom) by flow cytometry.

pathway. Unexpectedly, there was a slight decrease in decrease in LAMP1 expression at a concentration of 3μg/ml and higher. Other reports in the literature have shown α-CD137 stimulation alone on NK cells do not affect NK cytotoxicity [Wilcox et al., 2002]. LAMP1 is a surrogate marker that is expressed upon degranulation of cytotoxic molecules. However, it may not reflect the actual cytotoxicity of NK cells, meaning that the decrease in LAMP1 expression here may not mean a decrease in NK cytotoxicity, which may explain this discrepancy.

4.3.4 α-CD137 induced NK cell death is not dependent on TNF-α, TRAIL, Fas ligand, or activating cytokines

To further investigate the contradictory effects of α-CD137 treatment, in which stimulation with agonistic α-CD137 antibodies seem to stimulate the function of NK cells through increased IFN-γ production and proliferation but also result in the death of NK cells, I have investigated several common death pathways. TNF-α and TRAIL mediated death pathways are the best studied and common. Perforin and granzyme, and Fas-FasL mediated cytotoxicity are two mechanisms through which NK cells confer cytotoxicity against target cells. Direct FasL expression on NK cells or soluble FasL that have been released from NK cell membranes may bind to Fas receptors to induce caspase activation in cells that express Fas, including NK cells themselves [Eischen et al., 1998]. Splenocytes were cultured in wells with plate-bound isotype antibodies or α-CD137 in addition to soluble α-TNF-α, α-FasL, and α-TRAIL antibodies for 2 days. If α-CD137 stimulated NK cell death was due to any of these factors, the introduction of blocking antibodies would rescue NK cells from death. Results showed, however, that none of the treatments rescued NK cells from death,

suggesting that death does not occur through pathways activated by these molecules (Figure 7A). Blocking other cytokines that are known to play a role in NK activation, IL12, and IFN-γ, which is increased upon α-CD137 stimulation in NK cells, were also tested with similar results (Figure 7A). α-CD137 stimulation of NK cells from perforin knockout mice, which are deficient in perforin, Fas-lpr mice, which have non-functional and truncated Fas receptor, and TNF deficient mice, deficient in TNF-α, showed a similar lack of rescue of NK death, suggesting that these molecules are not critical in inducing death on NK cells through α-CD137 (Figure 7B). This provides evidence that the death of NK cells is not due to cell cytotoxicity mediated by Fas ligand, TRAIL, perforin, or TNF-α.

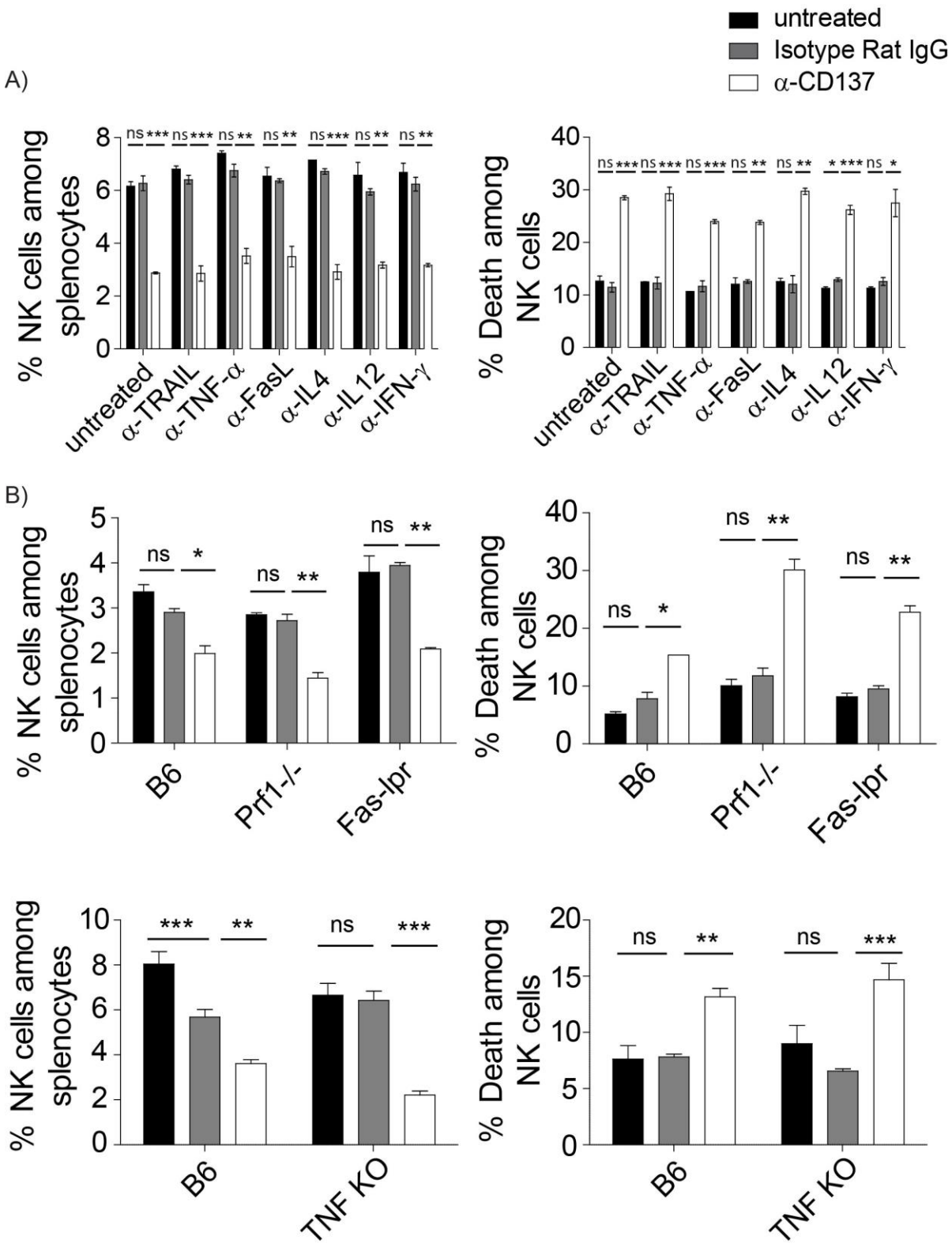
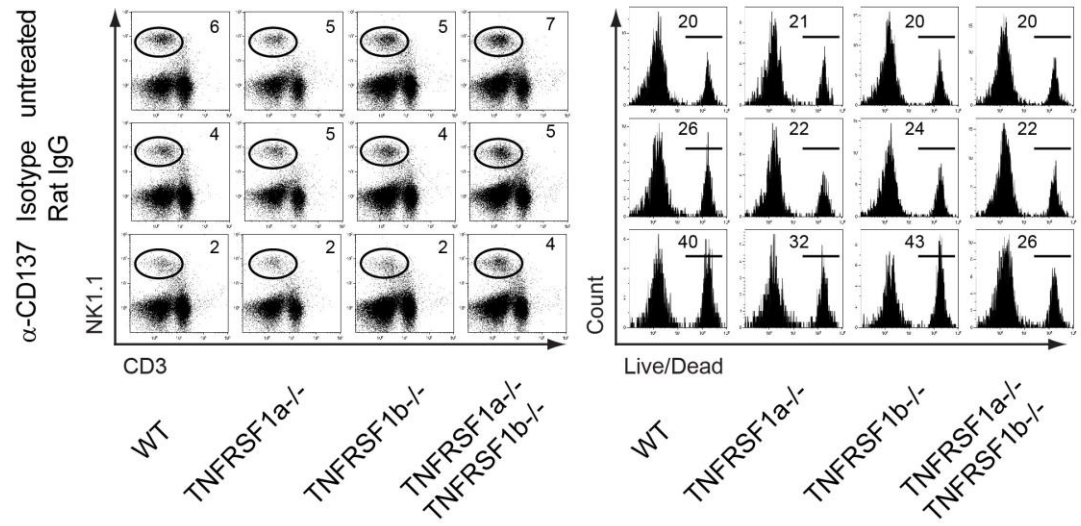

A)
untreated
Isotype Rat IgG
α-CD137
% NK cells among splenocytes
% Death among NK cells
untreated
α-TRAIL
α-TNF-α
α-FasL
α-IL4
α-IL12
α-IFN-γ
B)
B6
Prf1-/-
Fas-lpr
TNF KO
ns
*
**
***

Figure 8: α-CD137 induced NK cell death is not dependent on TNF-α, TRAIL, Fas ligand, or activating cytokines. Splenocytes were cultured in 96 well plates coated with α-CD137 antibodies (plates coated at a concentration of 10 μg/ml) with 10 ng/ml of IL15 and blocking antibodies for the indicated cytokines for 2 days. Cells were then stained and analyzed by flow cytometry for cell population and cell death. B) Splenocytes from B6, prf1-/-, and Fas-lpr mice were cultured in 96 well plates coated with α-CD137 antibodies (plates coated at a concentration of 10 μg/ml) for 2 days. Cells were stained and analyzed for cell population and cell death. One-way ANOVA and Tukey's post hoc test was performed. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data is representative of one independent experiment.

4.3.5 TNFR1 and TNFR2 double knockout NK cells are resistant to α-CD137 induced death

Splenocytes from TNFR1, TNFR2, and TNFR1/TNFR2 deficient mice were cultured ex vivo with 10 ng/ml of IL15 in 96 well plates coated with α-CD137 for 2 days, and analyzed by flow cytometry for the proportion of NK cells in total splenocytes and the percentage of death among NK cells. Representative dot plots are shown (Figure 8A). Previous data from TNF-α blocking antibodies and TNF deficient mice suggested that α-CD137 induced NK cell death was independent of the TNF-TNFR signaling pathway. Surprisingly, there was a slight reduction in the amount of NK cell death in the TNFR1 deficient cells and a significant rescue of death as well as NK proportion among splenocytes in the TNFR1 and TNFR2 double deficient mice (Figure 8B). This suggests that TNFR signaling was indeed involved in α-CD137 induced NK cell death. However, since TNF-α blocking did not seem to rescue NK cells from α-CD137 induced death, I investigated TNF-β (tumor necrosis factor β; also known as lymphotoxin-α), another ligand for TNFR1 and TNFR2, as a possible factor involved.

