A MORE EFFICIENT KILLING MACHINE: HOW CpG-OLIGODEOXYNUCLEOTIDES ENHANCE NATURAL KILLER CELL CYTOKINE PRODUCTION AND CYTOTOXICITY AGAINST LEUKEMIA INITIATING CELLS

by

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Abstract

Natural killer (NK) cells are lymphocytes that comprise part of the innate immune system and play a key role in the early defence against pathogenic organisms and cancer. CpG oligodeoxynucelotides (ODNs) are short synthetic ODN containing unmethylated CpG dinucleotide motifs that have immune-enhancing effects. NK cell-derived IFN-γ is essential for the effects of CpG ODNs, but how NK cells become activated by CpG ODNs remains unclear. We found that CpG ODN-mediated stimulation of NK cells requires IL-12 or IL-18. CpG ODNs did not stimulate IL-12-deficient mouse spleen cells and IL-12 neutralization almost completely inhibited IFN-γ production. Although IL-18 was undetectable in cultures, neutralization significantly dampened the IFN-γ response and addition of exogenous IL-18 greatly enhanced CpG ODN-mediated NK cell stimulation. IL-12 is mainly produced by Gr-1^+ monocytes and neutrophils, while what cells produce IL-18 remains unknown. We then tested the anti-leukemia effects of CpG ODN-stimulated NK cells. Studies with human acute myeloid leukemia (AML) patients have shown that haploidentical NK cells effectively kill AML blasts, but their ability to lyse leukemia initiating cells (LICs) has not been studied. Therefore, we tested NK cells from haploidentical F1 mice against the mouse AML cell line MN1. F1 mouse NK cells expanded in cultures in the presence of IL-15 and stimulated by CpG ODNs plus IL-18, effectively killed bulk MN1 cells in vitro and reduced the numbers of in vitro colony forming cells. NK cell-treated MN1 cells were also injected into irradiated B6 mice to test whether AML LICs were also killed. F1 mouse NK cells seemed to kill some AML initiating cells since mice receiving NK-treated MN1 cells survived significantly longer than those given untreated MN1 cells, but the frequency of LICs did not significantly differ between MN1 cells incubated with or without NK cells. For NK cells to be used as a

treatment for AML, we must find a way to induce a higher cytotoxicity in NK cells or to target them specifically towards LIC.

Preface

Some results on CpG ODNs stimulation of NK cells in Chapter 3 were sourced from Evette Haddad including Figure 2B and IFN-γ production by purified NK cells stimulated with IL-12 in Figure 3A.

All work on acute myeloid leukemia in Chapter 3 was completed in collaboration with Courteney Lai, a graduate student in the laboratory of Dr. Keith Humphries. The MN1 cell line was created in their lab and generously gifted to us for our work. I was responsible for performing all experiments with guidance and assistance from Courteney Lai.

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AML	acute myeloid leukemia
APC	antigen presenting cell
bDNA	bacterial DNA
BCG	Bacillus Calmette Guerin
BM	bone marrow
BMT	bone marrow transplant
CFSE	carboxyfluorescein succinimidyl ester
CpG ODN	Unmethylated cytosine phosphate guanine oligodeoxynucleotides
CMP	common myeloid progenitor
CR	complete remission
DC	dendritic cell
DLI	donor leukocyte infusions
ELISA	enzyme linked immunosorbent assay
EPO	erythropoietin
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FcR	receptor for Fc portion of antibody
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GITRL	glucocorticoid-induced tumour necrosis factor-related protein
GVHD	graft versus host disease
GVL	graft versus leukemia
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplant
ICAM	intracellular cell adhesion molecule
ICE	IL-1β converting enzyme
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
IL-12R	interleukin-12 receptor
ITAM/ITIM	immunoreceptor tyrosine-based activating/inhibitory motifs
ITD	internal tandem duplication
KIR	killer cell immunoglobulin like receptor
KO	knock out
LIC	leukemia initiating cell
LPS	lipopolysaccharide
LSC	leukemia stem cell
mAb	monoclonal antibody
MDS	myelodysplastic syndrome
MHC	major histocompatibility complex
MN1	meningioma (disrupted in balanced translocation) 1
NCR	natural cytotoxicity receptor
NK	natural killer

NKC	NK gene complex
NKG2D	NK group 2 member D
NKP	natural killer cell progenitor
NKT	natural killer T
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PO	phosphodiester
PRR	pattern recognition receptor
PS	phosphorothioated
qPCR	quantitative PCR
RAG	recombination activating gene
RAR/RXR	retinoic acid receptor/retinoic X receptor
RARE	retinoic acid response elements
SCID	severe combined immunodeficiency
SCT	stem cell transplant
SL-IC	SCID leukemia initiating cell
TCR	T cell receptor
TLR	toll like receptor
TNF-α	tumor necrosis factor-α
WBC	white blood cell
WT	wild type

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To my wonderful husband and family

Chapter 1 Introduction

1.1 NK Cells

Natural killer (NK) cells are lymphocytes that comprise a part of the innate immune system play a key role in the early defence against tumors and pathogenic organisms. They are the third major lymphocyte population, comprising approximately 2.5% of splenic leukocytes in mice. Even though the NK cell population is smaller than the T and B cell populations, large fractions of NK cells are stimulated by activating ligands, unlike T cells that can individually only respond to one antigen. NK cells are naturally cytotoxic, spontaneously killing transformed or infected cells without specific antigen recognition (Yokoyama, Kim, & French, 2004a). They were discovered in 1975 as antigen-non-specific cytotoxic lymphocytes (Kiessling, Klein, Pross, & Wigzell, 1975) and thought to protect the host by rejecting transformed, virus-infected or nonsyngeneic hematopoietic cells (Herberman & Ortaldo, 1981; Hercend & Schmidt, 1988; Trinchieri, 1989). The majority of NK cells are located in the peripheral blood, lymph nodes, spleen, liver, lung and bone marrow (BM), but they can be chemoattracted to inflammation sites (Ferlazzo et al., 2004). The absence of clonally expressed antigen receptors distinguishes NK cells from B- and T-cells. As their development does not require antigen receptor gene rearrangement, NK cells are present in recombination activating gene (RAG) deficient mice, unlike T and B cells (Yokoyama, Kim, & French, 2004a).

NK cells can be identified by combinations of cell surface markers. Since NK cells share many surface markers with various T cell populations, identification markers must exclude T cells (Vivier & Anfossi, 2004). A small population of lymphocytes termed NKT cells complicate the identification of NK cells since many express the NK1.1 epitope commonly used to isolate NK cells (Godfrey, Stankovic, & Baxter, 2010). The phenotypic definition of NK cells is also confounded by the fact that markers are not constant between species. In humans, mature NK cells are defined phenotypically by surface expression of CD56 and not CD3 (Robertson & Ritz, 1990). Human NK cells are heterogeneous and are subdivided by the level of CD56 expression and CD16 expression, with some NK cells being $CD56^{bright}CD16^{-}$ (cytokine producers) and some $CD56^{dim}CD16^{+}$ (cytotoxic). Murine NK cells, however, do not express CD56 (Caligiuri, 2008). Instead, mouse NK cells are defined by $CD3^{-}NK1.1^{+}$ in B6 mice. NK1.1 is one epitope that is shared by the activating receptor NKR-P1C in C57BL/6 mice and the inhibitory receptor NKR-P1B in SJL mice, but other mouse strains do not react with the anti-NK1.1 antibody (Carlyle et al., 2006). DX5 is a widely used pan-NK cell marker recognizing CD49b (α_2 integrin) on NK cells from all common mouse strains and also a small subset of T cells. DX5 can distinguish functional subsets of NK cells; most lymphocytes that express NK1.1 also express DX5, but some weakly cytotoxic $NK1.1^{+}DX5^{-}$ cells do exist (Arase, Saito, Phillips, & Lanier, 2001).

1.2 NK Cell Receptors

The function of NK cells is controlled by an array of germline-encoded inhibitory and stimulatory receptors. Most inhibitory receptors on NK cells are specific to major histocompatibility complex (MHC) class I molecules. The killer Ig-like receptor (KIR) superfamily of inhibitory receptors are expressed on human and primate NK cells, while rodents express inhibitory receptors from the Ly49 family (in the C-type lectin superfamily).

Both primates and rodents also express another inhibitory receptor, CD94/NKG2A, belonging to the C-type lectin family of type II transmembrane proteins (Smyth et al., 2005). NKG2D is a well-characterized activating receptor expressed on human and mouse NK cells, $\gamma\delta^+$ T cells, $CD8^+$ αβ T cells and activated macrophages. Ligands for NKG2D are expressed by stressed, infected and transformed cells and are structurally related to MHC class I molecules. Identified ligands include the MICA/MICB and ULBP proteins in humans and the Rae1 and H60 families in mice (Smyth et al., 2005). NKp46 (NCR1), NKp44 (NCR2), and NKp30 (NCR3) make up the natural cytotoxicity receptors (NCRs) on human NK cells (Moretta, Biassoni, Bottino, Mingari, & Moretta, 2000) and *NKP46* is conserved between human and mouse, making it the only unifying marker for NK cells across mammalian species (Walzer et al., 2007). Ligands for NCRs remain elusive, though the role of NKp46 in tumor immunosurveillance is established and in virally-infected cells, the hemagglutin and hemaglutinin-neuroaminidase molecules of influenza are recognized by NKp46 (Halfteck et al., 2009). NKp30 and NKp46 are expressed on both activated and resting NK cells, while NKp44 is expressed by activated NK and γδ T cells, but not resting NK cells (Biassoni, 2008).

NKDCs are a subset of NK cells expressing NK1.1, B220, CD11c and MHC class II. These cells are also sometimes termed IKDCs for Interferon-producing Killer DC. Originally thought to be a subset of DCs, more recent studies identified NKDCs as a subset of NK cells that are cytotoxic, potent producers of IFN-γ and exert anti-tumor responses in vivo (Chaudhry et al., 2006; Chaudhry, Kingham et al., 2006; Chaudhry et al., 2007).

1.3 Missing Self and Licensing in NK Cells

NK cell cytotoxicity is regulated by a balance of signals from inhibitory and activating receptors. NK cells are negatively regulated by the interaction of MHC class I molecules with inhibitory receptors containing cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Ly49 in mice and KIR in humans) (Lanier, 1998; Raulet, Vance, & McMahon, 2001). Under physiologic conditions, cells are protected from NK cell cytotoxicity by expressing adequate amounts of MHC class I on their surface (Ljunggren & Karre, 1990). During missing self recognition, NK cells recognize and eliminate cells that do not express MHC class I. This theory originated when it was observed that NK cells kill cell lines with no or reduced MHC class I (Karre, Ljunggren, Piontek, & Kiessling, 1986), mediate rejection of allogeneic lymphoma and BM grafts (Karre et al., 1986; Kiessling et al., 1977) and resist parental grafts in an Fl-hybrid mouse (Bennett, 1987; Cudkowicz & Bennett, 1971).

NK cells express multiple inhibitory receptors in seemingly random patterns. Mouse NK cell inhibitory receptors are encoded in the NK gene complex (NKC), including the Ly49 family. Some of the expressed inhibitory receptors do not even recognize any self MHC class I molecules expressed in the host (Fernandez et al., 2005). This is because inhibitory receptors are germ line encoded and expressed by overlapping subsets of NK cells in a stochastic or probabilistic manner (Raulet et al., 2001; Trowsdale, 2001; Yokoyama & Plougastel, 2003). Functional NK cells must express at least one receptor with self-specificity (Raulet et al., 2001), and NK cells in MHC class I-deficient hosts are hyporesponsive. The functionality of NK cells is thought to be achieved by inhibitory receptors interacting with host MHC class I molecules during a process called licensing (Bix et al., 1991; Furukawa et al., 1999; Vitale et al., 2002; Kim et al., 2005). Thus, NK cells are tolerant because licensed cells express inhibitory receptors for self-MHC Class I and unlicensed cells are hyporesponsive.

1.4 NK Cells and IFN-γ Production

The function of NK cells is broader than just cytotoxicity. They play an important role by secreting immunomodulating cytokines such as interferon-γ (IFN- γ) or tumor necrosis factor-α (TNF-α) that promote a T_H1 immune response (Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008). IFNs are separated into two main classes based on structure, function and source. IFN-γ is the sole type II IFN and can be produced by activated CD4 T, CD8 T, γδ T cells, NKT, and NK cells. NK cell IFN-γ production is important for early defence against pathogens, while T cell production of IFN-γ does not occur until days later (Frucht et al., 2001; Sen, 2001). IFN-γ production is mainly induced by the cytokines interleukin (IL)-12 and IL-18 (Akira, 2000; Dinarello, 1999; Fukao, Matsuda, & Koyasu, 2000; Otani et al., 1999), while being negatively regulated by IL-4, IL-10, transforming growth factor-β, and glucocorticoids (Fukao et al., 2000; Hochrein et al., 2001; Schindler, Lutz, Rollinghoff, & Bogdan, 2001; Sen, 2001). An inflammatory immune environment is created by IFN-γ that controls pathogen infection and tumour spread. Mice that are deficient for IFN-γ or its receptor are impaired in their resistance to bacterial, parasitic and viral infections (Buchmeier & Schreiber, 1985; Huang et al., 1993; Kamijo et al., 1993; Pearl, Saunders, Ehlers, Orme, & Cooper, 2001; Suzuki, Orellana, Schreiber, & Remington, 1988; van den Broek, Muller, Huang, Zinkernagel, & Aguet, 1995) and are compromised in tumor rejection (Dighe, Richards, Old, & Schreiber, 1994; Kaplan et al., 1998; Tannenbaum & Hamilton, 2000).

IFN-γ also plays a role in immunosurveillance (Ikeda, Old, & Schreiber, 2002). All these effects are achieved through promoting innate cell-mediated immunity, macrophage activation and upregulation of components of the Class I and II antigen presentation pathways, which increases specific cell-mediated immunity as seen in Figure 1 (Boehm, Klamp, Groot, & Howard, 1997; Mach, Steimle, Martinez-Soria, & Reith, 1996; Schroder, Hertzog, Ravasi, & Hume, 2004).

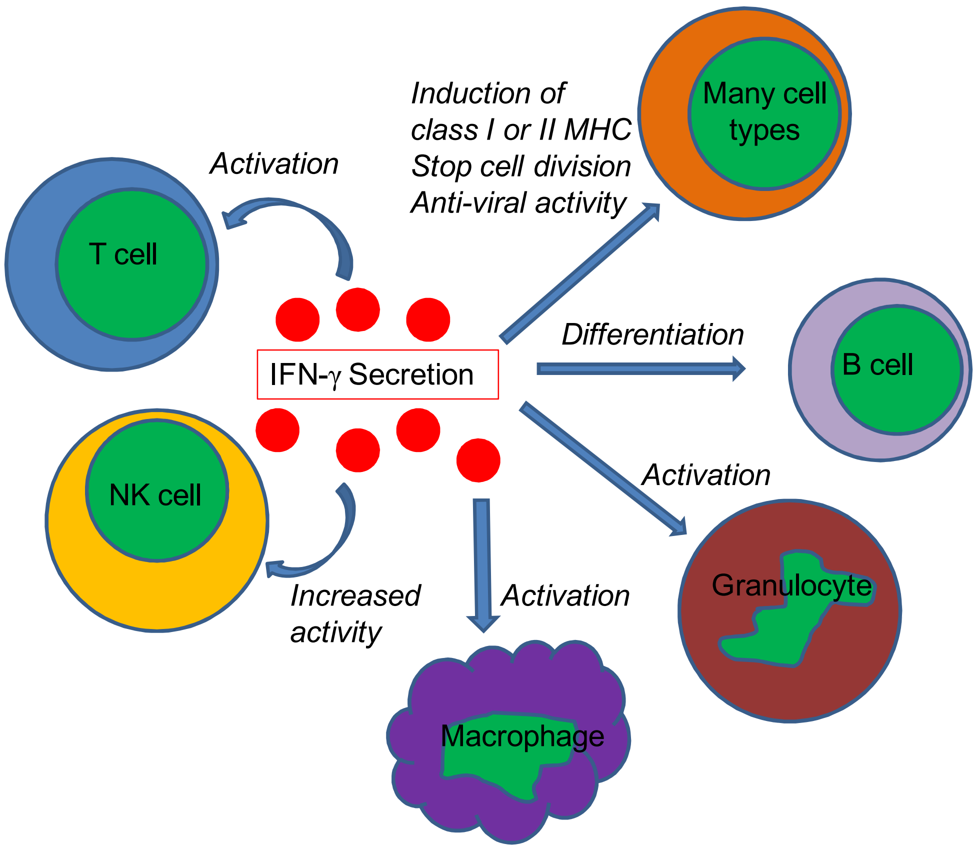


Figure 1. IFN-γ affects the actions of many cell types

IFN-γ is produced by NK and T cells. It activates NK cells, T cells, macrophages and granulocytes; affects the differentiation of B cells and upregulates MHC class I and II on many cell types.

1.5 Cytokines and NK Activation

1.5.1 IL-12

IL-12 is a T_H1 proinflammatory cytokine that has anti-tumor properties and is a strong inducer of IFN-γ production and lytic activity from T and NK cells (Trinchieri, 1995; Trinchieri, 1997). IL-12 is heterodimeric, consisting of two covalently linked chains: p40 and p35; only the p70 heterodimer is biologically active. IL-12 signals through the IL-12 receptor (IL-12R) formed from two subunits: IL-12Rβ1 and IL-12Rβ2 (Presky et al., 1996). IL-12, TNF-α, IL-18, IL-2 and IFN-γ all can control the expression of IL-12R and thus sensitivity to IL-12 (Gately et al., 1998). The main producers of IL-12 are phagocytic cells (monocytes, macrophages and neutrophils) and antigen presenting cells (APCs) such as DCs. Cell-mediated immunity, macrophage activation and production of opsonising immunoglobulin, G2a isotype, is enhanced by IL-12 signalling (Trinchieri & Gerosa, 1996). Resting NK cells express a low amount of IL-12R and require priming with IL-18 to respond (Haddad, Senger, & Takei, 2009). The three major effects of IL-12 on NK cells are to induce cytokine production, proliferation and enhance cytotoxic functions. In addition to its direct stimulatory effects on innate immunity, IL-12 also enhances antigen-specific T cell responses (Schmidt & Mescher, 2002).

1.5.2 IL-18

Interleukin-18 is a member of the IL-1 family of cytokines. It is translated as a pro-cytokine that must be processed by the protease, IL-1β converting enzyme (ICE)/caspase-1, to become active (Arend, Palmer, & Gabay, 2008; Dinarello, 1998). IL-18 mRNA is produced constitutively and ubiquitously by most cells including DCs, macrophages, B cells and neutrophils (Lorey, Huang, & Sharma, 2004; Nakanishi, Yoshimoto, Tsutsui, & Okamura, 2001; Sporri, Joller, Hilbi, & Oxenius, 2008). IL-18 activity is regulated by cleavage with caspase-1 or neutralization by IL-18 binding protein (Arend et al., 2008; Boraschi & Dinarello, 2006). IL-18 was first discovered as a strong inducer of IFN-γ from IL-2 stimulated mouse spleen (Nakamura, Okamura, Wada, Nagata, & Tamura, 1989). Deficiency of IL-18 is associated with impaired NK cell activity (Andoniou et al., 2005; Takeda et al., 1998). Resting NK cells express IL-18 receptor, and IL-18 can augment both murine and human NK cell cytotoxicity (Okamura et al., 1995; Ushio et al., 1996). On the other hand, IL-18 by itself cannot induce IFN-γ production by human NK cells since cytokine production by NK cells requires multiple signals (Carson et al., 1994). IL-18 synergizes with other cytokines, especially IL-12 and acts as a potent co-stimulator (Nakahira et al., 2002). IL-18 is also well known as an enhancer of the production of IL-12 and is thought to be involved in CpG ODN activation. Pre-treatment of mice with CpG ODNs and then stimulation with LPS increases serum IL-18 and IFN-γ production. Blocking of the IL-18 receptor abrogates IFN-γ production, leading authors to conclude that CpG ODNs potentiate NK stimulation by increasing IL-18 availability (Gould, Greene, Bhoj, DeVecchio, & Heinzel, 2004). IL-18 and CpG ODNs together can activate NK cells to release IFN-γ and enhance cytotoxicity, which can prevent the growth of tumors in vivo (Chaudhry, Kingham et al., 2006).

1.5.3 IL-15

IL-15 is critical for NK cell development. Significant levels of IL-15 are produced in the BM where many NK cells develop and mice deficient in IL-15 or IL-15Rα possess very small numbers of NK or NKT cells (Kennedy et al., 2000; Lodolce et al., 1998). The IL-15R complex includes the IL-15Rα, IL-2/IL-15Rβ, and the IL-2R (common) γ (γc) chains. The IL-2/IL-15Rβ-chain (CD122) is expressed on NK cells, cytotoxic CD8 T cells and NKT cells (Waldmann & Tagaya, 1999; Elpek, Rubinstein, Bellemare-Pelletier, Goldrath, & Turley, 2010). IL-15 is “trans-presented” as an IL-15/IL-15Rα complex on the surface of DCs or monocytes that trigger signalling through IL-15Rβ and γc on the target cell (Fehniger & Caligiuri, 2001). IL-15 is not just important for development; it plays an important role in NK cell homeostasis by maintaining antiapoptotic factors (Yokoyama, Kim, & French, 2004b). Soluble IL-15/IL-15Rα complexes are promising agents for tumor immunotherapy. Transient (>14 days) stimulation with these complexes promotes NK cell proliferation and increases their effector functions (Dubois, Patel, Zhang, Waldmann, & Muller, 2008; Elpek et al., 2010; Epardaud et al., 2008).

1.6 Cellular Interaction and NK Activation

The NK response to most pathogens requires contact dependant or independent signals (IL-12, IL-15, IL-18 and type I IFNs) from accessory cells such as DCs, monocytes, macrophages and neutrophils (Newman & Riley, 2007). These cells express many pattern recognition receptors (PRRs) that recognize certain molecular structures called pathogen-associated molecular patterns (PAMPs) that are present on pathogens, but not on self (Dempsey, Allison, Akkaraju, Goodnow, & Fearon, 1996; Kumar et al., 1997). Toll-like receptors (TLRs) are type I transmembrane proteins whose structure is conserved between insects and mammals (Anderson, 2000). TLR’s are able to recognize bacteria, viruses and fungi, but do not recognize multicellular parasites (Kopp & Medzhitov, 2003). The signalling molecule, MyD88, transduces signals from many TLRs and is required for cells to produce inducible inflammatory cytokines like IL-12, TNF and IL-6 (Kopp & Medzhitov, 2003).

1.6.1 Dendritic Cells

DCs are innate antigen presenting cells that patrol the periphery searching for pathogens, pick up antigens and process them for presentation. When they are activated by the presence of pathogenic antigens, they mature by upregulating MHC class II and co-stimulatory proteins and travel to the lymphoid organs to secrete chemokines and cytokines. DCs are mainly identified by their high expression of the β-integrin $CD11c^+$, which some other cells, including NK cells, B cells, macrophages, and neutrophils only express at much lower levels. There are three main types of DCs: the $CD8^-$ classic myeloid tissue DCs (Steinman & Cohn, 1973), the $CD8^+$ plasmacytoid pDCs that reside in T cell zones and make IFN-α in response to CpG ODNs or Poly I:C stimulation (Vremec et al., 1992) and the Langerhans-derived DC that are specialized for the epidermis (Steinman, Pack, & Inaba, 1997). Activated mDCs produce IL-12, IL-6 and TNF-α while pDCs secrete high levels of IFN-α (Vasilakos et al., 2000). IL-12 from DCs induces NK cell IFN-γ production, which creates a positive feedback loop that supports T_H1 cell mediated immunity (Martin-Fontecha et al., 2004). Tight synapses form between DCs and NK cells *in vitro* (Borg et al., 2004) to allow membrane bound receptors and ligands to pair for contact dependant signals and to directly deliver cytokines, such as the presentation of IL-15 by DCs to NK cells (Koka et al., 2004; Walzer, Dalod, Robbins, Zitvogel, & Vivier, 2005).