A)


α-CD137
Isotype Rat IgG
untreated
NK1.1
CD3
Count
Live/Dead
6
5
5
7
4
5
4
5
2
2
2
4
20
21
20
20
26
22
24
22
40
32
43
26
WT
TNFRSF1a-/-
TNFRSF1b-/-
TNFRSF1a-/- TNFRSF1b-/-

B)

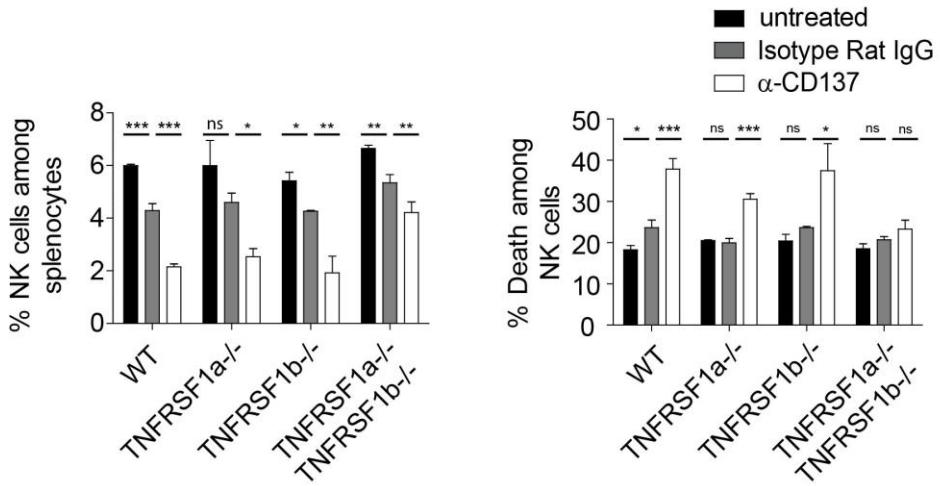

untreated
Isotype Rat IgG
α-CD137
% NK cells among splenocytes
% Death among NK cells
***
***
ns
*
*
**
**
**
*
***
ns
***
ns
*
ns
ns
WT
TNFRSF1a-/-
TNFRSF1b-/-
TNFRSF1a-/- TNFRSF1b-/-

Figure 9: TNFR1 and TNFR2 double knockout NK cells are resistant to α-CD137 induced death. Splenocytes from B6, TNFRSF1a-/-, TNFRSF1b-/-, and TNFR SF1a-/- TNFRSF1b-/- double knockout were cultured in 96 well plates coated with α-CD137 (plates coated at a concentration of 10 μg/ml) for 2 days. Cells were then stained and analyzed for cell population and cell death. A) Representative plots of NK cells among total splenocytes and percent death among NK cells. B) Percentage of NK cells and death among the NK cell population among total splenocytes after 2 days of stimulation with α-CD137 antibodies (plates coated at a concentration of 10 μg/ml). One-way ANOVA and Tukey's post hoc test was performed. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data is representative of one independent experiment.

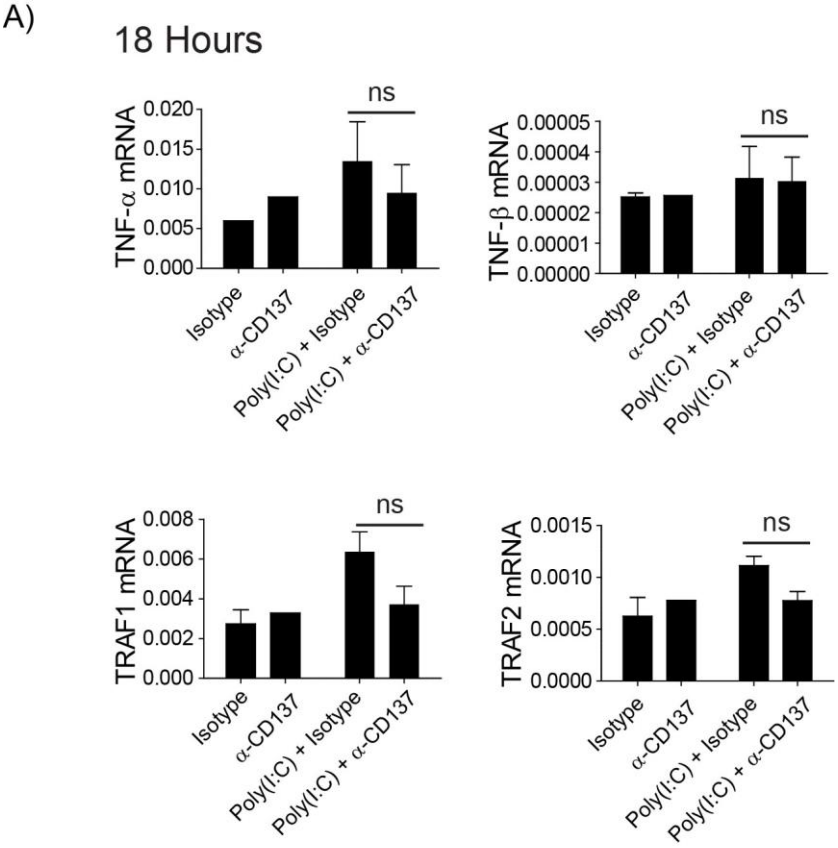
4.4 In vivo α-CD137 stimulation on NK cells during poly(I:C) induced inflammation

4.4.1 TNF-α and TNF-β mRNA levels are unchanged in the spleen at 18 and 48 hours post injection with poly(I:C)

Unfortunately, antibodies against TNF-β and methods for quantifying TNF-β are not readily available in the mouse system. Therefore, quantitative polymerase chain reaction (qPCR) was carried out to determine whether TNF-β mRNA levels were affected upon α-CD137 treatment. In addition, the effect of poly(I:C) in combination with α-CD137 treatment was investigated to test how α-CD137 treatment can affect the immune system during an inflammatory state. Poly(I:C) was used to create an inflammatory environment by stimulating TLR3, in which the effects of α-CD137 can be investigated. Wild type mice were injected with 200 μg of α-CD137 antibodies with a subsequent injection of 200 μg of poly(I:C) to mimic a viral infection through activation of TLR3. Splenocytes were taken from the mice after 18 hours and 48 hours. 30E6 splenocytes were then processed, and cDNA was obtained through reverse transcription PCR. The cDNA was then used to run a qPCR reaction using primers against TNF-α and TNF-β.

Results indicated that there was no difference in the expression of mRNA for TNF-α or TNF-β in mice treated with isotype control or α-CD137 on both 18 hours and 48 hours (Figure 9A, B). With poly(I:C) treatment at 48 hours, however, both mRNAs levels were decreased, although not statistically significant (Figure 9B). However, this is likely a consequence of the decrease in NK cell populations through α-CD137 induced NK cell death. Although not the main producers of TNF-α, NK cells are known to produce

A)


18 Hours
TNF-α mRNA
0.020
0.015
0.010
0.005
0.000
ns
TNF-β mRNA
0.00005
0.00004
0.00003
0.00002
0.00001
0.00000
ns
TRAF1 mRNA
0.008
0.006
0.004
0.002
0.000
ns
TRAF2 mRNA
0.0015
0.0010
0.0005
0.0000
ns
Isotype
α-CD137
Poly(I:C) + Isotype
Poly(I:C) + α-CD137

B)

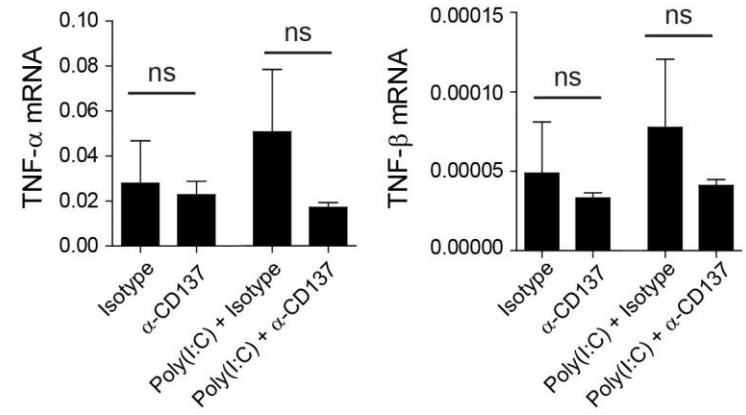

48 Hours
TNF-α mRNA
0.10
0.08
0.06
0.04
0.02
0.00
ns
ns
TNF-β mRNA
0.00015
0.00010
0.00005
0.00000
ns
ns
Isotype
α-CD137
Poly(I:C) + Isotype
Poly(I:C) + α-CD137

Figure 10: TNF-α and TNF-β mRNA levels are unchanged in the spleen at 18 and 48 hours post injection with poly(I:C). B6 mice were injected with 200 μg of isotype control rat IgG or α-CD137 antibodies 4 hours prior to 200 μg of poly(I:C) for A) 18 and B) 48 hours. Splenocytes were processed for mRNA and analyzed by qPCR for TNF-α, TNF-β, TRAF1, and TRAF2 mRNA. Unpaired student t tests were performed to calculate p values between indicated groups. n = 2 mice per group. *ns $p > 0.05$. Data is representative of one independent experiment.

significant amounts during viral infections, and a significant decrease in NK cell populations may be the reason for this decreased mRNA expression. In addition to TNF-α and TNF-β mRNA levels, TRAF1 and TRAF2 levels were slightly decreased 18 hours after treatment, although not statistically significant (Figure 9A). The first adaptor proteins to be recruited to the TNFR1 and TNFR2 intracellular domains are TRAF1 and TRAF2, and are vital in further signaling down this pathway. It is possible that α-CD137 signaling leads to the decreased expression of TRAF1 and TRAF2, tampering with the balance between survival and death maintained by the TNFR receptors.

4.4.2 α-CD137 stimulation decreases NK proportions in spleen, liver, and blood

Mice were injected with agonistic α-CD137 antibodies 4 hours prior to poly(I:C) injection intraperitoneally. On day 2 post treatment, cells were analyzed by flow cytometry for NK cell proportions. NK cell death was measured but no difference in death could be detected in contrast to in vitro experiments, likely due to the clearance of dead cells through constant surveillance by phagocytic cells. There were significantly decreased proportions of splenic NK cells upon α-CD137 treatment (Figure 10 top left). This was accompanied by similar decreases in the liver and blood, suggesting that the decrease is not due to the migration of NK cells out of the spleen, but caused by NK cell death (Figure 10 top middle and right). Among T cells, there were slight decreases in the spleen (not statistically significant) and blood (Figure 10 bottom left and right) and increases in the liver (not statistically significant; bottom middle) after α-CD137 administration and poly(I:C)

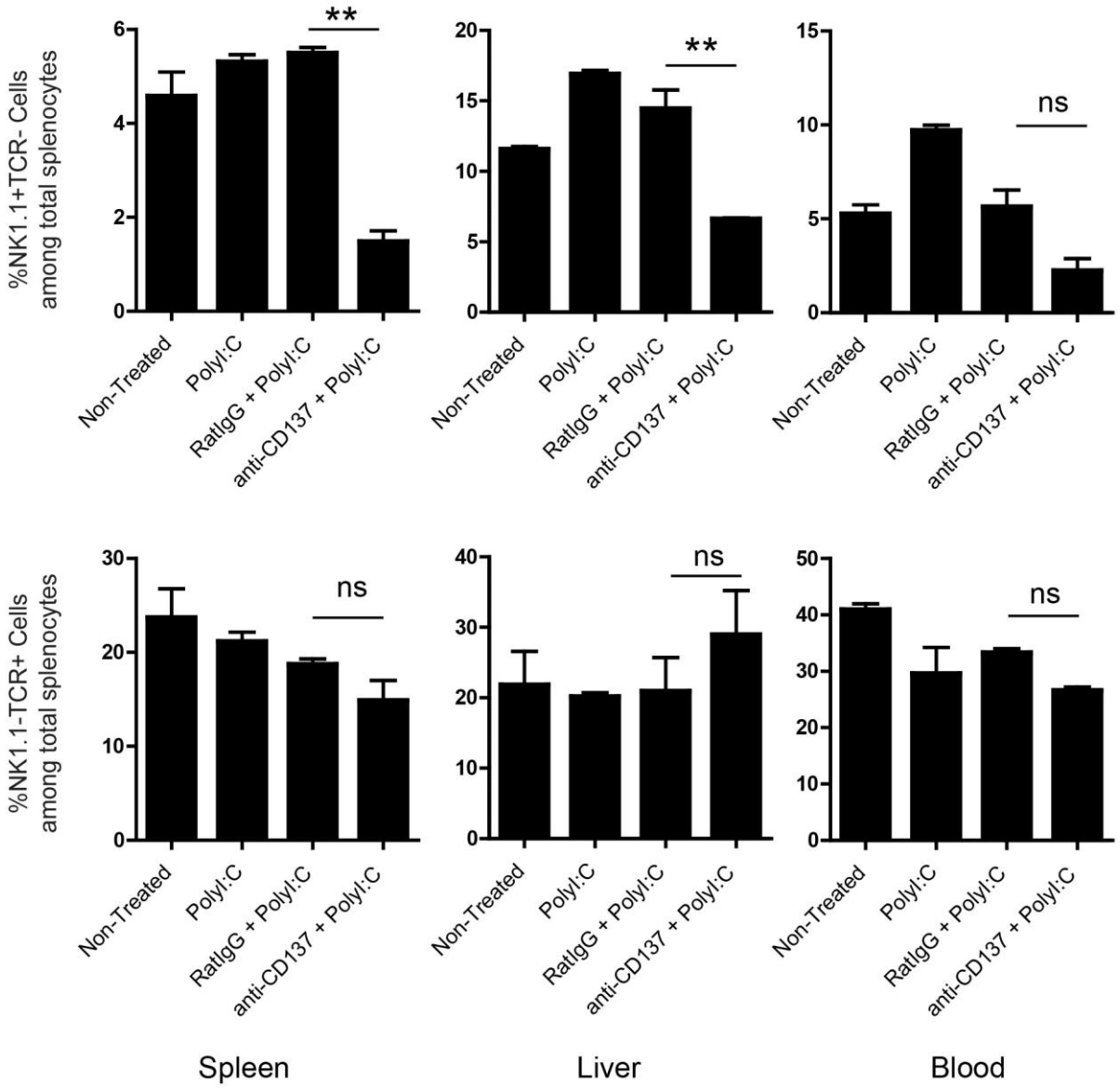

%NK1.1+TCR- Cells among total splenocytes
%NK1.1-TCR+ Cells among total splenocytes
**
ns
Non-Treated
Poly I:C
RatIgG + Poly I:C
anti-CD137 + Poly I:C
Spleen
Liver
Blood

Figure 11: α-CD137 stimulation decreases NK proportions in spleen, liver, and blood. B6 mice were treated with α-CD137 antibodies with a subsequent treatment with poly(I:C) 4 hours later. Organs were harvested after 2 days and cells were processed for flow cytometry. NK1.1+TCR- NK cells (top) and NK1.1-TCR+ T cells (bottom) in the spleen (left column), liver (middle column), and blood (right column). One-way ANOVA and Tukey's post hoc test was performed. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Data is representative of two independent experiments with n = 2-4 mice per group.

treatment. Although the changes in the spleen and liver are statistically not significant, it likely reflects the migration of T cells into the liver upon α-CD137 treatment.

4.4.3 α-CD137 treatment increases TNFR2 expression on NK cells upon treatment with poly(I:C)

Treatment with α-CD137 also slightly decreased the expression of Ly49H on NK cells (Figure 11 A, B). Although NK proportions were decreased in different organs along with decreased Ly49H expression among NK cells, proliferation was increased upon α-CD137 treatment (Figure 11A, B). However, α-CD137 treatment did not seem to further increase NK proliferation during inflammation induced by poly(I:C) treatment. An interesting observation involves the expression of TNFRs after α-CD137 and poly(I:C) treatment. Although α-CD137 treatment did not affect the expression of either TNFR1 or TNFR2 alone, when poly(I:C) was administered, the levels of TNFR1 was decreased slightly and TNFR2 increased drastically, suggesting a possible role of α-CD137 in altering their expression and signaling during inflammatory environments (Figure 11 A, B). Another possibility may be that the increase in TNFR2 was a compensatory response by NK cells upon α-CD137 stimulation, either by a lack of TNFR2 ligand, or by blocking TNFR2 signaling.

4.5 Effects of in vivo α-CD137 stimulation on the resistance against MCMV infection

Results from α-CD137 and poly(I:C) cotreatments revealed a decrease in NK proportions in different organs, the expression of Ly49H which binds m157, and the expression of TNFRs. The overall changes to NK cell phenotype and function seem to

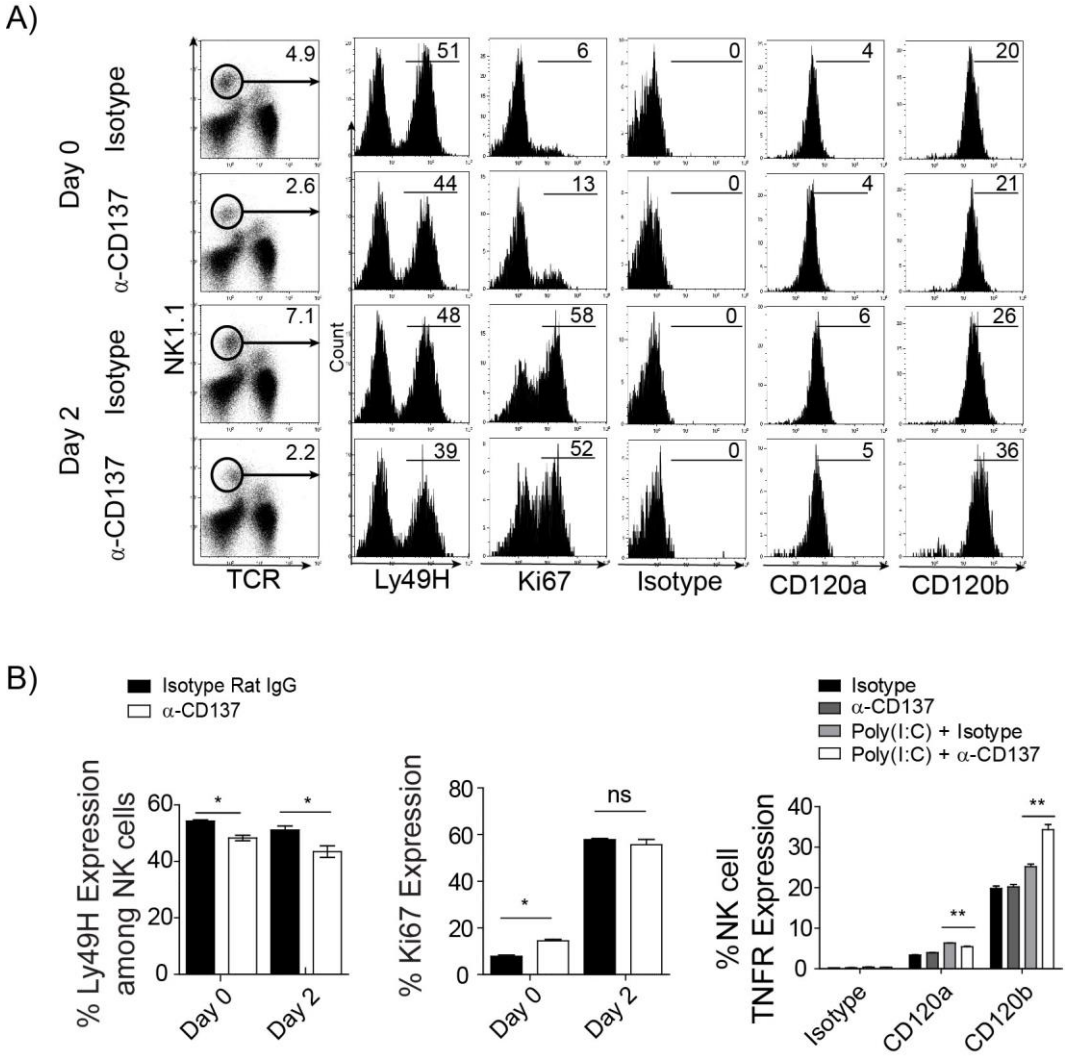

A)
Day 0
Isotype
α-CD137
Day 2
Isotype
α-CD137
NK1.1
TCR
4.9
2.6
7.1
2.2
Count
Ly49H
51
44
48
39
Ki67
6
13
58
52
Isotype
0
0
0
0
CD120a
4
4
6
5
CD120b
20
21
26
36
B)
Isotype Rat IgG
α-CD137
% Ly49H Expression among NK cells
60
40
20
0
*
*
Day 0
Day 2
% Ki67 Expression
80
60
40
20
0
*
ns
Day 0
Day 2
Isotype
α-CD137
Poly(I:C) + Isotype
Poly(I:C) + α-CD137
% NK cell TNFR Expression
40
30
20
10
0
**
**
Isotype
CD120a
CD120b

Figure 12: α-CD137 treatment increases TNFR2 expression on NK cells upon treatment with poly(I:C). B6 mice were treated with α-CD137 (200 μg) intraperitoneally and subsequently with poly(I:C) (200 μg) 4 hours later. A) Representative plots of NK cell functional markers and CD120 expression 2 days post injection with α-CD137 and poly(I:C). B) Ly49H expression among NK1.1+TCR- NK cells, Ki67 expression for measurement of proliferation, and TNFR expression after treatment. Unpaired student t tests were performed to calculate p values between indicated groups. Data is representative of two independent experiments with n = 3-4 mice per group. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

point towards an overall negative effect in the ability of NK cells to function properly during an inflammatory state. NK cell populations were decreased with less Ly49H expression, suggesting an impaired ability to detect and control MCMV infections. Even though poly(I:C) stimulation provided an estimation of the effects of α-CD137 treatment during infection, it cannot be substituted for a real viral infection. Viral infections stimulate the immune response through various cell types and immune receptors. Recognition of viral particles, interception of various cellular proteins by viral proteins, and continuous viral replication all contribute to a complete and wide range of immune responses during an infection. Studying α-CD137 treatment in vivo with MCMV would provide data on the full range of effects of α-CD137 treatment on the immune system in the context of infection, but also host-pathogens interactions. To test the effects of α-CD137 stimulation on MCMV resistance, B6 mice were treated with an intraperitoneal injection of 200 μg of agonistic α-CD137 antibodies and subsequently infected i.p. with 20,000 pfu of MCMV. Uninfected mice were treated with α-CD137 for 4 days and sacrificed. These mice were used as control for both day 1.5 and day 4 infections as a difference between α-CD137 treated mice on day 1.5 and day 4 was not expected. Infected mice were treated with α-CD137 for 4 hours followed by an infection for 1.5 days, then sacrificed.