1.6.2 Monocytes/Macrophages

Monocytes are mononuclear leukocytes that develop in the BM, are released in the bloodstream as non-dividing cells and later enter tissues to become macrophages. As they express many PRRs, monocytes are a target for adjuvants. Blood mononuclear cells express CD11b, CD11c, and CD14 in humans and CD11b and F4/80 in mice with a lack of B, T, NK, and DC markers. The immune response is maintained by the mutual activation of NK cells and monocytes, making both important mediators of inflammation (Welte, Kuttruff, Waldhauer, & Steinle, 2006). Monocytes also mediate terminal NK cell differentiation. Mutant mice that are deficient for the transcription factor T-bet lack $CD11b^{hi}CD27^{low}$ NK cells since T-bet deficient monocytes are unable to provide IL-15Rα-dependent support to the transition of $CD11b^{hi}CD27^{hi}$ NK cells to terminally differentiated $CD11b^{hi}CD27^{low}$ NK cells (Soderquest et al., 2011). Splenic NK cells reside in the red pulp of the spleen that is also the home of a reservoir of mouse monocytes (Swirski et al., 2009).

Monocytes are functionally and phenotypically heterogeneous. Geissmann *et al.* write "Monocyte subsets are notoriously pleomorphic and able to change surface markers and phenotypic characteristics because of local conditions" (Geissmann et al., 2008). There are two main subsets: the short-lived "inflammatory subset" ($CX3CR1^{lo}CCR2^{+}Gr1^{+}$) that homes to inflamed tissue, where it can trigger immune responses and differentiate into DCs *in vitro* and the "resident subset" ($CX3CR1^{hi}CCR2^{-}Gr1^{-}$) with a longer half-life, that home to noninflamed tissues and participates in surveillance (Auffray et al., 2007; Geissmann, Jung, & Littman, 2003). During infection, $Gr\text{-}1^{+}$ ($Ly6C^{+}$) blood monocytes move from BM to the bloodstream and differentiate via a MyD88-dependent mechanism into cells that produce TNF-α, IL-12, iNOS and upregulate major histocompatibility complex-II antigens, CD80, CD86 and CD11c (Dunay et al., 2008; Robben, LaRegina, Kuziel, & Sibley, 2005; Serbina et al., 2003; Tsou et al., 2007).

1.6.3 Neutrophils

Neutrophils are well known as phagocytic cells. They are usually the first-responders in an infection and establish foci of inflammation (Mayer-Scholl, Averhoff, & Zychlinsky, 2004; Segal, 2005). A high number of neutrophils circulate in peripheral blood, making up 50-70% of white blood cells (WBCs) (Witko-Sarsat, Rieu, Descamps-Latscha, Lesavre, & Halbwachs-Mecarelli, 2000). Stimulated neutrophils are capable of producing proinflammatory cytokines (TNFα, IL-1β, chemokines), anti-inflammatory cytokines (IL-1ra, TGFβ), immunoregulatory cytokines (IL-12p70, IL-18, mouse only IL-10, mouse only IFN-γ) and angiogenic factors. The amounts of immunoregulatory cytokines that neutrophils produce are less on a per cell basis than DCs or monocytes/macrophages (Cassatella, 1999; Fortin, Ear, & McDonald, 2009; Yin & Ferguson, 2009), but are still significant considering the number present in the body. IL-12 can be found as preformed proteins in neutrophils and are likely released soon after stimulation (Bennouna & Denkers, 2005; Mason, Aliberti, Caamano, Liou, & Hunter, 2002; Matzer, Baumann, Lukacs, Rollinghoff, & Beuscher, 2001). Neutrophils are short-lived (8-20 hours), but their survival is increased if cytokines (IFN-γ, GM-CSF, G-CSF, TNF-α) or microbial products (LPS, PGs, CpG DNA) are present (Luo & Loison, 2008). The ability to make all these products enables neutrophils to play roles in regulating inflammatory, immune, angiogenic, hematopoietic, wound-healing, antiviral, and antitumoral responses (Cassatella, Locati, & Mantovani, 2009; Nathan, 2006; Soehnlein, 2009). They can also regulate the activity of DCs (Boudaly, 2009), macrophages

(Silva, 2010), NK cells (Costantini & Cassatella, 2011) and T cells (Muller, Munder, Kropf, & Hansch, 2009).

Neutrophils act as accessory cells regulating IFN-γ production from NK cells in many mouse infection models including *B. Pseudomallei* (Easton, Haque, Chu, Lukaszewski, & Bancroft, 2007), *M. Tuberculosis* (A. M. Cooper, Solache, & Khader, 2007), *H. Influenzae* (Miyazaki et al., 2007) and *L. Pneumophila* (Denkers, Butcher, Del Rio, & Bennouna, 2004). During *L. Monocytogenes* and *Candida albicans* challenge, IL-12 from neutrophils is thought to be the key cytokine inducing IFN-γ (Emoto et al., 2003). However, all the above neutrophil depletion studies used the mAb, RB6-8C5, which depletes other Gr-1 expressing cells such as inflammatory monocytes.

1.7 CpG ODN and NK Cell Activation

1.7.1 Bacterial DNA

Pattern recognition receptor (PRR) pathways are valuable as they can be manipulated for cancer or infection therapy and to prevent undesirable immune activation. Bacterial DNA (bDNA) is one such manipulator that has long been studied for therapeutic potential. The immune system recognizes bDNA by the presence of a high frequency of unmethylated CpG dinucleotides (1:16 are unmethylated) while vertebrate DNA has very few (1:60 are unmethylated) (Krieg et al., 1995). Vertebrate DNA also has other undefined inhibitory effects since its presence will abolish the stimulatory effect of bDNA (Chen et al., 2001) and artificially unmethylated vertebrate DNA is not stimulatory (Sun, Beard, Jaenisch, Jones, & Sprent, 1997). One of the first utilizations of the immunostimulatory activity of bDNA was Coley's toxin, a bacterial vaccine, to combat inoperable sarcoma (Wiemann & Starnes, 1994). Extracts of an attenuated mycobacteria bacillus, Calmette Guerin (BCG), became standard therapy for human bladder cancer in the 1970's (Morales, 1978) and it was later discovered that the active component for NK activation was DNA (Tokunaga et al., 1984).

1.7.2 Structure of CpG ODN

CpG ODNs are short synthetically produced DNA containing cytosine-guanine dinucleotide motifs. These are synthetic mimics of bacterial DNA and activate a T_H1 immune response, which is dependent on the production of IL-12 (Klinman, 2004; Krieg, Love-Homan, Yi, & Harty, 1998). The stimulatory effects depend on the number and spacing of unmethylated CpG dinucleotides (Kuramoto et al., 1992), the bases flanking it (Hartmann & Krieg, 2000; Rankin et al., 2001), poly G sequences and backbone composition (Krieg, 2002). The normal phosphodiester (PO) DNA backbone is easily degraded (Zhao et al., 1993), but the modified phosphorothioated (PS) backbone is nuclease resistant (Stein, Subasinghe, Shinozuka, & Cohen, 1988) and readily binds to plasma membranes, increasing uptake (Zhao et al., 1993; Zhao, Waldschmidt, Fisher, Herrera, & Krieg, 1994). However, the PS backbone has less NK cell and macrophage stimulatory capability (Ballas, Rasmussen, & Krieg, 1996; Boggs et al., 1997).

1.7.3 Types of CpG ODN

Based on structural attributes, stimulatory targets and biological effects, synthetic CpG ODNs are categorized into three types: A, B and C. Because of the high variation between molecules with CpG motifs, they should each be considered a separate agent. Type-A CpG ODNs are characterized by a central palindromic region of unmethylated CpG motifs on a phosphodiester backbone that is capped at each end with a phosphorothioate poly-G string.

The best A-type CpG ODNs have PS modified ends with a PO center (Ballas et al., 1996). This type of CpG ODN can activate pDCs to secrete IFN-α (in humans and mice), stimulate IL-12 from macrophages (in mice only) and activate NK cells (Ballas et al., 1996; Kadowaki, Antonenko, & Liu, 2001; Krieg, Matson, & Fisher, 1996; Krug et al., 2001). The poly G motifs enhance uptake and activation of human NK cells to produce IFN-γ (Ballas et al., 1996; Kimura et al., 1994). Type-B CpG ODNs have a PS backbone with one or more CpG dinucleotides and no poly-G strings (Krieg, 2001). These are particularly good at activating B cell proliferation, maturation and secretion of IgM, while weakly stimulating IFN-α from pDCs. Type-B CpG ODNs strongly stimulate IL-12 production (Ballas et al., 1996; Boggs et al., 1997; Stacey, Sweet, & Hume, 1996). The newly described type-C CpG ODNs are structural and functional hybrids between A and B and elicit high levels of IFN-α, strong B cell stimulation and efficient secretion of IFN-γ. All types induce secretion of IL-6 (Vollmer et al., 2004).

1.7.4 Toll-like Receptor 9

CpG ODNs must be taken up by the cell to be recognized (Krieg et al., 1995). As CpG ODNs are large polyanions, they cannot diffuse across the plasma membrane, so uptake is an active process that is competable, saturable and sequence independent (except for the presence of poly G sequences) (Hacker et al., 1998; Krieg et al., 1995; Yamamoto, Yamamoto, Kataoka, & Tokunaga, 1994). TLR9 resides within endosomes and binds unmethylated viral and bacterial DNA and synthetic CpG DNA (Chuang, Lee, Kline, Mathison, & Ulevitch, 2002; Hemmi et al., 2000; Latz et al., 2004). TLR9 activation alters the NFκB pathway (El Kebir, Jozsef, & Filep, 2008). In humans, B cells, neutrophils, plasmacytoid DCs (pDCs) and NK cells express TLR9 (Hornung et al., 2002; Roda, Parihar, & Carson, 2005; Zamai et al., 2007). In mice, many more cell types express TLR9 including macrophages, myeloid DC, pDC and neutrophils (Akira, Takeda, & Kaisho, 2001; Gururajan, Jacob, & Pulendran, 2007; Tsuda et al., 2004). To date, TLR9 protein has not been found in murine NK cells.

1.7.5 NK Cell Activation

NK cells are activated by CpG ODNs to become cytotoxic and secrete high levels of IFN-γ (Cowdery, Chace, Yi, & Krieg, 1996), but the same NK cell may not necessarily engage in both cytokine secretion and cytotoxicity (Ballas et al., 2001). B and T cells are not necessary for NK cell activation by CpG ODNs, yet CpG ODNs do not stimulate highly purified NK cells, thus other assistance is required (Ballas et al., 1996; Ballas et al., 2001). Purified NK cell IFN-γ production requires either the presence of adherent cells, CpG ODN-conditioned supernatants or IL-12, type I IFNs and TNF-α (Ballas et al., 1996; Cowdery et al., 1996). Administration of CpG ODNs with doses of IL-12 induce activation marker expression on NK cells, induce IFN-γ and TNF-α production and increase cytotoxicity (Sivori et al., 2004), while neutralization of IL-12 inhibits the NK cell response (Chace, Hooker, Mildenstein, Krieg, & Cowdery, 1997). However, many studies looking into CpG ODN-induced activation of NK cells do not agree with these findings and are complicated by differences between types of ODN, whether murine or human NK cells were used and whether cytotoxicity or cytokine production was measured. Unfortunately, most studies of NK cell stimulation used B-type ODN's that do not induce as much IFN-γ production as A-type ODNs. IL-12 as a key cytokine in stimulation is agreed upon in many instances. DCs activated by type-B CpG ODN or bDNA can produce IL-12 (Blackwell & Krieg, 2003; Krug et al., 2001; Persson & Chambers, 2010; Schulz et al., 2000; Sparwasser et al., 1998), but monocytes and macrophages are also able to produce IL-12 in response to CpG ODN, especially when primed with IFN-γ (Chace et al., 1997; Cowdery et al., 1996; Cowdery, Boerth, Norian, Myung, & Koretzky, 1999; D'Andrea et al., 1992; Sparwasser et al., 1998; Sweet, Stacey, Kakuda, Markovich, & Hume, 1998). In one study, NKDCs (a subset of NK cells) were found to be the source of IL-12 when NK cells were stimulated with CpG ODNs and IL-18 (Chaudhry, Kingham et al., 2006). IFN-α production from DCs appears to be especially important for NK cell cytotoxicity (Blackwell & Krieg, 2003; Krug et al., 2001; Schulz et al., 2000) and some suggest that CpG ODN-induced IFN-γ from NK is primarily dependant on IFN-α from pDCs (Marshall, Heeke, Abbate, Yee, & Van Nest, 2006). In addition to cytokines, murine macrophages can produce many chemokines including MIP-1α, MIP-1β, RANTES, JE/MCP-1, and IP-10 when stimulated with CpG ODNs (Takeshita, Takeshita, Haddad, Ishii, & Klinman, 2000). Few studies have looked into the role of neutrophils. One study found that CpG-B ODNs can indirectly activate mouse neutrophils. When exposed to bDNA, trafficking, chemokine expression, adhesion molecule expression and phagocyte activity of neutrophils are promoted. Thus they may be an underappreciated accessory cell (Weighardt et al., 2000).

1.8 Therapeutic Applications of CpG ODNs

The anticancer effects of infections have been observed for a long time. Immunosurveillance is mediated by IFN-γ and lymphocytes that patrol the body searching for altered cells that could cause cancer. In this way, tumours are suppressed before they become a problem

(Shankaran et al., 2001). Since CpG ODNs increase IFN-γ production and lymphocyte activation, it may enhance this process. Treatment with CpG-B ODNs prevent disease progression in 75% of c-Myc transgenic mice that express high levels of Myc protein in B cells and usually sicken within four months of life from lymphoma (Wickstrom, 1997). CpG ODNs have been experimentally and clinically tested as adjuvants against cancer, microbial infection, allergy and asthma (Klinman, 2004; Vollmer & Krieg, 2009; Weiner, 2009). However, the clinical application of CpG ODNs is often much less successful than would be predicted from animal studies. A common argument for this effect is that TLR9 is differentially expressed in DCs between mice and men and human mDCs express low or no TLR9. However, one study has found comparable expression of TLR9 in DCs from mice and humans, so it remains unclear why CpG ODNs are not as successful a treatment in humans as in mice (Hoene, Peiser, & Wanner, 2006). In lymphoid malignancies, CpG ODNs can induce activation induced cell death that affects B cell malignancies very well (Weiner, 2009). Type-A CpG ODNs exert a profound NK activation and antitumor effect (Dow et al., 1999); systemic or local therapy protects C57BL/6 mice from a lethal challenge with B16 melanoma cells independently of T or B cells (Ballas et al., 2001). However, most studies still use type-B CpG ODNs even though A is very effective (Lipford, Sparwasser, Zimmermann, Heeg, & Wagner, 2000; Sparwasser, Vabulas, Villmow, Lipford, & Wagner, 2000).

1.9 Acute Myeloid Leukemia

AML is a heterogeneous clonal disorder marked by the accumulation of undifferentiated myeloid blasts with self renewal capabilities (Gilliland, 2001). Subtypes of AML are classified based on morphology, immunophenotype, cytogenic and molecular alterations. Genetic alterations are the basis for differentiated risk stratification. A favourable prognosis gives the realistic change of a cure with standard radiation and chemotherapy treatments, while for a high relapse risk prognosis, the best choice is allogeneic hematopoietic stem cell transplantation (HSCT) (Bacher et al., 2009). While the first treatment for AML is radiation for cytoreduction, all leukemic cells cannot be killed by radiation as it kills via first order kinetics. Increasing radiation doses will reduce the risk of relapse, but survival rate does not increase due to treatment-related mortality, making the need for alternative therapies high.

1.10 MN1

Meningioma 1 (MN1) is a 150 KDa nuclear protein that is highly conserved among vertebrates and has no homology to other proteins (Lekanne Deprez et al., 1995). MN1 locates to retinoic acid response elements (RAREs) and is a transcription cofactor of the retinoic acid receptor/retinoic X receptor (RAR/RXR) complex (van Wely et al., 2003). A common fusion partner of TEL in t(12;22) AML, MN1 is a single driver oncogene and oncoprotein that blocks myeloid differentiation (Buijs et al., 1995). High levels of this protein can cause leukemia initiation. In addition, MN1 is a collaborator protein that can enhance the leukemogenic activity of other mutations (Heuser et al., 2007). The *MN1* gene is also a target of reciprocal chromosome translocation (12;22)(p13;q12) in some AML patients (Buijs et al., 2000) and is overexpressed in AMLs specified by inv(16) (Ross et al., 2004; Valk et al., 2004). When MN1 is overexpressed in patients with AML with normal cytogenetics, it is a negative prognostic factor (Heuser et al., 2006). The presence of MN1 overexpression causes a rapid myeloproliferative disease that is oligoclonal rather than polyclonal, so additional genetic changes must occur for disease development (Carella et al., 2007). There are very few mouse AML cell lines in existence and this has been very detrimental in the past to performing NK cell studies in mice.

1.11 AML Stem Cells

Six hallmark properties define malignant populations: independence from outside growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion (Hanahan & Weinberg, 2000). The idea of cancer stem cells that act to populate cancers was postulated decades ago (Clarkson, 1969; Dick, 2008). We now know that for many cancers, growth and spread relies on a subset of cancer cells with enhanced self-renewal at the apex of a cellular hierarchy, and differentiation of these cells results in heterogeneity within the cancer (Dick, 2008; Guzman et al., 2007). Only a subset of primary human AML cells is able to engraft recipient mice and confer leukemia (Deshpande et al., 2006; Kirstetter et al., 2008; Lapidot et al., 1994; Somervaille & Cleary, 2006). To measure leukemia stem cell potential, leukemia-initiating cells (LICs) that initiate leukemia upon transplantation are quantified by limiting dilution transplantation (Heuser et al., 2009). Many believe that leukemia stem cells (LSCs) originate from the hematopoietic stem cell (HSC) pool rather than the committed progenitor pool (Bonnet & Dick, 1997), but the process of leukemogenesis disrupts cell differentiation so this link is hard to prove. AML LSCs are hierarchically organized with heterogeneity in the lifetime of the clones and self-renewal capacity, which is similar to normal HSCs. The initial transformed cell is likely in the HSC compartment as both the LSC and HSC compartments are structured the same (Hope, Jin, & Dick, 2004). The leukemic hierarchy is continuously replenished by rare LSCs (Wang & Dick, 2005). Normal HSCs are in the Lin^- $CD34^+CD38^-$ fraction (Bhatia, Wang, Kapp, Bonnet, & Dick, 1997; Conneally, Cashman, Petzer, & Eaves, 1997; Larochelle et al., 1996) and the $CD34^+CD38^-$ fraction of human AML samples are highly enriched for SCID leukemia initiating cells (SL-ICs), further supporting similarities in HSCs and LSCs (Lapidot et al., 1994; Rombouts, Martens, & Ploemacher, 2000). However, recent studies have found AML leukemia initiating cells (LICs) to be rather heterogeneous in phenotype and not all LICs are similar to normal HSCs (Hays, 2009). An alternative hypothesis to the transformation of HSCs into LSCs is that differentiated cells attain limitless proliferative capacity, such as when human translocation products are overexpressed in murine committed progenitors and AML develops (Cozzio et al., 2003; DiMartino et al., 2002; Krivtsov et al., 2006). In MN1-driven leukemia's, common myeloid progenitors (CMPs) were identified as the cells of origin and susceptibility of the cells to MN1-induced transformation was determined by the activity of MEIS1 and AbdB-like HOX protein complexes that play a role in haematopoiesis and development (Heuser et al., 2011).

Only a minor proportion of AML blasts are clonogenic progenitors (Griffin & Lowenberg, 1986; McCulloch, 1983) and only 1/250 000 $CD34^+CD38^-$ AML cells are LICs in the SCID-Hu mouse model (Lapidot et al., 1994; Hope et al., 2004) just like all normal clonogenic progenitors are not HSCs (Larochelle et al., 1996). Clonogenic progenitors rapidly proliferate while LSCs are slow dividers (Guan & Hogge, 2000; Guzman et al., 2001; Hope et al., 2004). LSCs can be selected from HSCs by CD123 (Frankel, Liu, Rizzieri, & Hogge, 2008; Jordan et al., 2000), CLL-1 (Moshaver et al., 2008), CD44, CD96, CD32 and CD25 expression (Hosen et al., 2007; Jin, Hope, Zhai, Smadja-Joffe, & Dick, 2006; Saito et al., 2010). Effective therapy must target the highly self-renewing long-term SCID leukemia initiating cells (SL-ICs) that are quiescent (Hope et al., 2004). Finding the origin of the LSC population would improve our understanding of the biology of the LSC which would explain why current therapies are often ineffective and lead us to better agents to eradicate AML.

1.12 Allogeneic Hematopoietic Stem Cell Transplant

Immune-based cancer treatments are primarily a means of sustaining remission and are best utilized after the disease has been bulk-reduced with chemotherapy. Allogeneic stem cell transfer (SCT) is the only real 'curative therapy' available for AML patients with a high risk of relapse. These are patients who exhibit unfavourable prognostic features at diagnosis, do not achieve complete remission (CR) after first induction cycle or are in their second/later CR (Burnett et al., 2002). The decision to undergo allogeneic SCT depends on disease risk and donor availability. A human leukocyte antigen (HLA) identical or matched unrelated (10/10 HLA match) SCT is recommended for a first remission with unfavourable cytogenetics or the presence of FLT3 or MLL mutations (Dohner et al., 2010). During late first remission or advanced disease, allogeneic SCT becomes an option (Ljungman et al., 2010). However, patients not in remission receiving SCT have a higher post-transplant risk of relapse, progression and death (Ciceri et al., 2008; Lang et al., 2004; Marks et al., 2006).

Seventy-five percent of patients do not have an HLA identical sibling. The genes for HLA are closely linked and inherited as haplotypes, so two siblings have a one-in-four chance of being identical. More people have a haploidentical relative than an HLA identical sibling and the immediate availability of a haploidentical donor makes allogeneic SCT an attractive option. Having a family member donor is associated with improved leukemia-free survival (LFS) (Ciceri et al., 2008). Other options include matched unrelated donors and unrelated umbilical cord-blood transfers. Post-treatment relapse still remains a major challenge. The relapse rate depends on many factors including remission status at transplant time, presence of FLT3 internal tandem duplication (ITD) mutation, poor-risk cytogenetics, underlying myelodysplastic syndrome (MDS) and treatment-related variables (Parmar, Fernandez-Vina, & de Lima, 2011). A second allogeneic SCT, after relapsing from a first SCT, has high morbidity, mortality and relapse rates with only 25% long-term disease-free survival (Arcese et al., 1993; Radich et al., 1993).

1.13 Graft Versus Host Disease and Graft versus Leukemia in Allogeneic Stem Cell Transplant

Initially, allogeneic SCT was not desirable due to problems with poor engraftment and high rates of graft versus host disease (GVHD) (Anasetti & Hansen, 1994; Beatty et al., 1985). GVHD is an affliction mainly caused by T cells that especially affects skin, intestines and liver (Korngold & Sprent, 1987; Ruggeri, Aversa, Martelli, & Velardi, 2006). A two-step cycle induces GVHD: conditioning-induced tissue damage leads to activation of recipient APCs that present recipient alloantigens to donor T cells, donor $CD4^+$ T cells then expand and release cytokines (TNF-α, IL-2, IFN-γ) that cause tissue damage and promotes differentiation of cytotoxic $CD8^+$ T cells which leads to more tissue damage (Ruggeri et al., 2006). GVHD is treated with immunosuppressants (Schleuning et al., 2009) or *ex vivo* expanded T regulatory cell infusions (Brunstein et al., 2011). In the past, HLA polymorphism and restrictions of serologic HLA-typing limited matching accuracy, and rates of rejection and GVHD were high (Szydlo et al., 1997). Now, DNA-based techniques for high-resolution matching reduce rejection and GVHD, but make it more difficult to find matches even though mismatches are more tolerated (Petersdorf et al., 2001). With current optimized conditioning regimens and improved graft selection, stable engraftment is the norm and GVHD is reduced (Aversa et al., 2005; Henslee-Downey et al., 1997; Henslee-Downey et al., 1997). T cell depletion by $CD34^+$ immunoselection especially improves outcomes (Klingebiel et al., 2010), but immune reconstitution takes longer (Ball et al., 2005; Seggewiss & Einsele, 2010). In a phase II study of T cell-depleted fully haplotype-mismatched HSCT, high engraftment rates and low GVHD led authors to conclude that haploidentical HSCT is a viable alternative source of stem cells for AML patients without matched donors (Aversa et al., 2005).