4.5.1 α-CD137 stimulation increases viral burden and decreases NK proportions on day 1.5 of MCMV infection

A decrease in the viral resistance against MCMV infection was seen from the increased viral burden in the spleen (Figure 12A). Two different surface markers for defining NK cells were used for MCMV infections: NK1.1 and CD49b. As expected, decreased

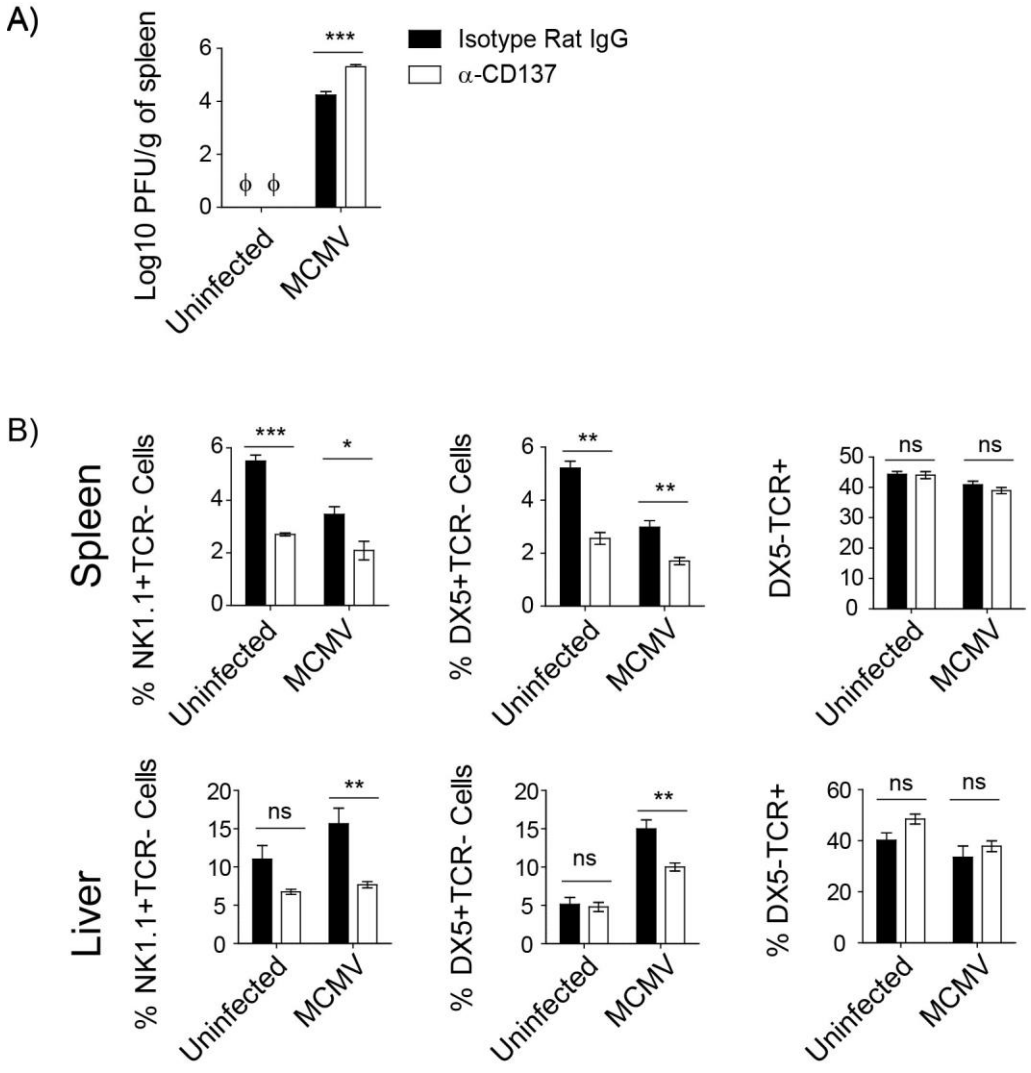
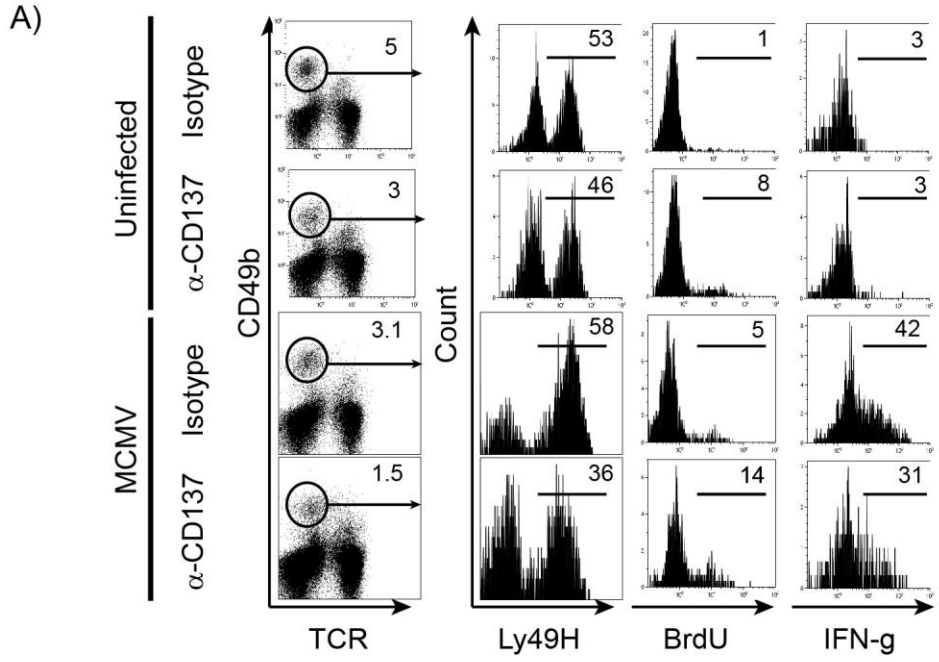

A)
Log10 PFU/g of spleen
6
4
2
0
***
ϕ ϕ
Uninfected
MCMV
Isotype Rat IgG
α-CD137
B)
Spleen
% NK1.1+TCR- Cells
***
*
% DX5+TCR- Cells
**
**
DX5-TCR+
ns
ns
Liver
% NK1.1+TCR- Cells
ns
**
% DX5+TCR- Cells
ns
**
% DX5-TCR+
ns
ns
Uninfected
MCMV

Figure 13: α-CD137 stimulation increases viral burden and decreases NK proportions day 1.5 post MCMV infection. Uninfected control mice were treated with α-CD137 antibodies (200 μg) intraperitoneally for 4 days. Experimental mice were treated with α-CD137 antibodies with a subsequent infection with MCMV (2E5 pfu) 4 hours later for 1.5 days. Spleen, livers, and blood were harvested for plaque assay and analysis by flow cytometry. A) Plaque assay of spleen to measure viral burden. B) NK cell and T cell proportions on day 1.5 post treatment and infection. Unpaired student t tests were performed to calculate p values between indicated groups. Data is representative of two independent experiments with n = 3-4 mice per group. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

levels of NK proportions were observed in the spleen, liver, and blood in infected mice, which explains the decrease in viral resistance (Figure 12B left and middle). In infected mice, NK cells decreased in the spleen and increased in the liver compared to uninfected mice, possibly due to cell migration in response to MCMV (Figure 12B). Upon α-CD137 treatment, both organs experience a significant decrease in NK cell proportions, likely due to cell death. This decrease is only seen in NK cells with T cell proportions remaining consistent between all groups (Figure 12B).

4.5.2 α-CD137 stimulation increases proliferation but not cytotoxicity of NK cells on day 1.5 post MCMV infection

In addition to the viral burden and NK cell proportions, NK cell function was also examined after α-CD137 treatment and MCMV infection. Mice were injected with BrdU 2 hours prior to sacrifice. Splenocytes were then harvested and stained, or incubated with brefeldin A for 4 hours to measure cytokine production. Representative plots for the expression of Ly49H and BrdU are shown (Figure 13A). Results again show a decrease in the expression of Ly49H among NK cells (Figure 13 A, B). Proliferation measured by BrdU, however, is significantly increased in both uninfected and infected mice, indicating that α-CD137 in fact does increase the proliferation of NK cells (Figure 13B). IFN-γ production was unchanged after α-CD137 treatment as was the ability of NK cells to confer cytotoxicity against target cells as shown by a killing assay against the MHC-I deficient YAC-1 cell line (Figure 13B). This indicates that the decrease MCMV resistance mediated by α-CD137 treatment was not due to the impairment of NK cell cytotoxicity or cytokine production, but through decreased NK cell availability and ability to recognize MCMV m157 through


A)
Uninfected
MCMV
Isotype
α-CD137
Isotype
α-CD137
CD49b
TCR
Count
Ly49H
BrdU
IFN-g
5
3
3.1
1.5
53
46
58
36
1
8
5
14
3
3
42
31

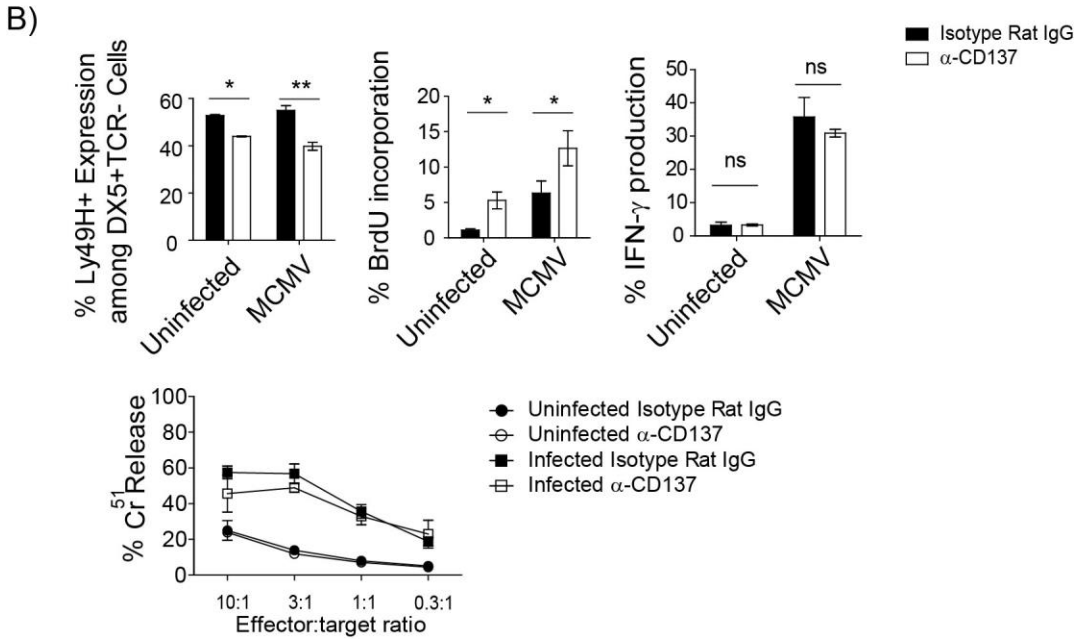

B)
Isotype Rat IgG
α-CD137
% Ly49H+ Expression among DX5+TCR- Cells
% BrdU incorporation
% IFN-γ production
Uninfected
MCMV
ns
% Cr51 Release
Effector:target ratio
10:1
3:1
1:1
0.3:1
Uninfected Isotype Rat IgG
Uninfected α-CD137
Infected Isotype Rat IgG
Infected α-CD137

Figure 14: α-CD137 stimulation increases proliferation but not cytotoxicity of NK cells on day 1.5 post MCMV infection. A) Representative plots of NK cell functional markers and BrdU expression 1.5 days post infection with α-CD137. B) Ly49H, BrdU expression for measurement of proliferation, IFN-γ expression for NK cell cytokine production, and killing assay for NK cell cytotoxicity. Unpaired student t tests were performed to calculate p values between indicated groups. Data is representative of two independent experiments with n = 3-4 mice per group, with the exception of the NK killing assay which was performed once. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Ly49H. This seems somewhat counterintuitive as α-CD137 increases NK cell proliferation, but at the same time decreases the proportion and availability of NK cells to combat the infection.

4.5.3 α-CD137 stimulation increases viral burden and increases NK proportions on day 4 post MCMV infection

Similar to the results from day 1.5 post infection, increased viral burden in the spleen was observed in infected mice (Figure 14A). The NK1.1+TCR- NK cell populations were slightly increased in the spleen and decreased in the liver, but there was a significant increase in the levels of CD49b+TCR- NK cell proportions in the spleen, liver, and blood 4 days post infection (Figure 14B). During MCMV infections, NK1.1 expression on NK cells was significantly downregulated by day 4 (Figure 14C). Among Ly49H+ NK cells, which are the most specific marker for NK cells, NK1.1 was downregulated while CD49b expression remains similar (Figure 14C). Therefore, while NK1.1 is usually an adequate marker for the NK cell population, it is an insufficient marker during MCMV infection as a large proportion of NK cells will express very low levels of NK1.1. CD49b was used as an alternative marker, although slightly less specific for NK cells. Nonetheless, most CD49b expressing cells are also positive for Ly49H on splenocytes from MCMV-infected mice on day 4. Thus, NK populations as measured with NK1.1 is likely not accurate, and the slight increase seen in the spleen is much more significant, which is more accurately demonstrated with the CD49b marker. When NK cell populations were measured with CD49b on day 4 of MCMV infection, although there was only a slight increase in NK cell populations in the liver as seen by CD49b, there was an extreme ~3 fold increase in the spleen in mice treated with α-

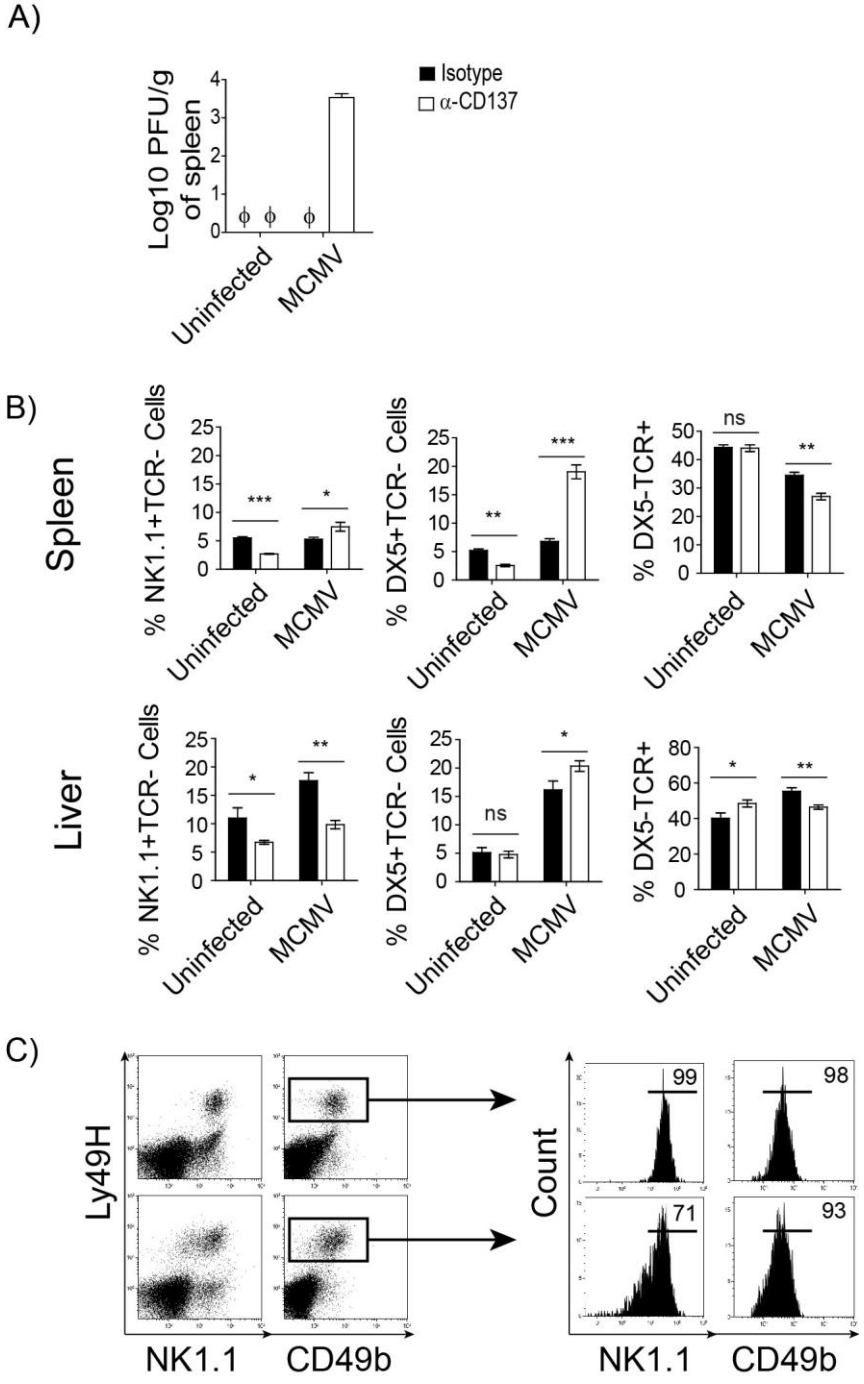

A)
Log10 PFU/g of spleen
Isotype
α-CD137
Uninfected
MCMV
B)
Spleen
Liver
% NK1.1+TCR- Cells
% DX5+TCR- Cells
% DX5-TCR+
ns
C)
Ly49H
NK1.1
CD49b
Count
99
98
71
93

Figure 15: α-CD137 stimulation increases viral burden and increases NK proportions on day 4 post MCMV infection. B6 mice were treated with α-CD137 antibodies (200 μg) intraperitoneally with a subsequent infection with MCMV (2E5 pfu) for 4 days. Spleen, liver, and blood were harvested for plaque assay and analysis by flow cytometry. A) Plaque assay of spleen to measure viral burden. B) NK cell and T cell proportions on day 4 post treatment and infection. C) Expression of NK1.1 and CD49b on Ly49H+ NK cells during MCMV infection. Unpaired student t tests were performed to calculate p values between indicated groups. Data is representative of two independent experiments with n = 3-4 mice per group. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

CD137. A slight decrease was also observed in the T cell population after α-CD137 treatment, but this was due to the drastically increased NK cell proportions (Figure 14B).

4.5.4 α-CD137 stimulation increases proliferation but not cytotoxicity of NK cells on day 4 post MCMV infection

NK cell function was also examined after α-CD137 treatment and MCMV infection. Mice were injected with BrdU 2 hours prior to sacrifice and splenocytes were then harvested and stained, or incubated with brefeldin A for 4 hours to measure cytokine production. Representative plots for the expression of Ly49H and BrdU, are shown (Figure 15 A). There was a decrease in the expression of Ly49H among NK cells in α-CD137 treated uninfected mice, but infected mice treated with α-CD137 had higher expression of Ly49H among NK cells (Figure 15B). Combined with the day 1.5 post infection data, it is likely that the residual NK cells from day 1.5 began to compensate for the increased viral burden in the mouse by increasing Ly49H expression among the NK cell population. Proliferation measured by BrdU was increased in both uninfected and infected mice upon α-CD137 treatment, showing higher proliferation in NK cells, similar to day 1.5 (Figure 15B). IFN-γ production by NK cells was slightly decreased, although statistically insignificant, upon α-CD137 treatment with or without infection. Cytotoxicity itself was unchanged, as killing assays against YAC-1 cells showed no change in killing ability of NK cells (Figure 15B). These data together suggest that the most significant modulation of NK cells by α-CD137 treatment is not the function of NK cells through changes in cytokine production and cytotoxicity, but through the regulation of cell survival and death as well as Ly49H expression, altering the amount of NK cells available to confer resistance against infection.

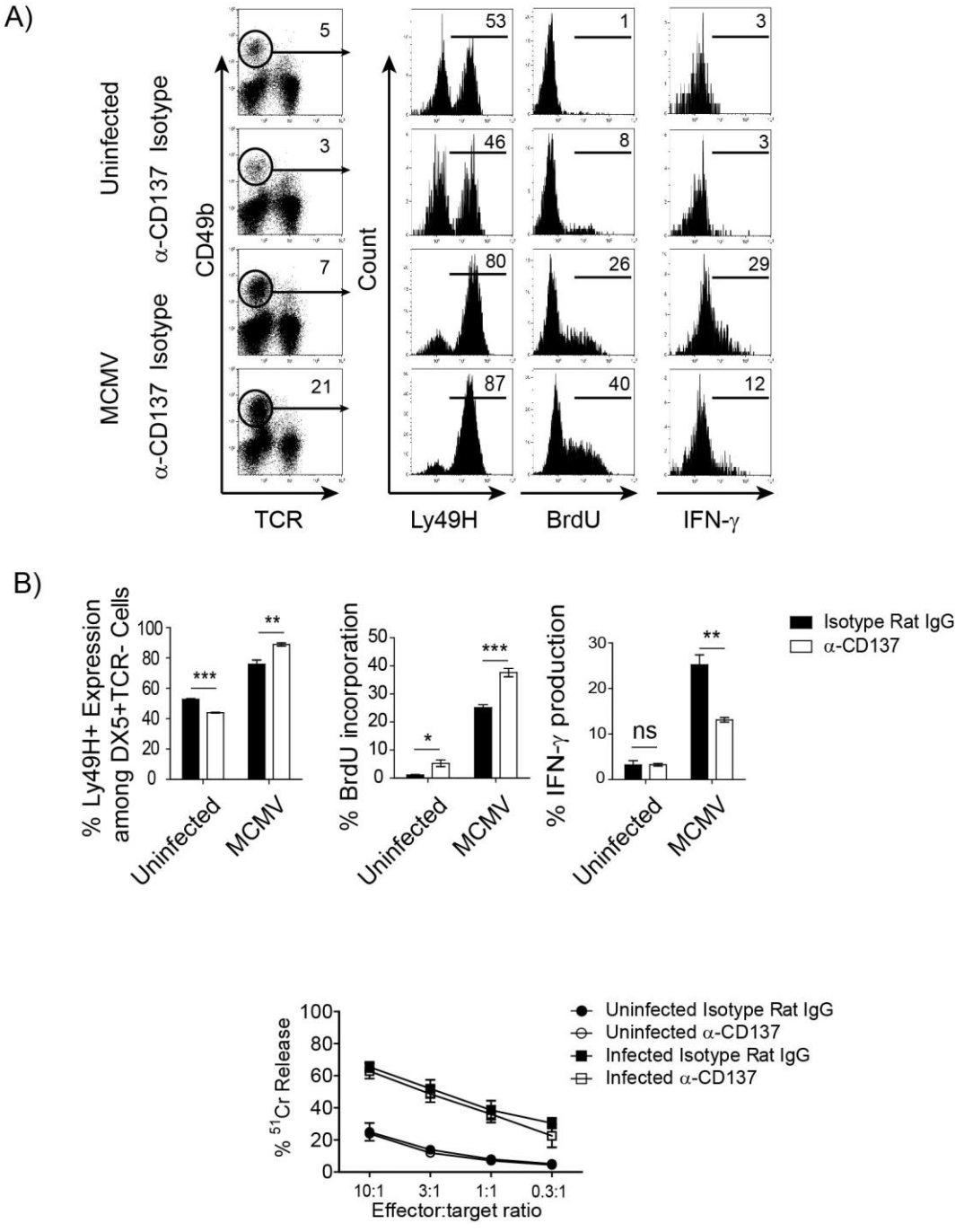

A)
Uninfected
MCMV
Isotype
α-CD137
Isotype
α-CD137
CD49b
TCR
Count
Ly49H
BrdU
IFN-γ
5
3
7
21
53
46
80
87
1
8
26
40
3
3
29
12
B)
% Ly49H+ Expression among DX5+TCR- Cells
% BrdU incorporation
% IFN-γ production
Uninfected
MCMV
***
**
*
ns
Isotype Rat IgG
α-CD137
% 51Cr Release
Effector:target ratio
10:1
3:1
1:1
0.3:1
Uninfected Isotype Rat IgG
Uninfected α-CD137
Infected Isotype Rat IgG
Infected α-CD137

Figure 16: α-CD137 stimulation increases proliferation but not cytotoxicity of NK cells on day 4 post MCMV infection. A) Representative plots of NK cell functional marker expression 1.5 days post infection with α-CD137 (200 μg) and infection with MCMV (2E5 pfu). B) Ly49H expression among NK1.1+TCR- NK cells, Ki67 expression for measurement of proliferation, IFN-γ expression for cytokine production, and killing assay for NK cytotoxicity after treatment. Unpaired student t tests were performed to calculate p values between indicated groups. Data is representative of two independent experiments with n = 3-4 mice per group, with the exception of the NK killing assay which was performed once. *ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

5. DISCUSSION

5.1 Summary of Data

α-CD137 antibodies are widely studied in terms of cell activation and tumor therapy. Its potent effects on increasing T cell and NK cell proliferation, survival, and anti-tumor function make it a prime target for developing methods for not only in vivo treatments, but also for in vitro procedures, such as expanding cells and preparing them for adoptive transfers. However, different procedures utilizing α-CD137 have varying degrees of success in terms of activating NK cell function. Furthermore, α-CD137 can, in certain instances, induce cell death. Although Fas ligand mediated cytotoxicity is an important mediator of α-CD137 induced T cell death, the mechanism of α-CD137 induced death in NK cells is unknown.