In addition to being more readily available, donor allogeneic cells exert a graft-versus-leukaemia (GVL) effect, where donor T and NK cells suppress and eliminate leukemia cells remaining after cytoreduction (Barrett, 2008). In one early experiment, leukemic mice were given either syngeneic or allogeneic marrow transplants. Mice that received syngeneic marrow relapsed and died, but those given allogeneic marrow did not relapse through a "process of immunity" (GVL). These same mice developed a "wasting syndrome" (GVHD) as well and the first link between GVL and GVHD was demonstrated (BARNES & LOUTIT, 1957). Anecdotal reports of CR being achieved after a flare of GVHD (Odom et al., 1978) or withdrawal from immunosuppressants also supports this link (Collins et al., 1992; Higano et al., 1990). The mismatch between inhibitory receptors for self-MHC class I molecules on donor NK clones and MHC class I ligands on recipient cells creates donor vs. recipient NK cell alloreactivity.

The presence of alloreactive NK cells correlates with improved survival after SCT as they promote engraftment, reduce GVHD and decrease relapse by killing remaining leukemic cells and recipient immune cells (Giebel et al., 2003; Ruggeri et al., 2002). The first direct evidence for GVL in humans was in a patient with relapsed CML that achieved CR after allogeneic bone marrow transplant (BMT) and IFN-α treatment (Kolb et al., 1990). Early "hybrid resistance" experiments performed in mice help us understand GVL and GVHD in HSCT; cells from F1 mice reject parental BM, but tolerate parental skin and organ grafts (Yu, Kumar, & Bennett, 1992). It was thought that nonhematopoietic tissues lack ligands to bind and activate NK cells. In mice, pretransplant infusions of alloreactive NK cells reduce GVHD so much that no high-intensity conditioning is needed (Ruggeri et al., 2002). This is because NK cells kill recipient DC that initiate GVHD by presenting host alloantigens to donor T cells and kill remaining recipient T cells that prevent engraftment (Ruggeri et al., 1999; Ruggeri et al., 2002; Shlomchik et al., 1999). NK cells increase in number and activity after allogeneic BMT (Hauch et al., 1990; Reittie et al., 1989) and engrafted stem cells repopulate an NK cell repertoire of donor origin that interact with HLA KIR ligands on donor hematopoietic cells to become licensed during development. These new NK cells are shaped to be self (donor) tolerant and recipient-alloreactive (Ruggeri et al., 1999; Ruggeri et al., 2007). But beneficial alloreactive NK cell responses are only detectable for a few months after transplantation as they become tolerant of the recipient HLA type (Ruggeri et al., 1999). T cells contribute to both GVHD and GVL (Sykes, Romick, & Sachs, 1990; Truitt et al., 1983; Truitt & Atasoylu, 1991), so depletion can increase relapse rates, but decrease GVHD (Okunewick, Kociban, Machen, & Buffo, 1994; Truitt & Atasoylu, 1991; Weiss et al., 1990). The major milestones of allogeneic SCT include: T cell depletion to prevent GVHD (Reisner et al., 1983), megadoses of T cell depleted stem cells to ensure engraftment (Aversa et al., 1994; Aversa et al., 1998; Bachar-Lustig, Rachamim, Li, Lan, & Reisner, 1995) and the discovery of alloreactive NK cells that eradicate leukemia cells, help engraftment, protect from GVHD and improve survival.

1.14 Adoptive NK Cell Therapy

Activated NK cells are able to effectively target and kill leukemic blasts (Hercend et al., 1986; Jiang, Cullis, Kanfer, Goldman, & Barrett, 1993; Lowdell et al., 1997; Pattengale, Sundstrom, Yu, & Levine, 1983; Whiteway, Corbett, Anderson, Macdonald, & Prentice, 2003) and inhibit leukemic progenitor colony growth (Mackinnon, Hows, & Goldman, 1990) through a GVL effect. Infusions of alloreactive NK cells are an attractive alternative to a complete HSCT. The effectiveness of donor leukocyte infusions has been confirmed in many studies of chronic myeloid leukemia (CML) (Drobyski et al., 1993; Helg et al., 1993; Hertenstein et al., 1993; Porter, Roth, McGarigle, Ferrara, & Antin, 1994; van Rhee et al., 1994), but for relapsed AML patients, only 9-20% achieve CR after adoptive immunotherapy. GVL appears to be more important in some diseases and less in others, possibly due to undefined antigens, costimulatory molecule expression or cell growth rate (Porter & Antin, 1999). GVHD is still a complication with donor leukocyte infusions (DLI), but not as much as with HSCT (Collins et al., 1992). The reduced GVHD may be due to a lack of a 'cytokine storm' caused by tissue damage from conditioning and infections experienced during HSCT (Antin & Ferrara, 1992). In a pilot study for haploidentical NK cell transplantation to treat AML, ten patients in their first CR treated with chemotherapy, then KIR-HLA mismatched NK cells plus IL-2 saw a significant engraftment and expansion of NK cells with no GVHD. There was 100% survival over 2 years (Rubnitz et al., 2010).

Expansion of transfused NK cells *in vivo* can enhance GVL without increasing GVHD. NK cells need IL-15 to survive and expand *in vivo* (M. A. Cooper et al., 2002). Conditioning with low-dose TBI or chemotherapy can increase IL-15, which increases circulating NK cell numbers and cytotoxicity (Miller et al., 2005; Szczepanski et al., 2010). Some clinical-grade strategies to expand NK cells *ex vivo* include culturing NK cells with irradiated Epstein-Barr virus-transformed lymphoblastoid cells (Berg et al., 2009) or modified K562 cells expressing IL-15 and 41BB ligand (Fujisaki et al., 2009). These methods can achieve a twenty to two-hundred fold expansion of pure but activated NK cells over several weeks that are fully functional and kill leukaemia and tumour targets. No clinical trials using *ex vivo* expanded NK cells have been performed yet but hopes for the future are to combine *ex vivo* and *in vivo* expansion. A major obstacle to successful NK cell treatment is inhibitory signals from binding MHC class I on targets, which protects them from NK lysis (Verheyden et al., 2009; Yan et al., 2008). AML cells can also escape by expressing less co-stimulatory molecules (Dermime et al., 1997) or expression of ligands like glucocorticoid-induced tumour necrosis factor-related protein (GITRL) that blocks NK function (Baessler et al., 2009). A majority of NK cells from AML patients possess a low NCR surface density and weak cytolytic activity against autologous leukemic cells that blocking MHC Class I does not help, but in some instances NCR bright NK cells are thwarted by AML cells down-regulating NCR ligands (Costello et al., 2002).

In the last decade we have advanced in our knowledge of the molecular and biochemical processes that contribute to malignant transformation of myeloid and lymphoid cells. Despite this, there have been no major breakthroughs for extending the long-term survival of patients of AML and lymphoma. This failure can usually be attributed to resistance to multiple chemotherapeutic drugs and morbidity and mortality from intensive chemotherapy (Dunussi-Joannopoulos, 2002). Attempts at novel immune approaches have so far not been effective enough for long-lasting results. We need to develop novel therapeutic strategies combining immunotherapy with other approaches to increase effectiveness without causing excessive toxicity in patients.

1.15 Thesis Objectives

A T_H1 immune environment helps to control infection and tumour spread, and IFN-γ is essential to establishing this environment. CpG ODNs are often used as adjuvants to enhance Th1 responses, and NK cell IFN-γ production is essential for the immune enhancing effects of CpG ODN. CpG ODN enhancement of NK cell cytotoxicity and IFN-γ production has been demonstrated in many studies. However, how NK cells become activated by CpG ODNs remains unclear. We hypothesized that NK cells require assistance from accessory cells in the form of cytokines or cell contact signals to respond to CpG ODNs. The goal of this study was to discover the mechanism of murine NK cell activation in response to CpG ODNs and apply it to treat AML.

We hypothesized that non-T, non-B accessory cells respond to CpG ODN stimulation and produce cytokines that subsequently activate NK cells. I tested what cytokines are required by neutralizing T_H1 cytokines as well as by analyzing cytokine-deficient mutant mice. To find the source of the cytokines, I depleted innate immune cell populations from unfractionated splenocytes and compared their stimulation to unmanipulated splenocytes or I stained stimulated splenocytes intracellularly for proteins of interest.

We took our knowledge further by applying the stimulation of NK cells with CpG ODNs to a mouse model of AML in order to discover if haploidentical NK cells are able to target leukemic stem cells and if we could enhance their cytotoxicity with CpG ODN stimulation. During an allogeneic SCT, donor allogeneic T and NK cells exert a graft-versus-leukaemia (GVL) effect, which eliminates remaining leukemia cells that cause relapse (Barrett, 2008). NK cells are especially important for improving survival as they promote engraftment, reduce GVHD and decrease relapse by killing leukemic cells and recipient immune cells (Giebel et al., 2003; Ruggeri et al., 2002). We aimed to test if haploidentical NK cells are able to target leukemic stem cells and if we could enhance their cytotoxicity using CpG ODN and cytokines.

Chapter 2 Materials and Methods

2.1 Mice

C57BL/6, Rag1KO (B6.129S7-*Rag1*^{tm1Mom}/J), IL-12 deficient (B6.129S1-*Il12a*^{tm1Jm}/J), IL-18 deficient (B6.129P2-Il18^{tm1Aki}/J) and CB6F1/J mice were all purchased from The Jackson Laboratory (Bar Harbour, ME) and bred or housed pathogen free in the animal facility of the BC Cancer Research Centre (BCCRC). All animal use was approved by the animal care committee of the University of British Columbia, and animals were maintained and euthanized under humane conditions in accordance with the guidelines of the Canadian Council on Animal Care.

2.2 Antibodies, Cytokines and Media

Anti-CD16/CD32 FcRγ (III/II) (2.4G2) (American Type Culture Collection, Manassas, VA) was purified from hybridoma supernatant and used to block all CD16 binding before staining with other antibodies. PE, FITC, allophycocyanin, or Peridinin-chlorophyll protein (PerCP)-Cy5.5 conjugated mAbs to NK1.1, Gr-1, Ly-6G, CD11c, CD3ε, CD19, B220, CD11b, CD4, CD8, Ter119, Sca-1, c-kit, CD44 and matching isotype controls were purchased from BD-Biosciences (Mississauga, ON). PE conjugated mAb to F4/80 was purchased from ebiosciences (San Diego, CA). The biotin conjugated mAb to CD34 was purchased from ebiosciences (San Diego, CA). Anti-ICAM1-1 mAb (YN1/1.7) was generated in our laboratory (Horley, Carpenito, Baker, & Takei, 1989), purified and biotin conjugated. Anti-MHC K^b mAb was purified from hybridoma supernatant and biotinylated in our lab. Mouse recombinant IL-12 was purchased from StemCell Technologies (Vancouver, BC) and mouse recombinant IL-18 was purchased from Biovision (Mountain View, CA). Mouse recombinant single chain IL-15/IL-15R complex was purchased from eBiosciences (San Diego, CA). 2216-CpG ODN were purchased from Cedarlane Laboratories (Burlington, ON). RPMI 1640 media (StemCell Technologies) supplemented with 10% qualified, heat-inactivated FBS (GIBCO®, Burlington, ON), penicillin and streptomycin (StemCell Technologies) and 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich, Oakville, ON) was used for all primary cell cultures. AML MN1 cells were grown in DMEM with 15% FBS for mouse myeloid colony-forming cells (StemCell Technologies) supplemented with 50 ng/ml mouse SCF, 10 ng/ml IL-3 and 10 ng/ml human IL-6 (StemCell Technologies). Methylcellulose-based medium containing FBS, BSA, human insulin, human transferrin, 2-mercaptoethanol, mouse stem cell factor, mouse IL-3, human IL-6, human erythropoietin and erythropoietin for mouse cells was purchased from StemCell Technologies and frozen in one-use aliquots for colony forming cell (CFC) assays.

2.3 Preparation of Primary Cell Cultures

Mouse spleens were passed through a 70-μm nylon sieve to prepare a single cell suspension, washed with PBS (2% FBS) and red blood cells lysed with ammonium chloride solution for 1 minute at RT. Cell were then washed twice with PBS (2% FBS) before use.

2.4 Purified NK Cell Cultures

Single cell suspensions of mouse spleen were blocked with 2.4G2 (anti-FcR) for 15 minutes and NK cells were isolated either with a custom negative isolation NK cell purification kit missing the CD24 mAb (StemCell Technologies) or FACs sorting. For sorting: cells were blocked with 2.4G2, washed and stained for NK1.1 plus CD3ε for 30 minutes and sorted to high purity as $CD3\varepsilon^{-}NK1.1^{+}$. Dead cells were excluded using propidium iodide at a final concentration of 5 μg/ml. For CpG ODN experiments, cells were FACS sorted and cultured in a 96-well plate at a density of 1.5×10^5 cells/ml in 200 ul of RPMI media with 2216-CpG (2 μg/ml) plus/minus IL-12 (1 ng/ml) or IL-18 (10 ng/ml) for 48 hours. Cell-free supernatants were frozen at -20°C and cytokine production measured by ELISA. CBLF1/J mouse NK cells were always isolated by negative selection kit and expanded in RPMI media (Stemcell Technologies) with recombinant murine IL-15/IL-15R complex (eBioscience) at 4-10 ng/ml for five to seven days before use.

2.5 Cell Depletion Cultures

Splenocyte suspensions were blocked with 2.4G2 and stained with monoclonal antibodies for 30 minutes, then washed with propidium iodide buffer. Unfractionated and depleted cultures were plated at a density of 8×10^6 (C57BL/6) or 4×10^6 (Rag1KO) in RPMI media with 2216-CpG (2 ug/ml) for 24 hours at 37°C and cell-free supernatants were frozen at -20°C. In restored cultures, depleted cells were added back at the same percentage they were before sorting. Cytokine production was measured by ELISA.

2.6 IFN-γ, IL-12 and IL-18 ELISA

IFN-γ and IL-12 ELISA kits were purchased from ebiosciences (San Diego, CA). Maxisorp plates were purchased from NUNC (Rochester, NY). The IL-18 kit with pre-coated plates was purchased from MBL (Woburn, MA). The IL-10 ELISA kit came from BD Biosciences (Mississauga, ON).

2.7 Intracellular Cytokine Staining

Splenocytes were cultured at a density of 4×10^6 cells/ml in 14 ml round bottom polypropylene tubes with 2216-CpG (2 ug/ml) for 10 hours at 37°C with Brefeldin A. Cells were surface stained with mAbs before being fixed and permeabilized using a Cytofix/CytopermTM Plus kit (BD Biosciences). Permeabilized cells were stained with allophycocyanin-conjugated IL-12 mAb and a FACSCalibur (BD Biosciences) was used for acquisition and FLOWJO software (BD Biosciences) for analysis.

2.8 IL-12qPCR

NK cells were highly purified (>99%) from Rag1KO splenocytes by negative selection kit (Stemcell), blocked with 2.4G2, stained with mAbs and FACs sorting of $NK1.1^+$ cells. Monocytes/macrophages were blocked with 2.4G2 and FACS sorted as $CD11b^+F4/80^+CD11c^-NK1.1^-$ cells (>95%). NK cells and monocytes/macrophages were cultured at 1.5×10^5 cells/ml in 200 µl of RPMI media with 2216-CpG (2 µg/ml) for 4 to 6 hours. RNA was extracted using the RNeasy Plus mini kit (QIAgen), treated with Turbo DNA free (Ambion) according to the manufacturer's instructions, and 0.1-0.7 µg RNA was used as a template for reverse transcription with random primers using Superscript III (Invitrogen) according to the manufacturer's instructions. We performed reactions with no reverse transcriptase as negative controls to ensure no DNA contamination was present. For quantitative PCR, the cDNA template was diluted 10-fold for *Il12* and 100-fold for *Gapdh* amplification. 3 µl of cDNA was used in each 10 µl reaction containing 1 x FAST SYBR green mix (Applied Biosystems) and 0.2 µM of each primer. Reactions were run on an Applied Biosystems 7500 Fast real-time PCR machine with 20 s initial denaturation at 95ºC followed by 40 cycles of 3 s at 95ºC and 30 s at 60ºC. We ensured that each primer pair only produced one PCR product by agarose gel electrophoresis. The ΔC(T) method of relative quantitation was used for analysis. Primer sequences were: Il12p40 forward 5´-TCTGAGCCACTCACATCTGC-3´, and Il12p40 reverse 5´-TTGGTGCTTCACACTTCAGG-3´, and Gapdh forward 5´-GACTTCAACAGCAACTCCCAC-3´, and Gapdh reverse 5´-TCCACCACCCTGTTGCTGTA-3´.

2.9 Transwell Culture

C57BL/6 splenocytes were stained with NK1.1 and CD3ε mAb; NK cells (NK1.1$^+$CD3ε$^-$) and the remaining splenocytes were separated using a BD FACS Aria cell sorter (BD Biosciences). NK-depleted splenocytes were cultured at a density of 2×10^6 cells/ml in a 24-well plate with NK cells (3.5×10^4) separated by cell-impermeable (0.4 μm pore size) transwell inserts (BD-Falcon) in a final volume of 800 μl. NK cells in the inserts and NK-depleted cells in bottom wells were stimulated with 2216-CpG (2 μg/ml) for 24 hours before cell-free supernatants were frozen at -20°C

2.10 Cell Lines

The MN1 AML cell line used in these experiments was kindly donated by the Humphries laboratory. Cells originated from retroviral transduction of C57BL/6 BM cells with human *MN1*. Frozen stocks at day 20 after the transduction were kept and expanded three days in culture before use (Heuser et al., 2007).

2.11 Cytotoxicity Assay

NK cells expanded with IL-15/IL-15R were further stimulated with IL-12 (1 ng/ml), IL-18 (1-10 ng/ml) and/or 2216-CpG (2 µg/ml) for 24 hours at 37°C before assay. Target MN1 or YAC1 cells were labelled with 0.5 µM Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) and then mixed with stimulated NK cells at varying ratios (1:5 to 1:100 Target:Effector) in a 96-well round bottom plate in 150 µl RPMI media and incubated at 37°C for four hours. 500 MN1 cells (calculated based on original live numbers) were removed from each condition and plated in duplicate on methylcellulose medium with recombinant cytokines and erythropoietin. Remaining cells were washed and stained with 7-Aminoactinomycin D (7-AAD) (Sigma-Adrich) and percentage of bulk dead target cells measured via a FACSCalibur (BD Biosciences) and FLOWJO software (BD Biosciences) used for analysis. Target cells incubated with no NK cells served as background death controls. Death of colony forming cells plated on methocellulose versus bulk cell killing was determined by counting colonies from control vs. NK-treated MN1 cells five days later and comparing to FACs data. For resistant colony analysis, colonies from control and NK-treated plates were washed off with PBS and re-grown in MN1 media for two days. Cells were then stained with mAbs for marker analysis by FACs and used in a second cytotoxicity assay as described above.

2.12 Stem Cell Assay

MN1 cells and NK cells were prepared in a cytotoxicity assay. Each dose of MN1 cells to be injected into one mouse was incubated with or without NK cells in separate wells and four doses worth were pooled for each condition. For example, the 2.5×10^5 cell dose had four wells with only MN1 cells and then four wells with MN1 cells plus NK cells. After incubation the four MN1 only and four MN1 plus NK cell wells were pooled. Cells were spun down and resuspended in PBS with 2% FCS .Control MN1 cells and NK-treated MN1 cells were injected intravenously into age matched 810 Gy-irradiated C57BL/6 mice along with a life-saving dose of 2×10^5 syngeneic BM cells from a C57BL/6 mouse. BM was prepared by flushing the femur and tibia with PBS (2% FBS) and lysing red blood cells with ammonium chloride. Mice were given water containing HCl plus Cipro and a dough diet for 2 weeks. Engraftment of donor cells was monitored by tail vein bleeds, lysing red blood cells with ammonium chloride and FACS analysis of GFP-expressing WBCs on weeks three and eight. When mice reached a humane endpoint, they were euthanized and tissue collected for analysis. Blood was collected from the femoral artery and counts with differential WBC analysis were performed using an ABC Vet Automated Blood Counter (VetNovations Canada). Cell suspensions of BM and spleen were prepared as described above. Lineage distribution was determined by FACS analysis. Remaining cells were frozen in FBS with 10% dimethysulfoxide (DMSO) (Sigma).

2.13 Statistical Analysis

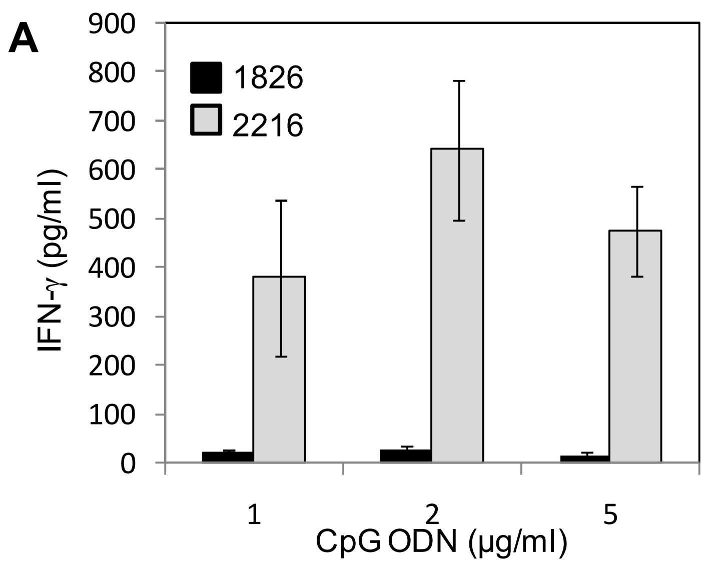
All statistical analyses of experimental mean values were performed using the Students' paired *t* test. Values of $p<0.05$ were considered significant.

Chapter 3 Results

3.1 CpG ODN Stimulation of NK Cell IFN-γ Production

3.1.1 IFN-γ Production in Bulk Splenocyte Cultures in Response to Type A and B CpG ODNs

The optimal type and concentration of CpG ODN to activate NK cells was determined by stimulating unfractionated splenocytes from naïve B6 mice with type-A CpG ODN 2216 (CpG-2216) or type-B CpG ODN 1826 (CpG-1826) for 48 hours at 37°C and assessing the concentration of IFN-γ secreted into the culture media.