To characterize CD137 expression on NK cells and the ability of NK cells to respond to α-CD137 stimulation, I have first investigated in an in vitro system. CD137 was expressed among most NK cells by day 1 and 2 when activated with IL2 and IL15 (Figure 1). This confirmed previous data with the addition of a dose dependency on the expression of CD137 [Wilcox 2002]. I have also shown that other NK activating cytokines observed during MCMV infection, such as IL12 and IL18, are also inducers of CD137 (Figure 1, 2). Not only do they induce CD137 expression, the combination of these cytokines induces the greatest expression when compared to just one cytokine alone (Figure 1). This suggests that CD137 expression is controlled and maximized by a multitude of activating cytokines involved in immune responses against pathogens. Although NK cells are typically able to respond to activating signals as quickly as 4 hours post stimulation with cytokines, such as IL2 and IL12, or by receptor activation, such as Ly49H and NK1.1, CD137 stimulation by

α-CD137 did not take effect within this short period of time (Figure 3). NK cell derived IFN-γ was increased by α-CD137 stimulation only after day 1 (Figure 3). It is likely that CD137 mediated effects require an extended period of time to take place.

In addition to NK cell IFN-γ production, NK cell death was also observed. This was also reported in the spleen and bone marrow, but with conflicting data regarding the liver [Niu et al., 2007][Lee et al., 2009][Choi et al., 2010]. Although the discrepancy in NK cell populations in the liver suggests a possibility in NK migration, the authors suggested that it was instead due to NK cell death. During α-CD137 treatment with either poly(I:C) or MCMV infection, NK cell populations were decreased in the spleen and liver, as well as in the blood (Figure 10, 12, 14). This suggests that α-CD137 mediated NK cell diminishment occurs in multiple organs, and thus unlikely that the lowered NK cell population is a result of NK cell migration to other organs. NK cells from mice treated with α-CD137 also expressed lower levels of CD11b [Choi et al., 2010]. It is possible that the decrease in CD11b may be linked to the developmental status of NK cells. This is further supported by data showing the suppression of NK cell development in the bone marrow and spleen after α-CD137 treatment, which was dependent on IFN-γ produced by peripheral T and NK cells [Choi et al., 2010]. In vitro stimulation of splenocytes or enriched NK cells with α-CD137 results in significant NK cell death independent of the generation and maturation of new NK cells or IFN-γ. Since CD137 stimulation of peripheral T and NK cells regulate NK cell maturation in the bone marrow through IFN-γ but NK cell death seems to be independent of IFN-γ [Lee et al., 2009][Choi et al., 2010], CD137 regulated maturation and death are likely regulated through different pathways. Furthermore, since CD137 mediated NK development is reportedly mediated mainly by peripheral NK and T cells, it likely takes place once the

adaptive immune response starts to develop. Therefore, it is possible that both mechanisms are involved in controlling NK cell populations upon α-CD137 treatment. However, it is likely that modulation of NK cell development by CD137 occurs during the transition from the innate immune system to the adaptive immune system, and not during the early phase of MCMV infection. As a co-stimulatory molecule, CD137 may rely on the presence of other cytokines and cell interactions to guide immune responses towards different outcomes. It is possible that CD137 mediated death occurs in the absence of specific co-stimulation seen in low inflammatory environments, such as in naïve mice, or very early during infection before which sufficient inflammatory milieu has built up, and CD137 mediated developmental suppression occurs during the transition into the adaptive immune response.

To identify the mechanism behind α-CD137 induced cell death, common death pathways were investigated. Neutralization of Fas ligand did not rescue NK cells from α-CD137 induced death, and NK cells from Fas-lpr mice, which have non-functional Fas receptors, also were unable to survive upon α-CD137 stimulation (Figure 7). ADCC through Fc receptors, as well as action by perforin, also did not play a role in α-CD137 induced NK cell death, as seen by coating α-CD137 antibodies on the bottom of well to prevent cell-cell interactions mediated by antibodies and Fc receptors (Figure 5, 7). Blocking antibodies against TNF, or α-CD137 treatment of TNF knockout mice, did not rescue NK cells from death (Figure 7). The treatment of NK cells from TNFR knockout mice with α-CD137, however, led to levels of death similar to that seen in untreated cells, suggesting that α-CD137 induced NK cell death is dependent on two TNFR receptors: TNFR1 and TNFR2 (Figure 8). The absence of these receptors completely abolishes α-CD137 induced NK cell death to levels comparable to that of control mice. The absence of both receptors was crucial

as the absence of only one did not significantly alter NK populations. The possible cause for this discrepancy is the inability of blocking antibodies to effectively neutralize a sufficient amount of TNF-α in the media. However, this does not explain the presence of α-CD137 induced NK cell death in TNF deficient mice. It is possible that TNF-β, a second ligand to TNFR1 and TNFR2, may serve a redundant role in TNFR signaling, and can cause NK cell death even in the absence of TNF-α. However, due to the unavailability of reagents for working with TNF-β in the mouse system, methods for detection and neutralization are limited. qPCR results show that by 18 hours, neither TNF-α nor TNF-β mRNA levels were significantly altered (Figure 9). By day 2, mRNA levels were decreased, although not statistically significant. The results are representative of total splenocytes and may not accurately reflect the state of NK cells. Currently, it is difficult to determine the role of TNF-β in α-CD137 induced NK cell death. The possibility that TNF-α and TNF-β controls the outcome of α-CD137 stimulation through TNFR signaling remains to be clarified. Although statistically insignificant, TRAF1 and TRAF2 levels are also slightly lowered at 18 hours (Figure 9). These adaptor molecules are crucial mediators downstream of CD137, TNFR1, and TNFR2 signaling, and may contribute to α-CD137 mediated NK cell death. The availability of TRAF molecules and their interaction with downstream proteins may be of key importance in deciding the fate of cell survival or death.

Alterations in NK cell proportions occur in α-CD137 treatment in mice. I have provided further evidence that this is caused by NK cell death from α-CD137 treatment, and that it is dependent on TNFR1 and TNFR2. However, the effects of α-CD137 on NK cells during inflammation are not clear, and the alteration of NK cell proportions in vivo by other groups have only been seen in sterile, in vivo conditions. The effects of α-CD137 treatment

during inflammation was tested by treating mice with poly(I:C) to stimulate TLR3. The stimulation of anti-viral responses through the stimulation of TLR3 by poly(I:C) and treatment with α-CD137 revealed that NK cell proportions were decreased on day 2, even in an induced inflammatory condition (Figure 10). The decrease in NK proportions was observed in the spleen, liver, and blood, further suggesting that in vivo α-CD137 induced NK cell death was due to death and not migration. Ly49H expression in NK cells was decreased with no change in proliferation (Figure 11). This was accompanied by a slight decrease in TNFR1 and significant increase in TNFR2 expression (Figure 11).

The treatment of α-CD137 followed by infection of mice with MCMV revealed similar effects as with poly(I:C) treatment. NK cell proportions were decreased, IFN-γ production was increased, and cytotoxicity was unchanged on day 1.5 of MCMV infection (Figure 12, 13). However, proliferation measured by BrdU expression in NK cells was increased (Figure 13, 15), which conflicts with data from α-CD137 and poly(I:C) stimulation where proliferation measured by Ki67 was not altered (Figure 11). It is possible that Ki67 expression was already saturated in poly(I:C) treated mice, and that further treatment with α-CD137 did not result in a significant detectable increase. BrdU likely allows for a more accurate depiction of the proliferative ability of NK cells than Ki67 due to a higher sensitivity, resulting in the detection of increased proliferation in MCMV infected mice upon α-CD137 treatment. As expected with a decreased NK cell proportion in mice on day 1.5 of MCMV infection, viral burden was increased upon α-CD137 stimulation (Figure 12, 14). Despite the decrease of NK cell proportions on day 1.5 of MCMV infection, they were greatly increased by day 4 (Figure 14). Even though viral burden in α-CD137 treated mice was still higher when compared to the rat IgG treated control group, NK cell

proportions were multiplied immensely by a factor of 4-5 (Figure 14). This is likely due to the overabundance of viral presence. Whether α-CD137 plays a role in the expansion of NK cells in this situation is unclear. This was still accompanied by increased IFN-γ production, high proliferation, and unchanged cytotoxicity (Figure 15).

The pro-survival and proliferative effects of Ly49H may contribute greatly to the fluctuations of the NK cell population during α-CD137 treatment and MCMV infection. Ly49H is a crucial NK receptor against MCMV and is a large factor in determining NK cell expansion during MCMV. Fluctuations in NK cell numbers in response to MCMV has been seen in perforin deficient and Ly49H deficient mice. In Ly49H deficient mice, the NK cell population is drastically reduced compared to B6 control mice during MCMV infection [Lee et al., 2009]. In perforin deficient mice, NK cell populations are increased significantly compared to B6 control mice during MCMV infection [Lee et al., 2009]. In both situations, Ly49H and perforin deficient mice have much higher viral burden in the spleen, likely due to the inability of NK cells to effectively combat MCMV infection. In the case of the Ly49H deficient mice, NK cells lack a method to recognize the m157 glycoprotein of MCMV and cannot receive sufficient activating signals, preventing the proliferation and control of viral replication [Lee et al., 2009]. In perforin deficient mice, the lack of the crucial cytotoxic molecule results in the recognition of MCMV but inability to clear infected cells [Lee et al., 2009]. This causes an immense proliferative signal cause by the uncontrolled viral replication of MCMV, but inability to clear the infection. Consequently, most NK cells in perforin deficient mice were Ly49H+, and the depletion of Ly49H in perforin deficient mice resulted in a reduction of NK cells and proliferation during MCMV infection, highlighting the importance of Ly49H in NK cell expansion [Lee et al., 2009]. Ly49H provides strong

survival and proliferative signals during MCMV infection, and the inability of NK cells to effectively combat MCMV results in an uncontrolled replication of MCMV [Lee et al., 2009].