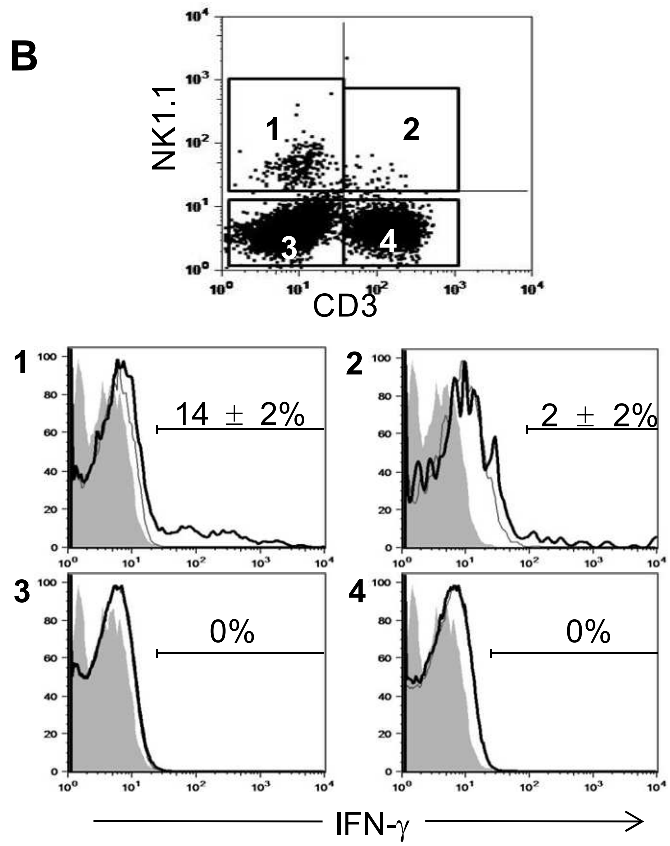


Figure 2. B6 mouse splenocytes IFN-γ production in response to type-A and -B CpG ODNs

(A) Unfractionated B6 splenocytes were cultured at 4×10^6 cells/ml with 1, 2 or 5 μg/ml of CpG-2216 or CpG-1826 in 96-well round-bottom tissue culture plates for 48 h. Cell free supernatants were assessed for IFN-γ by ELISA. Results are the mean ± SD of two independent experiments, each with triplicate cultures. (B) Unfractionated B6 splenocytes were cultured at 4×10^6 cells/ml with or without 2 μg/ml of CpG-2216 for 24 h, stained with mAbs to NK1.1 and CD3ε, fixed, permeabilized, stained for intracellular IFN-γ and analyzed by flow cytometer. NK cells ($NK1.1^+CD3\varepsilon^-$), NKT cells ($NK1.1^+CD3\varepsilon^+$), double negative ($NK1.1^-CD3\varepsilon^-$), and T ($NK1.1^-CD3\varepsilon^+$) cells were gated as shown by numbers in dot plots, and the staining of intracellular IFN-γ in each population is shown in histograms. Filled histograms show staining with isotype-matched control Ab, open grey and black histograms show IFN-γ staining for unstimulated and stimulated cultures, respectively. The data are representative of at least four independent experiments and the numbers indicate the percentages of positively stained cells (mean ± SD, n=4) (E. Haddad, unpublished data).

Wild type B6 mouse splenocytes released double the amount of IFN-γ in culture supernatants when stimulated with 2 μg/ml of CpG-2216, as compared to 1 μg/ml. The B-type CpG-1826 only induced a minimal amount of IFN-γ from B6 mouse splenocytes (Fig. 2). These results confirm past papers indicating that type-B CpG ODNs are poor stimulators of IFN-γ and NK cells, while type-A CpG ODNs optimally induce NK cell activity (Ballas et al., 1996). We chose 2 μg/ml (310nM) of CpG-2216 for subsequent experiments as it induced the most IFN-γ and is unlikely to cause the backbone effects that a higher ODN concentration above 330nM (i.e., above 2.12 μg/ml) can induce (Brummel & Lenert, 2005; Haas, Poe, Steeber, & Tedder, 2005; Vollmer & Krieg, 2009). Intracellular IFN-γ staining showed IFN-γ was mainly produced by NK cells and a small subset of NKT cells (Fig. 2B).

3.1.2 Purified NK Cell IFN-γ Production

Highly purified NK cells cannot become cytotoxic with CpG ODN stimulation alone (Ballas et al., 1996). We tested whether highly purified NK cells are able to directly respond to CpG-2216 and produce IFN-γ or lyse YAC-1 cells or whether they require additional help in the form of NK-activating cytokines such as IL-12 and IL-18, which are well known activators of NK cells (Haddad et al., 2009). IL-12 and IL-18 were previously titrated for stimulation of

NK cells in bulk splenocytes cultures, and 1 ng/ml IL-12 and 10 ng/ml IL-18 were found to be optimal concentrations.

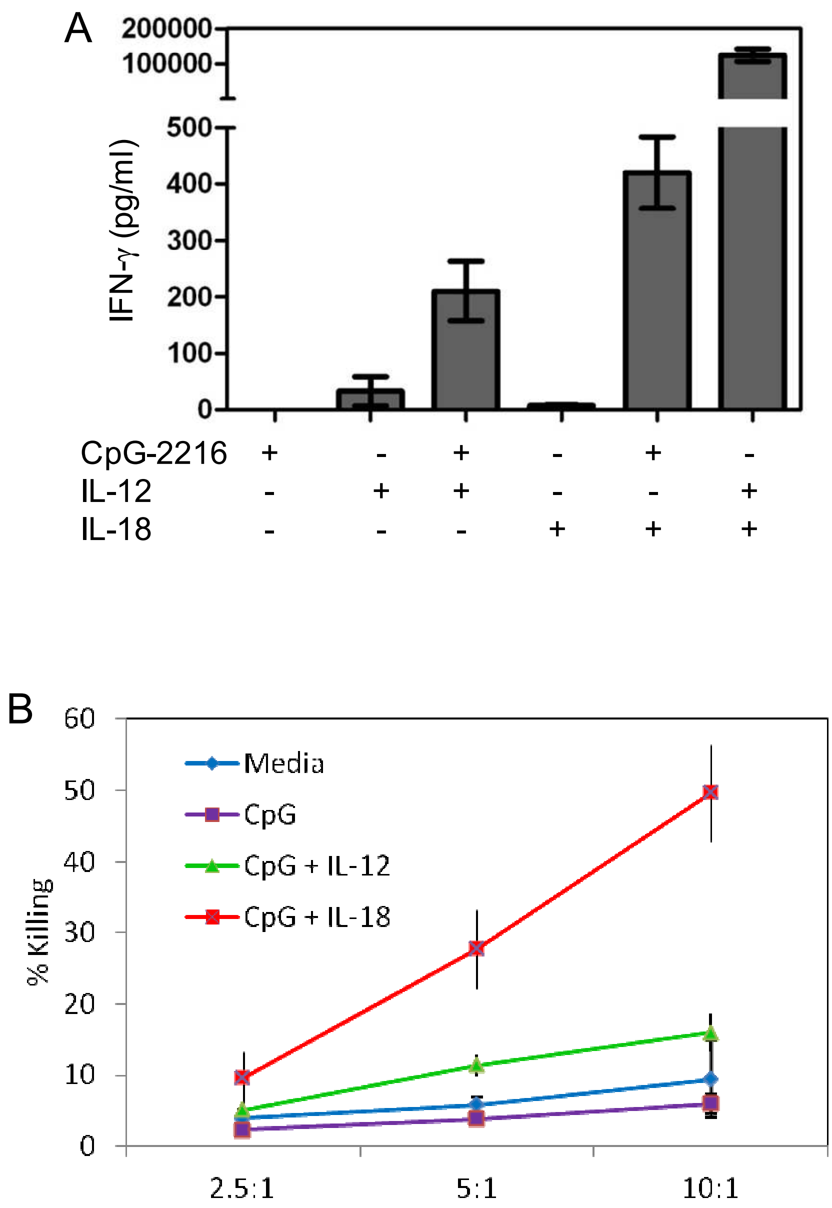


Figure 3. Purified NK cells require priming by IL-12 and IL-18 to respond to CpG ODNs

Highly purified (>99%) NK cells isolated by FACS sorting of NK1.1$^+$ cells from RAG1KO spleen were cultured at 1.5×10^5 cells/ml with either 2 μg/ml of 2216-CpG, 2216-CpG plus 1 ng/ml of IL-12, 10 ng/ml of IL-18 or IL-12 plus IL-18 in a 96-well round-bottom tissue culture plate. (A) After 48 h, cell free supernatants were assessed for IFN-γ by ELISA. Results are the mean ± SD of triplicate cultures and representative of three independent experiments, each with triplicate cultures. (B) After culturing 24 h, activated NK cells were cultured for four hours with CFSE-labelled YAC-1 cells at 2.5:1, 5:1 and 10:1 ratios in triplicates. YAC-1 cells without NK cells were used as control. The percentage of CFSE$^+$7AAD$^+$ cells out of CFSE$^+$ cells was measured by flow cytometry and background death in control wells subtracted. Dots represent mean ± SD from triplicate wells. The IL-12 and IL-18 only stimulation results in (A) are from Evette Haddad.

Purified NK cells did not respond to CpG-2216 alone, but if either IL-12 or IL-18 was added, NK cells weakly responded by secreting approximately 175 to 400 pg/ml of IFN-γ. The combination of IL-12 and IL-18 activated purified NK cells to secrete high levels of IFN-γ reaching 130 ng/ml (Fig. 3A). Only the combination of CpG-2216 with IL-18 resulted in significant cytotoxicity against the typical NK cell target YAC-1 (Fig. 3B).

3.1.3 Cytokines Required For CpG-2216 Stimulation of NK Cells

3.1.3.1 Neutralization of cytokines in RAG1KO spleen

Previous studies revealed that cell-free supernatants from CpG ODN-stimulated spleen cells are able to activate NK cells (Ballas et al., 1996; Cowdery et al., 1996; Marshall et al., 2006). Similarly, we found that IL-12 and IL-18 can either prime or co-stimulate purified NK cells to respond to CpG ODNs. Cell contact with other splenocytes was not required for NK cells to produce IFN-γ (data not shown). This led us to hypothesize that cytokines are produced in the spleen in response to CpG ODNs that stimulate NK cells. Neutralizing antibodies to IL-12, IL-18, IL-15 and IFN-α were added to unfractionated RAG1KO splenocyte cultures stimulated with 2 μg/ml of CpG-2216. The amounts of IFN-γ produced in CpG-2216 stimulated control cultures was compared to those containing neutralizing antibodies to determine what cytokines are required for CpG-2216-induced IFN-γ production by NK cells. We previously determined the optimal concentration of 0.5 μg/ml anti-IL-12 and 1 μg/ml anti-IL-18 antibody for neutralization. RAG1KO mouse spleen lacked T or B cells and NK cells comprised ~50% of splenocytes.

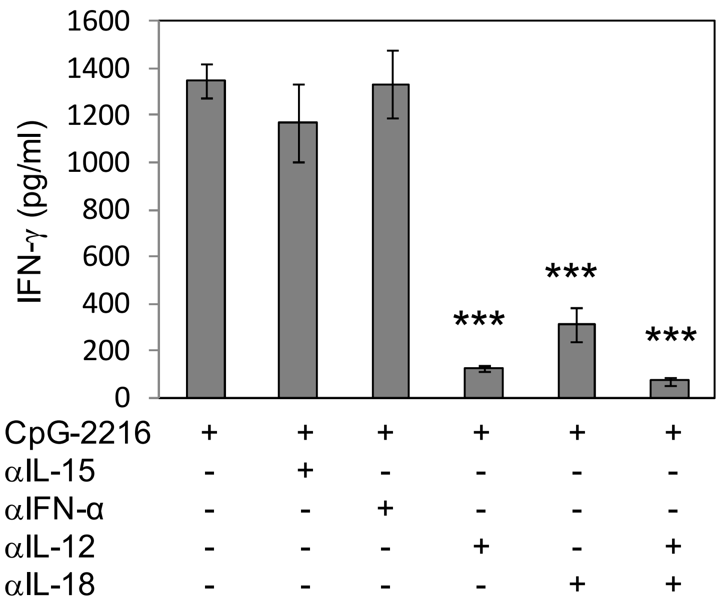


Figure 4. Neutralization of IL-12 and IL-18 reduces IFN-γ production in response to CpG ODN

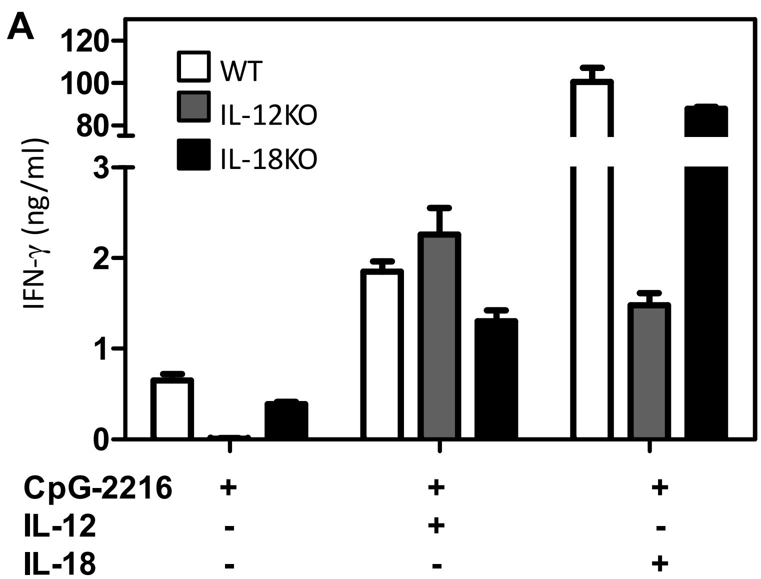
Unfractionated splenocytes from RAG1KO mice (4×10^6 cells/ml) were stimulated with 2 μg/ml CpG-2216, and neutralizing antibodies to IL-15 (αIL-15, 0.4 μg/ml), IFN-α (αIFN-α, 2.5 μg/ml), IL-12 (αIL-12, 0.5 μg/ml) or IL-18 (αIL-18, 1 μg/ml) were added before culturing in a 96-well U-bottom plate for 48 hours at 37°C. Cell free supernatants were assessed for IFN-γ by ELISA. Results are the mean ± SD of two independent experiments, each with triplicate cultures. ***, $p < 0.005$.

IL-18 neutralization reduced the IFN-γ in the supernatant to a quarter of control cultures and IL-12 neutralization almost completely abrogated IFN-γ production by NK cells to a mere 100 pg/ml. Neutralization of both IL-12 and IL-18 lowered IFN-γ levels to barely detectable levels (Fig. 4). IFN-γ production was also lowered to about 35% of control levels when IL-

18 was neutralized and almost non-detectable when IL-12 was neutralized in B6 splenocyte cultures (E. Haddad, unpublished results). IL-15 and IFN-α neutralization did not reduce IFN-γ production from CpG-2216 stimulated spleen.

3.1.3.2 CpG ODN stimulation of IL-12KO and IL-18KO mouse splenocytes

The role of IL-12 and IL-18 in CpG-2216 stimulation of murine spleen cells was further studied with IL-12KO and IL-18KO mice of the B6 background. Splenocytes from wild type B6 mice and knock-out mice were stimulated with CpG-2216, CpG-2216 plus IL-12 and CpG-2216 plus IL-18, and IFN-γ production from each culture was compared.



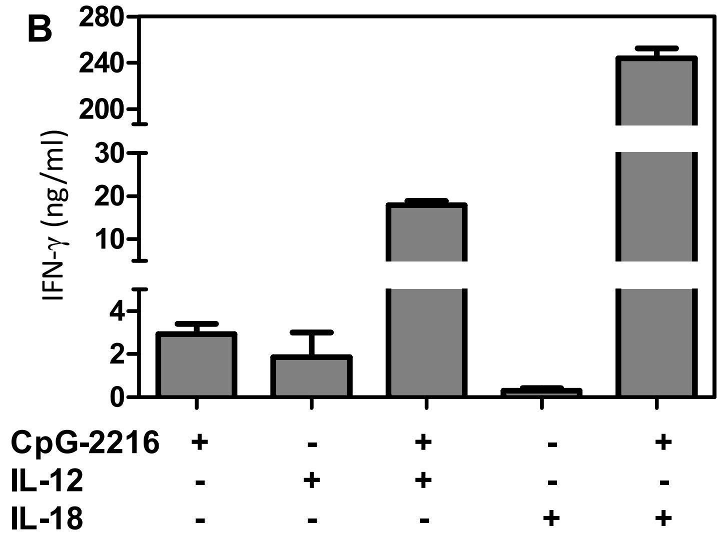


Figure 5. IL-12KO mouse splenocytes do not respond to CpG ODNs

(A) Unfractionated splenocytes (4×10^6 cells/ml) from B6 WT, IL-12KO or IL-18KO mice were stimulated with 2 μg/ml CpG-2216, CpG-2216 plus 1 ng/ml IL-12 or CpG-2216 plus 10 ng/ml IL-18 for 24 hours. Cell free supernatants were assessed for IFN-γ by ELISA. Results are the mean ± SD of triplicate cultures and representative of three independent experiments, each with triplicate cultures. (B) Unfractionated splenocytes from RAG1KO mice at 4×10^6 cells/ml were stimulated with 2 μg/ml CpG-2216, 1 ng/ml IL-12, CpG-2216 plus IL-12, 10 ng/ml IL-18 or CpG-2216 plus IL-18. Splenocytes were cultured in a 96-well U-bottom plate for 24 hours at 37°C. Cell free supernatants were assessed for IFN-γ by ELISA. Results are the mean ± SD of three mice, each with triplicate cultures.

IL-12KO splenocytes were unable to produce detectable amounts of IFN-γ in response to CpG-2216 stimulation whereas the response of IL-18KO splenocytes was about 50% of WT splenocytes (Fig. 5A). In the presence of exogenous IL-12, wild type, IL-12KO and IL-18KO splenocytes were stimulated by CpG-2216 and produced comparable levels of IFN-γ, which was significantly higher than the level produced by wild type splenocytes without exogenous IL-12. The addition of IL-18 also drastically increased the amount of IFN-γ produced by wild type and IL-18KO splenocytes, whereas it only modestly increased the IFN-γ produced by IL-12KO spleen cells. Thus, IL-12 is required for the stimulation of spleen cells by CpG-ODNs. Although IL-18 is also involved in the stimulation of splenocytes by CpG ODNs and the combination of CpG ODNs and IL-18 strongly stimulates splenocytes, IL-18 is dispensable. The high levels of IFN-γ produced in response to CpG-2216 and IL-18 prompted us to suspect that perhaps T cells were contributing to IFN-γ production. However, RAG1KO splenocytes (missing T and B cells) stimulated with CpG-2216 and IL-18 produce almost triple the IFN-γ of B6 splenocytes. Clearly, T and B cells are not required for IFN-γ production in response to these stimulations. Cells from RAG1KO spleen produced ~2 ng/ml of IFN-γ in response to stimulation with IL-12 alone, but this is increased ten-fold with the addition of CpG-2216. RAG1KO splenocytes respond minimally to IL-18 alone while IL-18 plus CpG-2216 induced a large amount (>200 ng/ml) of IFN-γ production (Fig. 5B).

3.1.4 Cellular Source of IL-12 and IL-18 in Response to CpG ODNs

3.1.4.1 IL-12 qPCR

Chaudhry *et al.* found that a subset of NK cells expressing CD11c, termed NKDC, are capable of making IL-12 in response to type-B CpG ODN stimulation (Chaudhry, Kingham et al., 2006). We tested whether NK cells produce IL-12 in response to CpG-2216 and IL-18 stimulation. NK cells were highly purified to >99% purity first by negative selection and then by FACS sorting of NK1.1$^+$ cells. Monocytes/macrophages purified by sorting CD11b$^+$F4/80$^+$CD11c$^-$NK1.1$^-$ cells were used as controls. Cells were stimulated with CpG-2216 (monocytes/macrophages) or CpG-2216 plus IL-18 (NK cells) for four to six hours before RNA was extracted and quantitative PCR performed to measure amounts of IL-12p40 mRNA produced compared to a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) control.

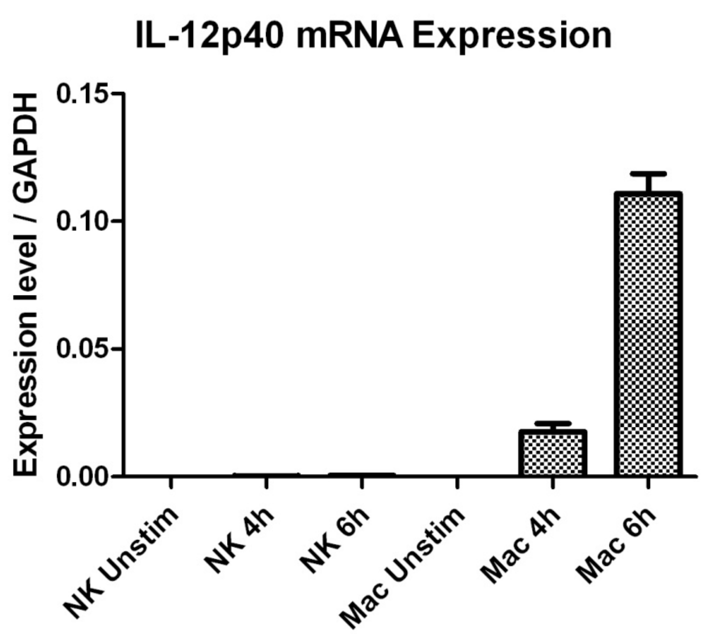


Figure 6. IL-12p40 mRNA expression from CpG ODN stimulated NK cells

Highly purified (>99%) NK cells were isolated using a negative selection kit followed by FACS sorting of NK1.1$^+$ cells from Rag1KO spleen. Monocytes/macrophages (Mac) were isolated by FACS sorting of CD11b$^+$F4/80$^+$CD11c$^-$NK1.1$^-$ cells. Cells were cultured at 1.5×10^5 cells/ml with media alone (unstim.), 2 µg/ml of CpG-2216 (monocytes/macrophages) or 2 µg/ml of CpG-2216 plus 10 ng/ml of IL-18 (NK cells) in a 96-well round-bottom tissue culture plate for 4 and 6 h before RNA was extracted and converted to cDNA for quantitative PCR analysis as described in the Material and Methods. Data were analyzed by the ΔC(T) method of relative quantitation, and are shown as a percentage of IL-12p40 gene expression normalized to GAPDH expression.

Highly purified NK cell cultures produced no significant amount of IL-12p40 mRNA in response to stimulation with CpG-2216 and IL-18 while control cultures of monocytes/ macrophages produced ~0.02 times at four hours and ~0.11 times the expression level of control GAPDH mRNA after six hours of stimulation with CpG-2216 (Fig. 6). The IL-12p40 and IL-12p35 chains are encoded on separate chromosomes and the two chains must be co-expressed for functional IL-12 to be released. We found no significant production of IL-12p35 from any culture. IL-12 was undetectable in the supernatants of highly purified cultures of NK cells stimulated with CpG-2216 plus IL-18 by ELISA, while small amounts of IL-12p70 (60 pg/ml) were detected in monocytes/macrophages stimulated with CpG-2216. Thus it appears that NK cells (including NKDCs) do not produce IL-12 when stimulated with type-A CpG ODN. The difference in our experiments and Chaudhry's that may account for the disparity of results is they used the type-B CpG ODN, 1826. Possibly, NK cells can produce IL-12 in response to type B CpG ODNs and not type A. IL-18 is constitutively produced in cells, so measuring mRNA levels by qPCR is not representative of what active protein is being released, leading us to decide not to run such an experiment. When we measured IL-18 in supernatants of CpG-2216-stimulated splenocyte cultures, IL-18 was undetectable. The detection limit of currently available IL-18 ELISA kits is 25 pg/ml.

3.1.4.2 FACS Depletions of Cell Populations from Spleen

We set out to determine what cells are required for IL-12 and IFN-γ production in B6 spleen by staining for and depleting probable accessory cell populations. DC ($CD11c^{+}NK1.1^{-}$), monocytes/macrophages ($F4/80^{+}$) and neutrophils ($Gr\text{-}1^{+}$) were depleted from bulk B6 spleen by FACS and the IFN-γ and IL-12 production was compared to unfractionated spleen cultures.

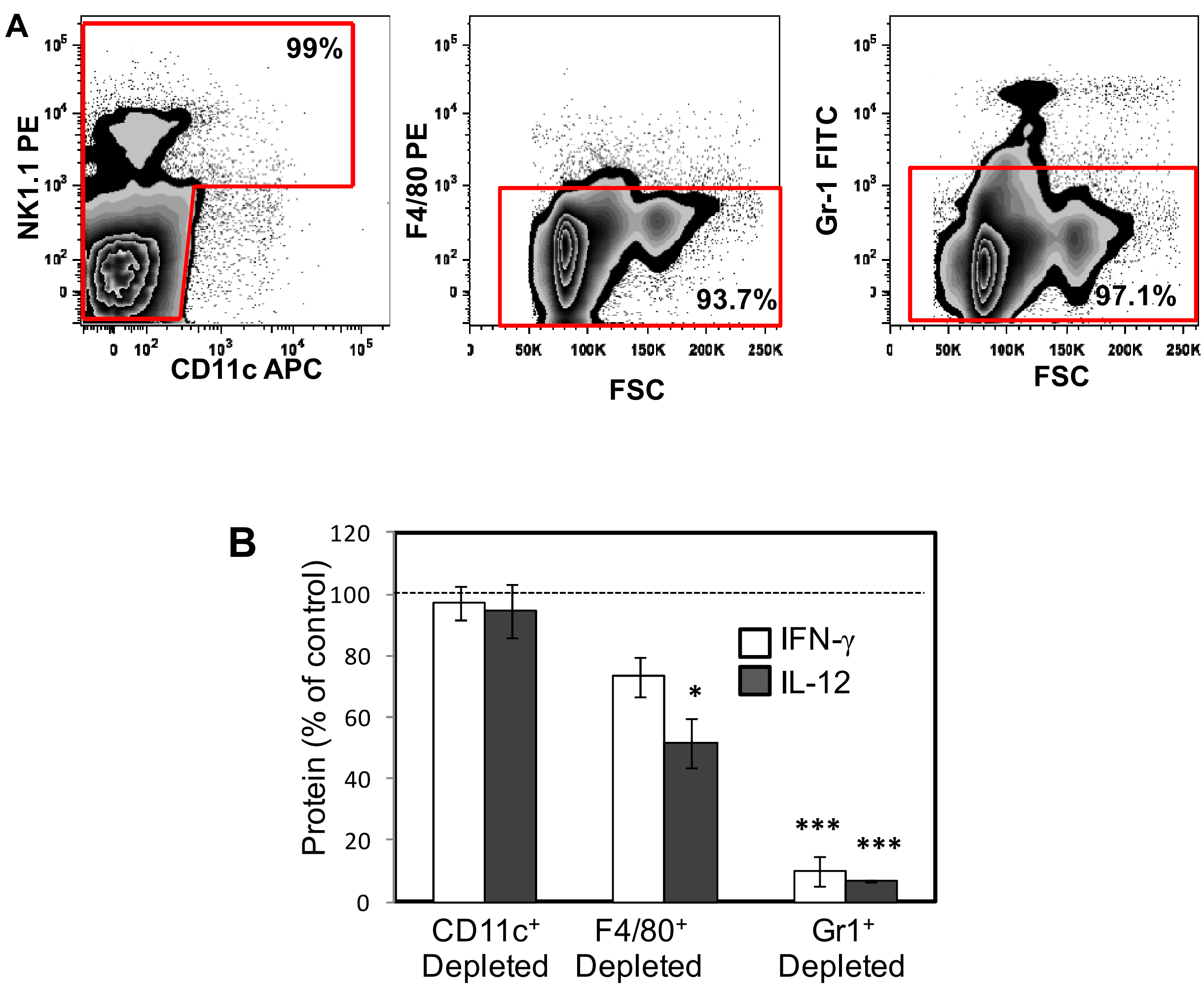


Figure 7. Cytokine production in depletion cultures.

(A) Splenocytes from B6 mice were sorted by FACS into $CD11c^{+}NK1.1^{-}$, $F4/80^{+}$ and $Gr1^{+}$ depleted populations. (B) Bulk and depleted splenocytes (2×10^6 cells/ml) were cultured with CpG-2216 (2 μg/ml) for 48 hours and cell-free supernatants assessed for IFN-γ and IL-12p70 by ELISA. Fold increases in IFN-γ or IL-12 production are shown, calculated as a percentage of cytokine production from depleted cultures divided by cytokine production from control cultures. The results shown are the mean ± SD and representative of three independent experiments, each with triplicate cultures. *, $p<0.05$; ***, $p < 0.005$.

Removal of DCs from B6 splenocytes did not affect the IFN-γ or IL-12 production in response to CpG-2216 (Fig. 9), indicating that DCs are not required for NK cells to respond to CpG ODNs. Depletion of monocytes/macrophages ($F4/80^{+}$) reduced the amount of IL-12 by ~50% and IFN-γ by ~30%. Depleting cells expressing Gr-1 significantly decreased IL-12 and IFN-γ production, bringing cytokine production down to only ~10% of the amount in control cultures. When $CD11b^{+}NK1.1^{-}$ cells were removed from RAG1KO splenocytes, no detectable level of IL-12 or IFN-γ was produced (not shown). It should be noted that most $Gr\text{-}1^{+}$ cells and $F4/80^{+}$ cells also express CD11b. Depletion of mast cells and basophils ($Fc\varepsilon R1\alpha^{+}$) had no effect on IFNγ/IL-12 production (data not shown).

$Gr\text{-}1^{+}$ cells likely included neutrophils ($CD11b^{+}F4/80^{-}Ly6C^{int}$ $Ly6G^{hi}$) and inflammatory monocytes ($CD11b^{+}F4/80^{+}Ly6C^{hi}Ly6G^{-}$) (Daley, Thomay, Connolly, Reichner, & Albina, 2008; Egan, Sukhumavasi, Bierly, & Denkers, 2008). To test which population is involved in the stimulation of splenocytes by CpG-2216, B6 splenocytes were depleted of $Gr\text{-}1^{+}F4/80^{+}$, Gr-1^{+}F4/80^{-} and Ly-6G^{+} populations by FACS and the IFN-γ and IL-12 production compared to unfractionated spleen cultures.

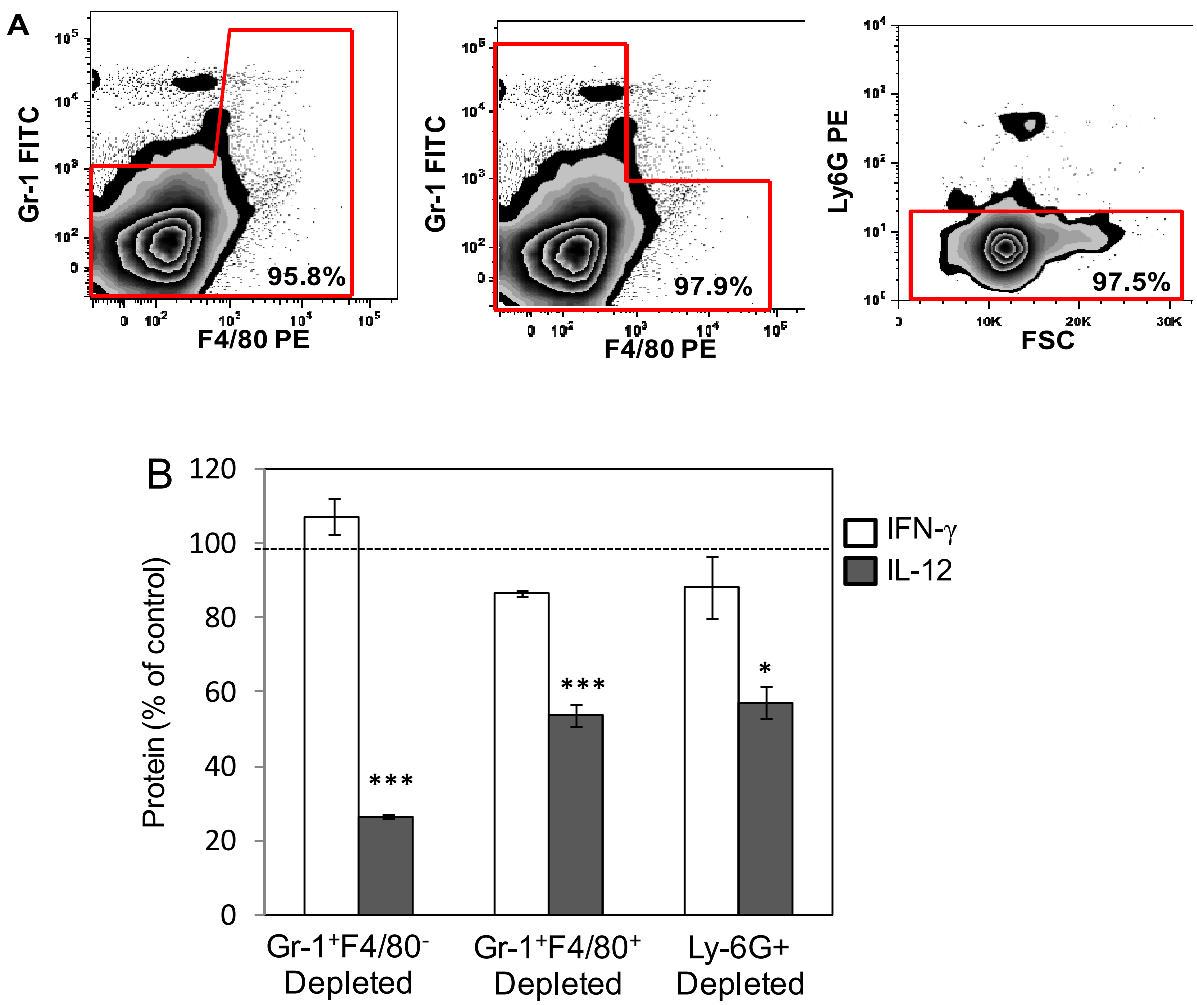


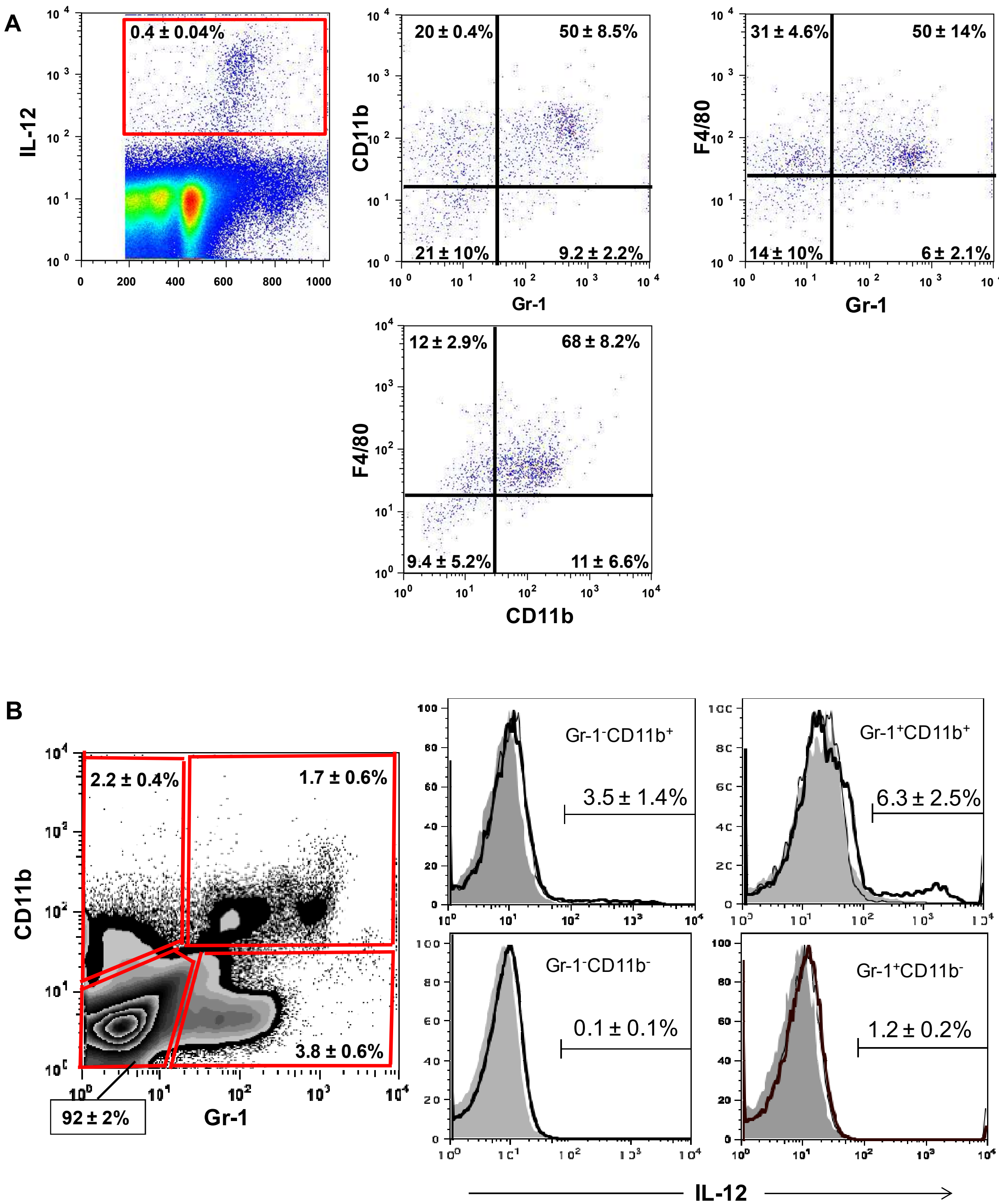
Figure 8. Monocytes and neutrophils contribute to IL-12 production

Splenocytes from B6 mice were FACs sorted into Gr-1^{+}, Gr-1^{+}F4/80^{-}, Gr-1^{+}F4/80^{+} and Ly-6G^{+} depleted populations. (A) FACS plots are representative of triplicate experiments. (B) Bulk and depleted splenocytes (2×10^{6} cells/ml) were cultured with CpG 2216 (2 μg/ml) for 48 hours and cell-free supernatants assessed for IFN-γ and IL-12p70 by ELISA. IFN-γ or IL-12 productions are shown as a percentage of cytokine production from depleted cultures divided by cytokine production from control cultures. The results shown are the mean ± SD of three independent experiments, each with triplicate cultures. *, $p<0.05$; ***, $p < 0.005$.

When Gr-1^{+}F4/80^{-} cells were removed, the IL-12 production was reduced by ~75% while IFN-γ production was not affected. Depletion of Gr-1^{+}F4/80^{+} cells also brought the IL-12 production down to a half of control but IFN-γ production was only reduced by ~10%. Ly-6G is considered to be a specific marker of neutrophils (Daley et al., 2008). Ly-6G^{+} cell depletion decreased the IL-12 production by a half whereas Gr-1^{+}F4/80^{-} cells, most of which are thought to be neutrophils, reduced IL-12 production to a quarter of control (Fig. 8). These results suggest that both neutrophils and inflammatory monocytes produce IL-12 in response to CpG-2216 and induce IFN-γ production by NK cells. Even though IL-12 production was decreased when either of these populations was depleted, the reduced levels of IL-12 seemed sufficient for the IFN-γ production by NK cells.

3.1.4.3 Intracellular Staining of IL-12 in CpG ODN-Stimulated Bulk Spleen Cells

To identify cell populations that actually produce IL-12 in response to CpG-2216, bulk spleen cells from B6 mice stimulated with CpG-2216 overnight were stained for surface antigens of interest, fixed and permeabilized and stained for intracellular IL-12.


A
0.4 ± 0.04%
IL-12
20 ± 0.4%
50 ± 8.5%
21 ± 10%
9.2 ± 2.2%
CD11b
Gr-1
31 ± 4.6%
50 ± 14%
14 ± 10%
6 ± 2.1%
F4/80
Gr-1
12 ± 2.9%
68 ± 8.2%
9.4 ± 5.2%
11 ± 6.6%
F4/80
CD11b
B
2.2 ± 0.4%
1.7 ± 0.6%
3.8 ± 0.6%
92 ± 2%
CD11b
Gr-1
Gr-1-CD11b+
3.5 ± 1.4%
Gr-1+CD11b+
6.3 ± 2.5%
Gr-1-CD11b-
0.1 ± 0.1%
Gr-1+CD11b-
1.2 ± 0.2%
IL-12

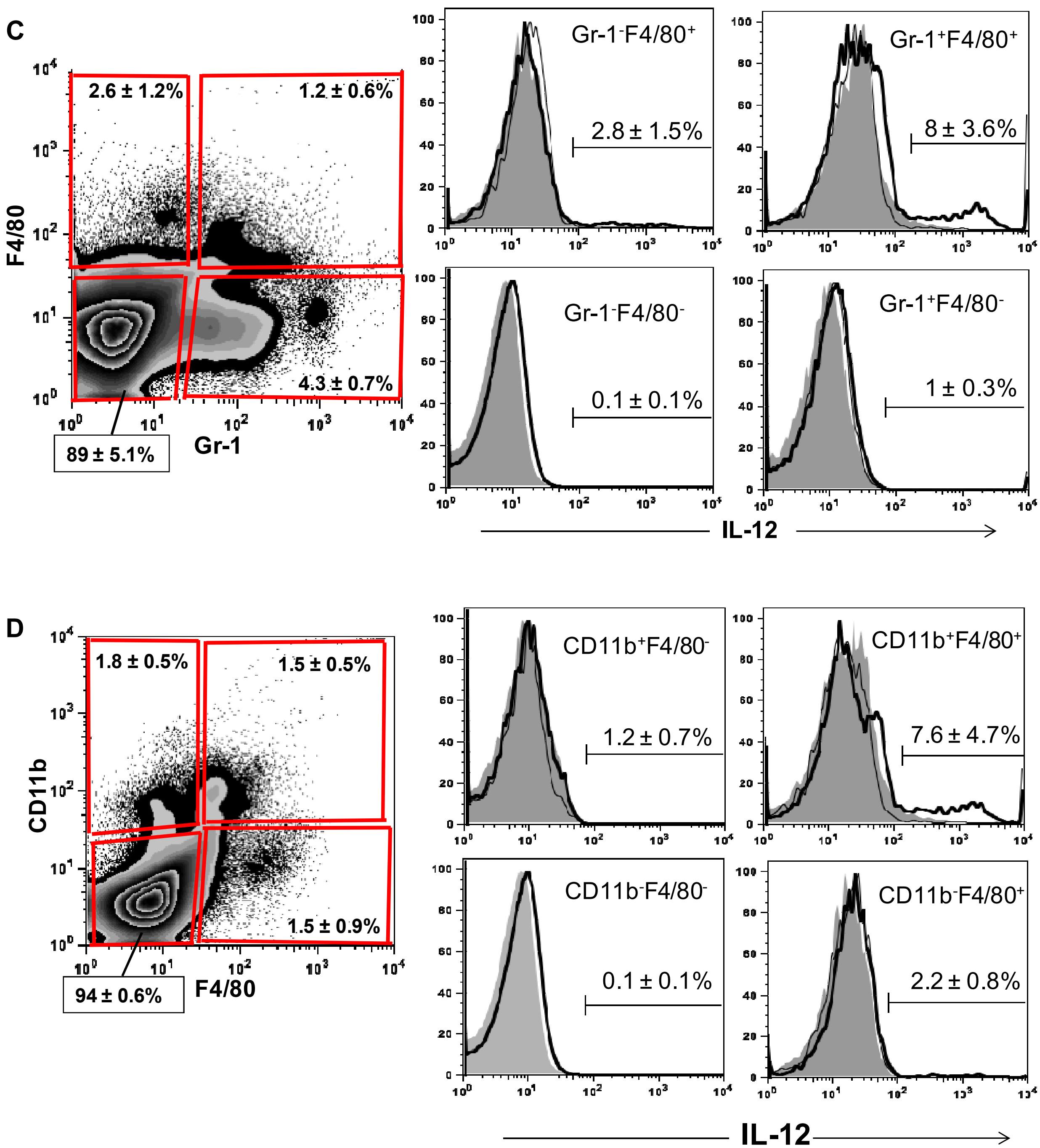


Figure 9. Intracellular IL-12 staining of CpG ODN stimulated splenocytes

Bulk splenocytes from B6 mice were cultured at 4 × 10^6 cells/ml with or without CpG-2216 (2 μg/ml) for 24 h, stained with mAbs to Gr-1, F4/80 and CD11b, fixed, permeabilized, stained for intracellular IL-12 and analyzed by flow cytometry. (A) All IL-12^+ cells were gated and analyzed for Gr-1, F4/80 and CD11b expression. (B) Splenocytes were gated by CD11b and Gr-1 expression and the percentage of IL-12^+ cells are plotted in histograms. (C) Splenocytes were gated by F4/80 and Gr-1 expression and the percentage of IL-12^+ cells are shown in histograms. (D) Splenocytes were gated by CD11b and F4/80 expression and the percentage of IL-12^+ cells are plotted in histograms. Filled histograms show staining with isotype-matched control Ab, open grey and black histograms show IL-12 staining for unstimulated and stimulated cultures, respectively. The lots are representative of three independent experiments. The numbers in the plots show the percentages (mean ± SD, n=3) of cells in quadrants and gates.

Only a small fraction (~0.4%) of splenocytes stimulated by CpG-2216 was positive for intracellular IL-12. Almost 60% of IL-12^+ cells were Gr-1^+ and most of them co-expressed CD11b or F4/80 although smaller fractions of the Gr-1^+ cells did not express CD11b or F4/80. The remaining ~40% of IL-12^+ cells were Gr-1^- and included CD11b^+ and F4/80^+ cells (Fig. 9A). The IL-12^+ cell population was also heterogeneous with respect to CD11b and F4/80 expression. The majority were double positive for CD11b and F4/80, the phenotype of monocytes/ macrophages. Various spleen cell populations defined by Gr-1, CD11b and F4/80 expression were also analyzed for IL-12^+ cells (Figures 9B-D). The percentages of IL-12^+ cells were the highest among Gr-1^+F4/80^+ and CD11b^+F4/80^+ cells while smaller fractions of Gr-1^-F4/80^+ and CD11b^-F4/80^+ cells were IL-12^+.

Though a small percentage (<2%) of $CD11c^+$ cells stained for IL-12 (data not shown), they all expressed $CD11b^+$, which Gr-1^+ pDCs do not express (Egan et al., 2008), thus ruling out pDCs. Among CpG-2216-stimulated splenocytes, a significant fraction of IL-12^+ cells was Gr-1^- and most F4/80 negative cells were IL-12^-. We also sorted IL-12^+ cells onto a slide and stained with Diff quick to determine cell types by morphology. Although cells were damaged from the permeabilization/fixation procedures required for intracellular staining, they appeared to mainly consist of monocytes. Very few of them had the morphology of neutrophils with segmented nuclei (data not shown).

3.1.4.4 Purified Cell Cultures

To further investigate the roles of Gr-1^+, F4/80^+, $CD11c^+$ and $CD11b^+$ cell populations in IL-12 production, we purified them by FACS, stimulated for two days with CpG-2216 and cell-free supernatants were assessed for IL-12 production.