The initial depletion of the NK cell population during α-CD137 treatment may provide a less than optimal resistance against MCMV (Figure 16). As resistance against the early phase of MCMV is mainly provided by NK cells, this resulted in a serious impairment in the anti-viral response by the day 1.5 of infection, allowing nearly uncontrolled viral replication. Without sufficient NK cells to clear the infection, MCMV could propagate with minimal resistance. Extreme viral progression in turn provided NK cells with an abundant source of the m157 ligand for the Ly49H receptor, greatly increasing signals for survival and proliferation. By day 4, this resulted in a reversal of death in NK cells and promoted the proliferation of the NK cell population to 4-5 fold compared to control groups. Although not examined at further time points, it is highly likely that this leads to the eventual clearance of infection, as there was no suppression of function of individual NK cells. It is unclear whether mice treated with α-CD137 and poly(I:C) would exhibit the same behavior, as poly(I:C) would be cleared from the body with time and does not provide direct ligands for NK cell proliferation. Thus, the fluctuations in the NK cell populations during α-CD137 treated MCMV infected mice is likely the result of a combination of α-CD137 induced cell death and Ly49H-m157 mediated proliferation (Figure 16).

5.2 Role of TNF-α and TNF-β in α-CD137 induced NK cell death

As seen by the rescue of α-CD137 induced NK cell death in mice deficient in TNFR1 and TNFR2, it is possible that the changes in NK cell populations depend on the expression

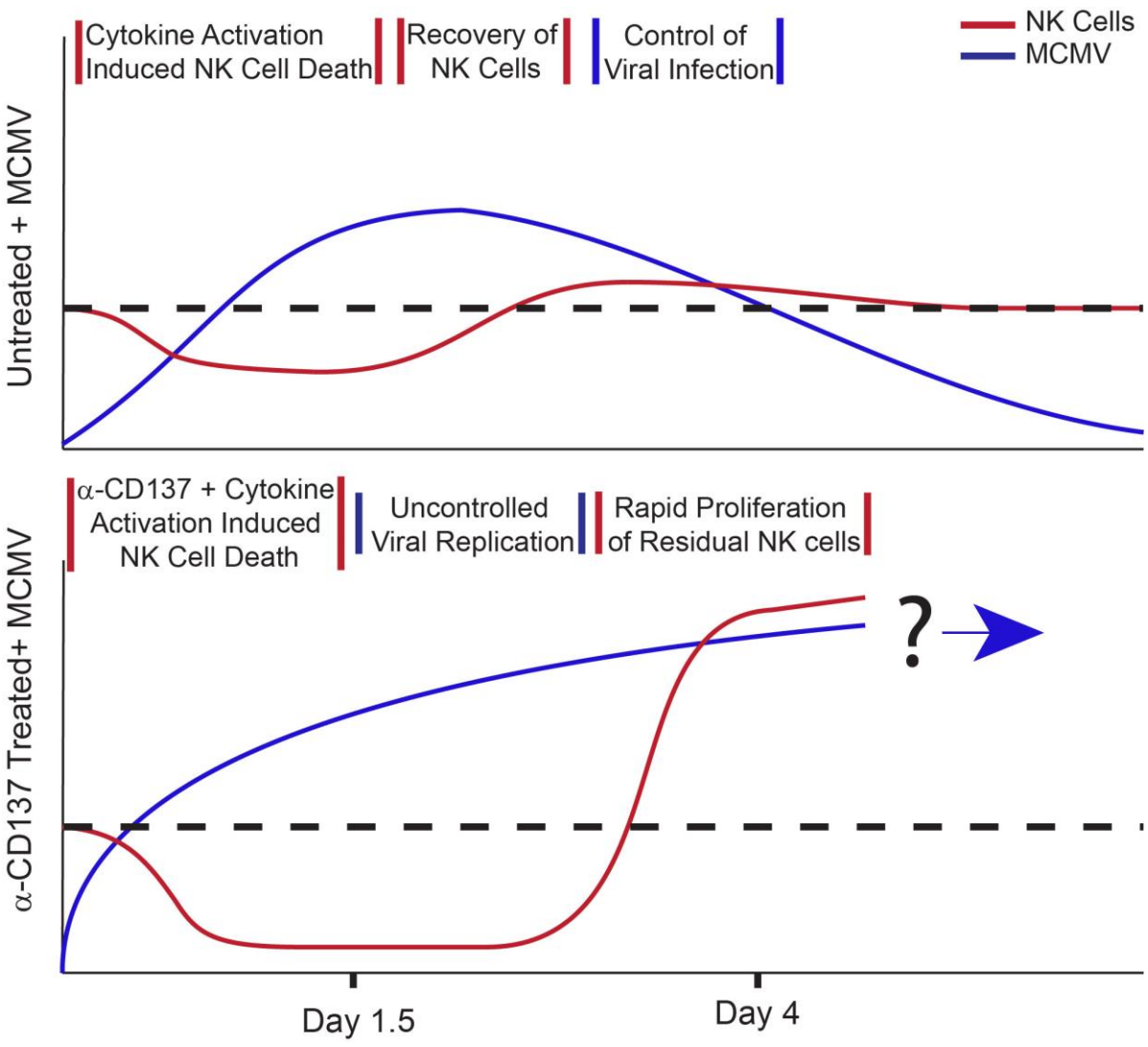

Cytokine Activation Induced NK Cell Death
Recovery of NK Cells
Control of Viral Infection
NK Cells
MCMV
Untreated + MCMV
α-CD137 + Cytokine Activation Induced NK Cell Death
Uncontrolled Viral Replication
Rapid Proliferation of Residual NK cells
?
α-CD137 Treated+ MCMV
Day 1.5
Day 4

Figure 17: Model Representation of the effects of α-CD137 treatment during MCMV infection. Top) NK cells in B6 mice infected with MCMV will undergo a moderate amount of activation induced cell death during the initial phase of the viral infection. As the infection progresses, the residual NK cells will recognize and proliferate to recover their numbers, largely due to Ly49H interactions with MCMV m157 glycoprotein. This increase in NK population will lead to the resistance of MCMV infection and the virus will be cleared. Bottom) NK cells in MCMV infected B6 mice treated with α-CD137 will exhibit increased activation induced cell death. Consequently, viral replication will be uncontrolled by the insufficient NK population, and will drastically increase viral burden in the mouse. The residual NK cells will eventually proliferate robustly in response to the significantly increased viral burden in the mouse, to several fold when compared to untreated mice.

of TNFR1 and TNFR2 on NK cells. In a simplified model, cell survival and activation are controlled by TNFR2 while cell death is controlled by TNFR1. The relative expression of these two receptors may control whether cells undergo death or survival. More TNFR1 expression may lead to cell death while more TNFR2 expression may lead to cell survival. However, there exists a concept of "ligand passing" which suggests that the higher association/dissociation rate of TNFR2 for TNF-α causes an increase in local concentration of TNF-α for TNFR1 receptors [Tartaglia et al., 1993]. This in turn increases TNFR1 and TNF-α binding, promoting a stronger signaling for TNFR1 [Tartaglia et al., 1993]. Additionally, even though TNF-α binds TNFR2, it is a poor activator of the actual TNFR2 pathway compared to membrane bound TNF-α, whereas TNFR1 is activated equally by both [MacEwan 2002 Br J Pharmacol] [Grell et al., 1995]. Thus, soluble TNF-α likely induces a stronger signaling through the TNFR1 pathway rather than TNFR2. Consequently, TNF-α induced by CD137 stimulation and released from the membrane may contribute towards cell death in this manner, as the activation of TNFR1 will be stronger. Membrane bound TNF-α has also been implicated in death. Endogenous production of membrane TNF-α increases upon NF-κB, CD40 and CD30 stimulation and can induce TNFR-1 mediated apoptosis through autotropic and paratropic signaling [Grell et al., 1999].

However, the absence of TNF-α does not seem to affect the induction of cell death NK cells, as they still undergo α-CD137 induced cell death in TNF-α deficient mice. This contrasts with NK cells from TNFR1/TNFR2 double deficient mice where they were rescued from α-CD137 induced NK cell death. This suggests a few possibilities as to how death occurs. First, death may not require TNF-α at all and relies only on TNF-β. Second, death may require only one of TNF-α or TNF-β, with TNF-α and TNF-β serving redundant

purposes in this regard. Third, there is a balance in TNF-α and TNF-β levels that guide TNFR1 and TNFR2 signaling towards either survival or death due to the different binding patterns and affinities of each ligand. In any case, the outcome of cell fate through TNFR1 and TNFR2 binding likely still depends on the expression of the two receptors. However, it is unknown whether the concept of "ligand passing' may apply also to TNF-β. Detecting TNF-β in the mouse system solely with qPCR provides a challenge, and further studies must be done to determine the importance of TNF-β in the role of α-CD137 induced NK cells death. In addition to TNF-α and TNF-β, it is also possible that unknown ligands for TNFR1 and TNFR2 still exist. Even though these receptors are extensively studied, undiscovered ligands remain a possibility. Finally, death may be a result of the interaction of downstream signaling molecules involving TNFR1, TNFR2, and CD137.

5.3 Role of TRAF in TNFR crosstalk

Downstream molecules may interact with each other and create "crosstalk" between TNFR1 and TNFR2 signaling. Even though TNFR2 does not contain death domains, it can still signal for death directly downstream of its signaling. This was first suggested when exclusive TNFR2 stimulation with monoclonal antibodies resulted in the apoptosis of HELA cells, suggesting that there may be crosstalk between TNFR1 and TNFR2 downstream [Bigda et al., 1994]. It is clear that signaling for both receptors share many similarities and it is thought that TRAF and cIAP molecules are important in the crosstalk between these receptors. Even though TRAF2 and cIAP molecules are important for downstream signaling, they have been reported to be depleted upon TNFR2 stimulation [Fotin-Mleczek et al., 2002]. TRAF2 degradation is carried out by ubiquitination by cIAP-1 and proteasomal degradation

[Li et al., 2002]. The depletion of these crucial proteins in the TNFR pathway can prevent TNFR1 from signaling downstream for cell survival and activation, and instead induce caspase-8 activation [Fotin-Mleczek et al., 2002]. Furthermore, TRAF2-cIAP1-2 complexes have been shown to prevent the activation of caspase-8 [Fotin-Mleczek et al., 2002], and in NF-κB null cells, which cannot inhibit caspase-8 activation, the induction of TRAF1, TRAF2, cIAP1, and cIAP2 all together were required to blocked TNF mediated apoptosis suggesting that these proteins are critical in directing TNFR signaling to survival instead of apoptosis [Wang et al., 1998]. In addition, TRAF1 can reverse the repression of TNFR2 on TNFR1 mediated NF-κB activation by protecting TRAF2 from degradation [Wicovsky et al., 2009]. Thus, TRAF and cIAP molecules are key components that determine the outcome of cell survival or death. Insufficient TRAF and cIAP molecules caused by degradation or competitive binding from different TNFRs may be a determining factor in cell death.

Crosstalk between TNFR receptors may not be limited just between TNFR1 and TNFR2. The similarities between the signaling of TNFR2 and CD137 suggest that stimulating CD137 can also lead to the degradation of TRAF2 molecules and possibly the activation of caspase-8 induced apoptosis in a TNFR1 dependent manner. In addition, it may also introduce further competition for TRAF binding to TNFR1 and TNFR2, resulting in the TNFR1 pathway to further signal for cell death. This additional degradation of TRAF2 and competition for TRAF binding introduced by strong CD137 stimulation may be the cause of TNFR1 downregulation and TNFR2 upregulation on NK cells during α-CD137 and poly(I:C) treatment in mice. The strong competition for TRAF and cIAP molecules may lead to the activation of apoptotic pathways through TNF-α mediated TNFR1 activation and crosstalk between TNFR1, TNFR2, and CD137. As the immune system recognizes a need for an

increased inflammatory state to prime cells for anti-viral activity, increased signals for survival such as poly(I:C) on TLR3 or m157 on Ly49H may cause the upregulation of TNFR2 for increased survival and activation signals, and downregulate TNFR1 for decreased death signals. This could be the cause for the increased NK cell populations seen on day 4 of α-CD137 treated and MCMV infected mice. It is known that the levels of TNFR1/TNFR2 expression can be an important determinant of the outcome of TNFR signaling, and it is plausible that the increased ratio of TNFR2:TNFR1 may lead to a balance of TNFR signaling preferring TNFR2 and survival. Many reports show that a decrease or absence of TNFR1 can have anti-apoptotic effects. In a retinal ischemia model, the depletion of TNFR1 prevented neurodegeneration [Fontaine et al., 2002], and an antagonist against TNFR1 blocked apoptosis in HeLa cells, suggesting that the absence of the receptor or signaling can prevent apoptosis [Kontermann et al., 2008]. Although not tested in mice treated with α-CD137 and infected with MCMV, it is likely that TNFR1 is downregulated and TNFR2 is upregulated, similar to what is seen in α-CD137 and poly(I:C) treated mice.