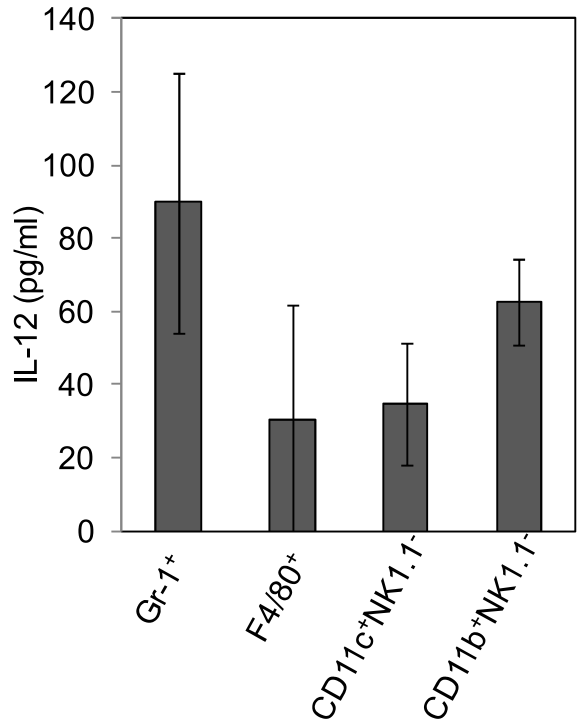


Figure 10. Purified cells produce small amounts of IL-12 when stimulated with CpG ODNs

Gr-1$^+$, F4/80$^+$, NK1.1$^-$CD11c$^+$ and CD11b$^+$NK1.1$^-$ cells were sorted by FACS from RAG1KO spleen and cultured at 1.5×10^5 cells/ml with 2 μg/ml of CpG-2216 for 48 h in a 96-well round-bottom plate. Cell-free supernatants were assessed for IL-12p70 by ELISA. The results shown are the mean ± SD of three independent experiments, each with triplicate cultures.

Very little IL-12 was produced by any of the purified cell populations after stimulation with CpG-2216. Gr-1$^+$ cells secreted the highest amount of IL-12 at an average of 90 pg/ml and CD11b$^+$ cells followed closely behind with an average of 63 pg/ml. These cell populations make up approximately 6% and 5% of spleen respectively. Unfractionated spleen cell culture of 4×10^6 cells/ml should contain 2.4×10^5/ml Gr-1$^+$ cells and 2×10^5/ml CD11b$^+$ cells and they produced an average of 300 to 500 pg/ml of IL-12, which was six to eight fold higher than that produced by purified Gr-1$^+$ cells and CD11b$^+$ cells cultured at 1.5×10^5 cells/ml. F4/80 and CD11c positive cell produced barely detectable levels of IL-12 (Fig. 10). These results suggest that cell-cell interaction and/or additional cytokines are required for optimal production of IL-12 by CpG-2216 stimulated Gr-1$^+$ cells and CD11b$^+$ cells. Addition of any of the purified populations to purified NK cells did not result in enhanced production of IL-12 or IFN-γ in response to CpG-2216.

3.2 NK Cell Killing of Acute Myeloid Leukemia Initiating Cells

3.2.1 Phenotype of the MN1-Overexpressing AML Cell Line

The above studies showed that NK cells are efficiently activated by CpG-2216 and IL-18 and not only produce a large amount of IFN-γ but they also kill the standard NK cell target YAC-1. Therefore, we tested whether the activated NK cells also efficiently kill leukemic cells. For this study, we used the murine AML line MN1, which was generated by retroviral gene transfer of the human oncogene *MN1* into B6 BM cells (Heuser et al., 2006), as a model AML cell line. The MN1 cell line was first analyzed for the expression of GFP, H-2K^b, ICAM-1 and various cell surface markers.

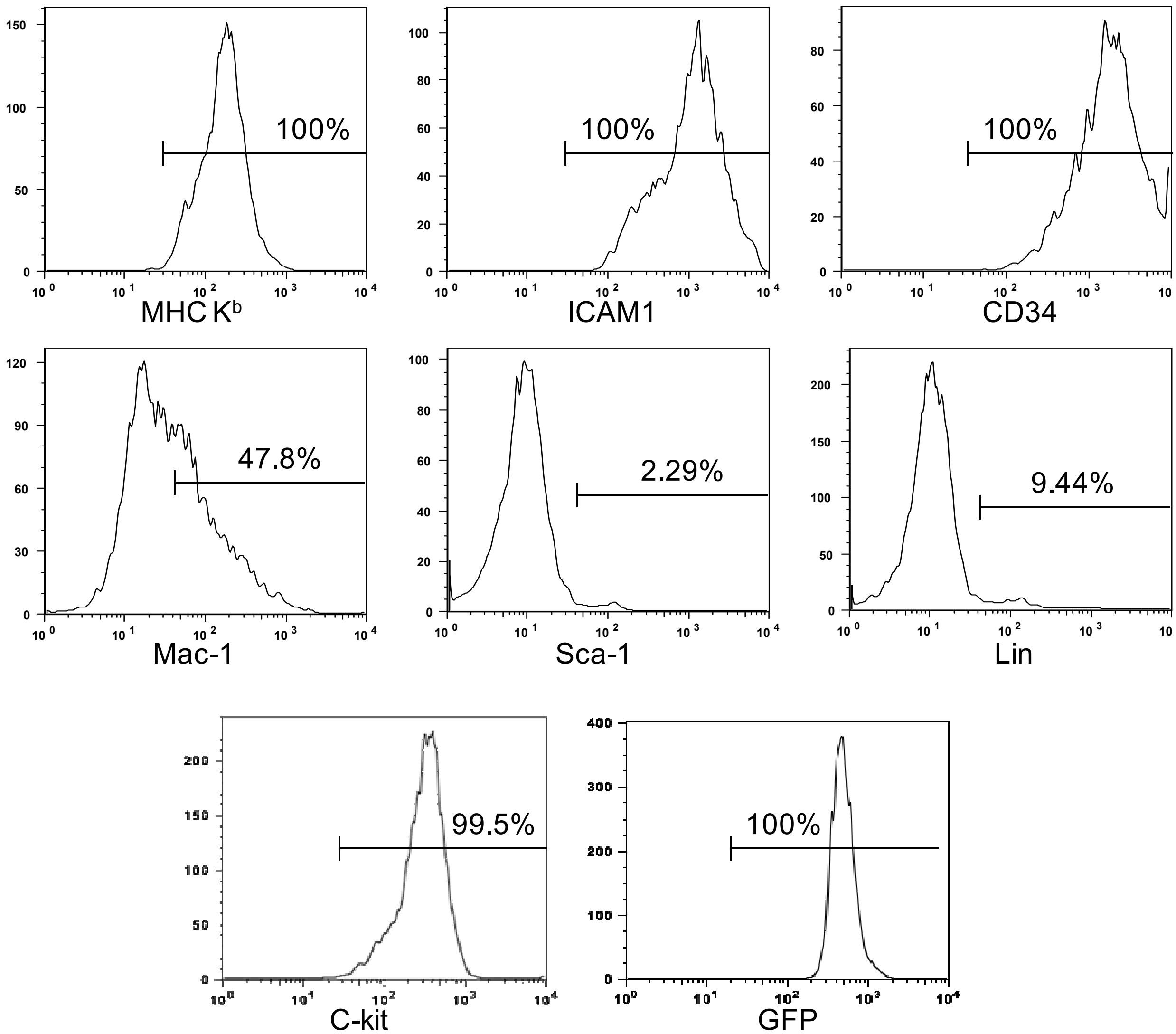


Figure 11. Phenotype of MN1-overexpressing AML cells

MN1 cells were stained with monoclonal antibodies and marker expression evaluated by flow cytometry. Dead cells were excluded by propidium iodide staining. Percentage of MN1 cells expressing markers is shown in histograms. Lineage mixture contained CD3, CD4, CD8, NK1.1, Ter119, Gr-1, CD19, CD11c and B220.

All MN1 cells expressed high levels of GFP confirming *MN1* gene expression, as the retrovirus used to generate the cell line was designed to express MN1 and GFP as a single transcript separated by an internal ribosome entry site. All MN1 cells expressed high levels of H-2K^b and ICAM-1. The former potentially inhibits NK cells expressing Ly49C/I, while the latter is critical for the binding of MN1 cells to NK cells (Fig. 11). Expression of various cell surface markers showed strong myeloid skew of MN1 cells. They expressed various levels of Mac-1 and only ~10% positive for lymphoid or erythroid lineage markers. Only ~2% of MN1 were positive for Sca-1, a marker for HSC in mice (Bradfute, Graubert, & Goodell, 2005) while over 99% expressed c-kit, which is expressed on normal immature hematopoietic progenitors (Bradfute et al., 2005). CD34, a marker used in stem cell isolation, was also expressed highly on MN1 cells (Nielsen & McNagny, 2008). The surface marker expression patterns of MN1 cells suggest that they are a mixture of immature myeloid cells of different levels of development.

3.2.2 Cytotoxicity of Haploidentical NK Cells against the MN1 Cell Line

As the high level of H-2K^b expression on MN1 cells suggested that they might be resistant to syngeneic (B6) NK cells, we tested NK cells from (C57BL/6 mice × BALB/c)F_1 mice (CB6F1 mice). This is analogous to treatment of human AML with haploidentical donor-derived NK cells, which are thought to be highly cytotoxic against AML cells due to mismatch between MHC class I on AML cells and inhibitory receptors on NK cells (Ruggeri et al., 2002). We confirmed that haploidentical NK cells are cytotoxic against MN1 cells by killing of bulk and in vitro colony-forming MN1 cells. After expansion and activation for four to seven days with recombinant mouse IL-15/IL-15R complex, the cytotoxicity of the NK cells against the MN1 cell line was assessed.

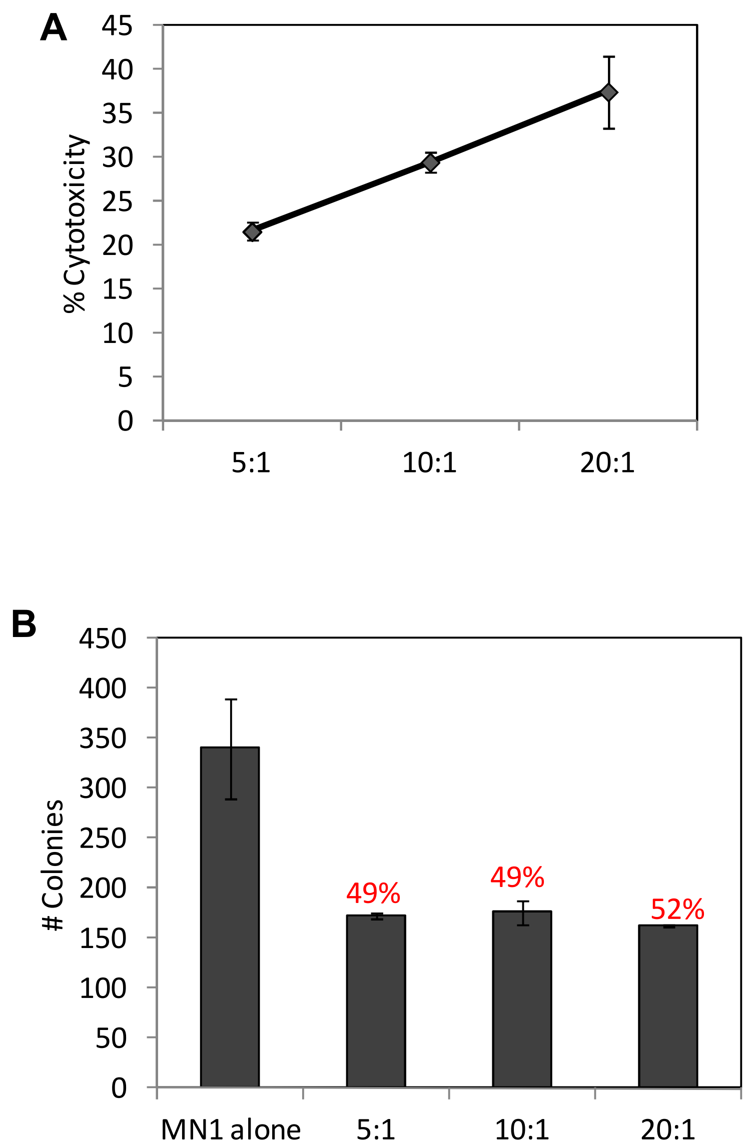


Figure 12. Cytotoxicity of haploidentical NK cells against MN1 cells

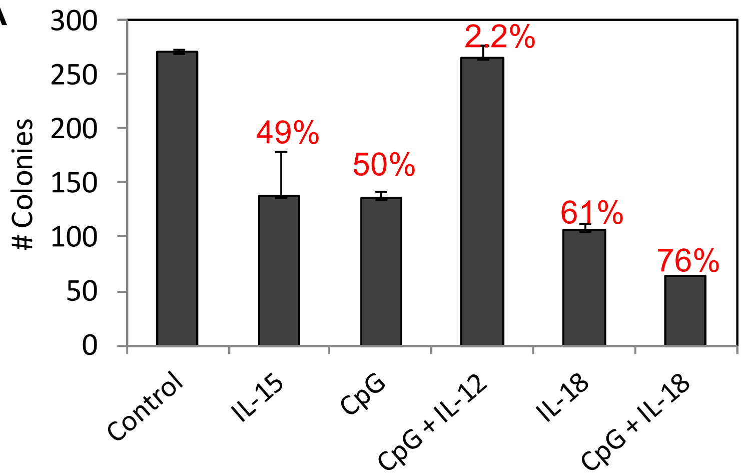
Cytotoxicity of haploidentical NK cells against (A) bulk and (B) colony-forming cells was substantiated. Target MN1 cells were labelled with CFSE before the assay. IL-15 expanded NK cells were mixed with MN1 cells at three ratios, 5:1, 10:1 and 20:1 NK:MN1 in triplicates and control with only MN1 were incubated for four hours at 37°C. Pre-calculated volumes for cells were taken from each condition and plated at 500 cells/plate with duplicate plates on methylcellulose medium to measure numbers of colony-forming cells. Colonies were counted five days later and numbers shown are the mean averages with standard deviations of duplicate plates for three independent experiments. To measure bulk cell killing, the percentage of $CFSE^{+}7AAD^{+}$ cells out of $CFSE^{+}$ cells was measured by flow cytometry and background death in control wells subtracted. (A) Diamonds represent averages plus bars for SD for triplicate wells. (B) Bars show mean ± SD colony numbers from three independent experiments. Numbers in red denote percent cytotoxicity.

IL-15-cultured haploidentical NK cells killed bulk MN1 cells with ~20% cytotoxicity at a 5:1 ratio of NK:MN1 and ~35% cytotoxicity at a 20:1 ratio (Fig. 12A). NK cells were less cytotoxic against MN1 cells than against the prototypic NK cell targets, YAC-1 or RMA-S cells (data not shown). The number of CFCs was reduced by ~50% (Fig. 12B). The number of CFCs was not further reduced as the ratio of NK cells to MN1 cells was increased, suggesting that some CFCs are resistant to NK cell killing.

3.2.3 Increasing Cytotoxicity of Haploidentical NK Cells

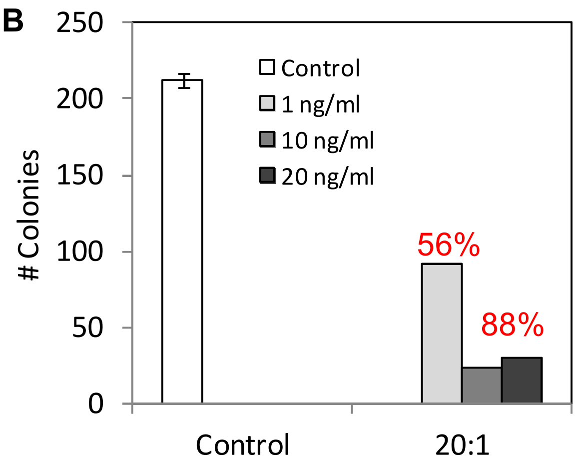
Recombinant IL-15/IL-15R efficiently expanded NK cells in culture, but the cytotoxicity of the cultured NK cells against MN1 was modest. Therefore, we further stimulated cultured NK cells with IL-12, IL-18 and CpG-2216 in the presence of IL-15/IL-15R.

A



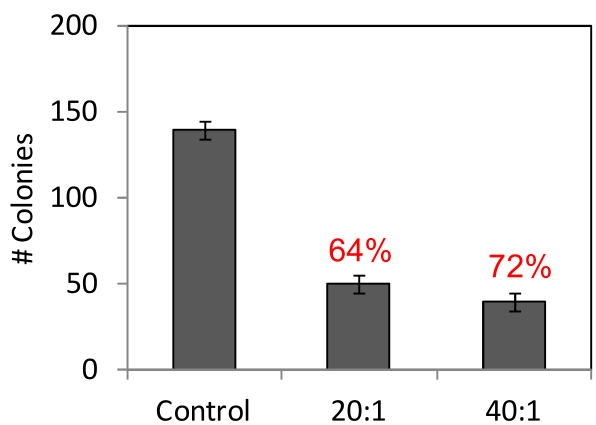
300
250
200
150
100
50
0
Colonies
49%
50%
2.2%
61%
76%
Control
IL-15
CpG
CpG + IL-12
IL-18
CpG + IL-18

B



250
200
150
100
50
0
Colonies
Control
1 ng/ml
10 ng/ml
20 ng/ml
56%
88%
Control
20:1

C



200
150
100
50
0
Colonies
64%
72%
Control
20:1
40:1

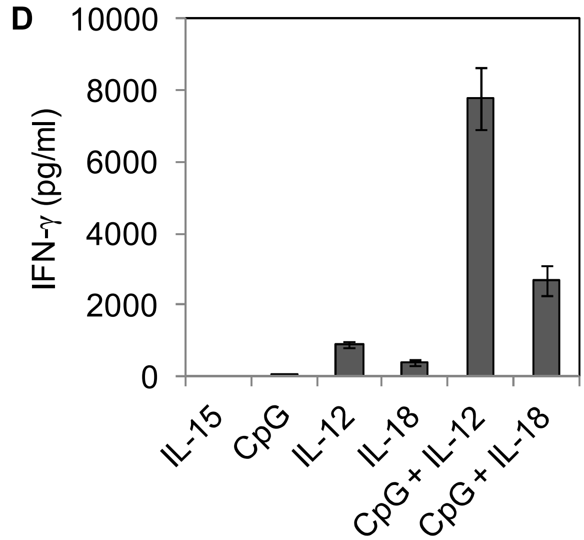


Figure 13. Haploidentical NK cells are most cytotoxic with CpG ODN and IL-18 stimulation

NK cells from CB6F1 mice were purified and expanded in cultures in the presence of IL-15/IL-15R (4 ng/ml) for 6 days. The cultured NK cells were further stimulated with IL-12 (1 ng/ml), IL-18 (1 ng/ml) and CpG-2216 (2 μg/ml) overnight in the presence of IL-15/IL-15R. (A) MN1 cells were incubated with stimulated NK cells at 1:20 ratio for 4 hours and then plated for colony formation. MN1 cells without NK cells were used as control. (B) IL-15/IL-15R expanded NK cells were activated with 2 μg/ml of CpG-2216 and 1–20 ng/ml of IL-18 in the presence of 4 ng/ml of IL-15/IL-15R, incubated with MN1 cells at 20:1 NK:MN1 ratio for 4 hours and plated for colony formation. (C) The expanded NK cells were activated with 4 ng/ml of IL-15, 1 ng/ml of IL-18 and 2 μg/ml of CpG-2216. MN1 and NK cells were incubated at 20:1 and 40:1 NK:MN1 ratio for four hours at 37°C. Pre-calculated volumes of MN1 cells from each condition were cultured at 500 cells/plate (with duplicate plates) on methylcellulose medium to measure numbers of colony-forming cells. Colonies were counted five days later. Bars show mean ± SD of two independent experiments each done in duplicates. Numbers in red denote percent cytotoxicity. (D) IFN-γ production by IL-15 expanded NK cells incubated for 24 hours with 4 ng/ml of IL-15, 2 μg/ml of CpG-2216, 1 ng/ml of IL-12 and 10 ng/ml of IL-18. Cell-free supernatants were assessed for IFN-γ by ELISA. The results shown are the mean ± SD of two independent experiments, each with triplicate cultures.

CpG-2216 alone had no significant effect on the killing of MN1 CFCs by the cultured NK cells. Unexpectedly, a combination of CpG-2216 and IL-12 inhibited NK cell cytotoxicity (Fig 13A) even though IL-15, CpG-2216 and IL-12 induced the highest amount of IFN-γ production from purified NK cells (Fig. 13D). IL-18 on its own enhanced the killing of CFCs by IL-15-cultured NK cells while a combination of CpG-2216 and IL-18 induced the highest cytotoxicity (Fig 13A). Increasing CpG-2216 concentration from 2 μg/ml to 5 μg/ml did not increase NK cell cytotoxicity (data not shown) while higher concentration of IL-18 (from 1 ng/ml to 10 ng/ml) further increased the killing of CFCs (Fig. 13B). Increasing the ratio of NK cells to MN1 cells to 40:1 did not increase NK cell cytotoxicity against CFCs (Fig. 13C), and even with increased NK cell cytotoxicity, some colony-forming MN1 cells remain resistant to NK cells.

3.2.4 Measuring NK Cell Killing of Leukemia Initiating Cells in Mice

Although haploidentical NK cells were capable of killing up to 88% of colony-forming MN1 cells, this is not a true representation of leukemia initiating cell (LIC) killing. Almost half of MN1 cells form colonies in vitro whereas the frequency of MN1 LICs that initiate AML upon transplantation into irradiated host mice has been estimated to be approximately 1 in 5000 cells (Heuser et al., 2009). To test if NK cells can kill MN1 LICs, we treated MN1 cells with cultured and stimulated NK cells from CB6F1 mice for 4 h in vitro and injected various dilutions of the cell mixtures into irradiated B6 mice (Fig. 14). The mice were observed for AML development for up to 12 weeks. Two experiments in mice were completed to measure LIC killing as outlined in Figure 14 below.

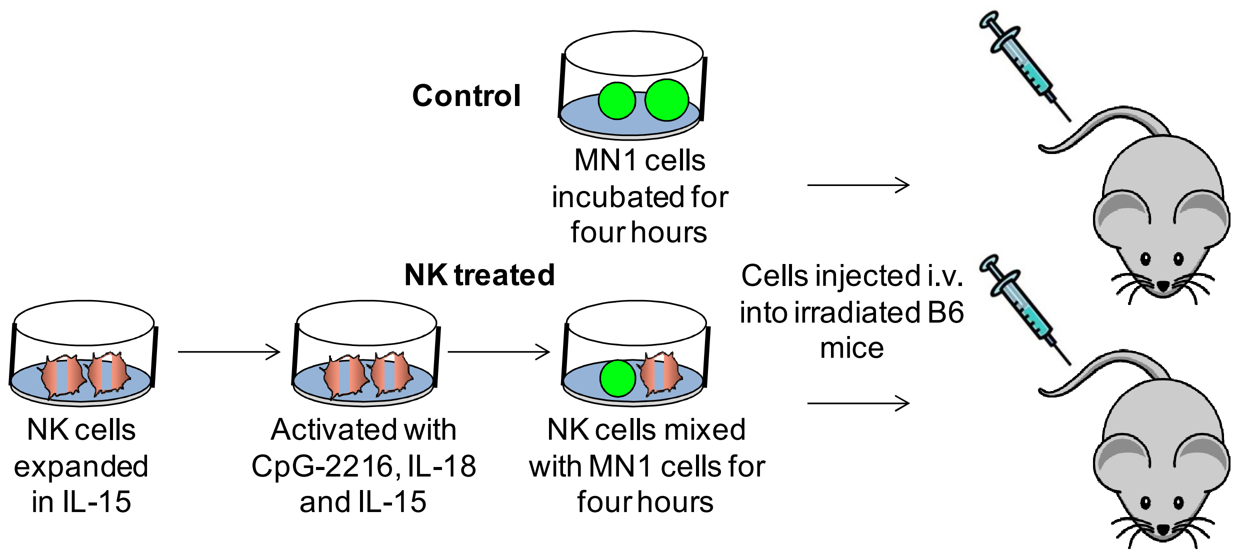
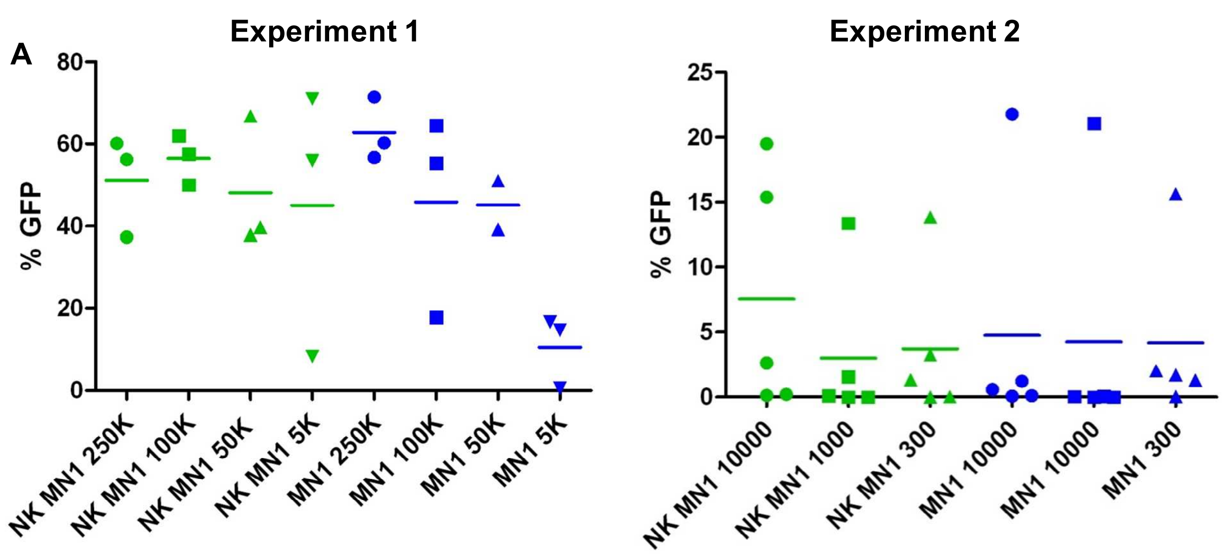


Figure 14. Leukemia initiating cell assay methods

Cytotoxicity assays were performed as above with MN1 cells incubated with or without NK cells in separate wells. IL-15 expanded NK cells were stimulated for 24 h with 6 ng/ml of IL-15, 2 µg/ml of CpG-2216 and 1 ng/ml (first experiment) and 10 ng/ml (second experiment) of IL-18 before mixing with MN1 cells pre-aliquoted to numbers used for each dose at a 20:1 (first experiment) or 100:1 (second experiment) NK:MN1 ratio. After four hours incubation in a 96-well round-bottom plate, either control MN1 cells or NK-treated MN1 cells were injected into irradiated B6 mice along with 2×10^5 B6 helper BM cells. Some cells were saved for a CFC assay to ensure NK cells were cytotoxic. For the first experiment, 2.5×10^5 to 5×10^3 MN1 cells or MN1 plus NK cells were transplanted into 7-week old irradiated B6 mice with three mice per dose. For the second experiment, 1×10^4 to 3×10^2 MN1 cells or MN1 plus NK cells were transplanted into 12-week old irradiated B6 mice with five mice per dose. 2.5×10^5 syngeneic BM cells were also injected to prevent BM failure from irradiation. Leukemia development was followed in transplanted mice.

3.2.4.1 Percentage of MN1 Cells in Peripheral Blood of MN1-Transplanted Mice

One mouse in the 5×10^5 dose control group was lost to BM failure due to irradiation. After three and eight weeks, peripheral blood was taken from tail veins and WBCs analysed by flow cytometry for percentage of GFP-expressing MN1 cells to confirm the engraftment of MN1 and leukemia development. All of the mice transplanted with high doses of MN1 cells in the first experiment and thirteen of the mice transplanted with low doses of MN1 cells in the second experiment perished by eight weeks and are not plotted in the eight week peripheral blood analysis.



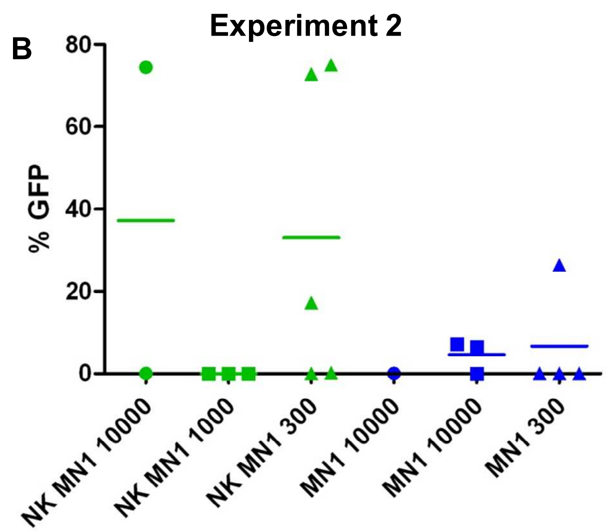


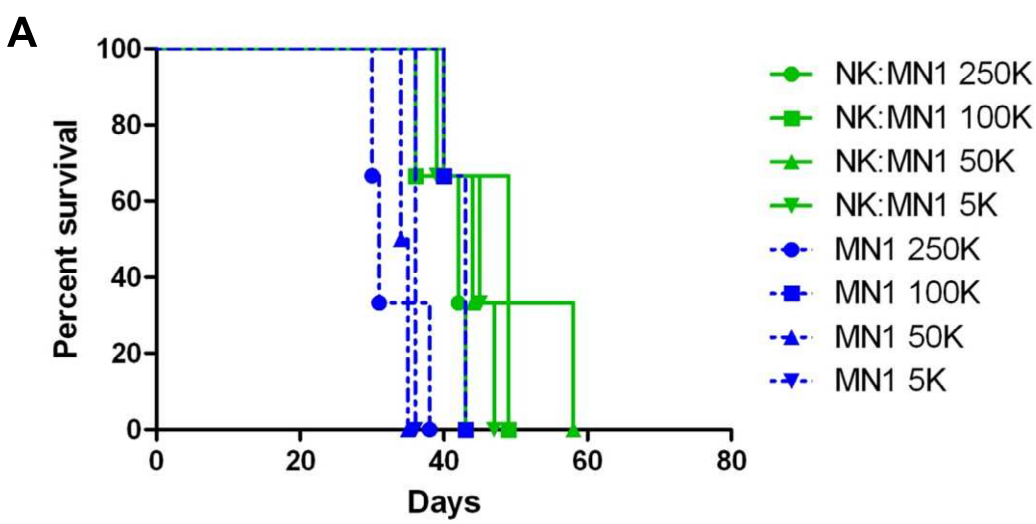
Figure 15. Peripheral blood analyses of transplanted mice

GFP expression of peripheral blood from the tail veins of mice transplanted with control and NK-treated MN1 cells. Peripheral blood was taken from the tail veins of mice at (A) three and (B) eight weeks after transplantation. Percentage of WBCs expressing GFP was assessed by flow cytometry. Each dot represents one mouse and the horizontal bar represents the average for all mice in the experimental group.

All mice in the first experiment show engraftment of MN1 cells. The percentage of WBCs expressing GFP was very high with averages for most experimental groups nearing 50%. Only four mice had a low GFP expression including one 5×10^4 NK treated mouse at 8.3%, one 1×10^5 control mouse at 18% and all three 5×10^4 control mice with percentages ranging from 0.48 to 17% (Fig 15A). In the second experiment, the percentages of GFP^+ WBCs in the peripheral blood of transplanted mice at three weeks were much lower. Eleven mice expressed GFP at lower than 1%, so MN1 cells may not have engrafted. Only one mouse per condition expressed GFP higher than 5% with the exception of the NK-treated 10,000 cell dose, which contained one mouse with 16% and another with 19% GFP^+ cells (Fig 15A). There does not appear to be any pattern to GFP expression in the peripheral blood. No differences in the average GFP expression were measured between the control and NK-treated group in either experiment. At eight weeks, the eleven mice still contained no GFP^+ WBCs in peripheral blood (Fig. 15B). At this late time point we can assume that mice were not engrafted by the MN1 cells injected. Four mice in the NK-treated group and three in the control expressed GFP.

3.2.4.2 Survival of MN1-Transplanted Mice and LIC Frequency

Over the next eleven weeks after transplantation, all transplanted mice in experiment one and nineteen out of thirty transplanted mice in experiment two perished. Mice were euthanized when they appeared hunched, thin and anemic. All mice but one that died early on of BM failure from irradiation in experiment one were confirmed to have died from leukemia. Survival of mice is plotted below in Figure 16.



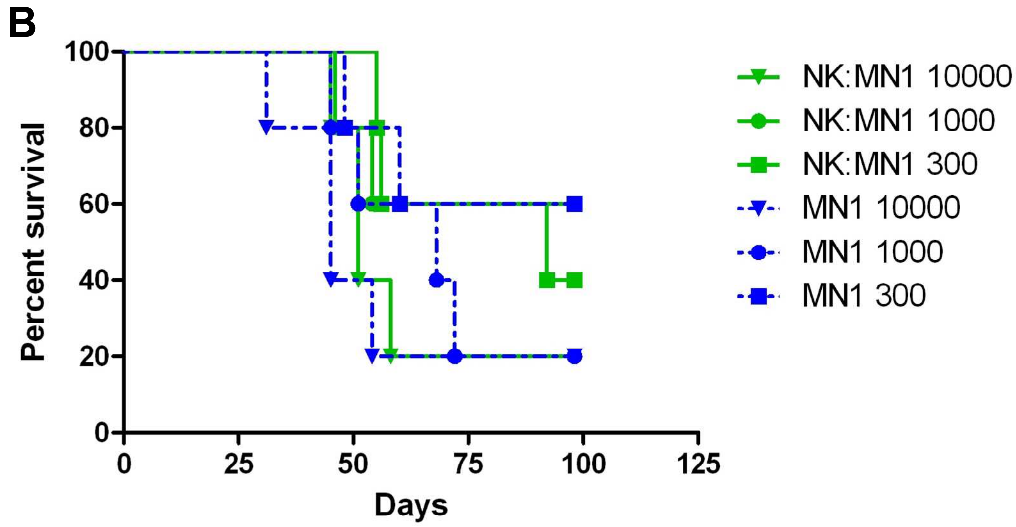


Figure 16. Survival curves of mice transplanted with MN1

Survival curves of control mice and experimental mice transplanted with (A) 2.5×10^5 to 5×10^3 and (B) 1×10^4 to 3×10^2 MN1 cells incubated with or without NK cells is shown over days. Each dot represents one mouse and each line represents each experimental group. Control mice that received MN1 only are in blue, mice that received MN1 cells incubated with NK cells are in green. 'K' in the sample title represents times one thousand.

All of the transplanted mice died from leukemia except for one that was lost to irradiation-induced BM failure in experiment one (Fig. 16A). Although we were unable to prevent leukemia by incubating MN1 cells with NK cells, these mice survived significantly longer than control mice in experiment one as the P values representing the difference between the survival curves calculated by the Log-rank test in Table 1 show.

Table 1. P values for survival curves of control versus NK-treated mice (Log-rank Test)

	Dose	P Value
Experiment 1	All curves combined	**0.0016**
	5, 000	**0.0455**
	50,000	**0.0389**
	100,000	0.3213
	250,000	1.0000
Experiment 2	All curves combined	0.3600
	300	0.6019
	1,000	0.3135
	10,000	0.6434

At high MN1 cell doses, no significant difference in survival was seen between control mice and mice receiving NK-treated MN1 cells. However, for the two lower 50,000 and 5,000 MN1 cell doses, mice receiving NK-treated MN1 cells lived significantly longer. If all the data for the control and NK-treated groups are combined for analysis, a significant P value of 0.0016 results. All mice did not perish from leukemia in experiment two; five of the control mice and six of the NK-treated mice survived leukemia-free (Fig. 20B). However, we calculated no difference between survival curves of NK-treated and control groups with a P value of 0.3600. The lack of engraftment in eleven of these mice indicates that no LICs were present in the transplanted cells. Because all the mice did not die from leukemia, we were able to calculate the LIC frequency for mice receiving MN1 and NK-treated MN1 cells by using the online extreme limiting dilution (ELDA) analysis tool developed by the Walter and

Eliza Hall Institute of Medical Research Bioinformatics department (Hu & Smyth, 2009). The results of this analysis are shown below in Table 2.

Table 2. Limiting dilution data

Group	Dose	Number of mice per dose	Number of leukemic mice	LIC Frequency, 95% confidence (range)
MN1 Control	10000	5	4	1 in 2188 (780 to 6143)
	1000	5	4	
	300	5	2	
NK-Treated	10000	5	4	1 in 2773 (1018 to 7554)
	1000	5	2	
	300	5	3	

When calculating the frequency of LICs by limiting dilution assay, the frequency decreased from 1/2188 to 1/2773 at a 95% confidence interval. However, the difference between these two groups was not significant with a P value of 0.715.

3.2.4.3 Analysis of Mouse Tissue after Death

Leukemia as cause of death in mice was confirmed by splenomegaly, high WBC count and low RBC count in peripheral blood (Table 3), as well as, infiltration of the spleen and bone marrow measured by percentage of GFP^+ cells (Fig. 17).

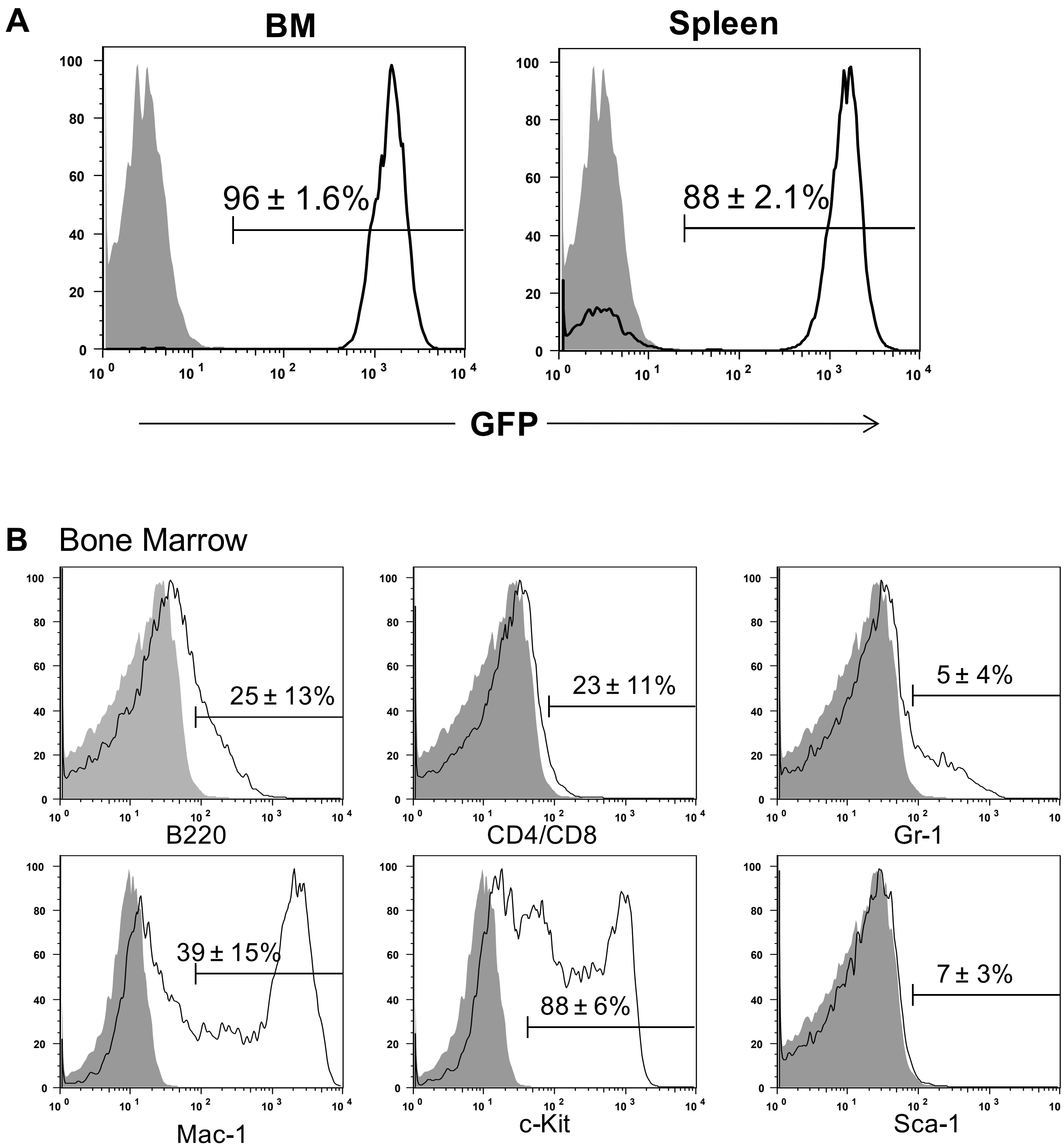
Table 3. Hematologic parameters and spleen weights of leukemic mice at time of death

Parameter	Experiment	MN1 Transplanted Mice		Healthy Mice*
		Control	NK-treated	
RBC × 10^3/mm^3, mean (range)	1	2.22 (1.50 – 3.34)	2.18 (1.15 – 3.11)	10.8 – 10.9
	2	3.08 (1.36 – 6.05)	3.23 (1.73 – 5.91)	
WBC × 10^6/mm^3, mean (range)	1	33.0 (8.00 – 80.0)	52.6 (20.1 – 80.0)	3.48 – 2.67
	2	50 (10 – 80)	72 (55 – 80)	
Spleen weight (g), mean (range)	1	0.55 (0.26 – 0.89)	0.48 (0.20 – 0.91)	0.072 – 0.081
	2	0.31 (0.12 – 0.45)	0.42 (0.27 – 0.65)	

*From hematology mouse phenome database on strain C57BL/6J, Jackson Laboratory, Bar Harbor, ME (www.jax.org).

At death, all transplanted mice had low RBC counts, high WBC counts and enlarged spleens compared to reported averages for healthy B6 mice, confirming the presence of leukemia. The high range in values for blood counts and spleen weights could in part be explained by the state of the mice when they were euthanized. Though we tried to euthanize all mice at the same stage of leukemia, mice developed symptoms of illness so quickly (within 24 hours) that it was very difficult to catch the leukemia at a standardized time-point. Low WBC counts resulted from several mice that had abdominal bleeding; blood from the abdomen contaminating blood from the femoral artery tended to lower WBC counts. No significant differences in these measurements resulted between the two groups (P values were all above 0.05).

Spleen and bone marrow GFP$^+$ cells taken from leukemic mice were analysed for primitive cell and differentiation markers: Mac-1, Gr-1, B220, CD4, CD8, cKit and Sca-1.


A
BM
Spleen
96 ± 1.6%
88 ± 2.1%
GFP
B Bone Marrow
25 ± 13%
23 ± 11%
5 ± 4%
B220
CD4/CD8
Gr-1
39 ± 15%
88 ± 6%
7 ± 3%
Mac-1
c-Kit
Sca-1

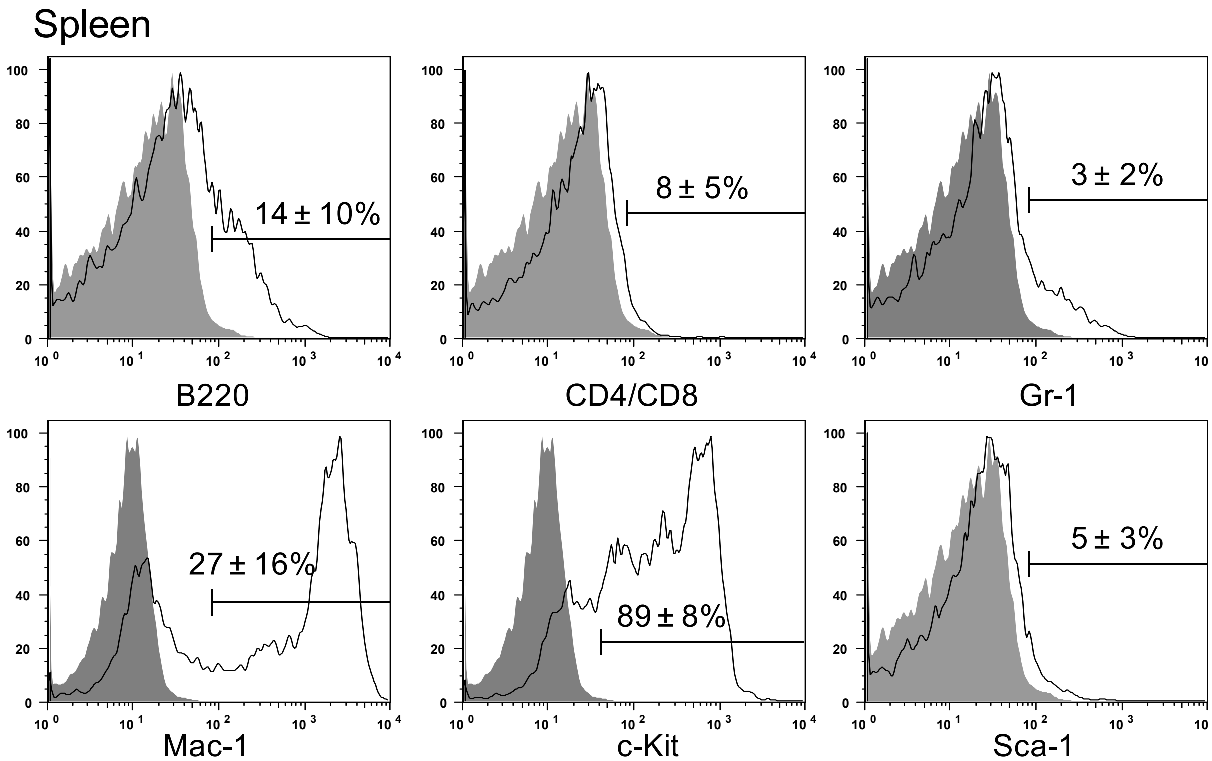


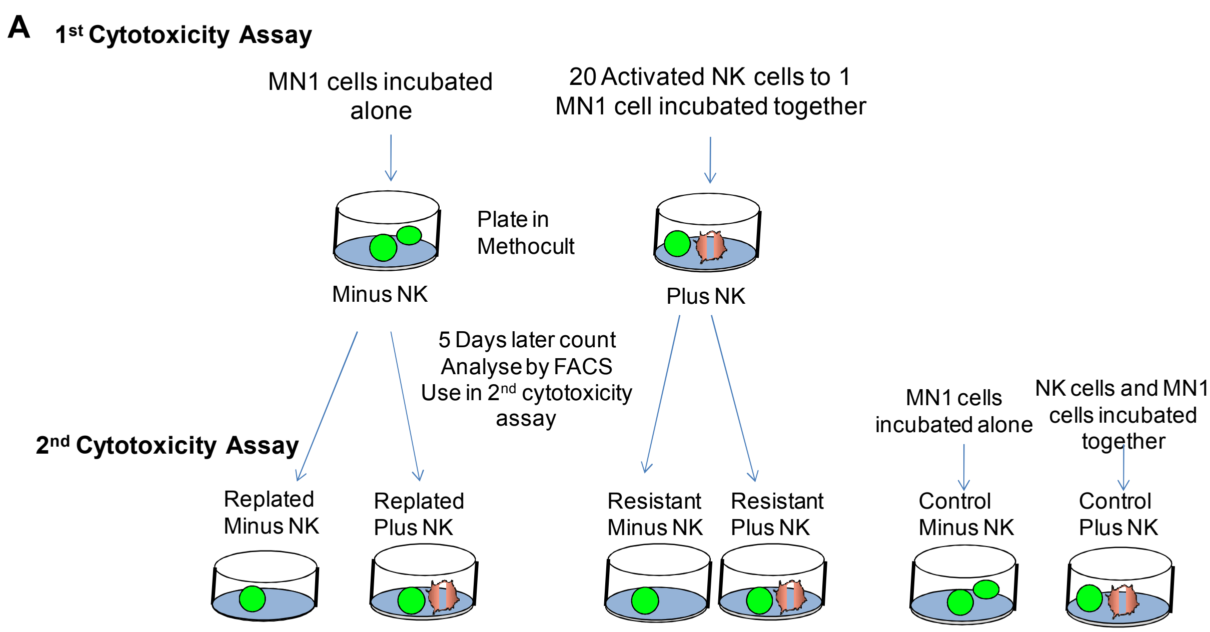
Figure 17. Spleen and bone marrow analysis of leukemic mice

Spleen and BM cells from leukemic mice at endpoint were analyzed by flow cytometry. (A) Open histograms show percentage GFP^+ MN1 cells from one representative mouse with mean ± SD for all mice in experiment one and shaded histograms show unstained healthy mouse BM (B) Cells were stained with mAbs to Mac-1, Gr-1, B220, CD4, CD8, cKit and Sca-1 and analyzed by flow cytometry. Open histograms show percentages for one representative mouse with mean ± SD for all mice in experiment one and shaded histograms unstained controls.