5.4 α-CD137 as a therapeutic

The tumor microenvironment utilizes several mechanisms to combat the immune system. Such methods include the production of TGF-β, regulatory cells, and cytokines in order to suppress NK cell function and proliferation, and modulation of NK cell receptors in order to escape recognition [Guillerey et al., 2016][Viel et al., 2016]. This results in poor recognition of tumors and the inability to clear them effectively. Agonistic α-CD137 antibodies were introduced as a way to improve tumor clearance, even for these poorly immunogenic tumors. Upon treatment with α-CD137 antibodies, “immunological ignorance”

can be broken for multiple tumors including Ag104A sarcoma, C3, TC-1, and B16-F10 [Melero et al., 1997] [Wilcox et al., 2002]. This anti-tumoral effect was demonstrated to be a result of increased T cell and NK cell function [Melero et al., 1997][Xu et al., 2004]. Consequently, monoclonal antibodies such as Urelumab and PF-05082566 are currently in clinical trials as tumor therapies. Monoclonal α-CD137 may provide improvements in adoptive NK cell therapies. A challenge in adoptive NK cell therapies, is maintaining a sufficient number of highly active NK cells after transfer. Highly activated NK cells are unable to survive for an extended amount of time when reintroduced in vivo due to activating cytokine levels. However, administering cytokines such as IL2 provides serious adverse effects in patients. α-CD137 is involved in increasing the proliferation and function of NK cells and may provide a method to ensuring survival upon reintroduction into patients [Wilcox et al., 2002]. However, this poses another problem as it may also lead to NK cell death before they have a chance to contribute to tumor control.

Although α-CD137 treatment has shown favorable effects in terms of anti-tumor therapy, it is apparent that there are situations where it can provide a lapse in the immune system's ability to combat pathogens due to cell depletion. The treatment of α-CD137 antibodies along with an infection with LCMV Armstrong resulted in a suppression of anti-viral responses which was caused by a lack of sufficient T cells [Zhang et al., 2007]. Administration of antibodies early during infection resulted in immune suppression, whereas administration on day 3 or later resulted in viral clearance [Zhang et al., 2007]. This suppression was dependent on DC cells and Fas upregulation on T cells [Zhang et al., 2010]. This shows a parallel between the death of NK cells during MCMV infection up to day 1.5 post infection. α-CD137 injection before MCMV infection results in NK death. Although

the time points for α-CD137 injection is not directly translatable to a viral infection in the context of NK cells, as NK cell activity peaks around day 1.5, it is possible that NK cell death occurs when a low amount of additional survival signals are present. This can explain why the NK cell population is drastically increased by day 4 of MCMV infection. MCMV provides NK cells with a strong anti-viral, proliferation, and activation signals, which results in the high expansion of NK cells. However, it is unclear whether this expansion is due to α-CD137 or solely due to Ly49H stimulation and the abundance of viral particles.

There is evidence that α-CD137 induced cell death may be prevented with added stimulation to cells. NK cells that were isolated from patients with EBV had higher expression of CD137, and EBV-NK cell lines were more resistant to cell death induced by chemotherapeutic agent etoposide when cultured with CD137L expressing CHO-CD137L cells [Yoshimori et al., 2014]. This suggests that stimulation of NK cells by viral infection may provide sufficient activation to prevent α-CD137 induced cell death. Furthermore, the requirement for a combinatory activation signal may be why most successful α-CD137 mediated NK therapies against tumors are combinatory with other monoclonal antibodies against tumor antigens, such as rituximab, cetuximab, and trastuzumab, or other therapies that regulate immune activation and cell survival. Monoclonal antibodies targeting specific tumor antigens bind to tumor cells and activate Fc receptors on NK cells, promoting activation and cytotoxic activity against the tumors. Thus, activation of NK cells through Fc receptors may provide sufficient signals to maintain cell survival in the context of α-CD137 stimulation. In the case of MCMV infection, this additional signal could be the presence of Ly49H. A significantly increased Ly49H signaling resulted in higher survival and proliferation of residual NK cells that initially underwent cell death in a low inflammatory

environment with α-CD137 treatment. It is unclear whether α-CD137 contributes to this expansion of NK cells or if it is just an effect of a higher abundance of Ly49H-m157 interaction. However, it is plausible to think that α-CD137 contributes significantly to NK cell expansion in conjunction with further survival signals. Therefore, α-CD137 treatment can induce strong activation or cell death in NK cells, which may be controlled by the presence or absence of specific activation signals. Although the exact mechanisms through which α-CD137 stimulation induces cell death remains unknown, it is possible that by providing specific activating signals, such as Ly49H, cell death can be prevented and improve NK cell responses during α-CD137 treatment.

6. CONCLUDING REMARKS

Agonistic antibodies towards the co-stimulatory receptor CD137 is a promising candidate for numerous tumor therapies. It has potent effects towards immune activation, increasing proliferation and function, anti-viral and anti-tumor resistance, specifically in T cells and NK cells. However, complications may arise from the administration of these antibodies that include dysregulation of immune cell homeostasis. It is important to understand the mechanism of such adverse effects in order to minimize complications during treatments. α-CD137 induced NK cell death is one such adverse effect that may prove problematic during treatments. I have demonstrated that α-CD137 induced NK cell death is independent of Fas ligand, perforin, and several NK activating interleukins. Although the role of the TNFR1 and TNFR2 ligands TNF-α and TNF-β are still unclear, I have shown that in the case of NK cells, α-CD137 induced NK cells death occurs through both TNFR1 and TNFR2 receptors. Downstream molecules of TNFR signaling, such as TRAF and cIAP molecules, likely play a major role in directing cells towards apoptotic pathways. α-CD137 induced NK cell death has shown to affect viral resistance immensely by altering NK cell populations. α-CD137 treatment caused a diminishment in MCMV resistance resulting in significantly increased viral burden in mice during infection. This was likely due to both a significant decrease in NK cell proportion and a decrease in Ly49H expression on NK cells during the early phase of infection. After 4 days, the abundance of viral particles likely led to the extreme propagation of residual NK cells. Whether this was solely due to increased viral burden and availability of the m157 ligand or also due to α-CD137 treatment remains to be determined. Although it is likely that the increased NK cell proportion in mice will cause a clearance of the infection, it is important to prevent this detrimental alteration of NK cell

populations to decrease complications that may arise in the early part of infections. Not only will increased viral burden be detrimental to the host, an overabundance of immune cells, such as NK cells, may cause collateral damage to the host system. The combination of additional NK stimulation may prove useful in this regard. Combined therapies utilizing blocking antibodies against immune checkpoint molecules referred to as "checkpoint inhibitors" are proving promising. Not only are checkpoint inhibitors against molecules such as PD-1 and CTLA-4 showing anti-tumor efficacy, but in combination with α-CD137, they are showing enhanced efficacy against tumors as well as decreased side effects from each treatment. As α-CD137 therapy undergoes more clinical trials and becomes a possible choice for standard tumor therapy, it is important that the treatment will not compromise the ability of the patients' immune systems to combat infections. To this end, it is imperative to increase the understanding of the full range of effects induced by α-CD137 treatment as well as the mechanism and signaling pathways of these effects. This will not only improve future clinical therapies, but also provide invaluable knowledge of immune signaling regarding the activation and death pathways of immune cells.

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8. CURRICULUM VITAE

Education

Master of Science Microbiology and Immunology
University of Ottawa, Department of Biochemistry, Microbiology and Immunology
Ottawa, ON
January 2015 – 2017

Bachelor in Microbiology and Immunology with a minor in computer science
McGill University, Department of Microbiology and Immunology
Montreal, QC
September 2010 - 2014

Research Experience

Master's Project
University of Ottawa, Department of Biochemistry, Microbiology and Immunology (January 2015 – 2017) Dr. Seung-Hwan Lee Laboratory
Investigation of agonistic anti-4-1BB antibody stimulation on Natural Killer cell function during Murine Cytomegalovirus Infection.

Lab Assistant
McGill University, Department of Natural Resource Sciences (2014) Dr. Brian Driscoll Laboratory
Characterization of Upstream Sequences in lpdA1, lpdA2, and pdhA Operons.
Investigation of non-coding regions in the three operons to test for regulatory sequences in Sinorhizobia meliloti.

Field Assistant, Lab Assistant
McGill University, Biology Department (2012 - 2014) Dr. Virginie Millien Laboratory
Fieldwork: Small Mammal Trapping and Processing in Gault Nature Reserve and other locations in Quebec.
Survey of mice populations to study parasite prevalence in order to assess the prevalence and migration of Borrelia and Lyme disease in Quebec
Preparation of mice skulls for morphometric studies

Lab Assistant
Carleton University, Molecular Plant Biology Lab (2008 - 2010) Dr. Shelley Hepworth Laboratory
Characterization of Floral-Meristem Identity Defects in pan-1 Mutants.
Characterization of Cauline-Leaf "Gap" Phenotype in bop 1 bop 2 Double Mutants.
Characterization of bop 1-4 bop 2-11 Double Mutants.
Characterization and isolation of Blade-On-Pediole (bop) mutants that are expressed specifically in lateral organ boundaries.

Lab Assistant

St. Boniface Hospital, Neurodegeneration Lab/Manitoba Science Academy (2009) Dr. Paul Fernyhough Laboratory
Potential Correlation between Type 2 Diabetes and AMPK Levels in Various Rat Tissues
Comparative study of AMPK levels in diabetic and WT rat sciatic nerves to identify a correlation between AMPK levels and diabetes and diabetes related neurodegeneration.

Lab Assistant
Atomic Energy of Canada Ltd. (AECL), Chalk River Laboratories/ Deep River Science Academy (2008) Dr. Marilyne Stuart Laboratory
The Effects of Environmental Stress on Cellular Growth
Observing Effects of Methylmercury, a byproduct of nuclear power at NRU, on the Growth of Human and Catfish Lymphocytes using various cell growth assays.

Presentations/

1. 29th Annual Spring Meeting of the Canadian Society for Immunology (2016)
   Poster presented: Modulation of natural killer cell function with agonistic a-4-1BB antibody stimulation
2. 5th Annual Symposium on Cytokines in Inflammation, ageing, cancer and obesity (2016)
   Poster presented: Modulation of natural killer cell function with agonistic a-4-1BB antibody stimulation
3. University of Ottawa, Department of Biochemistry, Microbiology and Immunology Poster Presentation Day, 3rd prize.

Awards

- University of Ottawa Biochemistry, Microbiology and Immunology Poster Day 3rd Place (2015)
- Canadian Nuclear Society Award for showing potential in research and development (2008)
- Youth Science and Technology Outreach Program Scholarship (2008)
- Michael Smith Award for top marks for a student registered in a single program (2008)
- Ontario Power Generation Scholarship (2008)