At death, the spleens of leukemic mice contained over 80% GFP^+ MN1 cells. Mice were very anemic with over 90% GFP^+ MN1 cells in the BM (Fig. 17A). GFP^+ BM and spleen cells expressed the phenotype of immature myeloid cells. Almost all GFP$^+$ spleen and BM cells were positive for the primitive cell marker c-Kit (CD117). Small percentages were positive for Sca-1, which is commonly expressed on HSCs. Mac-1 expression varied between mice from 20 to 40%. Low expression of lineage markers Gr-1, B220 and CD4/CD8 was measured in GFP$^+$ BM and spleen cells (Fig 17B).

3.2.5 Analysis of Resistant Colonies

To gain more insight into how some MN1 CFCs resisted NK cell killing, colonies from control and NK-treated plates were recovered and re-cultured. Cells were analyzed for lineage, c-Kit, Sca-1, Mac-1, ICAM1, MHC K^b and CD44 marker expression by flow cytometry. The re-cultured cells were also tested for sensitivity to NK cell cytotoxicity in the second round of NK cell cytotoxicity.



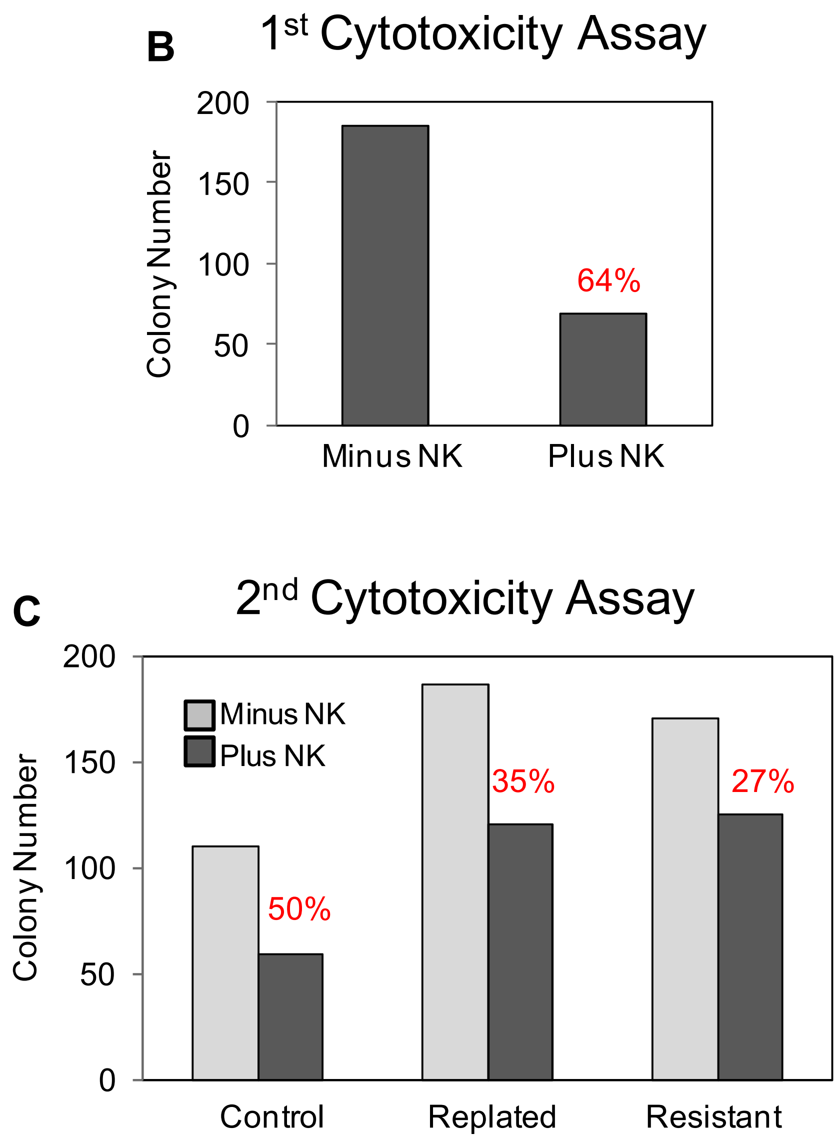


Figure 18. Cells from colonies are more resistant to NK cell killing

NK cell cytotoxicity against 'replated' and 'resistant' colonies from an initial cytotoxicity assay. (A) A diagram of methods for resistant colony analysis. (B) IL-15 expanded NK cells were activated with 4 ng/ml of IL-15/IL-15R, 1 ng/ml of IL-18 and 2 µg/ml of CpG-2216 for 24 h. Activated NK cells were mixed with MN1 cells at a 20:1 NK:MN1 ratio in a 96-well round-bottom plate along with a control well containing only MN1 cells. Cells were incubated for four hours at 37°C. Pre-calculated volumes for cells were taken from each condition and plated at 500 MN1 cells/plate with duplicate plates on methylcellulose medium. Colonies from control and NK-treated plates were counted five days later. (C) Cells in the colonies from 'B' were recovered, cultured for one day in media with cytokines before staining with mAbs for flow cytometry and being used in a second cytotoxicity assay as described above. 'Control' bars denote MN1 cells cultured normally, 'replated' bars and 'resistant' bars denote colonies cells from MN1 cells incubated with or without NK cells in the first cytotoxicity assay. Bars represent average colony number and numbers in red denote percent cytotoxicity.

The first round of cytotoxicity assay resulted in a 64% reduction in CFCs (Fig. 18B). We found no differences in marker expression on MN1 cells between control and NK-treated colonies (not shown). When MN1 cells that survived in the first round of NK cell treatment and formed colonies were treated by NK cells again, they appeared to be more resistant to NK cells killing (27% sensitive) than the original MN1 cells (64% sensitive) (Fig. 18C). However, MN1 cells from control plates that were not treated with NK cells in the first round of cytotoxicity were almost equally resistant to the second round of NK cell treatment. Therefore, the first round of NK cell treatment did not seem to significantly enrich NK cell-resistant MN1 cells. Instead, continued cultures in CFC assays seemed to result in more NK cell-resistant MN1 CFCs.

Chapter 4 Discussion

In this study, we have found that activation of NK cells by the A-type CpG ODN, CpG-2216, is rather complex and involves multiple cell types and cytokines. While CpG-2216 alone without exogenous cytokines stimulates NK cells in bulk splenocyte cultures and induces IFN-γ production, highly purified NK cells are not stimulated by CpG-2216 alone and require exogenous IL-12 or IL-18 to produce IFN-γ or become cytotoxic. Bulk splenocytes stimulated by CpG-2216 alone produce a significant amount of IL-12, and the neutralization of IL-12 almost completely inhibits the IFN-γ production. Moreover, IL-12-KO spleen cells produce very little IFN-γ in response to CpG ODN. These results indicate that IL-12 is critical for the stimulation of NK cells by CpG ODN in bulk splenocytes cultures. Our cell depletion experiments have shown that Gr-1$^+$ splenocytes are a critical source of IL-12 in bulk splenocytes cultures stimulated by CpG-2216. Most other studies on CpG ODN stimulation of NK cells thus far have suggested that DC and macrophages are critical accessory cells (Ballas et al., 1996; Chace et al., 1997; Cowdery et al., 1996; Marshall et al., 2006). However, in our study CD11c$^+$ cell depletion has no effect on IL-12 or IFN-γ production by CpG-2216-stimulated splenocytes, indicating that DCs are not a critical source of IL-12. Depletion of monocytes/macrophages (F4/80$^+$ cells) only partially reduces IFN-γ production in CpG-2216 stimulated spleen cells whereas depletion of Gr-1$^+$ cells almost completely inhibits IL-12 and IFNγ production by CpG-2216 stimulated splenocytes. Although Gr-1 is often used as a marker for neutrophils, the Gr-1 recognized by the mAb RB6-8C5 is expressed on both inflammatory monocytes and a subset of pDCs (Egan et al., 2008). As pDCs express CD11c, IL-12-producing Gr-1$^+$ cells likely exclude pDCs, leaving neutrophils and inflammatory monocytes as critical sources of IL-12 in the stimulation of NK cells in bulk splenocytes cultures. Gr-1^+ splenocytes are heterogeneous and can be divided into F4/80^+ inflammatory monocytes (Geissmann et al., 2003) and F4/80^- neutrophils (Daley et al., 2008), and depletion of either population results in partial reduction of IL-12 production but no significant effect on IFN-γ production by CpG-2216 stimulated splenocytes. The results suggest that both subsets produce sufficient IL-12 for the stimulation of NK cells by CpG-2216. Therefore, F4/80^+Gr-1^+ inflammatory monocytes and F4/80^-Gr-1^+ neutrophils seem to be the critical source of IL-12 in bulk splenocytes cultures stimulated by CpG-2216. While intracellular IL-12 staining of CpG-2216 stimulated splenocytes also shows that the majority of IL-12^+ cells are Gr-1^+, they are mostly Gr-1^+F4/80^+. However, some IL-12^+ cells are Gr-1^-F4/80^+CD11b^+. It remains to be determined whether the expression of Gr-1 and F4/80 changes when cells are stimulated by CpG ODN. Thus, Gr-1^+ inflammatory monocytes seem to be the main producers of IL-12 in response to CpG-2216, and the role of neutrophils (Gr-1^+F4/80^-) in the stimulation of NK cells is still unclear. It should also be noted that although purified Gr-1^+ cells are stimulated by CpG-2216 alone, the amount of IL-12 they produce is significantly smaller than that produced by bulk splenocytes. It suggests that optimal IL-12 production by CpG ODN-stimulated Gr-1^+ cells may require other cells or cytokines.

It has previously been reported that a subset of NK cells expressing CD11c, termed NKDC, produces IL-12 in response to the B-type CpG ODN, 1826 (Chaudhry, Kingham et al., 2006). However, we have found no production of IL-12p70 in the supernatant or IL-12p40 mRNA from highly purified NK cells, including CD11c^+ NK cells cultured with CpG-2216 alone or with CpG-2216 plus IL-18. It seems likely that the reported IL-12 production by

NKDC is due to contamination of non-NK cells. Ballas *et al.* found that highly purified NK cell (>99%) lytic activity would not be stimulated by CpG ODN alone (Ballas et al., 1996). IL-12 is also well established as being important for the NK cell response to bDNA and CpG ODNs. Ballas *et al.* found that a combination of IFN-α, TNF-α and IL-12 were involved in inducing the lytic activity of NK cells in response to CpG ODN as neutralization of these cytokines reduced activity (Ballas et al., 1996). However they found that induction of type I IFNs was the most pivotal step as NK cell cytotoxicity could still be activated in IL-12KO mice treated with type-B CpG ODNs. However, these studies looked only at the lytic function of NK cells and not cytokine production (Ballas et al., 2001).

Our results as well as other studies indicate that IL-12 is critical for the stimulation of NK cells by CpG ODNs. However, purified NK cells stimulated by CpG-2216 plus exogenous IL-12 produce only a very modest amount of IFN-γ, whereas they produce very large amounts of IFN-γ when stimulated by exogenous IL-12 and IL-18 without CpG-ODNs. Therefore, in addition to IL-12, another cytokine, most probably IL-18, is required for optimal stimulation of NK cells by CpG ODNs. Although IL-18 has not been detected in the supernatant of the bulk splenocyte cultures, IL-18 neutralization significantly inhibits IFN-γ production by CpG ODN-stimulated bulk splenocytes. It seems very likely that only a very small amount of IL-18, below the detection limit (25 pg/ml) of currently available ELISA kits, is produced, but the amount is sufficient to contribute to the stimulation of NK cells. What cells produce IL-18 in CpG ODN-stimulated bulk splenocytes cultures is still unclear. Functional IL-18 is generated by cleavage of the precursor pro-IL-18 by caspase-1 (Gu et al., 1997), and many cell types constitutive express IL-18 mRNA (Akira, 2000), making it difficult to identify the cells producing low levels of functional IL-18. We have not been able to detect IL-18-producing cells by intracellular staining after CpG-2216 stimulation of spleen cells, and the source of IL-18 in CpG-2216-stimulated splenocytes remains to be identified.

Purified NK cells stimulated by CpG-2216 plus IL-12 or IL-18 produce significantly more IFN-γ than those stimulated by IL-12 or IL-18 alone without CpG-2216, indicating that CpG-2216 directly co-stimulates NK cells. However, the amount of IFN-γ produced by the purified NK cells is much lower than those produced by bulk spleen cells stimulated in the same way. The combination of CpG-2216 and IL-18 in particular stimulates RAG1-KO splenocytes to produce very large amounts (~200 ng/ml) of IFN-γ whereas purified NK cells produce only less than 500 pg/ml even though the estimated number of NK cells in the bulk splenocytes cultures is comparable to that of purified NK cell cultures. This is likely due to IL-12 production by bulk splenocytes but not purified NK cells in response to CpG-2216. The combination of IL-12 and IL-18 is a very potent stimulator of NK cells, and purified NK cells produce ~100 ng/ml IFN-γ when both cytokines are added to the culture. Thus, NK cell stimulation by CpG-2216 in bulk splenocyte cultures may be mainly mediated by IL-12 and IL-18 produced by non-NK cells, possibly Gr-1$^+$ cells. CpG-2216 plus IL-18 can stimulate IL-12KO mouse spleen cells although they produce ~50 fold less IFN-γ than WT or IL-18KO splenocytes under the same condition. Taken together, these findings suggest that the stimulation of NK cells by CpG-2216 in bulk splenocytes cultures may be mediated by combined stimulatory signals generated by CpG-2216, IL-12 and IL-18. IL-12, produced by CpG-2216-stimulated Gr-1$^+$ cells, combined with CpG-2216 weakly stimulate NK cells without IL-18, as seen with IL-18 neutralization and IL-18KO mouse splenocytes. A small amount of IL-18, produced by unidentified splenocytes, augments the IFN-γ production. When a high dose IL-18 is added, the stimulatory signals generated from IL-18 and CpG-2216 is sufficient to weakly stimulate NK cells in IL-12KO splenocytes cultures. Thus, the amount of IFN-γ produced by CpG-2216-stimulated NK cells seems limited by the amount of IL-18 produced in splenocytes cultures. It further implies that a strategy to induce larger amounts of IL-18 will greatly enhance the stimulation of NK cells by CpG-2216.

While our study has suggested that both neutrophils and inflammatory monocytes are critical sources of IL-12, few studies have linked neutrophils and inflammatory monocytes with the NK cell response to CpG ODNs. Neutrophils are well-known first responders to infection, are able to produce IL-12 and IL-18 (Cassatella, 1999; Fortin et al., 2009; Witko-Sarsat et al., 2000; Yin & Ferguson, 2009), and are thought to be essential for the innate immune response to many infectious challenges of mice (A. M. Cooper et al., 2007; Denkers et al., 2004; Easton et al., 2007; Emoto et al., 2003; Feng et al., 2006; Miyazaki et al., 2007; Pedrosa et al., 2000; Sporri et al., 2008). A complication of these studies is they relied heavily on the depletion of neutrophils with the Gr-1 mAb RB6-8C5 that is not specifically expressed on neutrophils. Monocytes are able to secrete many cytokines including IL-12, IL-1, IL-6 and TNF-α and are essential to controlling many infections (Cowdery et al., 1996; Dunay et al., 2008). Two subsets have been recently described: the "inflammatory subset" ($CX3CR1^{lo}CCR2^{+}Gr\text{-}1^{+}$) that triggers immune responses in inflamed tissue and the "resident subset" ($CX3CR1^{hi}CCR2^{-}Gr\text{-}1^{-}$) that resides in non-inflamed tissues (Geissmann et al., 2003). Our results reveal an important role as cytokine producers for the inflammatory subset in NK cell activation after CpG-2216 stimulation.

For our study on anti-leukemia effects of CpG-ODN-activated NK cells, we chose the mouse AML line MN1, which was generated by overexpression of the human oncogene *MN1* in B6 mouse BM cells. MN1 has been well characterized by the Humphries laboratory, and the cell line rapidly induces a lethal AML in irradiated mice that closely resembles human AML (Heuser et al., 2009). Studies with human leukemias have shown that NK cells kill AML blasts (Moretta et al., 2000), and haploidentical NK cells are thought to be particularly effective anti-AML effector cells. Therefore, we used CB6F1 mouse NK cells to kill MN1 cells. In the F1 mice, but not B6 mice, NK cells that express inhibitory receptors specific for BALB/c MHC I ($H\text{-}2^d$), but not B6 MHC I ($H\text{-}2^b$) are expected to acquire killing functions by the process termed "NK cell licensing" (Yokoyama & Plougastel, 2003) and potentially kill MN1 cells as they are not inhibited by the $H\text{-}2^b$ MHC I expressed on MN1 cells. We were most interested in the ability of NK cells to eliminate the LICs within MN1 cells.

As expected from our study on naïve NK cells stimulation by CpG ODNs, the combination of CpG-2216 and IL-18 significantly enhances the cytotoxicity of NK cells isolated from CB6F1 mice and expanded in culture. However, identifying the killing of crucial leukemia initiating MN1 cells by NK cells is complicated by the heterogeneity of MN1 cells. Cell surface markers (Lin, Sca-1, CD34, c-Kit and Mac-1) on MN1 cells suggest that they are heterogeneous mixture of progenitors at various levels of development along the myeloid cell lineage. About 40% of MN1 cells form colonies in methyl cellulose cultures while only approximately 1 in 5,000 MN1 cells are LICs that initiate leukemia in irradiated mice (Heuser et al., 2009). While the in vitro expanded and stimulated NK cells kill 60-80% of in vitro colony forming MN1 cells, the killing of LICs seems less effective. Our LIC assay shows no significant changes in LICs frequency following in vitro treatment of MN1 with NK cells, possibly because of relatively small number of mice used in the LIC assay. It should be noted that mice injected with NK cell-treated MN1 cells survived longer than control mice injected with untreated MN1 cells. It is still unclear whether MN1 LICs are more resistant to NK cell killing than in vitro colony forming MN1 cells. However, it is clear that some in vitro colony forming MN1 cells and LICs are resistant to NK cell killing.

The mechanisms by which some MN1 cells resist NK cell-mediated cytotoxicity are currently unknown. All MN1 cells express high levels of the LFA-1 ligand ICAM-1 and the 2B4 ligand CD48. MN1 cells in colonies formed from MN1 cells that survived killing by NK cells express the same levels of ICAM-1 and CD48 as those of the original MN1 cells. Therefore, the apparent NK cell resistance of MN1 cells is not mediated by down-modulation of those ligands for the key NK cell receptors (Matsumoto, Nghiem, Nozaki, Schmits, & Penninger, 1998). If some of the MN1 cells are resistant to begin with, we expect most of the cells from the colonies formed from MN1 cells after a cytotoxicity assay to be enriched for NK-resistant cells. However, our analysis has shown no sign of the selection of NK cell-resistant MN1 by NK cell cytotoxicity. Instead, we have found that MN1 cells cultured in methylcellulose media for colony formation are more resistant to NK cell killing than the original MN1 cells. Our data have not revealed how this is so, but it is possible that resistant MN1 cells grow and expand more efficiently than susceptible cells. Alternatively, MN1 cells acquire resistance during expansion in this media. Currently, it is unknown whether MN1

LICs self-renew and differentiate to form colonies in vitro, so we cannot be sure the resistant MN1 cells expanded on methylcellulose are LICs. Whether or not the cells are already resistant or acquiring resistance, it may be due to several factors, including less expression of activating receptor ligands, overexpression of antiapoptotic molecules, direct interference with the perforin/granzyme pathway or active killing of NK cells (Igney & Krammer, 2002).

Alloreactive NK cells improve survival of AML patients by promoting engraftment, reducing GVHD and killing remaining leukemic cells (Giebel et al., 2003; Ruggeri et al., 2002). Studies have shown that NK cells are able to kill AML blasts, but the ability of NK cells to kill AML stem cells, quiescent cells that repopulate the leukemia and cause relapse, are only assumed based on decreased relapse rates from patients receiving allogeneic HSCT (Hercend et al., 1986; Jiang et al., 1993; Lowdell et al., 1997; Mackinnon et al., 1990; Pattengale et al., 1983; Whiteway et al., 2003). The GVL effect is more important in some cancers and less in others, likely due to undefined antigens, costimulatory molecule expression or cell growth rate (Porter & Antin, 1999). Our finding that killing of LICs in the AML MN1 cell line is ineffective has important implication to the NK cell based treatment of AML patients. Future experiments looking into ligands to NKG2D expressed on MN1 cells, the best characterized activating receptor that is present on the surface of all mouse and human NK cells, and NCRs (NKp30, NKp44 and NKp46) will help us understand MN1 cell resistance (Jamieson et al., 2002). Both mouse and human tumors often express NKG2D ligands, which possess structural homology to MHC class I (Cerwenka et al., 2000; Diefenbach, Jamieson, Liu, Shastri, & Raulet, 2000; Radaev & Sun, 2003). To mould NK cells into a better killing machine, we must continue to learn more about how NK cells interact with stem cells. Activating receptors and ligands need to be better characterized and once the mechanisms of NK cell activation are further revealed, we will be better able to develop an NK cell-based therapy.

Chapter 5 Concluding Chapter

The aim of this thesis was to elucidate the mechanism of murine NK cell activation in response to the TLR9 stimulant, CpG ODN, and apply this knowledge to NK cell treatments of AML. The results presented here demonstrate that a type-A CpG ODN, CpG-2216 best stimulates cytokine production from NK cells, but additional signals from IL-12 and IL-18 are required for the cells to respond to CpG ODN. DCs are not accessory cells to NK cell stimulation by CpG-2216 as expected; instead, it appears that Gr-1^+ monocytes produce the majority of IL-12 in splenocyte cultures with some assistance from neutrophils. AML is a disease of the bone marrow that shows much promise in being treated with infusions of allogeneic NK cells. An experimental AML cell line overexpressing MN1 that rapidly induces lethal AML in mice is susceptible to killing by haploidentical NK cells activated by CpG-2216, IL-18 and IL-15 in vitro. We found this cell line to be heterogenous, with variation in surface marker expression. Allogeneic NK cells were able to kill CFCs in vitro, but some resistant cells prevented complete cytotoxicity against this target. When irradiated B6 mice were transplanted with high doses of control or NK-treated MN1 cells, we found that all mice still perished from AML, but survival times were lengthened in mice receiving NK-treated MN1 cells. When transplanting irradiated B6 mice with lower numbers of MN1 cells, we saw a non-significant decrease in the LIC frequency and no increased survival time for mice receiving NK-treated MN1 cells. Based on these observations, we conclude that NK cells are not able to efficiently target and lyse LICs in this cell line. For these cells to be used as a treatment for AML, higher cytotoxicity against all AML cells or specific targeting towards stem cells is needed.

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